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# Androgenic Modulation in the Primary Ovarian Growth of the Japanese eel, *Anguilla japonica*

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*Anguilla japonica* seedling production is urgently required for eel aquaculture due to the species' severely dwindling population. This study aimed to understand androgenic modulation of the primary ovarian growth, a critical development phase in females, in this semelparous fish. Through histological analysis, primordial to primary follicle transition was observed before hormone injection, and eels injected with SPH + MT showed greater synchronous follicle development than those injected with SPH alone. An *in vivo* experiment revealed a positive correlation ($p < 0.05$, $r = 0.94$) between the mRNA expression of *ar*$\alpha$ and increasing gonadal somatic index (GSI) < 0.75% before SPH injection. Another positive correlation was seen between *ar*$\beta$ expression and GSI ($p < 0.05$, $r = 0.97$) after weekly SPH injections for three weeks. *fshr* expression was high in the SPH + MT-injected group. Significantly high *fshr* mRNA levels were found after weekly MT injections for two weeks ($p < 0.05$), whereas the expression levels dropped after flutamide injection. *ar*$\alpha$ and *ar*$\beta$ expressions revealed different patterns before and after SPH induction. In this study, androgen modulation was found with regard to ARs expressions during primary growth and the primordial to primary follicle transition prior to hormone induction. This modulation continuously affected *fshr* expression and vitellogenic development after SPH induction during ovarian growth in the Japanese eel.

**Key words:** Androgen, Androgen receptors, Primordial to primary transition, Semelparous eel, Ovary.

## BACKGROUND

Primary ovarian development in fish is similar to that of mammals (Kezele et al. 2002; Luckenbach et al. 2008), probably because the initial development of the ovary is conserved across evolution in vertebrates (Das and Arur 2017; Grier et al. 2016; Juengel et al. 2002). Initial ovarian development and recruitment critically affect female reproductive fertility and ovarian reserves (McGee and Hsueh 2000; Peters 1969; Ueno et al. 1989). During primary growth, primordial follicles are activated from their dormant state (Adhikari and Liu 2009), followed by a transition from primordial to primary follicles (Kezele and Skinner 2003). In this critical process, oocytes in the primordial follicles develop from the chromatin nucleolar stage to the perinucleolar stage, during which meiotic division is suspended in prophase I.

During the ovary's initial growth, mesonephron-derived development still remains after gonadal sex differentiation. Regulation of oocyte meiosis is species-dependent before the onset of puberty. In many species, the onset of immediate meiosis is initiated simultaneously with sex differentiation. In others, a prolonged period separates the gonadal sex differentiation and the onset of meiosis just prior to the puberty. This delayed meiosis is regulated by mesonephron-secreted meiosis-inducing substances and steroid hormones (progesterone and estradiol) (Byskov 1979; Dutta et al. 2016). In contrast, little or no steroid hormone can be detected immediately before meiosis begins. The initial development of the ovary is correlated with a low level of steroid hormone from mesonephron and ovigerous cords. In the Japanese eel

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life cycle, a long duration of prepuberty is observed prior to reproductive migration. Little is known about whether the regulation of meiosis affects this long hiatus in early ovarian development.

In semelparous eels, millions of ovarian follicles set out for development (Edel 1975). The initial ovarian development, which includes folliculogenesis and steroidogenesis, is an important preparation for previtellogenic (primary) development, and is believed to be regulated by many transcriptional factors (Pangas et al. 2006; Rajkovic et al. 2004; Sen and Hammes 2010; Shiina et al. 2006), intra-ovarian factors (Fortune 2003; McGee and Hsueh 2000; Skinner 2005), and steroid hormones (Juengel et al. 2002; Kezele and Skinner 2003; Vendola et al. 1999). Androgens have been reported to promote primary growth in primate ovaries (Vendola et al. 1999; Weil et al. 1998).

Two major androgens have been detected in adult teleosts. The first, testosterone (T), is thought to be an aromatizable precursor of estradiol ($E_2$). The other is non-aromatizable 11-Ketotestosterone (11-KT). Previous studies detected relatively high plasma levels of androgens in female migratory eels (Lokman et al. 1998). Several studies also showed that female eels were observed to synthesize 11-KT and suggest that the possible function of 11-KT is to affect lipid droplet accumulation and transportation in the previtellogenic (PV) ovary (Divers et al. 2010; Matsubara et al. 2003). Although other studies revealed that 11-KT on PV affect early vitellogenic development (Lokman et al. 2007; Setiawan et al. 2012), the modulation of androgen during primary growth still remains unclear in the previtellogenic ovary of female eels.

The function of GtH in primary ovarian development remains obscure. Some previous studies have shown that FSH promotes follicular cell growth in primordial follicles (Allan et al. 2006; Durlej et al. 2011; Roy and Albee 2000). In addition, there is a small elevation in basal FSH secretion during the further development of early follicular recruitment (Fortune 1994). The effects of FSH on GtH-dependent development during the pre-antral to antral follicle transition are generally positive (Gilchrist et al. 2001). On the other hand, some studies have reported that the actions of FSH on ovarian follicles can be modulated by locally produced sex steroids and intra-ovarian factors (Hillier 1994; Richards 1994). Studies of teleosts have reported that FSH mainly affects $E_2$ synthesis, vitellogenesis and vitelogenin uptake in ovarian follicles (Kayaba et al. 2008; Nagahama et al. 1993; Tyler et al. 1991). However, few functional studies have been conducted on FSH in PV ovary primary development.

Salmon pituitary homogenate (SPH) has been used to artificially induce whole ovary development since the 1970s (Yamamoto and Yamauchi 1974; Yamauchi et al. 1976). This study used SPH injection for three weeks as the main induction method to force ovarian development, and the androgenic effect on developmental difference was investigated via exogenous addition of MT. The characterization and calculation of follicle stages were histologically compared among treatments. The relevant gene expressions—*arα*, *arβ* and *fshr*—were investigated using relative RT-qPCR after *in vivo* experiments. Further experiments with flutamide injections were conducted to test whether AR-mediated actions affect gene expression in early ovarian development. To further understand how exogenous hormones influence relevant gene expression, an *in vitro* experiment was performed using developing ovarian tissue after *in vivo* weekly SPH injections for two weeks. FSH was treated as an intra-ovarian regulator of gene expression during short-term incubation. In addition, MT was added to evaluate whether androgen modulated relevant mRNA expression in the early stages of ovary development. We aimed to describe androgen modulation under SPH-forced early follicle development via histological observation, and *in vivo* and *in vitro* gene expression analyses.

## MATERIALS AND METHODS

### Animals

Japanese eels were purchased from an eel culture farm (Lukang, Taiwan) and ranged in weight from 500-650 g. Eels were kept in recirculating filtered freshwater at 20 ± 1°C with a 12:12-h light:dark photoperiod and feed was supplied twice daily. The eels undergo spontaneous starvation during their reproductive migration, so feeding was reduced during seawater acclimation. After approximately one week of seawater acclimation, gonadal maturation was induced by weekly intraperitoneal hormone injections. The condition of these animals was reviewed

by the Animal Research Committee of the National Taiwan University (approval number, NTU-103-EL-74). For each weekly injection, eels were anesthetized by light bathing in 2-phenoxyethanol (0.3 ppm; Sigma-Aldrich), and were euthanatized in 0.5 ppm for final tissue sampling (Borski and Hodson 2003). The physical condition was monitored through body weight measurements and exterior observations.

