Releasing prophase arrest in zebrafish oocyte: synergism between maturational steroid and Igf1

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Abstract

Binding of 17β-estradiol (E2) to novel G-protein coupled receptor, Gper1, promotes intra-oocyte adenylyl cyclase activity and transactivates epidermal growth factor receptor to ensure prophase-I arrest. Although involvement of either membrane progestin receptor (mPR) or Igf system has been implicated in regulation of meiosis resumption, possibility of concurrent activation and potential synergism between 17α,20β-dihydroxy-4-pregnen-3-one (DHP)- and Igf-mediated signalling cascades in alleviating E2 inhibition of oocyte maturation (OM) has not been investigated. Here using zebrafish (Danio rerio) defolliculated oocytes, we examined the effect of DHP and Igf1, either alone or in combination, in presence or absence of E2, on OM in vitro. While priming of denuded oocytes with E2 blocked spontaneous maturation, co-treatment with DHP (3 nM) and Igf1 (10 nM), but not alone, reversed E2 inhibition and promoted a robust increase in germinal vesicle breakdown (GVBD). Although stimulation with either Igf1 or DHP promoted Akt phosphorylation, pharmacological inhibition of PI3K/Akt signalling prevented Igf1-induced GVBD but delayed DHP action till 4–5 h of incubation. Moreover, high intra-oocyte cAMP attenuates both DHP and Igf1-mediated OM and co-stimulation with DHP and Igf1 could effectively reverse E2 action on PKA phosphorylation. Interestingly, data from in vivo studies reveal that heightened expression of igf1, igf3 transcripts in intact follicles corresponded well with elevated phosphorylation of Igf1r and Akt, mPRa immunoreactivity, PKA inhibition and accelerated GVBD response just prior to ovulation. This indicates potential synergism between maturational steroid and Igf1 which might have physiological relevance in overcoming E2 inhibition of meiosis resumption in zebrafish oocytes.

Introduction

Oogenesis in teleosts, as in other vertebrates, is under the precise control of neuroendocrine, endocrine and paracrine factors and at gonadal level principally by steroids, estrogens and progestins (Reinecke 2010). In fishes, as in other oviparous vertebrates, estrogens, mainly 17β-estradiol (E2), regulate the ovarian growth by hepatic synthesis of the yolk precursor protein, vitellogenin, which is ultimately sequestered by the growing oocytes through receptor-mediated endocytosis (Specker & Sullivan 1994). At the end of follicular growth, post-vitellogenic gravid oocytes remain arrested at the boundary of G2–M1 of meiotic cell cycle. Prior to ovulation, resumption of meiotic maturation is primarily regulated by the maturation-inducing steroids (MIS), which is 17α,20β-dihydroxy-4-pregnen-3-one (DHP) in majority of the fish species, but 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) in sciaenids and in marine perciform species (Khan & Thomas 1999, Nagahama & Yamashita 2008). Binding of MIS to its membrane progesterin receptor (mPRA; Zhu et al. 2003) activates various signal transduction pathways that ultimately culminate in activation of maturation-promoting factor or MPF (Schmitt & Nebreda 2002). Active MPF promotes progression from meiotic MI–MII through histone H1 kinase activation, chromosome condensation, spindle formation, germinal vesicle breakdown (GVBD) and release of the first polar body (Nagahama & Yamashita 2008).

While extensive studies over the past three decades clarified the role of gonadotropin (LH) and MIS, other hormones such as deoxycorticosterone and testosterone are also effective in regulation of meiotic G2–M1 transition in teleosts (Sundararaj et al. 1979, Nagahama & Yamashita 2008). Conversely, E2 has been shown as either ineffective or even inhibitory to oocyte maturation (OM) in several teleosts (Jalabert 1975, Sundararaj et al. 1979, Tokumoto et al. 2004). Moreover, E2 regulation of ovarian function in zebrafish has been shown to include up-regulation of gonadotropin receptors (Liu et al. 2011) and prevention of precocious OM through maintenance of cell cycle arrest at meiotic G2–M1 boundary (Pang et al. 2008, Pang & Thomas 2009, 2010). In addition to its role in epidermal growth factor receptor (Egfr) transactivation and MAPK activation, studies in Atlantic croaker and zebrafish oocytes have indicated that binding of E2 to its novel G-protein coupled membrane

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receptor Gper, promotes intra-oocyte adenylate cyclase activity and ensures prophase-I arrest (Pang et al. 2008, Peyton & Thomas 2011). Further, recent evidence demonstrates that catecholestrogen (17β-2-hydroxyestradiol) functions as a Gper antagonist and promotes GVBD response in zebrafish by blocking Gper-dependent E2 action (Chourasia et al. 2015).

Involvement of local autocrine/paracrine factors in ovarian function has been an area of active research in the recent past (Pang & Ge 2002, Park et al. 2004, Jamnongjit et al. 2005, Clelland & Peng 2009, Nelson & Van Der Kraak 2010a,b, Li et al. 2011, 2015). Specifically, expression of the insulin-like growth factor (Igf) system (i.e. Igf peptides, Igf receptors and Igf binding proteins) and their participation in regulation of ovarian physiology has been reported in teleosts (Reinecke 2010). While igf2 expression has been reported only in granulosa cells of the late stage follicles of tilapia (Schmid et al. 1999), Igf1 mRNA and/or peptide expression has been detected earlier in young and pre-vitellogenic oocytes of red seabream (Kagawa et al. 1995), gilthead seabream (Perrot et al. 2000) and tilapia (Schmid et al. 1999). Interestingly, expression of the gonad-specific Igf3 has been reported mainly in the follicular layer of full-grown oocytes in tilapia and zebrafish (Wang et al. 2008, Nelson & Van Der Kraak 2010b, Li et al. 2011). Further, human chorionic gonadotropin (hCG) stimulation of igf3 expression in zebrafish full-grown follicles suggests importance of Igfs as local paracrine factors in the ovary (Nelson & Van Der Kraak 2010b, Li et al. 2011). Moreover, earlier attempts have been made to correlate stages of follicular growth and development with relative abundance of Igf ligands in zebrafish ovary (Nelson & Van Der Kraak 2010a,b, Li et al. 2011).

