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Research paper

Spatiotemporal expression analysis of nuclear estrogen receptors in the zebrafish ovary and their regulation in vitro by endocrine hormones and paracrine factors

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ABSTRACT

Estradiol (E2) stimulates luteinizing hormone receptor (*lhcgr*) expression via nuclear estrogen receptors (nERs) in the zebrafish ovary. We have demonstrated that endocrine hormones such as gonadotropin (hCG) and paracrine factors such as epidermal growth factor (EGF) and pituitary adenylate cyclase-activating peptide (PACAP) can modulate E2-induced *lhcgr* expression in vitro. These observations raised a question on whether these hormones and factors exert their effects via regulating the expression of nERs. In this study, we first characterized the spatiotemporal expression profiles of three nER subtypes in the zebrafish ovary, including *esr1* (ERα), *esr2a* (ERβ2) and *esr2b* (ERβ1). All three nERs increased their expression at the pre-vitellogenic stage and peaked at mid- (*esr1* and *esr2a*) or late vitellogenic (*esr2b*) stage, followed by a significant decline at the full-grown stage. RT-PCR analysis showed that *esr1* and *esr2b* were exclusively expressed in the follicle layer while *esr2a* was expressed in both compartments. We then examined how E2, hCG, PACAP and EGF regulated the expression of nERs in cultured zebrafish follicle cells. E2 quickly increased *esr1* but reduced *esr2a* and *esr2b* expression from 1.5 to 12 h of treatment. Similarly, EGF down-regulated *esr2a* significantly at 1.5 h and this effect was further intensified at 24 h. hCG decreased the expression of all three nER subtypes with similar potency throughout the 24-h time-course. Interestingly, PACAP exerted a biphasic regulation on *esr2a*. Our present study suggests that nERs, especially *esr2a*, provide potential target points for other hormones and factors to modulate E2 activity during folliculogenesis in the zebrafish.

1. Introduction

In 1980s, estrogen receptor (ER) was first cloned and studied in the human (Green et al., 1986; Greene et al., 1986), chicken (Krust et al., 1986) and rat (Koike et al., 1987). One form of ER was believed to mediate various estradiol (E2) functions in vertebrates until 1996 when another ER was cloned in the rat ovary and prostate (Kuiper et al., 1996), which was designated as ERβ while the previous ER was renamed as ERα. In the following years, these two ERs have been extensively investigated and cloned in other species with several variants identified (Ascenzi et al., 2006; Nilsson et al., 2001).

As the key molecules in estrogen signaling, the regulation of nERs by various hormones or factors has been broadly reported in mammals (Pinzone et al., 2004). E2 itself exerts homologous regulation on nERs in multiple cells and tissues. It reduces expression of ERα in the MCF-7 cells (Donaghue et al., 1999) and mouse skeletal muscle cells (Baltgalvis et al., 2010) while diminishes both ERα and ERβ protein levels in human endothelial cells (Tschugguel et al., 2003). On the contrary, E2 increases ERα expression in the rat hypothalamic neurons (Dominguez and Micevych, 2010) and male rat pituitary (Friend et al., 1995). Furthermore, E2 up-regulates ERα but down-regulates ERβ expression in ovine vascular endothelial cells (Ihionkhan et al., 2002).

In addition to the homologous regulation by estrogens, the expression of nERs is also subject to regulation by endocrine gonadotropins and paracrine growth factors. ERβ co-expresses with LH receptor (LHCGR) in the granulosa cells of rat ovary, and hCG greatly decreases its expression (Byers et al., 1997). This hCG-induced down-regulation of ERβ involves a decrease in its mRNA stability (Guo et al., 2001). Similarly, hCG greatly reduces the expression of both ERα and ERβ in human granulosa-luteal cells

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(Chiang et al., 2000). As for growth factors, EGF (Cormier et al., 1989; Stoica et al., 2000a) and IGF-I (Stoica et al., 2000b) both down-regulate ERα as well as its binding with E2 in the MCF-7 cells.

In teleosts, there are three nER subtypes, *esr1*, *esr2a* and *esr2b*, which have been cloned and characterized in many species, including the zebrafish (Bardet et al., 2002; Menuet et al., 2004, 2002) and medaka (Chakraborty et al., 2011). Like nERs in mammals, different subtypes of nERs in teleosts do not seem to be functionally redundant. Lines of evidence indicate distinct functions of different nER subtypes in teleosts, including differential spatiotemporal expression (Chakraborty et al., 2011; Menuet et al., 2002), binding affinity with various ligands (Hawkins and Thomas, 2004; Menuet et al., 2002), receptor-reporter assay (Menuet et al., 2004; Sabo-Attwood et al., 2007), transactivation by E2 (Davis et al., 2007; Sabo-Attwood et al., 2007) and vitellogenin induction by nER subtype-specific activators (Leanos-Castaneda and Van Der Kraak, 2007). A recent knockdown study in the goldfish further supports distinct functional roles of nERs in fish (Nelson and Habibi, 2010).