### Histological observations of primary ovarian growth during hormonal injections

Thirty eels were randomly distributed throughout six tanks (500 liters / tank). Two tanks held each of the following three groups: control ($n$ = 10), weekly SPH injection for three weeks ($n$ = 10), and weekly SPH + MT injection for three weeks ($n$ = 10). All weekly hormone and drug injections were as follows: SPH was dissolved in a 0.9% saline solvent (20 mg/kg), MT (Sigma-Aldrich) was dissolved in a 1% dimethyl sulfoxide (DMSO) (3 mg/kg). The seawater control eels received a weekly saline injection followed by different sampling times based on the experiment. The seawater control eels received a weekly saline injection followed by different sampling times based on the experiment. A weekly hormone injection was carried out following the schedule (Table 1). Ovarian tissue was sampled after 72 hours from female eels, including controls (female, $n$ = 4; male, $n$ = 6), SPH-injected group (female, $n$ = 3; male, $n$ = 7) and SPH + MT-injected group (female, $n$ = 4; male, $n$ = 6), The gonadal somatic index (GSI) was calculated as (gonad weight/ body weight) × 100. Ovarian tissues were cut and fixed in solution (2% paraformaldehyde and 2.5% glutaraldehyde) for 16 hours. The fixed tissues were dehydrated by gradient alcohol concentrations (25%, 50%, 75%, 95% and 100%). After lamellae was embedded in the paraffin (EMS), 5-μm sections were stained using the hematoxylin and eosin staining (HE staining) method and then examined with light microscopy (Olympus corp., Japan) and photography analysis (SPOT Basic image capture software, Diagnostic Instruments Inc., USA).

### Microscopy examination

Ovarian follicle characteristic were examined using a light microscope (Olympus corp., Japan). For each tissue section, nucleated ovarian follicles were measured and categorized according to methods modified from previous studies (Abascal and Medina 2005; Fortune 2003; Menn et al. 2007) (Table 2). Ovarian tissue sections were stained using HE staining. Thirty nucleated ovarian follicles were selected from each tissue section for follicle stage numbering, and the calculation was repeated in three continuous sections for each individual. Follicle stage percentage was calculated from the repeated counts of the follicle number in IA, IB or IC follicles. The developmental levels from the earliest (IA) to the most advanced (IC) stage is presented as the depth of the grey tint (from light to dark) on the bar graph.

### *in vivo* investigation of *arα*, *arβ*, and *fshr* expression after weekly hormone injection during early ovarian development

Forty eels were randomly distributed throughout nine tanks (500 liters/tank). Three tanks held each of the following three groups: controls ($n$ = 14), weekly SPH injection for three weeks ($n$ = 13), and weekly SPH + MT injection for three weeks ($n$ = 13). All weekly hormone and drug injections were as follows: SPH was dissolved in a 0.9% saline solvent (20 mg/kg), MT (Sigma-Aldrich) was dissolved in a 1% dimethyl sulfoxide (DMSO) (3 mg/kg). The seawater control eels received a weekly saline injection followed by different sampling times based on the experiment. The seawater control eels a received weekly saline injection followed by different sampling times based on the experiment. A weekly hormone injection was carried out following the schedule (Table 2). Ovarian tissue was sampled from female eels 72 hours after the last injection in each group, including controls (female, $n$ = 6; male, $n$ = 8), SPH-injected group (female, $n$ = 9; male, $n$ = 4)

**Table 1.** Procedures for *in vivo* experiments

| Treatments | SPH (20 mg/kg) | MT (3 mg/kg) | Flutamide (6 mg/kg) |
|---|---|---|---|
| *in vivo* experiments (1 dose/ week) | | | |
| Seawater control | - | - | - |
| SPH | 3 doses | - | - |
| SPH + MT | 3 doses | 3 doses | - |
| *in vivo* experiments (1 dose/ week) with AR antagonist | | | |
| Seawater control | - | - | - |
| SPH | 2 doses | - | - |
| SPH + FLUT | 2 doses | - | (1 dose/ every 12 hour) *4 doses after 2 SPH injections |
| MT | - | 2 doses | - |
| MT + FLUT | - | 2 doses | (1 dose/ every 12 hour) *4 doses after 2 MT injections |

and SPH+MT-injected group (female, $n$ = 6; male, $n$ = 8). The gonadal somatic index (GSI) was calculated as (gonad weight/ body weight) × 100. Ovarian tissue was collected for total RNA extraction.

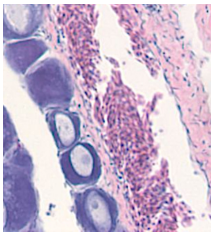
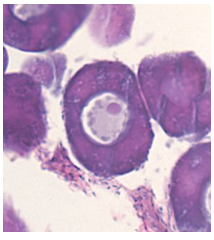
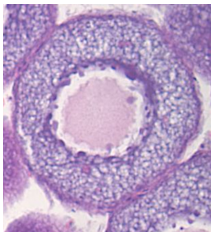
### *in vivo* investigation of *fshr* regulation following treatment with an AR agonist and antagonist in early ovarian development

Fifty eels were randomly distributed throughout ten tanks (500 liters / tank). Two tanks held each of the following five groups: controls ($n$ = 10), SPH ($n$ = 10), SPH+FLUT ($n$ = 10), MT ($n$ = 10), and MT + FLUT ($n$ = 10). All weekly hormone and drug injections were as follows: SPH was dissolved in a 0.9% saline solvent (20 mg/kg). MT (Sigma-Aldrich) was dissolved in a 1% dimethyl sulfoxide (DMSO) (3 mg/kg). FLUT (Sigma-Aldrich) was dissolved in a 1% DMSO (6 mg/kg) (Sigma-Aldrich). The seawater control eels received a weekly saline injection followed by different sampling times based on the experiment. Two weekly SPH injections were utilized to force ovarian growth. FLUT was utilized as an AR antagonist. In this experiment, eels received two weekly hormone injections, then a total of four FLUT doses every twelve hours (Table 2). Ovarian tissues from female eels including controls (female, $n$ = 4; male, $n$ = 6), SPH (female, $n$ = 4; male, $n$ = 6), SPH + FLUT (female, $n$ = 4; male, $n$ = 6), MT (female, $n$ = 7; male, $n$ = 3), and MT + FLUT (female, $n$ = 4; male, $n$ = 6) were sampled 6 hours after the last FLUT injection, respectively, and was collected for total RNA extraction and qPCR analysis.

### Detection of *arα* and *arβ* via *in vitro* incubation with FSH, MT, and FSH + MT after weekly SPH injection for two weeks

Ovarian tissues were taken 6 hours after the second SPH injection from female eels (GSI ≤ 0.9%). Tissue fragments were gently washed twice with eel Ringer's solution (150 mM NaCl, 3 mM KCl, 3.5 mM $MgCl_2$, 5 mM $CaCl_2$ and 10 mM HEPES; pH 7.4), and transferred into a basic culture medium (L-15 medium, 1.7 mM proline, 0.1 mM aspartic acid, 0.1 mM glutamic acid, 0.5% bovine serum albumin, and 10 mM HEPES; pH 7.4). Media contained either FSH (10 ng) (Sigma-Aldrich), MT (5 nM), both FSH and MT or no hormone (control). The final hormone concentration was achieved by serial dilutions with the basic culture medium, and all the L-15 media were filtered with 0.22-μm vacuum-