Physiological relevance underlying Igf function includes its effects on ovarian steroid production and development of OM competence (Kagawa et al. 1994, Patiño & Kagawa 1999, Weber & Sullivan 2000, 2001, Nelson & Van Der Kraak 2010a). In red seabream, mummichog, striped bass and in southern flounder, Igf1 and/or Igf2 has been shown to exert strong positive influence, albeit with varying intensity, on resumption of meiotic maturation either in denuded oocytes or most notably in intact follicles even in presence of steroidal-genesis inhibitors (Kagawa et al. 1994, Negatu et al. 1998, Weber & Sullivan 2000, Picha et al. 2012). While Igfs alone can induce GVBD, Igf2 has been shown to up-regulate mPRA and resumption of meiosis providing evidence behind integration of MIS and Igf signalling (Picha et al. 2012). Moreover, isolation of Igf1r and detection of high affinity Igf1 binding sites in the carp ovary (Maestro et al. 1997), coupled with expression of two different Igf receptor isoforms (Igf1ra and Igf1rb) in zebrafish oocyte (Nelson & Van Der Kraak 2010b) further strengthens the possibility of direct action of Igf on oocyte membrane and regulation of final OM (Reinecke 2010). Although gonadotropin regulation of major follicular events, e.g., biosynthesis of maturational steroid as well as Igf expression might involve common cellular signalling cascades, such as cAMP/PKA and/or MEK/MAPK (Nagahama & Yamashita 2008, Li et al. 2011), much less is known about interplay between these pro-maturational factors in teleost ovary.

In addition to the presence of E2 in circulation during the periovulatory period, earlier evidence has demonstrated gonadotropin stimulation of E2 production by full-grown ovarian follicles both in vivo and in vitro (Sundararaj et al. 1979, Nelson & Van Der Kraak 2010a, Sarkar et al. 2014). Moreover, in zebrafish with asynchronous ovarian development, it has been postulated earlier that relatively high level of endogenous estrogen is required to promote vitellogenesis and to support follicular growth (Pang & Thomas 2009), which could be sufficiently inhibitory for the daily spawning of this species. While studies in zebrafish have revealed participation of mPRA in MIS-induced OM that involves rapid activation of Gαi and decrease in cAMP (Hanna et al. 2006, Hanna & Zhu 2011), we observed involvement of PI3K/Akt signalling pathway is essential for insulin-induced OM in this species (Das et al. 2013). Moreover, hCG (analogue of Lh) stimulation of meiosis resumption in zebrafish ovarian follicles in vitro has been shown to require synthesis of maturational steroid, DHP, as well as Igf ligands, mainly Igf3 (Nelson & Van Der Kraak 2010a, Li et al. 2011, 2015). On the basis of these observations, we reasoned that potential interplay and integration of MIS- and Igf-mediated signalling pathways might have pivotal influence in overcoming the E2-mediated prophase-I arrest in zebrafish oocytes. Our results provide evidence in favour of physiological relevance behind potential synergism between maturational steroid and growth factor (Igf1) action facilitating release of E2 inhibition of OM and accelerated GVBD response in full-grown zebrafish oocytes in vivo and in vitro.

Materials and methods

Animals

Sexually mature zebrafish, obtained from local pet stores, were maintained in 601 glass aquaria, at 28 ± 1 °C and 14 h light:10 h darkness cycle with light turned on at 0600 h. Fishes were fed with live blood worms thrice daily and were acclimatized to laboratory conditions for at least 7 days prior to their use in experiments. All animal experiments were carried out following the guidelines of Institutional Animal Ethics Committee of Visva-Bharati University and approved by the committee according to Indian law.

Chemicals and antibody

Adenyly cyclase (Ac) inhibitor, SQ22536 and selective inhibitor of Akt, 10-DEBC hydrochloride were obtained from Tocris Bioscience (R&D Systems, Bristol, UK). Rabbit
polyclonal anti-MPRA was from Abcam (England, UK); anti-p-Akt (Ser473): sc-7985, anti-Akt: sc-8312, anti-p-PKAζ/β cat (Thr198): sc-32968, anti-p-PAκζ cat: sc-903, anti-IGF–IRβ: sc-713 and mouse monoclonal anti-p-Tyr (PY99): sc-7020 antibodies were from Santa Cruz Biotechnologies. Rabbit monoclonal anti-phospho-Akt (Thr308): #9275 antibody was from Cell Signalling Technology (Danvers, MA, USA). TRIzol reagent was purchased from Invitrogen, Life Technologies. Unless otherwise specified, hormones, reagents and antibodies were from Sigma–Aldrich. Anti-IGF–IR were from Rabbit polyclonal anti-MPRA (Maures et al. 2002).

**Oocyte preparation and in vitro culture**

For all in vitro experiments, fishes were autopsied in the evening (2000 h) when relatively lower percentage of oocytes matures spontaneously without exogenous stimuli (Hanna & Zhu 2011). Gravid females (n = 30–40) were anaesthetized by brief cold shock, decapitated, ovaries excised aseptically and placed immediately in oxygenated zebrafish Ringer (116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl₂, 5 mM HEPES; pH 7.2) supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) (Kondo et al. 1997). This preparation was also used as incubation medium in future in vitro experiments. The protocol for harvesting post-vitellogenetic intact follicles, enzymatic removal of the follicular layer, selection of oocytes divested of surrounding follicular cells, and with centrally placed germinal vesicle (GV) were as described earlier (Das et al. 2013). In brief, ovarian follicles were incubated in Ca²⁺ free zebrafish Ringer’s solution containing 0.001% collagenase type-IA (1.25 U/ml) for 1 h at 20 ± 1°C with mild agitation. Follicular cells were removed by repeated (30×) gentle pipetting through a narrow pipette (1 mm diameter) at 10 min intervals during incubation. Oocytes showing signs of damage or presence of fibrous amorphous follicle cells (incomplete denudation) were discarded manually. Complete removal of the follicular cells was ascertained by the absence of DAPI-positive nuclei surrounding the denuded oocytes (Peyton & Thomas 2011, Das et al. 2013) and their inability to undergo OM due to hCG stimulation (Das et al. 2013). Healthy, denuded oocytes of desired size (mean diameter ~550–600 µm) were washed thoroughly with fresh zebrafish Ringer and were cultured in a 24-well tissue culture plate (50 oocytes/ well) for the indicated time and hours at 25 ± 1°C under gentle agitation. DHP and E₂ were dissolved in ethanol and recombiant human IGF1 (Igf1) was dissolved in sterile water. Wortmannin (Wt, 10 µM), LY294002 (LY, 25 µM), 10-DEBC-hydrochloride (DEBC, 5 µM), forskolin (1 and 5 µM) were dissolved in DMDSO and dibutyl cAMP (dbcAMP, 0.1 and 1 mM) was dissolved in nuclease free water just before the experiment and added to the culture medium prior to either Igf1 or DHP addition. SQ22536 (SQ; 1–10 µM) was dissolved in DMso and was added to the culture medium either alone or in dbcAMP (1 mM)-primed oocytes. The doses of Wt, LY, DEBC, forskolin and dbcAMP used were as described earlier (Das et al. 2013, Maitra et al. 2014). All chemicals were prepared at 1000-fold stock and control wells received equal volume of solvent only. Triton X-100 mediated delivery of anti-IGF–IRβ into the oocytes was done according to the method described earlier (Maitra et al. 2014). In brief, Triton X-100 was diluted (0.1%, v/v) with 10 mM PBS; pH 7.2 and sonicated for 1 min and allowed to complex with anti-IGF–IRβ antibody (20 µg/ml) for 30 min each at 25 ± 1°C and at 4°C. The resultant complex (10 µl) was layered on oocytes in culture. Medium was replaced with fresh Ringer after 1 h. Oocyte viability was checked using Trypan blue and GVBD was monitored at 3 h following immersion in clearing solution (Lessman & Kavumpharut 1984) under an inverted microscope (Victory FL, Dewinter Optical, Inc., Italy) fitted with phase-contrast optics.

