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Epidermal growth factor differentially regulates activin subunits in the zebrafish ovarian follicle cells via diverse signaling pathways

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ABSTRACT

Epidermal growth factor (EGF) promotes oocyte maturation in the zebrafish and its effect is mediated via the activin system. However, the mechanisms by which EGF regulates activin subunits in the follicle cells remain unknown. The present study demonstrated that EGF controlled expression of three activin subunits (*inhbaa*, *inhbab* and *inhbb*) in the follicle cells via diverse signaling pathways. The expression of *inhbaa* and *inhbb* was often co-regulated via similar pathways. Suppression of MAPK3/1, p38 MAPK, PKC and PKA each blocked or partially reduced the stimulatory effects of EGF on the expression of *inhbaa* and *inhbb* while up-regulated that of *inhbab*. Conversely, inhibition of PI3K did not have any effect on the expression of *inhbaa* and *inhbb* but significantly suppressed the stimulatory effect of EGF on *inhbab*. In summary, EGF action in the zebrafish ovary involves activin system and its regulation of activin subunits is mediated by diverse signaling pathways downstream of EGFR.

1. Introduction

Epidermal growth factor (EGF) family plays important roles in vertebrate ovarian physiology (Park et al., 2004; Onagbesan et al., 2009; Ge, 2005), as supported by the evidence that an EGF receptor (EGFR) mutant with lower kinase activity leads to impaired oocyte maturation and ovulation in mice (Hsieh et al., 2007).

Our previous study in the zebrafish showed that EGF and transforming growth factor α (TGF-α), also an EGF family member, promoted oocyte maturation. Their stimulatory effects were likely mediated by the ovarian activin system because the activin-binding protein, follistatin (*fst*), could abolish the effects of EGF and TGF-α (Pang and Ge, 2002a). Further evidence showed that EGF increased the expression of activin subunits *inhbaa* and *inhbb* (Pang and Ge, 2002a; Wang and Ge, 2004a; Tse and Ge, 2010) while reduced that of *fst* in cultured follicle cells (Wang and Ge, 2004a). In order to elucidate the regulatory relationship of EGF and activin families in the zebrafish ovarian follicle, we have investigated the expression sites of EGF family members, EGFR and activin subunits in full-grown (FG) follicles. Interestingly, EGF family members including EGF (*egf*), TGF-α (*tgfa*), and heparin-binding EGF (HB-EGF, *hbegf*) were exclusively expressed in the oocyte except for betacellulin (*btc*), which was expressed in both compartments but with higher expression in the oocyte. In contrast, EGFR and activin subunits were only expressed in the follicle layer, whereas activin receptors were abundantly expressed in the oocyte (Tse and Ge, 2010). These results suggest a potential bi-directional paracrine communication network between the oocyte and follicle layer in the follicle. The EGF ligands released from the oocyte may bind to EGFR on the follicle cells, resulting in an increased expression of activins, which in turn act on the oocyte to promote oocyte maturation (Pang and Ge, 1999, 2002b).

Three activin subunits (βA1, *inhbaa*; βA2, *inhbab*; and βB, *inhbb*) exist in the zebrafish (DiMuccio et al., 2005; Wang and Ge, 2003; Wittbrodt and Rosa, 1994), and they exhibit distinct expression profiles during zebrafish folliculogenesis. The expression of *inhbaa* is low at the primary growth (PG) stage; however, its level increases significantly when the follicle is recruited to enter the previtellogenic stage (PV), reaches the peak at the mid-vitellogenic (MV) stage and declines when the follicle approaches late vitellogenic (LV) or full-grown (FG) stage. The expression of *inhbb* shows little change during follicle growth; however, its level surges dramatically *in vivo* in the follicles undergoing final maturation (Wang and Ge, 2004b). In contrast to *inhbaa* and *inhbb*, the expression of *inhbab* increases steadily during folliculogenesis, reaching the highest level in the maturing follicles (DiMuccio et al., 2005). These findings strongly suggest differential roles for different activin subunits in regulating zebrafish follicle growth and maturation. One interesting issue to address is the regulatory mechanisms underlying the differential expression of these activin subunits. As an ovarian growth factor that regulates activin in the zebrafish ovary, EGF could be one of the regulatory factors that contribute to the differential control of the activin system in the follicle. To elucidate the

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mechanisms by which EGF regulates activin subunits, we analyzed several signaling pathways involved in EGF-EGFR signaling in cultured zebrafish follicle cells.

EGFR belongs to receptor tyrosine kinase family (Citri and Yarden, 2006; Yarden and Sliwkowski, 2001) and its binding by EGF family ligands induces autophosphorylation at the cytoplasmic domain, which triggers various downstream signaling pathways (Saito et al., 2004). Among various signaling cascades, MAPK3/1 (ERK1/2), p38 MAPK, PKC and PI3K have been shown to be activated by EGF and play various roles in the ovary of vertebrates. Of these EGFR signaling pathways, MAPK3/1 pathway is the best studied. Recent studies showed that knockout or blockade of MAPK3/1 in the granulosa cells impaired oocyte maturation and terminal differentiation of the cells *in vivo* and *in vitro* (Fan et al., 2009a; Su et al., 2002; Reizel et al., 2010). Inhibition of MAPK3/1 signaling partially abolished EGF- or LH-induced oocyte maturation (Reizel et al., 2010; Hsieh et al., 2011) but completely suppressed FSH-induced oocyte maturation in cumulus-oocyte complex (Su et al., 2002). In addition to regulating oocyte maturation, EGF-induced MAPK3/1 signaling also regulates steroidogenesis (Seger et al., 2001; Woods et al., 2007). Other pathways have also been implicated in EGF signaling in the ovary of different species. EGF has been reported to activate PI3K/Akt in human granulosa cells (Zhang et al., 2010), PKC in the chicken ovary (Woods et al., 2007), and p38 MAPK in ovarian cancer cells (Zhou et al., 2007). To understand the intracellular signaling mechanisms that underlie EGF stimulation of activin subunits in the zebrafish ovary, we undertook this study using pharmacological approach, Western blot and immunohistochemical (IHC) staining, and our results provide evidence that EGF signaling in the ovary involves activin system and it controls the expression of three activin subunits via diverse signal transduction pathways.