**Table 2.** Categorization of primary ovarian development in the previtellogenic ovary

| Stage | Characterization |
|---|---|
|  | Stage IA (primordial follicle stage): Oocyte with chromatin-nucleolus and strongly basophilic cytoplasm (deep blue) are displayed with few oil droplets. Development of the oocyte surroundings cannot be observed, and the diameter of the follicles is < 50 μm |
|  | Stage IB (primordial-primary follicle transition stage): The nucleus of the oocyte is transferred from chromatin-nucleolus to perinucleolus and oil droplets accumulate in the cytoplasm. The diameter of the follicles is < 150 μm |
|  | Stage IC (primary follicle stage): All of the ovarian follicles arrest in the perinuleolus stage, and the weak basophilic background of cytoplasm can be seen. The number of oil droplets is increasing in the cytoplasm. Thicker follicular surroundings are shown around the oocyte. The diameter of the follicles is < 200 μm |

driven sterile filters (Merck Millipore Corp.). Ovarian tissue lamellae were cut from ovarian tissue fragments, just submerged in culture media, and incubated at 20 ± 0.5°C. After a 2-hour pre-culture step, ovarian lamellae were randomly distributed throughout the culture dish. Subsequent *in vitro* studies began. To examine serial mRNA variation within 24-h tissue cultures, the ovarian lamellae were incubated in the basic culture medium after the pre-culture step, then sampled after 0.5, 1, 6, 12, and 24 hours. For hormone treatment, the ovarian tissue was incubated with FSH, MT, or FSH + MT media after the pre-culture step. Ovarian tissue lamellae were sampled in triplicate after 1- and 12- hour incubations for total RNA extraction using 0.5 ml ice-cold Trizol solution (Thermo Fisher Scientific, USA). The cDNA library was constructed using pAW109 RNA (Thermo Fisher Scientific, USA) as a standard for relative qPCR.

### Total RNA extraction and cDNA synthesis

Total RNA was purified from homogenized ovarian tissue using Trizol reagent (Thermo Fisher Scientific, USA) according to established procedures. Quantification, purity, and RNA integrity were evaluated by absorbance at 260 and 280 nm using a NanoDrop ND-1000 UV-visible spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis. High-quality RNA with an A260/A280 ratio above 1.8 was used for cDNA synthesis. Total cDNA for the real-time polymerase chain reaction (PCR) was generated from 1 μg of total RNA utilizing the ImProm-II reverse transcription system (Promega Corp.) with random and poly-T primers.

### Quantitative reverse transcription PCR

Ovarian cDNA was serially two-fold diluted to determine PCR efficiency, and the standard was around E > 95%, $R^2$ > 0.95. The cDNA was also four-fold diluted for the real-time PCR reaction. Relative-quantitative real-time PCR analysis using SYBR Green detection was performed on an iQ5 PCR reactor (Bio-Rad, USA) using standard software settings. These included an adaptive baseline for background detection and a moving average and amplification-based threshold settings with the built-in FAM/SYBR filter (excitation wavelength: 492 nm; emission wavelength: 516 nm). Reactions were performed using 5 μl cDNA, 10 nmol forward and reverse primers, and 10 μl SYBR Green Master Mix (Kapa Biosystems, USA) for a total volume of 20 μl. The amplification conditions were 95°C for 3 minutes, followed by 40 cycles of 95°C for 20 seconds and then 59°C for 30 seconds. The melting curve was detected from 55°C to 95°C holding at 30 seconds for each 0.5°C, and a single peak was confirmed as a single qPCR product. Amplification of the eels' 18S rRNA was set as an internal standard for *in vivo* mRNA expression. In addition, M-CSF amplicons were amplified from pAW109 cDNA using GM20 (5'-TCGGACGCAGGCCTTGTCATG-3') and AW111 (5'-GAACAGTTGAAAGATCCAGTG-3') primers as a standard for *in vitro* mRNA expression. The primers for *arα*, *arβ*, *fshr*, and 18S rRNA are shown in table 3.

### Statistical Analyses

Statistical analyses were performed with SPSS Statistics v. 22 (IBM, USA). Significant differences between treatment groups were evaluated using one-way analysis of variance (ANOVA) after testing for normality and variance homogeneity. Statistical differences among groups were analyzed using LSD *post hoc* tests. The significance level was set at $\alpha$ = 0.05, and $p < 0.05$ indicated significant difference. Correlations between mRNA expression levels and GSI

**Table 3.** Primers for the quantitative reverse transcription PCR

| Gene name | Accession number | Primers | Amplicon |
|---|---|---|---|
| AR$\alpha$ | AB023960 | Forward: 5'-CAGCACCTCCTAGACATTGTG-3'<br>Reverse: 5'-CTCAGACGATCTCCTAGTTCGT-3' | 194 bp |
| AR$\beta$ | AB025361 | Forward: 5'-GGAACCCAAGAAAGTGTC-3'<br>Reverse:5'-GCAACCTCATTCGTCAAG-3' | 131 bp |
| FSHR | AB360713 | Forward: 5'-ATCACCGTGTCACACTCCAA-3'<br>Reverse: 5'-CTGGCGAGGATGAAGAAGTC-3' | 122 bp |
| 18SrRNA | AY695889 | Forward: 5'-CGCAAGACGGACGAAAGCGAAA-3'<br>Reverse: 5'-CGGATCGCTAGTTGGCATCGTT-3' | 128 bp |

were analyzed using Spearman's rho correlation.

## RESULTS

### Histological comparison among the control, SPH, and SPH + MT groups

Initial previtellogenic ovaries generally contained the ovarian follicle, which contained a chromatin-nucleolus oocyte with strong basophilic (deep blue) cytoplasmic contents and several oil droplets (Table 2). In addition, a thin and flattened follicular layer surrounded the oocyte with thick connective tissue (Fig. 1A, GSI = 0.42%; 1B, GSI = 0.46%; 1C, GSI = 0.5%; 1D, GSI = 0.75%). Slow initial growth was observed with IA and IB follicles in the control group.

The three weekly SPH and SPH + MT injections forced further follicular development. From these conditions, the hues of ovarian follicles turned a weak basophilic color (light violet) with greater oil droplet accumulation. IC follicles were especially evident in the ovaries of SPH + MT-injected eels. In the SPH-injected eels, IA (primordial follicles, small follicles) and IB follicle were observed in the ovaries (Fig. 1E, GSI = 0.63%; 1F, GSI = 1.02%; 1G, GSI = 1.07%). In contrast, IB (primordial to primary transition) and IC (primary follicle) follicles were apparent in the ovaries of the SPH + MT-injected eels (Fig. 1H, GSI = 0.93%; 1I, GSI = 1.34%; 1J, GSI = 1.38%; 1K, GSI = 1.41%).

Initial ovarian development was characterized based on previous studies (Abascal and Medina 2005; Fortune 2003; Menn et al. 2007), and the percentage of ovarian follicle stage was displayed (Fig. 2). Clear increases in GSI were observed after the SPH and SPH + MT injections ($p < 0.05$; Fig. 2A). In the control group, the ovarian composition showed 13-41% in IA follicles (Fig. 2B) and 59-87% in IB follicles (Fig. 2C), with a GSI ranging from 0.42-0.75% (Fig. 3A). In the SPH-injected group, the ovarian composition (GSI = 0.63-1.07%; Fig. 3B) was 7.8-11% in IA follicles (Fig. 2B), 84-90% in IB follicles (Fig. 2C) and 7.8% in IC follicles (Fig. 2D). In the SPH + MT-injected group, the composition of ovaries (GSI = 0.93-1.41%; Fig. 3C) was 6-7% in IA follicles (Fig. 2B), 81-100% in IB follicles (Fig. 2C) and 9-18% in IC follicles (Fig. 2D). In hormone-responded eels (GSI > 1%), ovarian follicles were seen in the IA, IB and IC stages. Although an eel (GSI = 0.93%) showed GSI < 1% with developing IB follicles, greater IC follicles were observed in most of