**Kinetics of in vivo induction of OM in gravid females**

Gravid females and adult males were kept separately for at least 7 days prior to their use in the experiment (Westerfield 2000). On the day before sampling, fishes were released at the ratio of one female:two males in 40 l aquaria at 1800 h. Since in this condition females start releasing eggs within 30 min of morning light signaled at 0600 h, the next day females (three to five fish for each time point) were autopsied at 0400, 0500 and 0600 h. Ovaries were removed and full-grown follicles were separated in Ringer’s solution by gentle pipetting. Separated follicles were randomly assorted into three groups (200/group) and were processed immediately for preparation of follicular extract, RNA isolation and for determination of relative proportion of oocytes at various stages of maturation and ovulation microscopically. The same experiment was repeated thrice and ovulated eggs were excluded from the present study.

**Preparation of oocyte extract**

Denuded oocytes from in vitro study and follicles from in vivo experiment were harvested at appropriate time intervals, washed (3×) and homogenised in chilled oocyte extraction buffer (100 mM sodium β-glycerophosphate, 20 mM HEPES, 15 mM MgCl₂, 5 mM EGTA, 100 µM p-PMSF, 3 µg/ml leupeptin, 1 mM DTT and 1 µg/ml aprotenin; pH 7.5; Hirai et al. 1992). Supernatant was separated by spinning at 17 500 g for 20 min at 4°C and was either used immediately or stored at −80°C until further use.

**Electrophoresis, immunoblot analysis and immunoprecipitation**

Following the determination of protein concentration (Lowry et al. 1951), oocyte lysates (50 µg/well) from control and treated groups were resolved in 15% SDS–PAGE, transferred to Hybond-P PVDF membrane (GE Healthcare Biosciences, Buckinghamshire, UK) using transfer buffer (25 mM Tris, 193 mM glycine, 20% methanol; pH 8.5) for 1 h. Membranes were incubated in blocking solution in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween-20; pH 7.6) containing 5% BSA overnight at 4°C, incubated in primary antibody diluted (1:500) in blocking buffer for 4 h at room temperature, washed with TBST (3×) and incubated in alkaline phosphatase tagged anti-rabbit IgG (1:1000). Bands were developed...
by adding BCIP-NBT, recorded in Gel Doc apparatus (Bio-Rad) and imported into Adobe Photoshop. Band intensities were quantified using the freely available ImageJ Software and expressed as an arbitrary unit.

For immunoprecipitation, ~100 μg of the proteins from zebrafish ovarian follicles were incubated overnight at 4 °C with 1 μg of anti-IGF–IRb antibody. Around 50 μl of protein A agarose (Pierce, Thermo Scientific, Rockford, IL, USA) was added to each tube and incubated with gentle mixing for 2 h at room temperature followed by addition of 500 μl of IP buffer (25 mM Tris, 150 mM NaCl; pH 7.2) and centrifugation at 2500 g for 3 min at 4 °C. The supernatant was discarded and this step was repeated two more times. Immunoprecipitates were boiled in 5× SDS sample buffer and resolved in 15% SDS–PAGE then transferred to Hybond-P PVDF membrane followed by primary and secondary antibody incubation and colour development as described previously in immunoblot analysis.

**Total RNA extraction, cDNA synthesis and RT-PCR**

Total RNA was extracted from ovarian follicles (~200 follicles/time group from three different donor fish) using TRIzol reagent according to the manufacturer’s protocol. RNA pellet was reconstituted in 20–30 μl of nuclease free water (Fermentas, GENETIX, New Delhi, India), followed by quantification using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Integrity of the RNA samples (A260/280 between 1.8 and 2.2) was checked electrophoretically in denaturing agarose gel followed by ethidium bromide staining. PCR amplification of products were electrophoresed on 2% agarose gels along with tham, MA, USA). Integrity of the RNA samples (A260/280 between 1.8 and 2.2) was checked electrophoretically in denaturing agarose gel followed by ethidium bromide staining. PCR amplification of products were electrophoresed on 2% agarose gels along with

**Statistical analysis**

The ratio between expression levels of igf1, igf2a, igf2b and igf3 to that of b-actin (internal control) were calculated and expressed as fold change compared with the reference group. All results are mean ± S.E.M. of at least three independent experiments using oocytes from different donor fish. Data were analyzed by one-way ANOVA (P<0.001), followed by Duncan’s post hoc test for multiple group comparisons, P<0.05 was considered as statistically significant.

**Results**

**E2 inhibition of spontaneous maturation in denuded zebrafish oocyte**

Priming with graded levels of E2 (1–1000 nM) could successfully block spontaneous maturation in dose- and duration-dependent manner in denuded oocytes in vitro (Fig. 1A and B). Further, as shown in Fig. 1B (inset), negligible or very low levels of p-ERK1/2 was detected

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**Table 1 List of zebrafish oligonucleotide primers and their accession numbers used in the semi-quantitative RT-PCR assay.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’–3’)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>igf1</td>
<td>F: CAGCAAACCGACAGGATAGG R: CAGCTCTGAAGACGAGATCG</td>
<td>NM_131825</td>
</tr>
<tr>
<td>igf2a</td>
<td>F: CCGTCTGCAAGAGATGATAC R: TCAGTGAGGCCATGTTGTT</td>
<td>NM_131433</td>
</tr>
<tr>
<td>igf2b</td>
<td>F: AACCTGCAAGTCACAGAGGG R: GGACCTCCCTTTAATGGCGC</td>
<td>NM_001001815</td>
</tr>
<tr>
<td>igf3</td>
<td>F: ACAGCTGGGCAGGACAAGACTAG R: GCCTGCTCAAGTTGTCACTT</td>
<td>NM_001115050</td>
</tr>
<tr>
<td>igf1ra</td>
<td>F: ACTCATCTCGTGGCTGCTGTTCC R: TGGCTATGTATTTATCCACAGCTTT</td>
<td>NM_152968</td>
</tr>
<tr>
<td>igf1rb</td>
<td>F: ATCTCCGCCCCGTACTTGGT R: CCTGTAAATGTCGCTCTGC</td>
<td>NM_152969</td>
</tr>
<tr>
<td>mpfa</td>
<td>F: CATTTCTGGGCTTAATTGTC R: GCCAACATCTGCCTCCTCAC</td>
<td>AY1499121</td>
</tr>
<tr>
<td>b-actin</td>
<td>F: GGTATGGCAGGAAAGACACAG R: AGAGTCCATCACGATACCAG</td>
<td>NM_181601</td>
</tr>
</tbody>
</table>

