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Expression of androgen receptor mRNA in the ovary of Japanese eel, *Anguilla japonica*, during artificially induced ovarian development

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ABSTRACT

In order to elucidate how androgens may mediate their effects on ovarian growth, we investigated the mRNA levels of two subtypes of androgen receptor (*ara* and *arb*) in the ovary of feminized Japanese eel (*Anguilla japonica*) during artificially induced ovarian development by quantitative real-time reverse transcriptase polymerase chain reaction and in situ hybridization. *Ara* mRNA levels were high from the late oil droplet stage to the late vitellogenic stage, whereas *arb* mRNA levels were high from the late oil droplet stage to the midvitellogenic stage. Both *ar* mRNAs were predominantly observed in the follicle cells and the epithelial cells of the ovigerous lamellae in all stages. In the oil droplet stage, oogonia exhibited intense signals for *ar* mRNAs. There was no obvious difference in localization pattern between *ara* and *arb* in all ovaries examined, irrespective of maturational stage. It was difficult to identify the follicle cell types that were positive for *ar* mRNA during ovarian development. Only in post-ovulatory follicles could theca and granulosa cells be clearly identified, and *ar* signals were observed in both layers. The predominant localization of *ar* mRNA in the follicle cells suggests that androgens play important roles in oocyte growth by acting on these cells in this species. We have shown the expression profile and localization of *ar* mRNA during ovarian development for the first time in an oviparous vertebrate.

1. Introduction

Oogenesis, the developmental progression of primordial germ cells to mature ova, is cyclically stimulated and requires sequential programs of growth that are controlled by a wide variety of endocrine and locally-acting paracrine and autocrine factors (Lubzens et al., 2010). As in other vertebrates, gametogenesis in teleost fish is regulated primarily by gonadotropins (GTHs). Typically, GTHs do not act directly, but rather, work through the gonadal biosynthesis of steroid hormones which in turn mediate various stages of gametogenesis (Nagahama, 1994). One class of steroid hormones, the androgens or C19-steroids, are generally considered to play a central role in regulating testis differentiation, development of secondary sexual characters and sexual maturation in male teleosts (Borg, 1994). This role is typically fulfilled by 11-ketotestosterone (11KT), a testosterone (T) metabolite that has been identified as the most potent androgen in teleost fish (Miura et al., 1991; Borg, 1994). However, this steroid is not confined to males, as 11KT has also been identified in blood of several female fish; it is, in fact, the major androgen in some genera e.g., the Anguillidae (Lokman et al., 2002). Serum levels of 11KT in female eel are as high as or higher than T levels. Moreover, serum 11KT levels increase gradually along with gonadal development in artificially maturing female Japanese eel (*Anguilla japonica*), suggesting an important role for this steroid in female sexual maturation (Matsubara et al., 2005).

Several studies have been conducted to identify the physiological roles of 11KT in female eels. Rohr et al. (2001) reported that implantation of 11KT in immature female shortfinned eel (*Anguilla australis*) resulted in changes reminiscent of the developmental transformation known as "silvering". These were considered to be puberty-related by Aroua and co-workers (2005). In vitro culture has proven that 11KT directly promotes late perinucleolar oocyte growth in ovarian fragments from shortfinned eel (Lokman et al., 2007). In addition, Endo et al. (2008) showed that 11KT is necessary for the very low density lipoprotein (VLDL)-mediated accumulation of oil droplets into oocytes in cultured ovarian fragments from previtellogenic Japanese eel. Recently, 11KT has also been reported to stimulate growth of primary oocytes in cod (Kortner et al., 2008, 2009). These studies indicate that 11KT is involved in the control of previtellogenic oocyte growth and acts

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directly on the ovary, at least in some teleost. However, the mechanisms underlying the regulation of oocyte growth by 11KT have not been fully clarified.

Androgens generally act on target cells through a nuclear androgen receptor (Ar). The Ar is a ligand-dependent transcription factor that belongs to a large family of nuclear receptors (Mangelsdorf et al., 1995). To date, one or two *ar* gene (or genes) has been described in many teleost species (Douard et al., 2008; Ogino et al., 2009). Functional analysis revealed that there is variation in Ar function with regard to ligand selectivity and transactivation potential between species and subtypes (Olsson et al., 2005; Hossain et al., 2008; Ogino et al., 2009). From a cDNA library of Japanese eel testes, two subtypes of *ar* (*ara* and *arb*) were isolated (Todo et al., 1999; Ikeuchi et al., 1999). The ligand-dependent transactivation function of both eel Ar subtypes is quite similar and is limited to androgens only. The naturally occurring androgens, 11KT and 5α-dihydrotestosterone (DHT), are most potent in this regard. T and 11β-hydroxytestosterone are also effective, but their potencies are lower than that of 11KT (Ikeuchi et al., 1999). These results suggest that the major native ligand of eel Ars is 11KT. Thus, it is believed that 11KT acts on the eel ovary through Ars. The presence of *ar* gene transcripts in the ovary has been shown in Japanese eel (Ikeuchi et al., 1999) and shortfinned eel (Lokman et al., 2007). Recent studies have also shown *ar* transcripts in differentiating ovary of sea bass (Blázquez and Piferrer, 2005) and zebrafish (Hossain et al., 2008), as well as developing ovaries of cyprinid fish (Liu et al., 2009), but the expression profile and the cellular localization of ovarian *ar* mRNAs during ovarian development have not been examined in eels or any other non-mammalian vertebrate.

In order to elucidate the mechanisms of androgenic action in ovarian growth, we investigated the expression of *ara* and *arb* mRNA in the ovary of the Japanese eel during artificially induced ovarian development. Changes in mRNA levels of both *ar*s were examined by quantitative real-time reverse transcriptase polymerase chain reaction (qPCR). In situ hybridization (ISH), using probes specific to each *ar*, was carried out to further determine the cellular localization of *ar* mRNAs in the ovary.