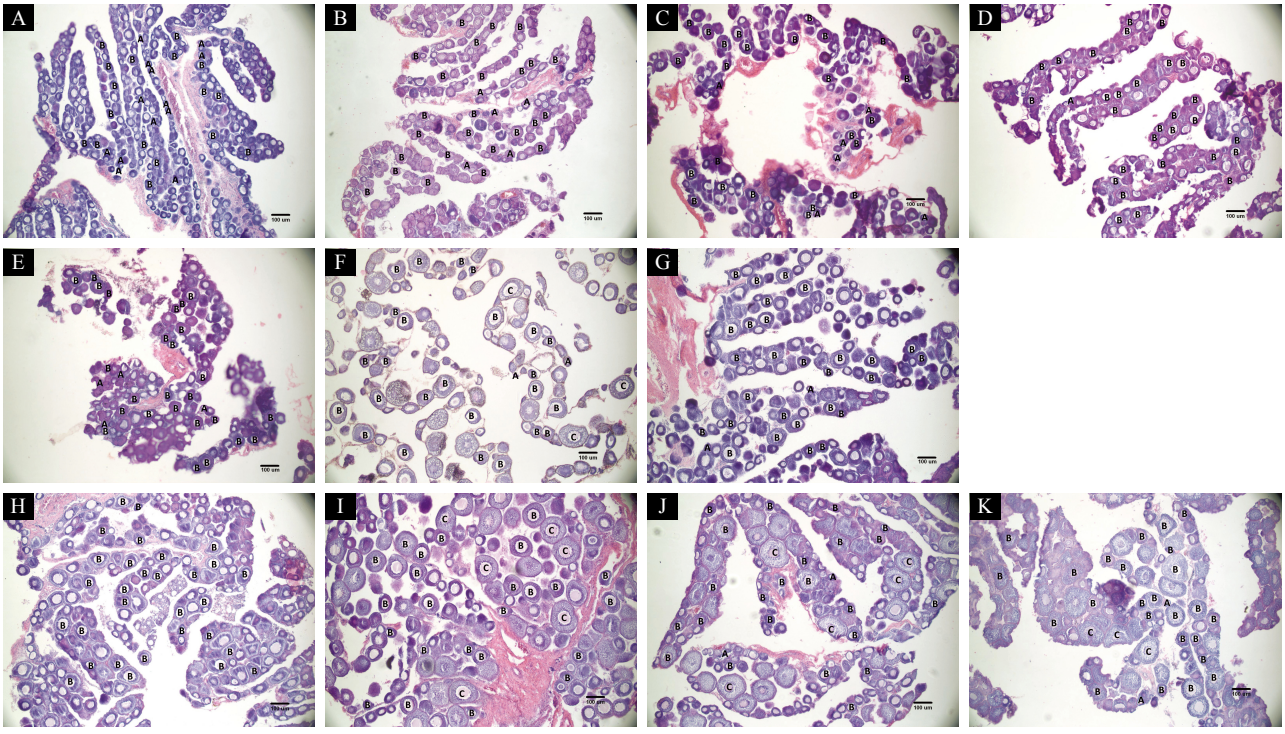



**Fig. 1.** Histological comparison of ovarian development among the control and three weekly SPH- and SPH + MT- injected groups. Histological analyses are shown in controls (A, GSI = 0.42%; B, GSI = 0.46%; C, GSI = 0.5%; D, GSI = 0.75%), weekly SPH injection for three weeks (E, GSI = 0.63%; F, GSI = 1.02%; G, GSI = 1.07%) and weekly SPH + MT for three weeks (H, GSI = 0.93%; I, GSI = 1.34%; J, GSI = 1.38%; K, GSI = 1.41%). Stage IA follicles are labeled as A; Stage IB follicles are labeled as B; Stage IC follicles are labeled as C. Ovarian tissue was sampled 72 hours after the third injection. Sections underwent HE staining. 10-fold magnified LM images were obtained by digital camera photography. Scale bar = 100 μm.

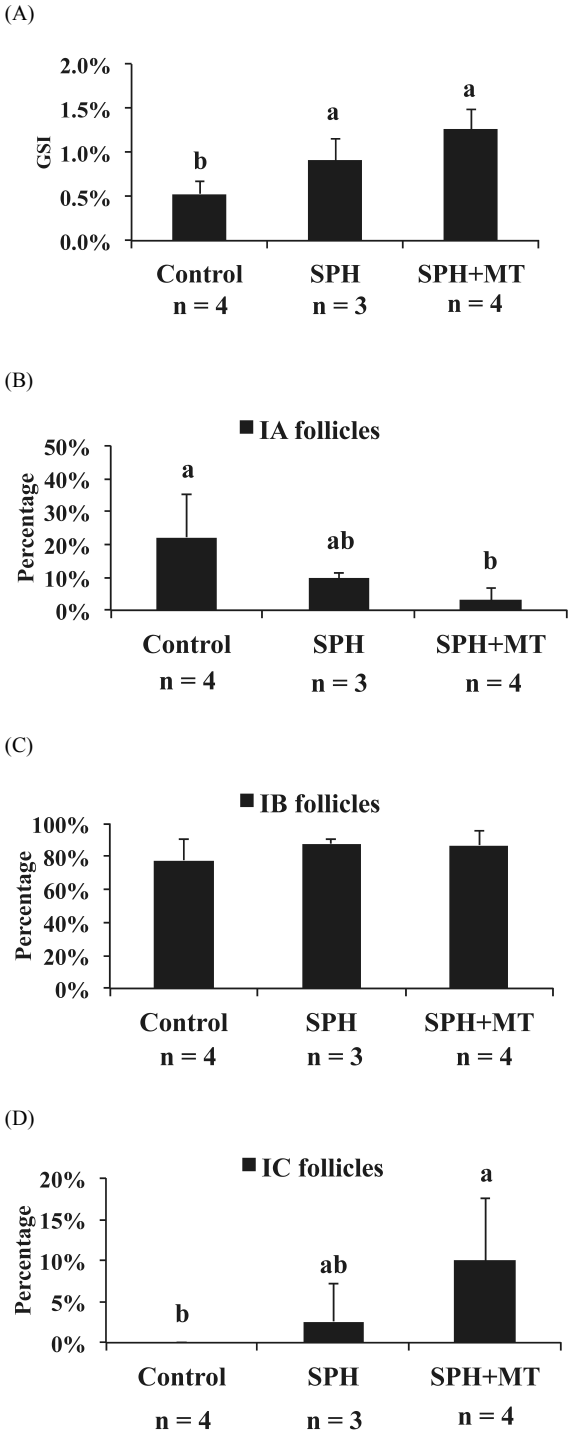



**Fig. 2.** Calculation of follicle stage among female eels undergoing hormonal induction of ovary development. (A) Ovarian development was demonstrated by GSI, and GSI percentage was calculated as mean ± SD (control, $n = 4$; SPH, $n = 3$ and SPH + MT, $n = 4$). Stage IA, IB, and IC follicles were categorized as the characteristics for follicle stage calculation among female eels in the control, weekly SPH-injected, and weekly SPH + MT-injected groups. Each stage calculation is displayed in (B) stage IA follicles, (C) stage IB follicles, and (D) stage IC follicles. Significant differences are compared using one-way ANOVA and LSD *post hoc* tests; $p < 0.05$.

the SPH + MT-injected eels. In contrast to SPH-injected eels, no statistical difference was found between SPH and SPH + MT (Fig. 2D). However, the individual (GSI > 1%) showed greater IC follicles after weekly SPH + MT injections (Fig. 3B, C).