*a Nelson & Van Der Kraak (2010b), †Pikulkaew et al. (2010), ‡Kishida & Callard (2001).
in unstimulated control and 1 nM E2-treated groups (inset, lanes 1 and 2, Fig. 1B). However, significant increase in p-ERK1/2 was observed due to either 10 or 100 nM of E2 as early as 30 min of incubation (inset, lanes 3 and 4, Fig. 1B). Further, compared to 100 nM E2-treated group, no significant variation in GVBD response and ERK1/2 phosphorylation was observed due to 1000 nM E2 (Fig. 1B). On the preceding basis, two different doses of E2, i.e., 10 and 100 nM were selected for subsequent experiments.

**Figure 1** Effect of 17β-estradiol inhibition on spontaneous maturation and ERK1/2 phosphorylation. Representative photomicrographs of intact ovarian follicles (upper panel) compared to the surface images of denuded oocytes (lower panel) viewed through light (left) or fluorescent microscopy (right) after staining with DAPI (A), inset, follicular cell nuclei stained positive for DAPI are shown at higher magnification. Solid arrows indicate selection of post-vitellogenic denuded oocytes of desired size class (~550–600 μm) used in the present study, scale bar ~200 μm. Denuded oocytes were treated either with graded levels of E2 (1–1000 nM) or not (control), GVBD were scored at indicated time intervals and expressed in percent (B). Results are mean ± S.E.M. of three independent experiments. Data were analyzed by one-way ANOVA (P < 0.001) and Duncan’s test (P < 0.05). Groups with same lowercase letter are not significantly different and those with different letters differ significantly; # P < 0.01; Fig. 1B). More importantly, co-treatment with very low doses of DHP and Igf1 (at 3 and 10 nM respectively) for 3 h, could trigger a robust increase in meiotic G2–M1 transition either in absence or in presence of E2 (Fig. 2B) and the data was comparable to the GVBD response either due to DHP (30 or 300 nM) or Igf1 (100 nM) at 3 h of incubation (Fig. 2A), indicating possibility of an additive

**Effect of DHP- and Igf1-stimulation on E2-mediated prophase arrest**

Though co-treatment with hCG and Igf1 could trigger significant increase in synthesis of DHP in zebrafish ovary (Nelson & Van Der Kraak 2010a), possibility of any synergistic action between maturational steroid- and Igf-mediated signalling cascades in alleviating E2-mediated prophase arrest has not been investigated earlier. In order to test this possibility, we examined GVBD response due to DHP and Igf1, either alone or in combination, in presence or absence of E2. While DHP and Igf1 could trigger GVBD response in a dose-dependent manner (Fig. 2A), priming of denuded oocytes with E2 (10 and 100 nM) could attenuate spontaneous maturation and prevent either DHP (3 nM) or Igf1 (10 nM)-mediated GVBD response significantly (P < 0.01; Fig. 2B). More importantly, co-treatment with very low doses of DHP and Igf1 (at 3 and 10 nM respectively) for 3 h, could trigger a robust increase in meiotic G2–M1 transition either in absence or in presence of E2 (Fig. 2B) and the data was comparable to the GVBD response either due to DHP (30 or 300 nM) or Igf1 (100 nM) at 3 h of incubation (Fig. 2A), indicating possibility of an additive
Inhibition of Igf1-, but not DHP-induced oocyte maturation (3 h) in IGF1Rβ immunodepleted cells (A) and (B) PI3K/Akt regulation of Igf1- and (C) maturational steroid-induced GVBD response. Denuded zebrafish oocytes primed with either Wrt (10 μM), LY (25 μM) or DEBC (5 μM) or not (Con, vehicle only), were stimulated with either with Igf1 (10 nM) till 3 h or DHP (3 nM) till 6 h and GVBD was scored microscopically. Oocyte lysates from control and treated groups were subjected to immunoblot analysis using anti-p-Akt (Ser473) antisera that specifically recognize active form of Akt, phosphorylated at Ser473, anti-Akt immunoblot served as loading control (D). Corresponding densitometric analysis are shown above the immunoblot, values are mean ± S.E.M. and are analyzed by one-way ANOVA followed by Duncan's multiple range test; a,bP<0.001 against Igf1- or DHP-stimulated group of respective time intervals. Groups with same lowercase letter above the bars are not significantly different and those with different letters differ significantly. Immunoblot data are representative of at least three separate experiments from different donor fish showing identical results.

Influence by two altogether separate signalling pathways leading to withdrawal of prophase arrest and resumption of meiotic maturation.

Relative importance of PI3K/Akt signalling on DHP and Igf1-induced GVBD response

Regulation of intra-oocyte signalling events by DHP and Igf1 was investigated. Pre-incubation with anti-IGF-1Rβ antibody could prevent Igf1, but not DHP-induced GVBD response (Fig. 3A). Further, pharmacological inhibition of PI3K/Akt signalling, by either 10-DEBC hydrochloride, a selective Akt inhibitor or by two mechanistically different and highly selective PI3K inhibitors, Wrt and LY, attenuates Igf1-induced OM significantly (P<0.01; Fig. 3B). Interestingly, priming with either LY or DEBC could delay maturational steroid-induced GVBD response till 4–5 h of incubation (middle and right panels, Fig. 3C) and Wrt in particular almost completely blocked DHP action on OM up to 3 h (left panel, Fig. 3C). Immunoblot analyses revealed that while priming with Igf1 (10 nM) promoted a significant increase in phospho-Akt (Ser473) as early as 15 min that reached the peak level between 60 and 90 min of incubation (right panel, Fig. 3D), appreciable increase in Akt phosphorylation (activation) was observed at 60 min of DHP (3 nM) stimulation (left panel, lane 3, Fig. 3D).

