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Androgen induces olfactory expression of prostaglandin E_2 receptor Ep1 in the burrow-living fish *Bostrychus sinensis*

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ABSTRACT

It is well documented that androgens modify olfactory processing in vertebrates. In fish, several lines of evidence indicate that androgens increase olfactory sensitivity to prostaglandin pheromone, but the molecular mechanism is still unclear. Our previous studies showed that prostaglandin E_2 (PGE_2) is a sex pheromone in the burrowing-living fish Chinese black sleeper (*Bostrychus sinensis*) and that the PGE_2 receptor 1 (Ep1) in the olfactory rosette is a candidate receptor for sensing sex pheromone PGE_2. In the present study, we found that testosterone (T) and 11-ketotestosterone (11-KT) exhibited stimulatory effects on the expression of *ep1* in the olfactory rosette *in vivo* and *ex vivo*. Moreover, the androgen receptor (Ar) agonist R1881 had similar effects to 11-KT on the expression of *ep1 ex vivo*, suggesting the up-regulatory effect is mediated by Ar. The amount of *arα* transcripts (˜1500 copies/100 ng total RNA) was greater than that of *arβ* (˜300 copies/100 ng total RNA) in the olfactory rosette, and the expression levels of *arα* increased with spermatogenesis and peaked at late meiosis stage. Moreover, activated Arα but not Arβ transactivated a 2k bp *ep1* promoter in HEK293T cell, and some OSNs exhibited co-localization of *arα* mRNA and Ep1 protein signals. Taken together, our results suggest that Arα, but not Arβ, plays a crucial role in mediating the androgen-induced up-regulation of *ep1* expression in *B. sinensis*. The present study is the first to shed light on the molecular mechanisms whereby androgens enhance responsiveness to prostaglandin sex pheromones in teleosts.

1. Introduction

Fish commonly employ pheromones to regulate a variety of functions including reproductive communication [1]. Reproductive pheromones induce both primer effects, such as changes in the endocrine or physiological state of conspecifics, and releaser effects such as rapid behavioral responses [2,3]. For example, in male goldfish (*Carassius auratus*), the pheromone 4-pregnen-17α,20β-dihydroxy- progesterone (DHP) triggers both primer increases in GnRH-II mRNA level in the telencephalon, luteinizing hormone (LH) in the plasma and milt volume [4–6] and releaser effects on socio-sexual behaviors [43]. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a key regulator of ovulation in many teleost (see review [7]), is another well-known teleost reproductive pheromone inducing both primer effects on milt production and releaser effects on sexual behavior in male fish [8,9]. However, in some teleost species including medaka (*Oryzias latipes*), prostaglandin E_2 (PGE_2) is involved in the ovulatory process [10,11]. In these fish species, PGE_2 instead of $PGF_{2\alpha}$ might serve as a sex pheromone.

Numerous studies showed that pheromonal responsiveness, including electro-olfactogram (EOG) responses, vary with reproductive status ([12–16]). It is believed that this effect of reproductive status on pheromonal responsiveness is regulated by endocrine factors [1,15,17]. Androgens are important gonadal hormones mediating the reproductive activity in male fish [18,19] and also an endocrine factor modulating pheromonal responsiveness ([12–16]). In *Epalzeorhynchos bicolor*, *Carassius auratus*, *Danio rerio* and juvenile *Puntius schwanenfeldi*, androgen treatment increases the magnitude and sensitivity of the EOG response to pheromones prostaglandins [5,15]. However, the molecular mechanism for the regulatory effects of androgens on odorant receptors is unclear. One possibility is that androgens bind receptors found in the olfactory epithelium [20], leading to increases in the number of pheromone receptors and/or varying sensitivity of the receptors [5].

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The biological activity of androgens is mediated by the androgen receptor (AR) which belongs to the nuclear receptor family, and exerts its function as a ligand-activated transcription factor to regulate the transcription of androgen target genes. The main ligand for the Ar in teleost fishes is 11-KT, while in humans testosterone (T) and the even more potent hormone 5α-dihydrotestosterone are the active androgens [3,21,22]. Unlike most vertebrates, which express a single AR gene, two distinct Ar genes have been identified in many teleosts, *e.g.* Arα and Arβ [23–25]. The subtype designated Arβ is more closely related to AR from mammals, birds and reptiles, while Arα exhibits sequence divergence [23–25]. Arα and Arβ have different transactivation potencies in response to androgens [24,25], and they exhibit unique expression patterns in some species [26,27]. Moreover, the sequence similarity between the DNA-binding domain (DBD) of Arα and Arβ is only around 70%, which suggests different biological functions of Arα and Arβ in teleosts.