### Detection of *arα*, *arβ*, and *fshr* expression after three weekly artificial hormone injections

Relative *fshr* expression levels in the *in vivo*

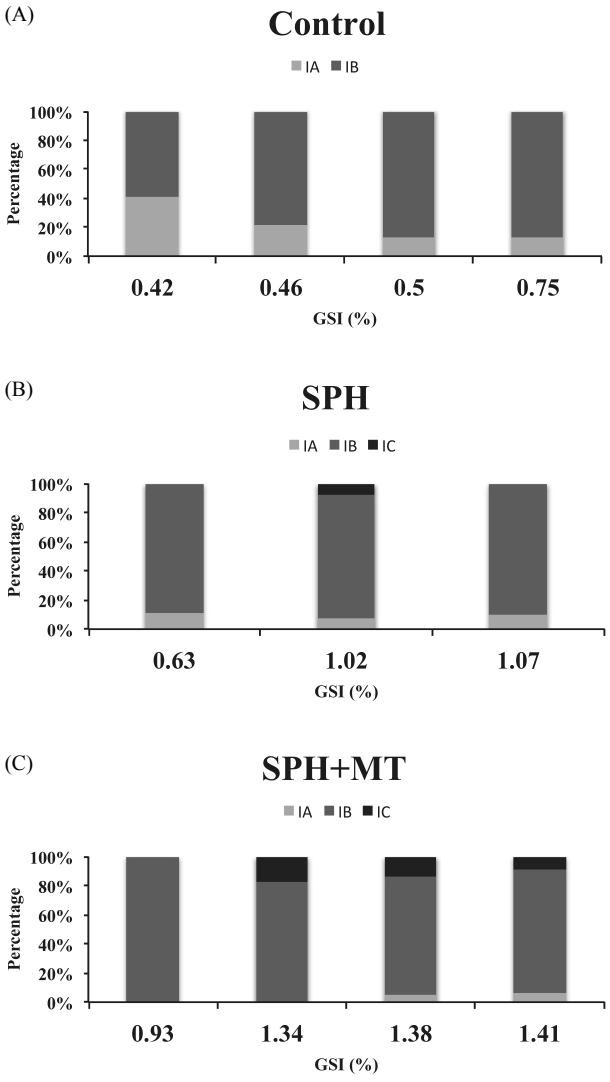



**Fig. 3.** The ovarian composition of female eel undergoing hormonal induction in three stages: IA, IB, and IC (the grey tint from light to dark). Stage was categorized as the characteristics for follicle stage calculation. GSI was measured and is shown in each individual. The ovary composition is shown in each percentage bar. The stage composition is shown among female eels in (A) the control group, (B) weekly SPH injection for three weeks and (C) weekly SPH + MT injection for three weeks.

hormone induction experiments increased significantly ($p < 0.05$) in the SPH + MT injection group compared to other groups (Fig. 4A). *arα* expression levels were not significantly different among groups (Fig. 4B), whereas *arβ* showed high levels in the SPH-injected group ($p < 0.05$; Fig. 4C). The above data were analyzed by plotting mRNA expression against GSI data. Limited ovarian development was observed (GSI < 1.2%) among the six eels in the control group (Fig. 5A, D and G), and *arα* expression showed a significant positive correlation with slight increases in GSI ($p < 0.05$, $r = 0.94$). In addition, a non-significantly negative trend was seen between *fshr* expression and GSI (Fig. 5A). Following the three weeks of SPH injections (Fig. 5B, E and H), further development of eels' ovaries was observed, and *arβ* showed a strong positive correlation with GSI ($p < 0.05$, $r = 0.97$) after SPH injection. Although increased GSI was detected in the SPH + MT group (Fig. 5C, F and I), it was negatively correlated with *arα*, *arβ*, and *fshr* expression levels. Individuals in the SPH + MT group showed high levels of *fshr* expression compared to the control and SPH groups.

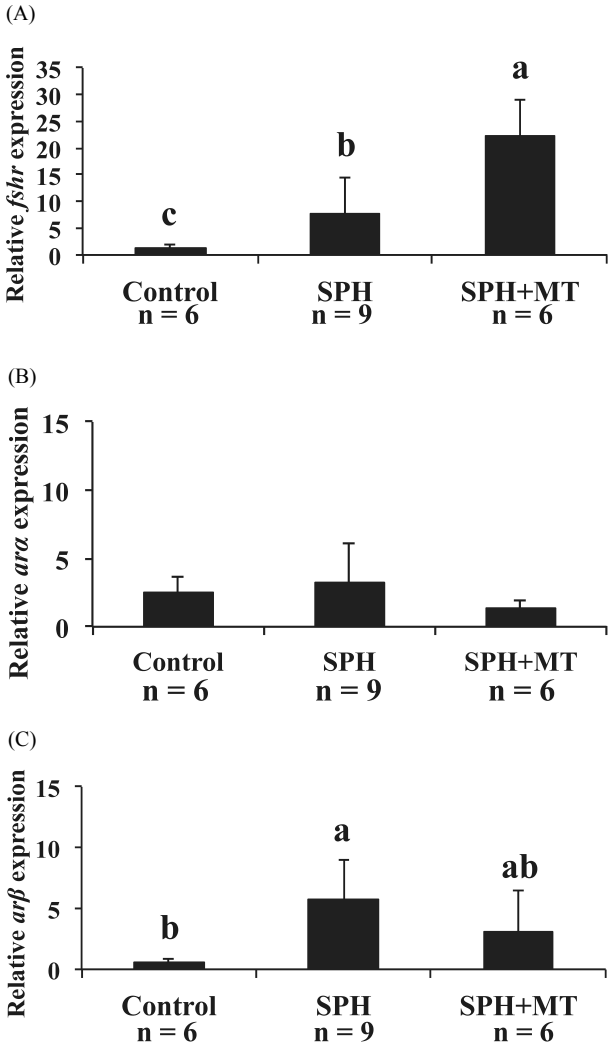



**Fig. 4.** *in vivo* mRNA expression of *arα*, *arβ*, and *fshr* after weekly hormone injection for three weeks. Relative mRNA expression of (A) *arα*, (B) *arβ*, and (C) *fshr* among the control ($n = 6$), SPH ($n = 9$) and SPH + MT ($n = 6$) groups. The mRNA of 18S rRNA was used as the internal standard for relative mRNA normalized quantification. The mRNA expression data from female eels' ovaries were collected and calculated as mean ± SD. Statistically significant differences are identified by one-way ANOVA and LSD *post hoc* tests. $p < 0.05$ is considered significant.

### *in vivo fshr* expression after treatment with AR agonist and antagonist

The above weekly hormone injection experiments demonstrated relatively high levels of *fshr* in the SPH + MT group. In this study, MT and FLUT function as an agonist and antagonist for AR, respectively. Expression levels of *fshr* significantly increased following weekly MT and SPH injections for two weeks ($p < 0.05$; Fig. 6A, B), while *fshr* levels dropped after 4 doses of FLUT in the SPH + FLUT ($p < 0.05$; Fig. 6A) and MT + FLUT (Fig. 6B) groups.