Effect of cAMP/PKA regulation of DHP and/or Igf1 induced meiotic G2–M transition

In teleosts, high intra-oocyte cAMP is considered as a pre-requisite for prophase-I arrest and attenuate MIS action on OM in a variety of teleosts species (DeManno & Goetz 1987, Haider & Baqri 2002, Khan & Maitra 2013). Next we examined effects of Ac inhibition or cAMP modulators on spontaneous maturation and DHP/Igf1-mediated GVBD response. As shown in Fig. 4A, priming with Ac inhibitor, SQ22536 alone
could promote meiotic maturation. In contrast, forced elevation of intra-oocyte cAMP by either forskolin or dbcAMP could abrogate DHP or Igf1 action in a dose-dependent manner (Fig. 4B).

Since high cAMP represents counter-regulatory action on DHP- and Igf1-mediated meiotic maturation, participation of PKA was hypothesized. The catalytic sub-units of PKA remain phosphorylated on its activation loop (phospho-PKAc) allowing proper substrate recognition and catalysis (Moore et al. 2002). In the present study, endogenous PKA activity was assessed indirectly by immunoblot analysis using p-PKA antibody (Khan & Maitra 2013, Maitra et al. 2014). As shown in Fig. 5A, elevated phosphorylation of PKA (active) was detected in un-stimulated control (lane 1) as well as in oocytes primed with graded levels of E2 (lanes 2, 3 and 4). Although stimulation with either DHP (3 nM) or Igf1 (10 nM) was sufficient to promote p-PKAc dephosphorylation (inactivation) (lanes 5 and 6, Fig. 5A), pre-incubation with E2 (at both 10 and 100 nM) could significantly reverse DHP- or Igf1 action on PKA inhibition (lanes 8, 9, 11 and 12, Fig. 5A). Further, co-treatment with DHP and Igf1 could rescue negative regulation by E2, at both the dose levels tested, as evident from rapid dephosphorylation of p-PKAc (lanes 10 and 13, Fig. 5A) indicating the pivotal influence of active PKA in the maintenance of E2-mediated prophase-I arrest and involvement of both maturational steroid and Igf1 in rapid dephosphorylation of p-PKAc in maturing oocyte. More importantly, the time kinetics data reveal that co-treatment with DHP and Igf1, but not alone, could successfully reverse negative regulation by E2 and promoted accelerated GVBD response at all the time points tested (lower panel, Fig. 5B). This suggests an additive influence of the two disparate signalling strategies which might lead to withdrawal of prophase arrest and resumption of meiotic maturation in this species. Interestingly, no significant variation in GVBD response was observed due to co-stimulation with DHP and Igf1 in oocytes either primed or not with two different doses of E2 (Fig. 5B).

Expression profile and phosphorylation status of molecular markers associated with in vivo OM

Since co-treatment with DHP/Igf1 was more potent in overcoming E2 inhibition of OM and also triggered rapid dephosphorylation of phospho-PKA in vitro, next we examined the physiological relevance of potential involvement of maturational steroid and growth factor-mediated events in withdrawal of meiotic arrest in gravid females in vivo. Ovaries were harvested at three different time intervals just prior to ovulation, with spawning induced in the laboratory condition (Fig. 6A). Follicles were separated manually and assayed for transcript abundance for igf ligands (igf1, igf2a, igf2b and igf3) through qRT-PCR in relation to relative proportion of oocytes undergoing GVBD in vivo. Further, presence of Igf receptor (igf1ra and igf1rb) and mpra transcripts was checked by semi-quantitative RT-PCR and immunoblot analyses were performed to detect anti-mPrA immunoreactive protein as well as phosphorylation status of Igf1rb, Akt and PKA using phospho-specific antibodies.

As shown in Fig. 6, while at 0400 h (~2 h of light signal on), ovaries contained mainly GV intact (immature) oocytes; substantial increase in percentage of

![Figure 5](image-url)
immunoblot served as internal loading control. Corresponding densitometric analysis are also shown above the immunoblot; values are mean ± S.E.M.; n = 3 experiments with three to five females for each time point per experiment. Representative photomicrograph of follicle-enclosed oocytes in clearing solution (upper panel, B) at indicated time points. Scale bar 500 μm. RNA samples from ovarian follicles (~200 follicles/time point from three to five different donor fish) were reverse transcribed and transcript abundance for Igf1 (igf1, igf2a, igf2b and igf3) was checked through qRT-PCR; b-actin served as endogenous control (C). Values are the mean ± S.E.M. of three separate experiments as mentioned earlier. Ovulated eggs were excluded from the present study. Besides, expression of igf1ra, igf1rb and mpra (left upper, D) was checked through semi-quantitative RT-PCR at indicated time points. RT(-) RNA without RT; L, DNA ladder. Further, protein samples extracted from intact follicles harvested at various time points prior to onset of ovulation, was incubated either without (-) or with anti-Igf-IRβ antibody (+), followed by probing with anti-p-Tyr antibody (left middle, D) or subjected to immunoblot analysis using anti-mPRA (left lower, D), anti-p-Akt (both Ser473 and Thr308) (right upper, D) and anti-p-PKAcβ/γ antibody (right lower, D). Anti-b-actin, anti-Akt and anti-PKAc immunoblot served as internal loading control. Corresponding densitometric analysis are also shown above the immunoblot; values are mean ± S.E.M. of three independent experiments. Data were analyzed by one-way ANOVA and Duncan’s multiple range test; *P<0.05 and **P<0.001 compared to data at −2 h of light signal on at 0400 h.

Discussion

Prophase-I arrest and its timely release play major roles to accomplish follicular growth, acquisition of maturational competence and production of fertilizable female gametes. Earlier E2 has been shown to prevent growing oocytes undergoing GV migration and GVBD was observed at 0500 h (−1 h; Fig. 6B). Moreover, at 0600 h (0 h), i.e., at the time of light signals were on, more than 90% of oocytes underwent GVBD (Fig. 6B).

Although there was a significant (P<0.01) increase in transcript for igf1 and igf3 (1.5- and 3 folds respectively) at 0500 h (−1 h), igf2a did not undergo any significant change at indicated time intervals (Fig. 6C). In contrast, a sharp increase was noticed in igf2b at 0600 h (Fig. 6C) when the majority of the oocytes completed G2–M1 transition (Fig. 6B) and ovaries were filled with oocytes arrested at MII stage (data not shown).

