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

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Abstract

Binding of 17 β -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 progesterin receptor (mPR) or Igf system has been implicated in regulation of meiosis resumption, possibility of concurrent activation and potential synergism between 17 α ,20 β -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.

Reproduction (2016) 151 59–72

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 17 β -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 17 α ,20 β -dihydroxy-4-pregnen-3-one (DHP) in majority of the fish species, but 17,20 β ,21-trihydroxy-4-pregnen-3-one (20 β -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

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 (17 β -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 steroidogenesis 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 G α_i 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 60 l glass aquaria, at 28 $\pm$ 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

Adenylyl 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-PKA $\alpha/\beta/\gamma$ cat (Thr198): sc-32968, anti-PKA α cat: sc-903, anti-IGF-IR β : 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 β was raised in rabbit against a peptide mapping at the C-terminus of IGF1R β of human origin, which is identical to the corresponding region of zebrafish Igf1 receptor (Maires *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 anesthetized 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 CaCl_2 , 5 mM HEPES; pH 7.2) supplemented with penicillin (100 IU/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) (Kondo *et al.* 1997). This preparation was also used as incubation medium in future *in vitro* experiments. The protocol for harvesting post-vitellogenic 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 Ca^{2+} free zebrafish Ringer's solution containing 0.001% collagenase type-IA (1.25 U/ml) for 1 h at $20 \pm 1^\circ\text{C}$ with mild agitation. Follicular cells were removed by repeated (30 $\times$) 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 $\sim 550-600\ \mu\text{m}$) 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 $25 \pm 1^\circ\text{C}$ under gentle agitation. DHP and E_2 were dissolved in ethanol and recombinant human IGF1 (Igf1) was dissolved in sterile water. Wortmannin (Wrt, 10 μM), LY294002 (LY, 25 μM), 10-DEBC-hydrochloride (DEBC, 5 μM), forskolin (1 and 5 μM) were dissolved in DMSO 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 Wrt, 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-IR β 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-IR β antibody (20 $\mu\text{g}/\text{ml}$) for 30 min each at $25 \pm 1^\circ\text{C}$ 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 & Kavumpurath 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 $\times$) and homogenised in chilled oocyte extraction buffer (100 mM sodium β -glycerophosphate, 20 mM HEPES, 15 mM MgCl_2 , 5 mM EGTA, 100 μM p-PMSE, 3 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM DTT and 1 $\mu\text{g}/\text{ml}$ aprotinin; 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 $\mu\text{g}/\text{well}$) 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 $\times$) 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-IRβ 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. One microgram of total RNA from each sample was reverse transcribed using Verso RT cDNA Synthesis Kit (#AB1453, Thermo Scientific) at 42 °C for 1 h in 20 µl reaction mix containing cDNA synthesis buffer, anchored oligo dT primer, deoxyNTPs, nuclease free water, RT enhancer and verso RT with significantly attenuated RNase H activity. In order to rule out the possibility of DNA contamination, RT(–) samples containing all other components previously mentioned, except RT, were prepared in parallels. Gene-specific primers for *Igf* receptor isoforms, and *mpira* (Table 1) were used for PCR amplification taking 1 µl of RT product (template cDNA) in a final reaction volume of 25 µl using 2× PCR master mix (#K0171, Fermentas) containing Taq polymerase, dNTPs and MgCl₂. After an initial denaturation step for 3 min at 95 °C, PCR amplification was carried out in Veriti 96-Well Thermal Cycler (Applied Biosystems) with the cycling profile of 30 s at 94 °C, 30 s at 52–56 °C (depending on the primer sets used), 30 s at 72 °C for 30 cycles followed by a final extension for 10 min at 72 °C. Amplified products were electrophoresed on 2% agarose gels along with 100 bp DNA ladder (Fermentas) and visualized by ethidium bromide staining. PCR amplification of *b-actin* was used as an internal loading control.