2. Materials and methods

2.1. Animals and artificial maturation

Glass eels purchased from a commercial eel supplier were feminized with estradiol-17β (E2; 10 mg/kg diet) for 4–6 months (Tachiki et al., 1997), and cultivated in fresh water until used for experiments at the breeding facilities at Aichi Fisheries Research Institute (Hazu, Aichi, Japan) or Hokkaido University, Faculty of Fisheries (Hakodate, Hokkaido, Japan). Since eels do not mature under normal culture conditions (Kagawa, 2003), artificial maturation was conducted at Hokkaido University to obtain maturing ovaries and eggs according to the method of Ohta et al. (1997). The eels were gradually acclimated to seawater 1 week before the beginning of maturation induction. A total of 39 fish were used in the qPCR experiment, 7 of which were terminally sampled as pretreatment controls (605–900 g). Prior to induction of sexual maturation, most eels had an ovary whose oocytes were in the late oil droplet or early vitellogenic stage (mean diameters of follicles of the largest size class measured 182–236 μm in pretreatment controls). Gonadal development in remaining fish was induced by repeated injections with salmon pituitary extract (SPE: 30 mg acetone-dried pituitary powder per kg body weight) once a week until completion of vitellogenesis. Females were terminally sampled at the early vitellogenic (n = 8, 665–970 g), mid vitellogenic (n = 4, 675–1020 g), late vitellogenic (n = 6, 605–850 g), or migratory nucleus stage (n = 5, 705–1660 g). Control fish (n = 6, 410–815 g) were obtained after 8 weekly injections with eel Ringer solution (150 mM NaCl, 3.0 mM KCl, 3.5 mM $MgCl_2$, 5.0 mM $CaCl_2$, 10 mM Hepes, pH 7.4) only. Euthanasia was done 3 days after an SPE or Ringer injections. To obtain post-ovulatory ovaries and ovulated eggs, females whose oocytes reach the migratory nucleus stage (assessed by cannulation; Ohta et al., 1997) received 17, 20β-dihydroxy-4-pregnen-3-one (DHP) injections at a dose of 2 mg per kg body weight to induce both oocyte maturation and ovulation. After DHP injection, fish were kept at 23 °C, and ovulated fish were sampled soon after ovulation (n = 3, 600–840 g). After anesthesia with 2-phenoxyethanol, ovaries were removed and weighed for calculation of gonadosomatic index (GSI: gonad weight as percentage of total body weight). In order to stage the ovaries, the mean diameters of follicles corresponding to the largest size class were calculated and stages determined on the basis of Kazeto et al. (2000) as follows: oil droplet stage for follicles <200 μm in diameter; early vitellogenic stage for follicles 200–400 μm; midvitellogenic stage for follicles 400–600 μm; late vitellogenic stage for follicles 600–800 μm and migratory nucleus stage for follicles 800–1000 μm. Ovarian fragments and eggs were snap-frozen in liquid nitrogen and stored at −80 °C until later use for total RNA extraction (see below). For ISH, ovarian tissue in the oil droplet (n = 9), early vitellogenic (n = 4), midvitellogenic (n = 4), late vitellogenic (n = 3), migratory nucleus (n = 2) and post-ovulatory stage (n = 1) were analyzed. Some samples used for ISH were from the fish used in the experiment outlined above, others were from fish treated with 40 mg salmon pituitary homogenate per kg body weight, described previously (Matsubara et al., 2005). Some of the ovarian samples in the oil droplet stage that were analyzed by ISH were from the fish reared in fresh water.

2.2. qPCR for eel androgen receptor

Total RNA was isolated with ISOGEN (Nippongene, Tokyo, Japan) according to the manufacturer's instruction. Samples for qPCR were reverse transcribed with ReverTra Ace (Toyobo, Tokyo, Japan) using oligo (dT) primer and 1 μg total RNA in 20 μl reactions. The transcript abundance for *ara* and *arb* was determined by qPCR as described by Kazeto et al. (2008). *Ara* and *arb* were quantified by multiplex qPCR, using TaqMan Univeral PCR Master Mix (PE Applied Biosystems, Foster City, CA) and Taqman probes (Sigma–Aldrich, St. Louis, MO) on an ABI Sequence Detection System 7000. Each reaction contains 75 ng total RNA equivalent. The transcript abundance of *ara* and *arb* was presented in non-normalized form and expressed as a fold-change in abundance relative to the pretreatment control group. Gene-specific primers and probes were designed from the cDNA sequences of *ara* (GenBank Accession No. AB023960) and *arb* (GenBank Accession No. AB025361) using Primer Express Software ver. 2.0 (PE Applied Biosystems). In order to avoid genomic DNA amplification, primers and probes were designed to anneal to separate exons. Sequences of primers and probes are listed in Table 1.

2.3. In situ hybridization for eel androgen receptor

Products of 434 bp for eel *ara* and 494 bp for eel *arb* were amplified by PCR using specific primers (listed in Table 1) from plasmids that contained the full-length eel *ar* CDS (Ikeuchi et al., 1999). The amplicons were then ligated into pGEM-T Easy Vector (Promega, Madison, USA). The *ar* cDNA-harboring vectors were linearized using *Spe* I (Takara, Shiga, Japan) and sense and antisense digoxigenin (DIG)-labeled cRNA probes were generated using an RNA labeling kit (Roche Diagnostics, Basel, Switzerland). Freshly dissected ovarian fragments were fixed with 4% paraformaldehyde in 100 mM phosphate-buffer (pH 7.4) at 4 °C overnight. Fixed ovarian fragments were embedded in paraffin, sectioned (5 μm) and