The Chinese black sleeper, *Bostrychus sinensis*, belongs to the family Eleotridae, suborder Gobioidei. This species is a burrowing animal and inhabits intertidal zones [28]. As a seasonal breeding fish, females and males live in individual burrows during the non-spawning season, but spawn as a pair inside the same burrow during the spawning season [29]. This spawning behavior and burrow-living habit suggest that mature males and females may employ sex pheromones to synchronize gametogenesis, spawning and fertility, since visual communication seems less efficient inside the burrow. Our previous studies showed that the levels of PGE_2 were obviously higher than those of $PGF_{2\alpha}$ in ovarian extract and urine at sex maturation stage [32], which suggested that PGE_2 may be involved in the ovulatory process, and serves as a sex pheromone in *B. sinensis*. This hypothesis was further proved by our study which showed that nests with a PGE_2-releasing tube attract more males and females and have in a higher spawning rate than control nests [29]. In addition, both mature male and female *B. sinensis* display greater EOG response to PGE_2, compared to immature fish [30], which indicates that PGE_2 is a putative sex pheromone in *B. sinensis* and that the olfactory sensitivity to PGE_2 depends on the reproductive status. According to transcriptomic data, three subtypes of PGE_2 receptor mRNAs (*ep1*, *ep2* and *ep4*) were expressed in the *B. sinensis* olfactory rosette. The *ep1* mRNA levels in the olfactory rosette of mature fish were significantly higher than those in immature fish [31], suggesting that the distinct olfactory sensitivities to PGE_2 might be due to the changes of Ep1 levels [31]. However, it is not clear if androgens contribute to the up-regulation of *ep1* expression in male fish.

In the present study, we first examined the *in vivo* and *ex vivo* effects of androgens on *ep1* expression in the olfactory rosette of male *B. sinensis*. Thereafter, we cloned two different *B.sinensis ar* cDNAs and investigated their roles in the effects of 11-KT on *ep1* expression.

2. Material and methods

2.1. Experimental fish and chemicals

Male adult *B.sinensis* (150–169 mm; 72–108 g) were collected from aquaculture farms in Dadeng Island, Fujian, China. Stock solutions of 11-KT (Steraloids, Newport, USA), methyltrienolone (R1881) (Sigma–Aldrich, China) and T (Dalian Meilun Biotech Co., Ltd, China) were prepared by dissolving the respective chemical compounds in 100% ethanol and stored at -20 ℃ before use. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Xiamen University.

2.2. Co-injection of hCG and LHRH-A3

Male fish at late meiosis stage were randomly divided into two groups with 8 individuals each. Fish were then acclimated for one day before the experiment. In the experimental group each fish was injected intraperitoneally with 200 µl phosphate-buffered saline (PBS) containing 700 IU human chorionic gonadotropin (hCG) and 7 µg LHRH-A3 (Ningbo Shansheng Pharmaceutical Co., China), an efficient technique to induce maturation in male *B. sinensis* [32,33]. A second injection was administrated 24 h after the first injection. The olfactory rosettes were surgically dissected and immediately frozen in liquid nitrogen 24 h following the second injection. Frozen samples were stored at –80 ℃ until analyses.

2.3. In vivo exposure to androgens

Plastic tanks (15 l) with seawater at a salinity of 15 ppt were used as the experimental containers. Fish at stage III were released into tanks and then acclimated for one day before the experiment. The fish were not fed during the experiment. Vehicle or androgens (final concentrations: 5, 50 or 100 ng/ml 11-KT [or T] in 6.67×10^{-7}% [v/v] ethanol) were applied to the fish tanks. After exposure for 24 h and 48 h, the fish were anesthetized and the olfactory rosettes were collected to determine the progestin receptors and *ep1* mRNA levels. Total RNA was extracted using the RNAzol reagent (Molecular Research Center Inc., Cincinnati, USA). The same amount of total RNA (1 µg) was used for synthesis of the first strand cDNAs using the RevertAid first strand cDNA synthesis kit (Thermo Scientific, USA). Real-time qPCR was performed as described in Section 2.14.