### Expression of *arα* and *arβ* in ovarian tissue cultures with and without hormones

Ovarian tissue was cultured eight hours after the second weekly SPH injection. *arα* and *arβ* expression levels were scanned within a 24-hour *in vitro* incubation period (Fig. 7A, B). *arα* and *arβ* expression levels were relatively lower and stable under the no-treatment condition (Fig. 7A, B). Therefore, the cultured tissue was sampled after 1 and 12 hours of incubation, and non-treated samples were set as time-match controls. After 1 hour of incubation, *arα* and *arβ* showed high expression levels in FSH + MT-treated tissue (Fig. 8A, C). Furthermore, *arα* and *arβ* showed different mRNA expression patterns after the 12-hour incubation. *arα* expression remained at a significantly high level ($p < 0.05$) in FSH + MT-treated tissue, and a slight increase in *arα* ($p < 0.05$) was also observed in FSH-treated tissue (Fig. 8B). In addition, *arβ* expression dropped after 12 hours in the FSH + MT-treated tissue, whereas the expression was maintained at a higher level in the 12-hour MT-treated tissue (Fig. 8D).

## DISCUSSION

Significantly high levels of androgens are detected in the plasma of both wild (Lokman et al. 1998) and SPH-injected female eels (Matsubara et al. 2005) at

the onset of natural spawning migration. In teleosts, androgens are the major hormones that affect male reproduction (Miura et al. 1991). Androgens were previously thought to be a potent precursor for estrogen synthesis, and increasing evidence has revealed that androgens affect reproductive fertility in females (Kortner et al. 2009; Prizant et al. 2014; Walters et al. 2008). The current study shows that androgen/AR action

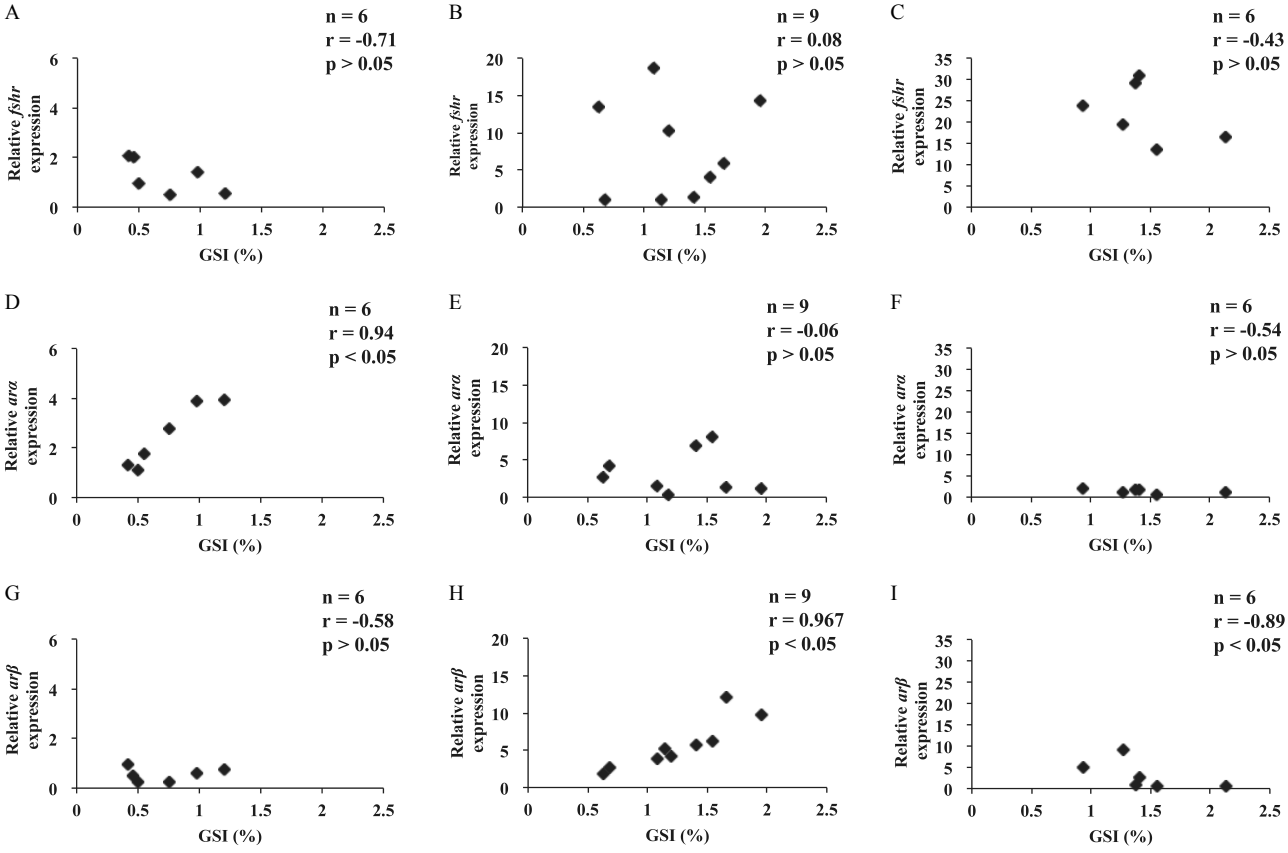



**Fig. 5.** Correlations between gene (*arα*, *arβ*, and *fshr*) expression and GSI. Correlations between gene expression and GSI are displayed. (A, B, and C) are correlations between *fshr* expression and GSI. (D, E and F) are correlations between *arα* expression and GSI. (G, H, and I) are correlations between *arβ* expression and GSI. The three groups displayed are the control (A, D, and G; $n = 6$), weekly SPH injection for three weeks (B, E and H; $n = 9$) and weekly SPH+MT injection for three weeks (C, F and I; $n = 6$). The correlation between mRNA expression and previtellogenic ovary growth condition was analyzed using Spearman's rho correlations. GSI is plotted against the mRNA expression level. $p < 0.05$ indicates significant correlation, and r-value represents the positive or negative correlation coefficient.

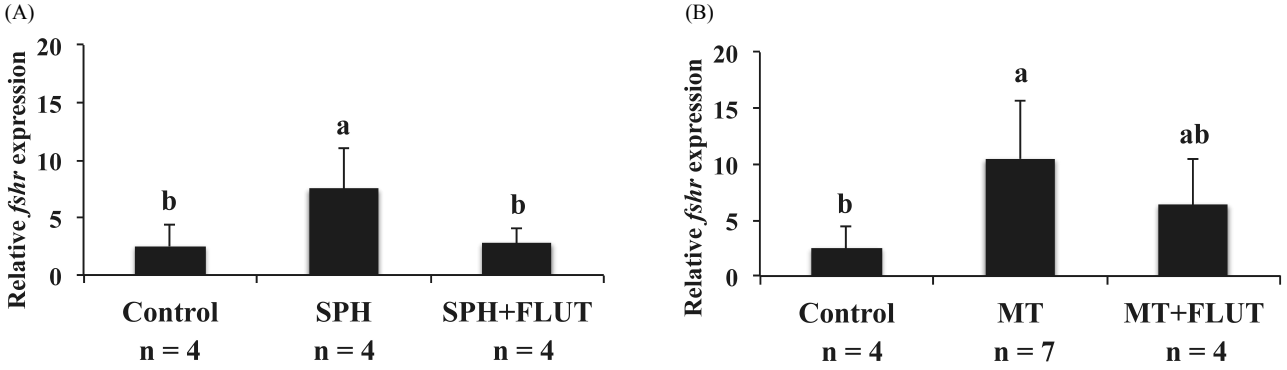



**Fig. 6.** *in vivo* mRNA expression of *fshr* following AR's agonist and antagonist treatments. Ovarian tissue was collected from female eels. (A) Controls, $n = 4$; SPH, $n = 4$; SPH + FLUT, $n = 4$. (B) Controls, $n = 4$; MT, $n = 7$; MT + FLUT, $n = 4$. The mRNA expression data from female eels' ovaries are presented as the mean ± SD, and statistically significant differences were determined via one-way ANOVA and LSD *post hoc* tests ($p < 0.05$).