While at all three sampling hours, transcripts for igf1ra and mpra were detected in isolated follicles, albeit in varying amounts, immunoblot analysis revealed elevated mPRA at protein level and Tyr phosphorylation of the anti-Igf1r immune-precipitate at 0500 h (−1 h; left panel, Fig. 6D). Besides, dual phosphorylation of Akt at Ser473 and Thr308 concomitant with marked dephosphorylation of phospho-PKAc at right panel, Fig. 6D) correlated well with elevated GVBD response (Fig. 6B) during this time in vivo.

oocytes to mature precociously and binding of E2 to its cognate cell surface receptor Gper was implicated in maintenance of meiotic arrest in Atlantic croaker and zebrafish oocytes in vitro (Pang et al. 2008, Pang & Thomas 2009, 2010). Although, significant increase in Gper, at both mRNA and protein level, has been reported earlier in late-vitellogenic oocytes (Pang & Thomas 2010), currently no data is available to correlate the follicular growth and physiological level of E2 either in circulation or in the ovary. As a result, we had to test effect of graded levels of E2 (1, 10, 100 and 1000 nM) on incidence of spontaneous maturation in defolliculated zebrafish oocytes. Present data demonstrate that E2 priming could attenuate meiosis resumption (spontaneous maturation) in defolliculated post-vitellogenic oocytes (average diameter: 550–600 μm) in dose- and duration-dependent manner. This is in agreement with earlier reports in zebrafish that showed a significant reduction in meiotic maturation due to E2 at 20 and 100 nM (Pang & Thomas 2009, 2010, Peyton & Thomas 2011). Moreover, available information indicates that priming with E2 (20 nM) could effectively promote Gper expression and down-regulate mpra, but could exert its effect at protein levels (Gper or mPRA) only at 100 nM concentration (Pang & Thomas 2010). Earlier stimulation of zebrafish ovarian fragments as well as follicular cells in culture with 50 nM of E2 has been shown to promote lhcr expression (Liu et al. 2011). Since earlier evidence suggests that E2 at 100 nM transactivates Egfr, promotes ERK1/2 phosphorylation and ensures prophase-I arrest in

Reproduction (2016) 151 59–72

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zebrafish oocytes (Peyton & Thomas 2011), we hypothesized that degree of ERK1/2 phosphorylation could further serve as a means for selection of the dose of E2. Present results reveal a dose-dependent increase in p-ERK1/2, the most effective being 10 and 100 nM of E2. Subsequently, two different doses of E2 have been considered; while 10 nM would be more physiological, overcoming the strong inhibitory effect even at 100 nM of E2 by maturation steroid and/or Igf1 could reasonably be more suitable to test our hypothesis.

While higher doses of DHP (30 and 300 nM) or Igf1 (100 nM) promotes relatively higher percentage of oocytes undergoing GVBD compared to either 3 nM of DHP- or 1 nM of Igf1-treated groups respectively, present data demonstrate that priming with E2 attenuates maturation steroid (DHP)- as well as growth factor (Igf1)-induced OM in a dose-dependent manner. Since receptors are probably fully occupied at lower concentration, activation of signalling molecules for prolonged period due to higher concentration of ligands seems unlikely. Previously, participation of mPR in MIS stimulation of meiotic G2-M1 transition has been studied in great detail in several teleosts including zebrafish (Zhu et al. 2003, Pace & Thomas 2005, Nagahama & Yamashita 2008, Hanna & Zhu 2011). Besides, earlier evidences have established that in addition to its role in positive regulation of mPR expression (Peng & Thomas 2010), DHP at higher concentration can activate nuclear progestin receptor (nPR) to promote genomic mechanism for OM (Hanna & Zhu 2011) or reduce Gper levels in the oocytes (Pang & Thomas 2010). Alternatively, while relative abundance of igf2a, igf2b and igf3 outweighs igf1 expression in the zebrafish ovary (Nelson & Van Der Kraak 2010b, Li et al. 2015), recently it has been demonstrated that hCG stimulation promotes a robust increase in igf3 expression and igf1 to a lesser extent prior to resumption of meiosis (Li et al. 2015). Besides, binding of Igf1 to insulin and/or insulin/Igf1 hybrid receptor may activate signalling molecules at higher magnitude (Belfiore et al. 2009), suggesting that activation of maturation steroid and Igf-mediated signalling pathways together may engage in complex interaction for alleviating prophase arrest in this species. Although its strong negative influence on MIS-induced maturation events in zebrafish and Atlantic croaker has been reported earlier (Pang et al. 2008, Pang & Thomas 2009), E2 inhibition of Igf1 action on meiosis resumption in defolliculated oocytes is being reported for the first time. Attenuation of both maturation steroid and growth-factor action on GVBD response possibly hints at the physiological relevance and universality of E2 action in maintenance of prophase-I arrest. Given that the substantial amount of E2 might be present in zebrafish ovary to support asynchronous mode of follicular growth, it would be interesting to examine the level of DHP and/or Igfs that could override strong negative influence of E2 under physiological conditions in future.

Though gonadotropin (Lh) regulation of maturation steroid (progestins) synthesis prior to meiotic G2–M1 transition has been reported earlier (Khan & Thomas 1999, Nagahama & Yamashita 2008), a growing body of literature indicates involvement of local paracrine factors (e.g. activin and Igf family members) in mediating Lh action in teleosts ovary (Pang & Ge 2002, Li et al. 2015). While Egf has recently been shown to act as an essential mediator of Lh action during mammalian OM (Park et al. 2004, Jamnongjit et al. 2005), presence of an active Igf system consisting of Igf ligands (Igf1, Igf2a, Igf2b and Igf3), receptors (Igf1ra and Igf1rb) and Igf binding proteins have been reported earlier in zebrafish ovary (Nelson & Van Der Kraak 2010b, Reinecke 2010, Li et al. 2015). Even though Igf1 could induce GVBD in mummichog and striped bass denuded oocytes or follicles when incubated with steroidogenesis inhibitors (Negatu et al. 1998, Weber & Sullivan 2000), Igf2 has been shown to up-regulate synthesis of mPra in southern flounder full-grown follicles (Picha et al. 2012). Moreover, available data indicate that while it requires almost 16 h for relatively high dose (2 μM) of Igf3, Igf1 at 100 nM could trigger a significant increase in meiotic maturation within the first 6 h in zebrafish full-grown and mid-vitellogenic follicles in vitro (Nelson & Van Der Kraak 2010a, Li et al. 2015). Further, in vitro incubation of zebrafish ovarian follicles with Igf1 alone or in combination with hCG stimulates synthesis of DHP, but not E2 (Nelson & Van Der Kraak 2010a). These observations prompted us to use Igf1 in the in vitro incubation study. Earlier, Igf1 has been shown to act as the most potent inducer of GVBD in red seabream, Pagrus major oocytes and more importantly in the presence of DHP; low doses of Igf1, Igf2 or insulin (which alone were ineffective) could promote GVBD response significantly (Kagawa et al. 1994). However, possibility of synergism between maturation steroid- and Igf-mediated signalling cascades in alleviating E2-mediated prophase arrest has not been investigated earlier. Present results show that co-treatment of DHP and Igf1 overcome the inhibitory effect of E2 irrespective of the doses of E2 used (10 and 100 nM). It has been established earlier that fish with synchronous ovarian development spawn once in a year and contain higher E2 concentration prior to spawning and ovulation (Specker & Sullivan 1994, Khan & Thomas 1999). However, in teleost like zebrafish, with asynchronous development of ovary contains higher level of circulatory E2 for the maintenance of the growing vitellogenic oocytes throughout the year. Clearly the present data support the hypothesis that the synergism between the maturation steroid and local paracrine factor like Igf may play the pivotal role in overcoming the E2-mediated prophase-I arrest possibly
in both these groups of fish. Moreover, results of the present study corroborate previous reports showing positive correlation between Igf2 and 20β-S in regulation of meiosis resumption in full-grown follicles of southern flounder, another teleost with asynchronous ovarian development (Picha et al. 2012). Taken together, these observations suggest that E2- and DHP/Igf-mediated pathways, initiated at the oocyte surface, may engage in a complex dialogue in maintenance of prophase arrest and resumption of meiotic maturation respectively.