2.4. Olfactory rosette exposure to sex steroids ex vivo

Olfactory rosettes of male fish were removed, washed three times with Hank's balanced salt solution in a 24-well plate (NEST, USA), and then incubated individually in basal culture medium consisting of 15 mg/ml Leibovitz's L-15 medium (Invitrogen, USA), supplemented with 10 mM Hepes (Merck, Germany), 0.5% w/v BSA (MULT Sciences, China), 200 U/ml penicillin and 200 mg/l streptomycin (Invitrogen, USA); pH was adjusted to 7.4 with NaOH. Olfactory rosette samples were incubated for 24 or 48 h in basal culture medium containing 0.5 or 5 ng/ml 11-KT, or 0.0001% (v/v) ethanol as control. Incubation was carried out in a humidified air atmosphere at 26 °C.

In addition, to study whether the stimulatory effects of 11-KT on *ep1* transcript levels were mediated *via* Ar, olfactory rosette samples were incubated for 48 h in basal culture medium with 5 ng/ml R1881 or 0.0001% (v/v) ethanol. At the end of incubation, the olfactory rosettes were collected for gene expression analysis as described above.

2.5. Cloning of androgen receptor cDNAs and 5′-flanking regions of the ep1 gene

Total RNA of testis was extracted using the RNAzol reagent (Molecular Research Center Inc., Cincinnati, USA) and reverse transcribed into first strand cDNA using the SMARTer® RACE cDNA amplification kit (Clontech, China) following the manufacturer's instructions. The partial sequences of *B.sinensis ar*s were obtained from brain and olfactory rosette transcriptomic data (unpublished). In order to confirm the sequence, two primers were designed (Supplemental Table 1). Then the PCR amplification was carried out in 20 µl volume under the following cycling conditions: 94 ℃ for 3 min (1 cycle); 94 ℃

for 30 s, 56 ℃ for 30 s and 72 ℃ for 1 min (35 cycles) followed by a final extension step at 72 ℃ for 10 min. All PCR products were purified from agarose gel and sub-cloned into vector pmd19-t (TAKARA, Japan), and then transformed into *Escherichia coli* DH5α (Promega, Madison, WI, USA). The plasmid DNA of several positive clones was prepared for DNA sequencing (Invitrogen Ltd, Guangzhou, China). Based on the partial cDNA sequence obtained, gene-specific primers were designed for further extension by 5′- and 3′-RACE. The first PCR amplification for 5′ or 3′ RACE was performed using a universal primer in the kit and a gene specific primer. If no specific band was obtained, these initial 5′ or 3′ RACE products were diluted and used for nested PCR amplifications with gene-specific nested primers, in combination with a nested universal primer. All RACE reactions were carried out following the manufacturer's instructions. RACE products were sub-cloned and sequenced. The 5′-flanking regions of the *ep1* gene were isolated using the Universal Genome Walker Kit (Clontech) according to the manufacterer's recommendations.

2.6. Phylogenetic analysis of androgen receptors

After obtaining the two *B.sinensis ar* cDNAs, a BLAST homology search was performed using the deduced amino acid sequences. The alignment of known and *B.sinensis* Ars was performed using MEGA 7.0 program and the Clustal W method. Then a phylogenetic tree was constructed using the neighbor-joining method with a bootstrap value of 1000 trials for each position.

2.7. Tissue-specific expression of ar transcripts

Four adult male and female fish were anesthetized and humanely decapitated. The brains were trimmed to collect the olfactory bulb, telencephalon, diencephalon, mesencephalon, cerebellum, and medulla oblongata separately. The olfactory rosette, gill, heart, intestine, liver, spleen, skin, muscle, and gonad were also collected and immediately dipped into liquid nitrogen and stored at −80℃ until further analysis. Total RNA extraction and cDNA synthesis were conducted as described in Section 2.3 and real-time qPCR was performed as described in Section 2.14.