2. Materials and methods

2.1. Chemicals

All common chemicals were obtained from Sigma–Aldrich (St. Louis, MO), Invitrogen (Carlsbad, CA) and USB (Cleveland, OH) unless otherwise specified. Recombinant human EGF was obtained from PeproTech (Rocky Hill, NJ). All pharmacological drugs including AG1478, U0126, SB203580, GF109293X, LY294002 and H89 were from Merck (Whitehouse Station, NJ).

2.2. Antibodies

Antibodies against MAPK3/1 (#9102) and p-MAPK3/1 (#4370, #9101), p-p38 MAPK (#9211), p-Akt (#4060), Akt (#9272) as well as β-actin (#4967) were purchased from Cell Signaling Technology (Danvers, MA). Anti-p1173-EGFR was obtained from Millipore (Billerica, MA). HRP-conjugated bovine anti-rabbit IgG secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Animals

Zebrafish were purchased from local fish stores and maintained in aquaria (42 L) with continuous water flow (~30 L fresh water per day) at 28°C on the photoperiod of 14L:10D. The fish were fed with commercial tropical fish food three times per day and allowed to adapt at least 2 weeks before use. All experiments were performed under license from Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

2.4. Primary zebrafish follicle cell culture

The primary follicle cell culture was performed according to our previous report (Pang and Ge, 2002a). Briefly, about 30 female zebrafish were anesthetized by ice shock and decapitated before dissection. Follicles were mechanically dispersed in 60% L15 medium (Gibco, Carlsbad, CA) and full-grown follicles were removed by sieving with a metal mesh. The follicles passing through the mesh, which were mostly early follicles up to the mid-vitellogenic (MV) stage, were washed four times with M199 and cultured in M199 with 10% FBS (Hyclone, Logan, UT) at 28 °C in 5% CO_2 for 6 days for follicle cell proliferation. The medium was changed on the third day of incubation. The follicle cells were harvested by trypsinization and 2.5×10^5 cells were planted in each well of 24-well plate for 24 h before treatment. The cells were washed with serum-free M199 twice before treatment and all the treatments were carried out afterwards in serum-free M199. The drugs were added to cultured follicle cells at different times towards the end of 4-h incubation period so that the total incubation time was the same (4 h) for all treatments (Liu et al., 2011).

2.5. Follicle incubation

Follicle incubation was similar to primary follicle cell culture except that after sieving the follicles with the metal mesh, the follicles in 60% L15 medium were evenly distributed in 24-well plate. The follicles were incubated at 28 °C.

2.6. Immunoblotting

Follicle cells were lysed with 40 μl 1X SDS sample buffer containing 62.5 mM Tris–HCl at pH 6.8, 1% w/v SDS, 10% glycerol and 5% -mercaptoethanol, and the lysate was heated at 95 °C for 10 min and then centrifuged for 5 min at RT. The samples (7.5 μl each) were separated on 12.5% polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were blocked with 5% non-fat milk in 1X TBST for 1 h at RT. After washing three times with 1X TBST, the membranes were incubated with primary antibodies (1:1000) in 5 ml of 1X TBST with 5% non-fat milk overnight at 4 °C. They were washed with 1X TBST three times followed by incubation with the HRP-conjugated secondary antibody (1:2000) in 5 ml 1X TBST for 1 h at RT. After washing, the membranes were incubated with the Developing Solution (Western Blotting Luminol Reagent; Santa Cruz Biotechnology) and signals detected on the Lumi-Imager F1 (Roche, Mannheim, Germany).

2.7. Immunohistochemistry

Zebrafish follicles were dispersed as described above and distributed to 2 ml tubes with 400 μl 60% L15. Follicles were maintained at 28 °C and washed three times with 200 μl 60% L15 at 10 min interval to remove endogenous ovarian factors that might be released during dissection and follicle dispersal. Follicles were then treated with EGF or vehicle for 20 min followed by fixation with 400 μl Bouin's solution overnight at 4 °C. After embedded in paraffin, the follicles were sectioned at 5 μm and mounted on Superfrost Plus slides. The sections were deparaffinized and rehydrated, and antigen retrieval performed at sub-boiling temperature for 10 min using 10 mM sodium citrate buffer. The endogenous hydrogen peroxidase was inactivated by treating the slides with 3% H_2O_2 for 10 min. The sections were then washed with 1X PBS three times at 5 min interval before blocking for 1 h with normal horse serum at room temperature. Sections were incubated overnight at 4 °C with 100 μl p-MAPK3/1 or p-Akt antibody diluted at 1:100 in blocking solution with normal horse serum, washed with 1X PBS three times, and then incubated with 100 μl secondary