The expression level of nERs greatly affects the performance of E2 action as high nER expression sensitizes the cellular response to E2 (Nelson and Habibi, 2010). Although nERs have been reported in a variety of fish species, the information on their regulation is limited and most studies are concerned about the homologous regulation by E2 in the hepatocytes. In the goldfish, E2 induces the expression of all three nERs (ERα, ERβ1 and ERβ2) in hepatic and gonadal tissues (Nelson et al., 2007; Soverchia et al., 2005), and the E2-stimulated hepatic ERα expression requires the presence of ERβ1 and ERβ2 (Nelson and Habibi, 2010). A separate study, however, showed contradictory results regarding the effects of E2 on ERβ1 and ERβ2 in goldfish liver and testis (Marlatt et al., 2008). In addition to goldfish, homologous regulation of nERs also exists in other teleost species. E2 increases the hepatic expression of ERα while showing no effect on ERβ in tilapia (Davis et al., 2007). In the zebrafish liver, E2 significantly stimulates ERα expression via ERα itself and ERβ2 but differentially reduces ERβ1 expression (Menuet et al., 2004).

Recently, we have identified E2 as a strong stimulator of *lhcgr* expression in the zebrafish ovary (Liu et al., 2011). Interestingly, this E2-induced *lhcgr* expression could be modulated by EGF (Liu and Ge, 2013), hCG and PACAP (unpublished). These results, together with the reports in mammals, have led us to hypothesize that EGF, hCG and PACAP may influence E2-stimulated *lhcgr* expression by controlling the expression of nERs, therefore modulating the responsiveness of zebrafish ovarian follicle cells to E2. To test this idea, we characterized the spatiotemporal expression profiles of all three nER subtypes (*esr1*, *esr2a* and *esr2b*) in the follicle and during folliculogenesis. This was followed by investigating the effects of E2, EGF, hCG and PACAP on the expression of nERs in cultured zebrafish follicle cells where all three nERs are expressed.

2. Materials and methods

2.1. Animals

Adult zebrafish (*Danio rerio*) were maintained in flow-through aquaria of 60 L at 28C under 14L:10D photoperiod control. All fish were fed with the tropical fish feed Otohime S1 (Marubeni Nisshin Feed Co., Tokyo, Japan) three times a day. All experiments were performed with endorsement of the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong and the Animal Ethics Committee of University of Macau.

2.2. Hormones and chemicals

All common chemicals were obtained from Sigma-Aldrich (St. Louis, MO), USB Corporation (Cleveland, OH), GE Healthcare (Waukesha, WI), or Merck (Whitehouse Station, NJ). 17β-estradiol (E2; Sigma-Aldrich) was first dissolved in absolute ethanol as stock. Human choriogonadotropin (hCG; Sigma-Aldrich), zebrafish pituitary adenylate cyclase-activating polypeptide$_{38}$-2 (PACAP$_{38}$-2; ChinaPeptides, Shanghai, China) and human epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ) were dissolved in water as stocks (stored at −80C) and diluted with medium to desired concentrations before use.

2.3. Staging ovarian follicles

Various stages of ovarian follicles were isolated for expression analysis. We divided follicles into six stages based on their size and morphology, including primary growth (PG, stage I), previtellogenic (PV, stage II), early vitellogenic (EV, early stage III), mid-vitellogenic (MV, mid-stage III), late vitellogenic (LV, late stage III) and full-grown (FG, stage III) (Wang and Ge, 2004b; Zhou et al., 2011).

2.4. Isolation of follicle layer from oocyte

The somatic follicle layer was separated from the oocyte according to our previous reports (Tse and Ge, 2010; Zhou et al., 2011). Briefly, FG follicles were isolated from the zebrafish ovary and incubated in Ca^{2+}/Mg^{2+}-free Cortland medium for 20 min to loosen the connection between the oocyte and the surrounding follicle layer. The follicle layer was peeled off as a whole from the oocyte with fine forceps. The separated follicle layers, the denuded oocytes and the intact FG follicles were pooled for RNA extraction and RT-PCR analysis with primers previously reported for *ef1a*, *lhcgr*, *esr1*, *esr2a* and *esr2b* (Liu et al., 2011) and *gdf9* (Liu and Ge, 2007).

2.5. Primary cell culture and drug treatment

The primary follicle cell culture and drug treatment schemes were based on our previous report (Liu et al., 2011). Briefly, vitellogenic ovarian follicles (mostly PV, EV and MV stages) were isolated from the zebrafish ovary and incubated in M199 (Gibco-BRL, Gaithersburg, MD) with 10% FBS (Hyclone, Logan, UT) for six days with the medium changed once on the third day. Subsequently, the proliferated follicle cells were trypsinized and subcultured into 24-well plates at density of 2×10^5 cells per well. After 24-h subculture for cell attachment, the medium was changed to starve the follicle cells in M199 without serum for another 24 h. Treatments were carried out during the following 24-h period after the starvation. Therefore, all cells were incubated for the same period of time.

2.6. Total RNA extraction and real-time qPCR

Cells from each well were lyzed by TRI Reagent (Molecular Research Center, Cincinnati, OH) to extract total RNA according to manufacturer's protocol. Since the number of cells in each well was strictly controlled, the entire total RNA extracted from each well was reverse transcribed to obtain cDNA by M-MLV reverse transcriptase (Invitrogen, Grand Island, NY). Real-time qPCR was performed on C1000 Thermal Cycler CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA) using primers reported previously (Liu et al., 2011).

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