Real-time quantitative PCR

Real-time quantitative PCRs were set up in a 7500 Real-Time PCR System (Applied Biosystems) using gene-specific primers from earlier reports (Table 1). For both target and reference genes, reaction mix (final volume: 20 µl) contained 2 µl of

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

Gene	Sequence (5'–3')	Accession no.
<i>igf1</i> ^a	F: CAGCAAACCGACAGGATATGG R: CAGCTCTGAAAGCAGCATTCCG	NM_131825
<i>igf2a</i> ^a	F: CGCCTGCCATGGATGATTAC R: TCAGTGAGCGCATCGTTGTT	NM_131433
<i>igf2b</i> ^a	F: AACCTGCCAAGTCAGAGAGGG R: GGACCTCTGTTTAAATGCCG	NM_001001815
<i>igf3</i> ^a	F: ACGCTGCGGACGAGAAGTAC R: GCTGCTCCAGGTTTGCTAT	NM_001115050
<i>igf1ra</i> ^a	F: ACTCATACTCTGGGCTCTCTGTCC R: TCGGTATGTATTATCCACAAGCTCTT	NM_152968
<i>igf1rb</i> ^a	F: ATCCTCCCGGCTTACTGTT R: CCTGTCAATTGTTTCGGTCTTGT	NM_152969
<i>mpira</i> ^b	F: CATGGCTTCCGGTTAATTGTC R: GCCCAATCTGCTCCATCAC	AY149121
<i>b-actin</i> ^c	F: GGTATGGGACAGAAAGACAG R: AGAGTCCATCACGATACCAG	NM_181601

^aNelson & Van Der Kraak (2010b). ^bPikulkaew *et al.* (2010). ^cKishida & Callard (2001).

sample cDNA template diluted (1:10) with nuclease-free water, forward and reverse primers (250 nM of each) and SYBR Green PCR Master Mix (SYBR Green Dye, dNTPs, passive reference I, AmpliTaq Gold DNA Polymerase; Applied Biosystems). PCR conditions were optimized to generate >95% efficiency. After an initial enzyme activation step at 95 °C for 10 min, the amplification reaction was carried out for 40 cycles with the cycling profile of 95 °C for 15 s, 52 °C for 30 s and 72 °C for 30 s. Melt curve analyses were performed at the end of reaction to check specific amplification of the target. Each sample was run in triplicate and the CT value was corrected using corresponding endogenous control, *b-actin* gene and calculations were based on the 2^{–ΔΔCT} method (Livak & Schmittgen 2001).

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

Figure 1 Effect of 17 β -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 μ m) used in the present study, scale bar ~200 μ m. Denuded oocytes were treated either with graded levels of E_2 (1–1000 nM) or not (control), GVBD were scored at indicated time intervals and expressed in percent (B). Results are mean $\pm$ 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. Oocyte lysates (30 min) of indicated dose groups were analyzed by SDS-PAGE and subjected to immunoblot analysis using phospho-specific anti-ERK1/2 antibody that specifically recognise the activated form of the protein (inset, B). Anti-ERK1 immunoblot served as internal loading control. Data are representative of two independent experiments with identical results.

in unstimulated control and 1 nM E_2 -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 E_2 as early as 30 min of incubation (inset, lanes 3 and 4, Fig. 1B). Further, compared to 100 nM E_2 -treated group, no significant variation in GVBD response and ERK1/2 phosphorylation was observed due to 1000 nM E_2 (Fig. 1B). On the preceding basis, two different doses of E_2 , i.e., 10 and 100 nM were selected for subsequent experiments.

Effect of DHP- and Igf1-stimulation on E_2 -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 E_2 -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 E_2 . While DHP and Igf1 could trigger GVBD response in a dose-dependent manner (Fig. 2A), priming of denuded oocytes with E_2 (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 E_2 (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

Figure 2 Effect of a 3 h treatment with maturational steroid (DHP) and insulin-like growth factor 1 (Igf1) on 17 β -estradiol (E_2)-induced meiotic prophase-I arrest *in vitro*. Defolliculated zebrafish oocytes were primed with E_2 (10 and 100 nM), followed by stimulation either without or with increasing concentration of DHP (A) and Igf1 (B), either alone or in combination (C), and scored for GVBD. Data (mean $\pm$ S.E.M.) of at least three independent experiments from different donor fish were analyzed by one-way ANOVA ($P < 0.001$) and Duncan's *post hoc* test ($P < 0.05$). Groups with same lowercase letter above the bars are not significantly different and those with different letters differ significantly; # $P < 0.01$ compared to groups with or without E_2 stimulation.