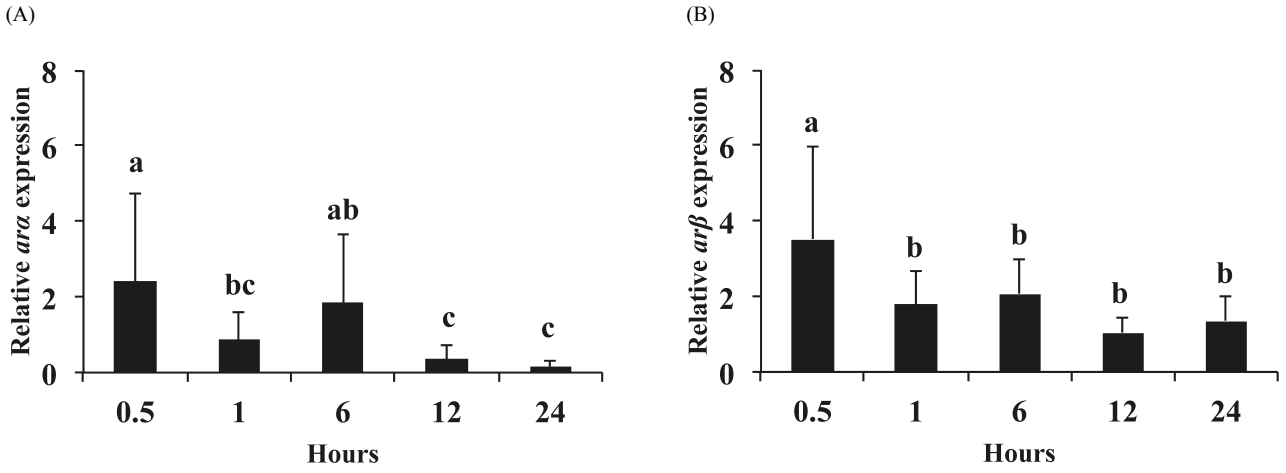



**Fig. 7.** *in vitro* detection of *arα* and *arβ* expression during ovarian tissue culture within 24 hours. The serial time course of mRNA expression was screened within 24 hours. mRNA expression patterns of (A) *arα* and (B) *arβ* within 24 hours in L-15 medium tissue culture without treatment. Quantitative mRNA expression data are presented as the mean ± SD, and statistically significant differences were determined by one-way ANOVA and LSD *post hoc* tests ($p$ <0.05).

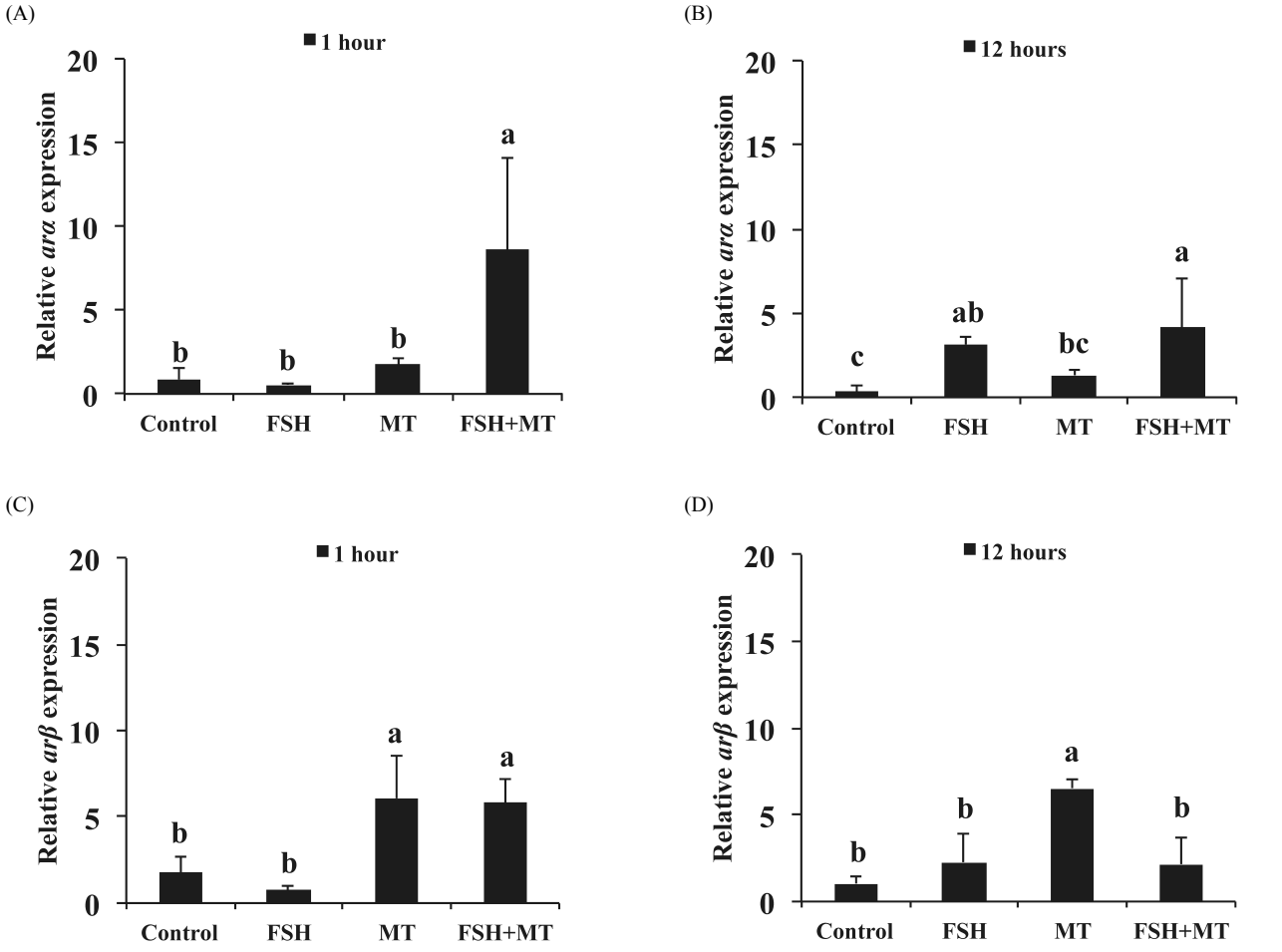



**Fig. 8.** *in vitro* detection of *arα* and *arβ* expression during ovarian tissue culture with and without hormone treatment. mRNA expression was detected after FSH, MT, or FSH + MT treatments. *ara* expression is shown after (A) 1 and (B) 12 hours of hormonal treatments; *arβ* expression is shown after (C) 1 and (D) 12 hours of hormonal treatments. Relative mRNA expression data are presented as mean ± SD, and statistically significant differences were determined by one-way ANOVA and LSD *post hoc* tests ($p$ < 0.05).

affects gene expression in early previtellogenic ovary development. This androgenic modulation was studied through *in vivo* or *in vitro* MT/ flutamide treatment. The interaction between androgen/AR and FSH/ FSHR actions is seen in early ovarian development, and androgen/AR actions may play an important role in modulating primary follicle development and maintaining ovarian follicle growth.

The regulation of initial ovarian development is functionally and structurally conserved across evolution in vertebrates (Das and Arur 2017; Grier et al. 2016). However, in the viviparous ovary, rigorous selection of dominant follicles takes places before antral follicle formation (McGee and Hsueh 2000). On the other hand, millions of ovarian follicles maintain further vitellogenic development for the ovarian maturation in semelparous eels. The effect of primary growth on ovarian fecundity and fertility remains to be studied in oviparous eels.