2.8. Differential expression of ar transcripts in the olfactory rosette during spermatogenesis

From December 2014 to July 2015, six male adult fish were anesthetized and humanely decapitated each month. The olfactory rosettes were collected and immediately dipped into liquid nitrogen and stored at -80 ℃. Total RNA extraction and cDNA synthesis were conducted as described in Section 2.3 and real-time qPCR was performed as described in Section 2.14. The testes were removed, weighed to calculate the gonadosomatic index (GSI), and fixed in Bouin's reagent overnight for conventional histology examination. Briefly, the fixed testes were dehydrated through a graded series of ethanol concentrations (70–100%), embedded in paraplast (Leica, Germany), sectioned (5 μm) on a retracting microtome (Leica, Germany) and stained with hematoxylin. Testicular development was classified into five stages according to analyses of histology sections: stage I (spermatogonial proliferation stage), stage II (early meiosis stage), stage III (mid meiosis stage), stage IV (late meiosis stage) or stage V (maturation stage) [33,34].

2.9. Plasmid construction

A 2176-bp fragment representing the 5′-flanking sequences of the *B. sinensis ep1* gene was obtained by PCR using the forward and reverse *ep1* promoter primers (Supplemental Table 1). The PCR product was digested with *Nhe* I and *Nco* I (New England Biolabs, USA) gel-purified and subcloned into the *Nhe* I- and *Nco* I-cut pGL3-basic vector (Promega, USA).

The full-length open-reading frames of the *B. sinensis arα* and *arβ* were obtained by PCR using a pair of primers (Supplemental Table 1) containing two restriction enzyme cutting sites, namely *Hind* III and *Eco* RI at their ends. After double digestion with *Hind* III and *Eco* RI restriction endonucleases, the digested products were cloned into pcDNA3.1 + vector (Invitrogen). Using the same strategy but with different primers (see Supplemental Table1), *B. sinensis arα* and *arβ* open-reading frames were cloned into pEGFP-N1 plasmid for subcellular localization. All constructs were verified by sequencing and all plasmid DNA used in the present study were prepared using the QIAfilter Plasmid Mini Kit (Qiagen, USA).

2.10. Cell culture and transient transfection experiments

Cell culture materials and transfecting agent were purchased from Life Technologies (USA) unless specified. Human embryonic kidney cells (HEK 293T) were used for the transactivation assays. Cells were maintained in phenol red-free Dulbecco's modified eagle's medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin at 37 ˚C with 5% CO_2.

For transient transfection, lipofectamine 3000 (Life Technologies) were used according to manufactures' protocols. For example, 1.0×10^5 cells per well was seeded on 24-well plates 12 h prior to transfection. Cells were then transfected with 300 ng firefly luciferase reporter plasmid (either the 2176-bp 5′-flanking sequence of the *B. sinensis ep1* gene in PGL3-basic or MMTV-luc) [22], 100 ng of Arα or Arβ expression vector, and 30 ng of the pRL-TK vector containing the *Renilla* luciferase reporter gene (as a control for transfection efficiency). After 6–8 h, the medium was aspirated and replaced with complete growth medium.

2.11. Luciferase assay

After transfection, the medium was replaced with luciferase assay medium (DMEM without phenol red, supplemented with 10% charcoal-stripped FBS,) containing steroid or vehicle. After 24–36 h of incubation at 37 ˚C, the cells were harvested to determine firefly and *Renilla* luciferase activities. Firefly and *Renilla* luciferase activities were measured using the dual luciferase assay system (Promega, USA) on a luminometer (Promega, USA). Firefly luciferase data were normalized to *Renilla* luciferase data. After normalization for transfection efficiency, induction factors were calculated as the ratios of the average luciferase value of the steroid-treated samples *versus* vehicle-treated samples.

2.12. Ep1 antibody production and Western blot

Mouse polyclonal antibody for western blot and immunohistochemistry was generated against a synthetic 17-amino acid-long peptide (CEASSLHITEKNASNKI) derived from the C-terminal domain of *B. sinensis* Ep1 linked to keyhole limpet hemocyanin by five intradermal injections (200 μg per injection in Freund's adjuvant) (Supplemental Fig. 1). Western blot assay was performed as previous

described [33]. In brief, total protein samples were extracted by immediately placing freshly excised tissues into 2 × SDS buffer, denatured by boiling for 5 min, and then cooled on ice. Protein samples were loaded and separated using a 12% SDS-PAGE gel and were transferred onto a PVDF membrane. The membrane was blocked in TBST containing 0.1% (v/v) Tween-20 and 5% (w/v) defatted milk powder at room temperature (RT) for 1 h, before the membrane was incubated with Ep1 antibody (1:1000, v:v) at 4 °C for 12 h. After five washes with TBST, the membrane was incubated at RT for 1 h with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:1000, v/v). A chemiluminescence detection kit (TransGen, China) was used to detect signals on the PVDF membrane.