Data of the present study demonstrate that priming with anti-IGF–IRβ antibody attenuates Igf1, but not DHP-induced GVBD response. This provides evidence in favour of specificity of different receptor-mediated signalling cascades at the oocyte surface. While Igf1 receptor has been purified and high affinity Igf1 binding sites were demonstrated in amphibian oocytes as well as in carp ovary (Hainaut et al. 1991, Maestro et al. 1997), the possibility of all four Igf ligands acting through Igf1r in zebrafish oocytes cannot be ruled out at this moment since transcripts for two Igf1 receptor isoforms (igfr1a and igfr1b) have been detected earlier (Nelson & Van Der Kraak 2010b). Moreover, immunoblot analysis reveal that while Igf1 triggers a robust increase in Akt phosphorylation (Ser473), DHP stimulation also promotes a significant increase in Akt activation as early as 15 min of in vitro incubation. While it abrogates Igf1 action on OM, pharmacological inhibition of PI3K/Akt signalling could significantly delay DHP-induced GVBD response till 4–5 h of incubation. More importantly, Wrt in particular completely blocked OM up to 3 h indicating potential involvement and importance of PI3K/Akt signalling in DHP regulation of meiosis resumption in this species. Previously, inhibition of PI3K/Akt pathway has been shown to abrogate maturational steroid-induced GVBD response in Atlantic croaker, striped bass (Weber & Sullivan 2001, Pace & Thomas 2005) and starfish oocyte (Sadler & Ruderman 1998). While microinjection of constitutively active Akt mRNA could induce meiosis in absence of 1-methyladenine, Akt is phosphorylated within a few minutes of 1-methyladenine addition, the natural MIS in this species (Okumura et al. 2002). Moreover, in mouse cumulus-free oocytes p-Akt has been detected at 20 min and Akt/PKB phosphorylation precedes onset of GVBD both in vivo and in vitro (Kalous et al. 2006). However, earlier evidence has established that PI3K inhibition either delays (Bagowski et al. 2001) or is without effect on progesterone-stimulated meiotic maturation in Xenopus, suggesting PI3K-mediated signalling might be an auxiliary pathway for progesterone action in this species (Andersen et al. 2003, Mood et al. 2004). Besides, insulin/IGF1 stimulation of meiotic G2–M1 transition in Xenopus and zebrafish oocytes has been shown to require Akt activation (Andersen et al. 2003, Das et al. 2013). Interestingly, pharmacological inhibition of PI3K could attenuate insulin/Igf1-mediated GVBD response in southern flounder and catfish follicle-enclosed oocytes (Picha et al. 2012, Hajra et al. 2016). Although growth factor-mediated Akt activation requires receptor tyrosine kinase activation, MIS regulation of Akt phosphorylation may primarily involve activation of GPCR in oocytes (Sadler & Ruderman 1998, Pace & Thomas 2005). Besides, rapid activation of Akt could be triggered through the Gβγ subunit of heterotrimeric G-protein that might dissociate upon ligand (DHP) binding to membrane GPCR (mPR) followed by the activation of class 1B PI3K (Stephens et al. 1997).

Moreover, present data reveal that while Ac inhibition alone is sufficient to promote meiosis progression, forced elevation of intra-oocyte cAMP abrogates both DHP- and Igf1-induced GVBD response in a dose-dependent manner. Previously, treatment of Atlantic croaker ovarian membranes by GPER agonist has been shown to increase Ac activity and also reversed the down-regulation of Ac activity by 20β-S (Pang et al. 2008). While stimulation with catecholestrogen has been shown to down-regulate intra-oocyte cAMP level (Chourasia et al. 2015), MIS action, presumably through mPRα, attenuates Ac activity and intra-oocyte cAMP level by activating the inhibitory G protein, Gαq (Zhu et al. 2003). Earlier we have reported that PI3K/Akt pathway is indispensable for insulin-induced meiotic maturation in denuded zebrafish oocyte, where PDE3 might act as possible a down-stream target (Das et al. 2013). Although involvement of a CAMP-independent PI3K/Akt signalling cascade has been proposed in starfish, striped bass and Atlantic croaker during MIS-induced maturation (Sadler & Ruderman 1998, Weber & Sullivan 2001, Pace & Thomas 2005), high CAMP could block insulin-induced GVBD response in zebrafish oocytes (Maitra et al. 2014). Taken together these data suggest that CAMP-mediated signalling might have strong negative influence on both maturational steroid and Igf regulation of meiotic G2–M1 transition in zebrafish oocytes.