Figure 3 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, * $P < 0.001$ against Igf1-treated and * $P < 0.05$ against 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-IGF1Rβ 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-M1 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

Figure 4 cAMP regulation of GVBD response in zebrafish oocytes. Defolliculated zebrafish oocytes were primed with either cell permeable dbcAMP or adenylyl cyclase (Ac) activator forskolin or not followed by stimulation with either increasing concentration of Ac inhibitor SQ22536 (A) or Igf1/DHP (B) and scored for GVBD at 4 h. Values are mean ± S.E.M. of three independent experiments. Data are analyzed by one-way ANOVA followed by Duncan's multiple range test. Groups with same lowercase letter above the bars are not significantly different and those with different letters differ significantly; * $P < 0.001$ compared to Igf1- or DHP-stimulated group.

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-unit 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 $\alpha/\beta/\gamma$ cat (Thr198) 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 E_2 (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 E_2 (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 E_2 , 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 E_2 -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 E_2 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 E_2 (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 E_2 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 Igf1r β , 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 Effect of DHP (3 nM) and Igf1 (10 nM) stimulation, either alone or in combination, on E_2 (10 and 100 nM) action on PKA phosphorylation (A) and kinetics of meiotic G2-M1 transition (B) *in vitro*. Oocyte lysates from indicated groups and time intervals were subjected to immunoblot analyses with anti-p-PKA $\alpha/\beta/\gamma$ cat (Thr198) antibody that recognizes the catalytic sub-unit of PKA phosphorylated on its activation loop. Anti-PKAc immunoblot served as loading control. Corresponding densitometric analysis are shown below the immunoblot, values are mean $\pm$ s.e.m. of three independent experiments. Data are analyzed by one-way ANOVA followed by Duncan's multiple range test. Groups with same lowercase letter above the bars are not significantly different and those with different letters differ significantly; * $P < 0.05$ compared to 10 nM and # $P < 0.001$ compared to 10 or 100 nM E_2 -treated groups.

Figure 6 Schematic representation of induction of captive breeding in gravid females (A). Relative proportion of immature (GV intact) and maturing oocytes (undergoing GVBD) *in vivo* (B). Data are mean $\pm$ 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 μ m. RNA samples from ovarian follicles (~ 200 follicles/time point from three to five different donor fish) were reverse transcribed and transcript abundance for Igf ligands (*igf1*, *igf2a*, *igf2b* and *igf3*) was checked through qRT-PCR; *b-actin* served as endogenous control (C). Values are the mean $\pm$ 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-IR β 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-PKA α / β / γ c antibody (right lower, D). Anti-b-actin, anti-Akt and anti-PKA α c immunoblot served as internal loading control. Corresponding densitometric analysis are also shown above the immunoblot; values are mean $\pm$ 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.

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 *igf1rs* 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-PKA α c (right panel, Fig. 6D) correlated well with elevated GVBD response (Fig. 6B) during this time *in vivo*.

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 E_2 has been shown to prevent growing

oocytes to mature precociously and binding of E_2 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 E_2 either in circulation or in the ovary. As a result, we had to test effect of graded levels of E_2 (1, 10, 100 and 1000 nM) on incidence of spontaneous maturation in defolliculated zebrafish oocytes. Present data demonstrate that E_2 priming could attenuate meiosis resumption (spontaneous maturation) in defolliculated post-vitellogenic oocytes (average diameter: 550–600 μ m) 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 E_2 at 20 and 100 nM (Pang & Thomas 2009, 2010, Peyton & Thomas 2011). Moreover, available information indicates that priming with E_2 (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 E_2 has been shown to promote *lhcr* expression (Liu *et al.* 2011). Since earlier evidence suggests that E_2 at 100 nM transactivates Egfr, promotes ERK1/2 phosphorylation and ensures prophase-I arrest in

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 maturational 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 maturational 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 progesterin 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 maturational 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 maturational 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 maturational 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 maturational 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 maturational 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 levels during the growth phase (vitellogenic phase) of the ovary with relatively much lower 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 maturational 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 20 β -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-IR β 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 (*igf1ra* and *igf1rb*) 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 G $\beta\gamma$ 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 20 β -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 mPRa, attenuates Ac activity and intra-oocyte cAMP level by activating the inhibitory G protein, G α_i (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

(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 E_2 (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 E_2 -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 seatrout 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*

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 μ M), 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 μ M) increase in Igf3-induced OM has been demonstrated in zebrafish (Li *et al.* 2011), suggesting the