2.13. In situ hybridization and immunohistochemistry

The localization of *arα* mRNA in the olfactory rosette was investigated by *in situ* hybridization as described previously [34], except that mixed probes were used to enhance probe sensitivity and permeability [35]. Briefly, gene-specific products were amplified by PCR using specific primers containing the T3 or T7 RNA polymerase promoter sequence attached at their 5′ends (Supplemental Table 1). The PCR products were then gel purified and served as a template for synthesizing digoxigenin-labeled cRNA probes (Roche, Swiss). Olfactory rosettes of male fish were dissected and fixed in 4% w/v paraformaldehyde in PBS at 4 °C overnight, followed by immersion in 25% w/v sucrose in PBS at 4 °C until sinking, and then embedded in optimal cutting compound (Tissue-Tek™, Sakura, USA) by freezing in liquid nitrogen. Cryostat sections were cut at 10 μm, and 3 probes were added at a final concentration of 1000 ng/ml in total. Bound DIG probes were detected with anti-DIG conjugated with POD (Roche) followed by Fluorescence (TSA™ Fluorescein System, PerkinElmer). Then the slides were washed in PBS and mounted with VectaShield containing DAPI (Vector Laboratories, Burlingame, CA, USA)

The localization of Ep1 protein in the olfactory rosette was examined using immunohistochemistry. Paraffin-embedded olfactory rosette sections were incubated with primary antiserum Ep1 (1:1000) at 4 °C for 18 h before labeling by the ABC method (Vector Laboratories Inc., Burlingame, California, USA Burlingame, CA, USA). Then a TSA Fluorescein System (PerkinElmer, USA) was used to detect the proteins of interest, followed by mounting.

For colocalization of *arα* mRNA and Ep1 protein, the cryostat sections were first used for localization of the *arα* mRNA using *in situ* hybridization. Thereafter, the sections were incubated in 1% H_2O_2 for 60 min to deactivate the HRP, and Ep1 protein was localized by immunohistochemistry.

2.14. Real-time qPCR

Specific primers for measuring the expression of target genes were designed and examined for their specificity and amplification efficiency on serial dilutions of respective target gene plasmid DNA (10^3–10^8 copies/μl) (Supplemental Table 1). All qPCR were performed using premix solution (Fermentas). Ct values were determined in a 7500 fast real-time PCR system (Applied Biosystems, USA) using default settings and baseline, and thresholds were adjusted manually. The relative mRNA levels of the target genes were determined using the comparative Ct method [36] with the *β-actin* gene used as an internal control. The absolute mRNA levels of *arα* and *arβ* were determined based on a standard curve generated by measuring known concentrations of a serial diluted plasmid containing corresponding target gene, and the amplification efficiencies for *arα* and *arβ* are close.

2.15. Statistical analysis

All data were presented as means ± standard error of the mean (SEM). Depending on the experimental setup, data were analyzed using either Student's *t*-test or one-way ANOVA followed by Fisher's PLSD post hoc test to assess statistical differences among the individual groups. The statistical analyses were run using the SPSS (version 21.0) statistical software package.

3. Results

3.1. 11-KT and T stimulate the expression of ep1 transcripts in the olfactory rosette

3.1.1. In vivo

The expression of *ep1* transcripts in the olfactory rosette significantly increased after co-injection of hCG and LHRH-A_3, suggesting that gonadal hormones might be involved in the regulation of *ep1* expression (Fig. 1A). In addition, after fish had been exposed to 11-KT for 48 h, only the highest concentration of 11-KT (100 ng/ml) up-regulated the *ep1* expression (Fig. 1B). Similarly, the expression of *ep1* transcripts increased after 48 h exposure to 100 ng/ml T (Fig. 1B).