In female eels, primordial follicle and the primordial to primary transition follicle are generally found in the ovary (GSI < 0.75%), and this condition is always observed during the previtellogenic developmental state. In hormone-responding eels (GSI > 1%), the SPH + MT-injected eels showed higher GSI than the SPH-injected individuals. Although primordial to primary transition follicles were found in SPH-injected and SPH + MT-injected eels, more primary follicles were observed with the main stage of the primordial to primary transition follicle in SPH + MT-injected eels.

Previous studies on primates have revealed that the pool of primordial follicles is a critical source of developing follicles over the entire reproductive life (Hansen et al. 2008). Relevant gene deficiencies of primordial-follicle activation lead to premature ovarian failure and female infertility (Reddy et al. 2008; Skinner 2005). Gonadotropins (GtHs) are the major element of pituitary hormones that affect ovarian development. Recently, sequences of FSHR cDNA were isolated and identified in female Japanese eels' ovaries (Kazeto et al. 2012). In teleosts, FSHR-deficient female fish are infertile because vitellogenesis is inhibited from the earlier stage of ovarian development (Murozumi et al. 2014). In female zebrafish, the expression of FSHR mRNA is detected in the primary growth follicles (Kwok et al. 2005); furthermore, disrupted *fshr* expression causes ovarian development to fail (Zhang et al. 2015). In mammals, several studies have reported that FSHR is present during early follicle growth in hamsters, pigs, baboons, and cows (Goxe et al. 1993; Roy and Albee 2000; Zachos et al. 2003; Wandji et al. 1992).

Extreme variation in *fshr* expression was seen among individuals of SPH-injected eels. In contrast, *fshr* expression levels were significantly higher after SPH + MT injection, but showed an insignificant, negative correlation with ovary development. In SPH + MT-injected eels, high *fshr* expression levels were observed, and the primary follicles were apparent in developed ovary.

Similar regulation was shown in a study of FSHR expression following flutamide treatment in porcine ovaries, and researchers proposed that FSH action is essential for primordial follicle development (Durlej et al. 2011). Another study also reported that FSHR is involved in primordial follicle recruitment (Allan et al. 2006; Balla 2003). In addition, a highly significant positive correlation between FSH and granulosa cell AR mRNA expression was observed in the primate ovary, in which FSHR mRNA levels increased with androgen treatment (Weil et al. 1999). In a study of the porcine ovary, high levels of AR and FSHR mRNA are observed in small antral follicles and their expression levels decreased with increasing P450 aromatase mRNA and follicular growth (Słomczyñska and Tabarowski 2001).

This study reveals that MT (an androgen and AR agonist) might regulate *fshr* expression. *fshr* expression levels were increased via two weekly MT injections, whereas mRNA levels were lowered via FLUT treatment after two weekly hormone injections. These experiments demonstrate that the eel's ARs regulate androgens in *fshr* expression.

In addition, a previous study reported that AR mRNA levels significantly increased during primordial to primary follicle growth in the bovine ovary (Hampton et al. 2004). Moreover, *in vitro* studies of bovine ovaries have demonstrated that ovarian follicles failed to enter primary growth since most are still not arrested in meiotic prophase I (Yang and Fortune 2008). This study suggests that oocytes have achieved meiotic prophase I arrest before the primordial to primary follicle transition. A similar function was reported in primate studies, in which androgen treatments regulated significant increases in primordial to primary follicle recruitment (Vendola et al. 1999). This process may be mediated by IGF-I signaling (Vendola et al. 1999). Another study of primates has also shown a positive correlation between AR gene expression and cell proliferation, and a negative correlation between AR gene expression and programmed cell death (Weil et al. 1998).

In Japanese eels, testosterone (T) has been reported to have a positive role in ovarian development (Lin et al. 1991). Our previous study revealed that ovarian follicle numbers during long-term SPH + MT induction may be similar to those of the control group (Wang and Lou 2007). In addition, 11-KT synthesis has been reported in female eels, and 11-KT may affect

lipid droplet accumulation and transportation in the previtellogenic (PV) ovary (Matsubara et al. 2003). A recent study showed that T supplements inhibit PTEN, and this inhibition occurs during the primary oocyte transition (Huang et al. 2012). Androgen actions emerge during ovarian development in female eels.

Two types of ARs have been isolated by cDNA cloning in Japanese eels (Ikeuchi et al. 1999; Todo et al. 1999). According to a report (Tosaka et al. 2010), these two ARs were found and detected in the follicular layer ovaries. However, there is no difference between the two ARs' expressions. In the present study, *arα* expression showed a significant positive correlation with increased GSI before SPH injection, whereas the expression of *arβ* exhibited a significant positive correlation after SPH injection. Observations of the control and SPH-injected eel ovaries indicate that *arα* and *arβ* may carry out respective functions during early previtellogenic development. *arα* was strongly expressed during the primordial to primary follicle transition, and *arβ* was expressed in SPH-forced ovarian development. In addition, both *arα* and *arβ* were suppressed with increased GSI after SPH+MT injection; nevertheless, synchronous follicle development is apparent in the ovaries. AR expression may be constantly maintained, and function as the their basic activation in SPH + MT-treated ovary.

Expression of *arα* and *arβ* showed high levels in the one-hour FSH + MT treatment. In addition, high *arβ* expression levels were shown in the MT treatment after one hour of incubation. It is still unclear whether autoregulation of AR expression (Bagamasbad and Denver 2011) occurs in the teleost ovary.

Early ovarian development is regulated by numerous important intra-ovarian factors (Adhikari and Liu 2009), while AR*α* and AR*β* are crucial for this mechanism. Androgens play important roles in initiating ovarian development and in modulating synchronous development by ARs in female eels. The innate expression of two ARs (AR*α* and AR*β*) in the eel ovary differs from that of only one AR in other species (Douard et al. 2008; Ogino et al. 2009). Thus, their distinct functions in the eel ovary needs to be verified.

## CONCLUSIONS

The present study demonstrates that androgen action modulates initial development in the previtellogenic ovary. Although SPH injection is a pivotal method for ovarian development, greater synchronous development is observed in the MT co-treated ovary. Moreover, the androgen/ ARs action significantly affects *fshr* expression. *in vivo* expression of both *arα* and *arβ* showed significantly different patterns before and after SPH injections. These results suggest that androgen actions may be involved in the global functions of FSH/FSHR and modulate the development from early previtellogenic to vitellogenic ovary. Androgen may modulate the fecundity and fertility in the semelparous eels. The adoption of additive androgen with SPH injection is necessary to further understand the artificial induction of eel gonadal development and maturation.

### List of abbreviations

SPH, Salmon pituitary homogenate.
GtH, Gonadotropin.
AR*α*, Androgen receptor alpha.
AR*β*, Androgen receptor beta.
FSHR, Follicle-stimulating hormone receptor.
FSH, Follicle-stimulating hormone.
MT, 17*α*-methyltestosterone.
FLUT, Flutamide.
DMSO, Dimethyl sulfoxide.
GSI, Gonadal somatic index.
PV, Previtellogenic stage.

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**Authors' contributions:** SCL and SWL designed the experiments and wrote the manuscript. SCL carried out the field experiments. SWL revised the manuscript and performed data analysis.

**Competing interests:** The authors declare that they have no competing interests.

**Availability of data and materials:** All the data are provided within the manuscript.

**Consent for publication:** Not applicable.

**Ethics approval consent to participate:** The condition of the experimental animals was reviewed by the Animal Research Committee of the National Taiwan University (approval number NTU-103-EL-74).

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