On the basis of the preceding, it was reasoned that DHP and Igf1-mediated signalling events might have additive influence and possibly converge at some point to down-regulate a factor which when active ensures prophase arrest. Previously, high CAMP has been shown to activate PKA which subsequently block synthesis of cyclin B and resumption of meiosis (Conti et al. 2002). Moreover, potential antagonism between CAMP and receptor tyrosine kinase-regulated signalling axes is evident from the fact that high CAMP/PKA could successfully abrogate insulin-mediated MEK/MAPK signalling axis in zebrafish oocytes (Maitra et al. 2014). Taking cue from these reports, we reasoned involvement of PKA in the cross-talk between E2, MIS and Igf-mediated signalling events that may act in tandem to regulate prophase arrest and resumption of meiotic maturation in this species. Present data demonstrate that E2 could significantly promote PKA phosphorylation.
Overcoming E2-inhibition of meiosis resumption

expression has been reported earlier (Li et al. 2015), we observed elevated expression of igf1 due to hCG stimulation (P Ghosh, D Das & S Maitra, unpublished observation). More importantly, available information indicates that while Igf1 at low dose (100 nM) could trigger a significant increase in meiosis resumption within 6 h, priming with either Igf2a, Igf2b or Igf3 at apparently supra-physiological concentration (2 μM), promotes GVBD response in ~60–80% of full-grown zebrafish follicle-enclosed oocytes after 17 h (Li et al. 2015). Moreover, a dose-dependent (from 0.2 nM to 2 μM) increase in Igf3-induced OM has been demonstrated in zebrafish (Li et al. 2011), suggesting the

Figure 7 Proposed model behind maintenance of prophase-I arrest and how DHP- and Igf-mediated signalling events may act synergistically to overcome E2 inhibition of meiosis resumption in zebrafish oocytes. Pioneering work by Pang & Thomas (2010) and Peyton & Thomas (2011) have demonstrated earlier that in addition to its role in Ac activation to synthesize cAMP, binding of E2 to Gper at oocyte surface transactivate Egfr to induce MAPK activation; both of which are involved in the maintenance of E2-mediated prophase-I arrest. Conversely, DHP action at the oocyte surface, possibly through mPRA, activates Gaα, which in turn attenuates Ac activity and prevents cAMP production (Zhu et al. 2003, Pang & Thomas 2010). While DHP stimulation promotes Akt phosphorylation (albeit in lower amount), Igf1 stimulation triggers a rapid and strong activation of PI3K/Akt/PDE3 cascade (Das et al. 2013) allowing breakdown of intra-oocyte cAMP. Thus inhibition of cAMP synthesis coupled with a rapid clearance of cAMP (that could possibly come through gap-junction complexes) leads to down regulation of PKA; a pre-requisite for resumption of meiosis (Conti et al. 2002, Nagahama & Yamashita 2008).

In conclusion, the physiological relevance of E2-mediated meiotic arrest and synergism between steroid- and growth factor-mediated signalling events allowing meiosis resumption might have evolved over time for better regulation of reproductive function necessary for successful perpetuation of a species. Dotted lines represent MAPK-mediated signalling pathway, activation of which represent diametrically opposite outcome in species-specific manner (Kajiura-Kobayashi et al. 2000, Pace & Thomas 2005, Peyton & Thomas 2011, Khan & Maitra 2013, Maitra et al. 2014).

(activation). Though low dose of either DHP or Igf1 alone is sufficient to promote p-PKAc dephosphorylation (inactivation) almost to the basal level and marginal increase in GVBD response, incorporation of E2 (10 and 100 nM) in the culture medium could reverse both these effects. On the contrary, co-treatment with DHP and Igf1 (at comparable dose) could successfully abrogate E2-mediated PKA phosphorylation concomitant with accelerated GVBD response. Although DHP- or insulin-mediated down-regulation of intra-oocyte PKA activity has been reported earlier in perch, Anabas testudineus (Khan & Maitra 2013) and zebrafish (Maitra et al. 2014), the present data could successfully demonstrate potential synergism between maturational steroid and Igf-mediated signalling in PKA inhibition prior to the release of prophase-I arrest and final OM.

Most importantly, participation of both DHP- and Igf-mediated signalling events in the maturation process under physiological condition is further evident from the fact that; i) heightened expression of igf1 and igf3; ii) elevated Tyr phosphorylation of anti-Igf1r immunoprecipitated protein; iii) mPRA expression at protein and possibly at mRNA level and iv) robust increase in Akt activation congruent with down-regulation of phospho-PKA (inhibition) in follicular lysates at 0500 h (~1 h before morning light signals on) correlates well with substantial increase in percentage of oocytes undergoing GVBD between 0500 and 0600 h, i.e., prior to ovulation in vivo. While mPRA as well as Igf1r have been identified in zebrafish oocyte (Nelson & Van Der Kraak 2010b, Hanna & Zhu 2011), a close association between the putative mPRA and OM in sea trout and zebrafish has been indicated earlier as rapid increase in receptor levels occurred in fully grown oocytes in vitro during hCG-induced GVBD (Zhu et al. 2003, Hanna & Zhu 2011). Besides, involvement of Igf1r in hCG-induced meiotic maturation has been demonstrated in zebrafish oocytes (Li et al. 2015). Further, involvement of DHP and/or Igf1 in activation of down-stream signalling molecules like Akt phosphorylation and PKA down-regulation, during the natural course of GVBD response, provides evidence in favour of synergistic action between MIS and Igf1.

While priming with anti-IGF1R antibody in vitro fails to inhibit DHP stimulation of meiotic maturation in denuded oocytes, present data provide evidence in favour of functional relevance of Igf1r (through Tyr phosphorylation) in Igf1- and/or Igf3-mediated maturation process in full-grown follicles in vivo. Although there has been significant increase in both Igf3 as well as Igf1 transcripts just prior to onset of ovulation, it is pertinent to reflect upon which form of Igf(s) is either necessary or essential for releasing prophase-I arrest in zebrafish. Interestingly, all four Igf ligands (Igf1, Igf2a, Igf2b and Igf3) have been shown to induce meiosis resumption with varying degrees of potentialities in zebrafish oocytes (Nelson & Van Der Kraak 2010a, Li et al. 2015). Although a robust increase in igf3
behind release of E₂-mediated prophase arrest in cascade (Maitra MAPK activation, potentially through Ras/Raf/Mek its involvement observed earlier in A. testudineus 2015, unpublished observation), suggesting contrary to OM (D Das, P Ghosh, S Pal, P Nath, S Hajra & S Maitra 2013). MAPK activation might have marginal influence in lifting prophase arrest in zebrafish oocyte.

Interestingly, significant increase in Akt phosphorylation was observed in Igf1 as well as DHP-induced oocytes and pharmacological inhibition of PI3K/Akt abrogated Igf1-induced GVBD and attenuates DHP action till 4–5 h of incubation. Moreover, high intracellular cAMP attenuates both DHP and Igf1-mediated GVBD and co-stimulation with DHP and Igf1 at physiological concentration, but not alone, could effectively reverse E₂ action on PKA activation and G2 arrest. These observations provide a basis for integration between two disparate signalling strategies at the molecular level. Interestingly, apparent increase in mPRα at both mRNA and protein level as well as elevated expression of igf1 and igf3 coupled with IGF1R phosphorylation and significant increase in Akt activation (phosphorylated at Ser473 and Thr308) in maturing follicles in vivo potentially hint at participation and functional relevance of both DHP and Igf mediated signalling prior to natural course of OM in this species.

**Declaration of interest**

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

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