3.1.2. Ex vivo

To examine whether 11-KT and T act directly on the olfactory rosettes to regulate *ep1* mRNA levels, an *ex vivo* olfactory rosette culture system was used. The results from *ex vivo* experiments showed that the transcript levels of *ep1* slightly increased after 0.5 ng/ml 11-KT exposure, and significantly increased after 5 ng/ml 11-KT or T exposure (Fig. 1C). 11-KT showed stronger stimulatory effects on *ep1* expression than T *ex vivo*. Treatment with R1881 (an Ar agonist) at 5 ng/ml had similar effects to 11-KT on the *ep1* expression, indicating the up-regulation was mediated through nuclear Ar pathway (Fig. 1D).

3.2. Characterization of B. sinensis Arα, Arβ and ep1 gene 5'flanking regionflanking region

The cDNAs of the Arα- and Arβ-encoded proteins consist of 698 and 722 amino acid residues, respectively (Genbank MK158225 and MK158226). The domain structure of the *B. sinensis* Ars is typical of nuclear hormone receptors [37], comprising an amino-terminal domain that displays considerable variation in amino acid sequence, a highly conserved DBD, a hinge region, and a highly conserved ligand-binding domain (LBD) (Supplemental Fig. 2A,B). Moreover, the α-helixes of *B. sinensis* Arα and Arβ that interacts directly with nucleotides in the androgen response element (ARE) in the DNA major groove were predicted and compared, and one amino acid difference was found (Supplemental Fig. 2C). The phylogenetic analysis showed that *B. sinensis* Arα and Arβ were clearly grouped with teleost Arα and Arβ, respectively (Fig. 2).

For the 5′flanking region of the *ep1* gene, a genomic fragment of 2176 bp upstream of the transcription initiation site was obtained after several rounds of genome walking (Genbank MK158227).