Figure 7 Proposed model behind maintenance of prophase arrest and how DHP- and Igf-mediated signalling events may act synergistically to overcome E_2 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 E_2 to Gper at oocyte surface transactivate Egfr to induce MAPK activation; both of which are involved in the maintenance of E_2 -mediated prophase-I arrest. Conversely, DHP action at the oocyte surface, possibly through mPRa, activates $G_{\alpha i}$, 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 E_2 -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).

required concentration of Igf3 is much higher to saturate Igf1r, the possible physiological receptor of Igf3. Further, Igf3, Igf2a and Igf2b at 2 μ m concentration has been reported to exhibit GVBD response up to 60% of denuded oocytes after 4 h (Li *et al.* 2015). However, in the present study at the same time point we find that Igf1 at 10 nM is sufficient to promote meiosis resumption in more than 75% oocytes. Taken together these results tempted us to believe that though mRNA abundance of Igf1 is much lower in the zebrafish ovary, Igf1 could be the possible isoform that actually helps in promoting meiosis resumption since the receptor isoform involved in mediating all Igfs function is essentially the same one, i.e., Igf1r. However, in the future it would be interesting to examine the specificity as well as the affinity of binding between various Igf ligands (Igf2a, Igf2b and Igf3) and Igf1r at the oocyte surface. Clearly, proteomic analysis of Igf(s) expressed prior to re-initiation of meiotic G2–M1 transition under physiological condition, as well as selective knock out of individual Igf ligands or targeted knock down using morpholino *in vitro* may provide significant insight to determining participation of specific Igf isoforms(s) or their overlapping action in the maturational process in zebrafish.

Finally, the model in Fig. 7 illustrates the mechanism behind release of E₂-mediated prophase arrest in zebrafish oocyte, most importantly synergism between DHP and Igf1 during G2–M1 progression. As reported earlier, cAMP from follicular cells through gap junction (Nagahama & Yamashita 2008) or E₂ action at the oocyte surface through Gper1/G α_s /Ac/cAMP cascade (Pang & Thomas 2010) promotes PKA activation and ensures prophase-I arrest (Conti *et al.* 2002). Besides, transactivation of Egfr system by Gper1 could stimulate MAPK activation (Peyton & Thomas 2011).

Although the molecular mechanism is not yet clearly understood, E₂-mediated activation of MAPK might have strong negative influence on meiosis progression in teleost oocytes. While participation of G $\beta\gamma$ subunit of heterotrimeric G-protein has been implicated in DHP stimulation of PI3K/Akt and MAPK activation (Hanna *et al.* 2006), down regulation of cAMP/PKA pathway involves mPRA/G α_i - mediated attenuation of Ac (Zhu *et al.* 2003). On the other hand, Igf1 action at oocyte surface requires participation of Igf1r, and triggers rapid activation of Akt-mediated signalling cascade in denuded oocytes *in vitro*. Earlier we demonstrated that recombinant human insulin (rh-insulin) could promote MAPK activation, potentially through Ras/Raf/Mek cascade (Maitra *et al.* 2014), as well as PI3K/Akt/PDE3 signalling in denuded zebrafish oocyte, allowing further reduction in cAMP/PKA and promoting OM (Das *et al.* 2013). Although it could attenuate rh-insulin-mediated GVBD, MEK inhibition failed to prevent DHP action on OM (D Das, P Ghosh, S Pal, P Nath, S Hajra & S Maitra 2015, unpublished observation), suggesting contrary to its involvement observed earlier in *A. testudineus* (Khan

& 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 intra-oocyte 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 mPRA 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.

Funding

The work is supported by Department of Biotechnology (grant number BT/29/NE/TBP/2010) and University Grants Commission (F. no. 39-681/2010 (SR)) to S Maitra and financial assistance to D Das from INSPIRE Program, Department of Science and Technology, New Delhi, Government of India.

Acknowledgements

The authors express their sincere thanks to the reviewers and editorial board members for their valuable suggestions and critical review of the manuscript. Technical assistance was provided by Mr Sudip Hajra, Ms Pritha Ghosh, Ms Poulomi Nath and by Dr Disha Banerjee from Thermo Fisher, India for qRT-PCR. The authors are also thankful to Head, Department of Zoology, Visva-Bharati University, Santiniketan, India for providing instrument facility.

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Received 23 April 2015

First decision 18 June 2015

Revised manuscript received 4 October 2015

Accepted 22 October 2015