3.3. Tissue specific expression patterns of ars and their expression in the olfactory rosette during spermatogenesis

Different tissue specific expression patterns of *arα* and *arβ* were

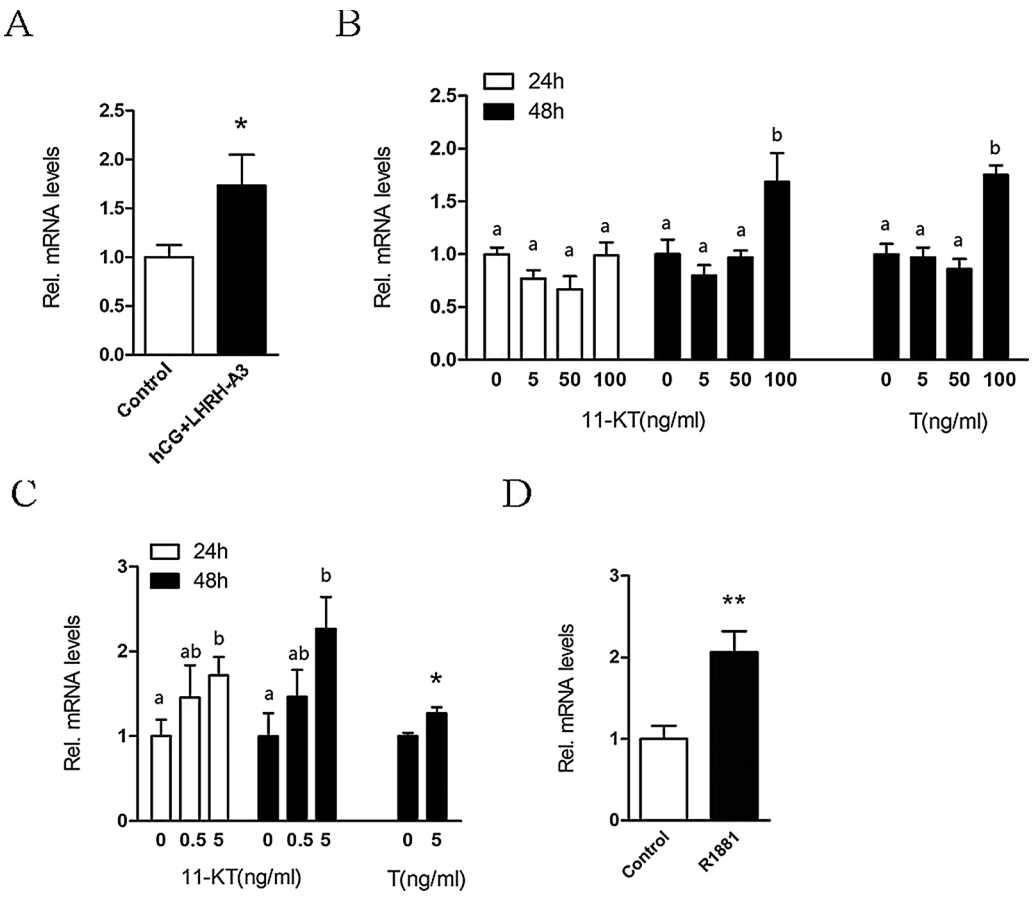

Fig. 1. Effects of the androgenic steroids 11-ketotestosterone (11-KT) and testosterone (T) on the expression of *ep1 in vivo* and *ex vivo* in male *B. sinensis*. A: Expression change of *ep1* in the olfactory rosette of nearly mature male (at stage IV) after co-injection of hCG and LHRH-A3. *In vivo* (B) and *ex vivo* (C) effects of androgens on the expression of *ep1* in the olfactory rosette of immature male fish (at stage III). D: *Ex vivo* effects of the androgen receptor agonist R1881 on the expression of *ep1* in the olfactory rosette of immature male fish (at stage III). All data are expressed as the mean ± SEM (n = 6). $*p < 0.05$; $**p < 0.01$. Bars marked with different letters are significantly different from each other ($p < 0.05$).

observed (Fig. 3A, B). Importantly, both *arα* and *arβ* were expressed in the olfactory rosette. The expression levels of *arα* exhibited significant differences during spermatogenesis (Fig. 3C). Expression levels of *arα* were relatively low at stages I and II, and reached a peak at stage IV. Then it slightly declined at stage V. In contrast, the expression levels of *arβ* fluctuated during spermatogenesis (Fig. 3C). It is worth mentioning that the *arα* transcript (about 1000 copies per 100 ng total RNA) was more abundant than *arβ* transcript (about 300 copies per 100 ng total RNA) in the olfactory rosette of male fish (from stage III to V).

3.4. Transactivation of the B. sinensis Arα and Arβ on MMTV and ep1 promoters, and subcellular localization of Ars in HEK293T cells

Dose-dependent, Arα-mediated activation of the MMTV promoter was shown for 11-KT, T and R1881 (Fig. 4A). Similarly, 11-KT, T and R1881 were all potent in activating the Arβ (Fig. 4B). In addition, the levels of transcriptional activation of the MMTV promoter by Arα were higher than those obtained by Arβ (Supplemental Fig. 4).

The responsiveness of the *ep1* promoter to 11-KT was assessed in HEK293T cells co-transfected with the *B. sinensis* Arα or Arβ expression vector. In the presence of Arα, 11-KT treatment significantly increased the *ep1* promoter activity at 2 and 5 ng/ml, and the increase was concentration-dependent (Fig. 4C). However, no effects were observed in the presence of Arβ (Fig. 4C) or zebrafish Ar (Supplemental Fig. 5).

The subcellular localization of the *B. sinensis* Arα and Arβ proteins was investigated using fluorescent protein fusions. Arα exhibited constitutive nuclear localization, while Arβ seemed to be located primarily in the cytoplasm and translocated into the nucleus upon 11-KT stimulation (Supplemental Fig. 3).

3.5. Cellular localization of arα and Ep1 in the olfactory rosette

The positive signals for Ep1 protein were located in the dendritic knobs in the apical surface of the olfactory epithelium (Fig. 5A). The *arα* mRNA signals were widely distributed in the olfactory epithelium, while almost no signal was present in the central core (Fig. 5B). It is clear that many olfactory sensory neurons (OSN) expressed arα. Even though the Ep1 protein was found in the dendritic knob while arα mRNA was observed in the cytoplasm around nucleus (no overlapped area), it is clear that Ep1 and Arα were co-expressed within one single OSN (Fig. 5C–F).

4. Discussion

It is well documented that androgens modify olfactory processing in vertebrates. For example, treatment of female mice with androgen on the day of birth is sufficient to produce the male-typical olfactory preference for female-soiled bedding [38]. It has been reported in several teleost species that androgens increase the olfactory sensitivity in response to prostaglandin pheromone [5,15], but the mechanism is still unclear. One possibility may be that androgens increase olfactory epithelium thickness as well as surface area, which results in higher

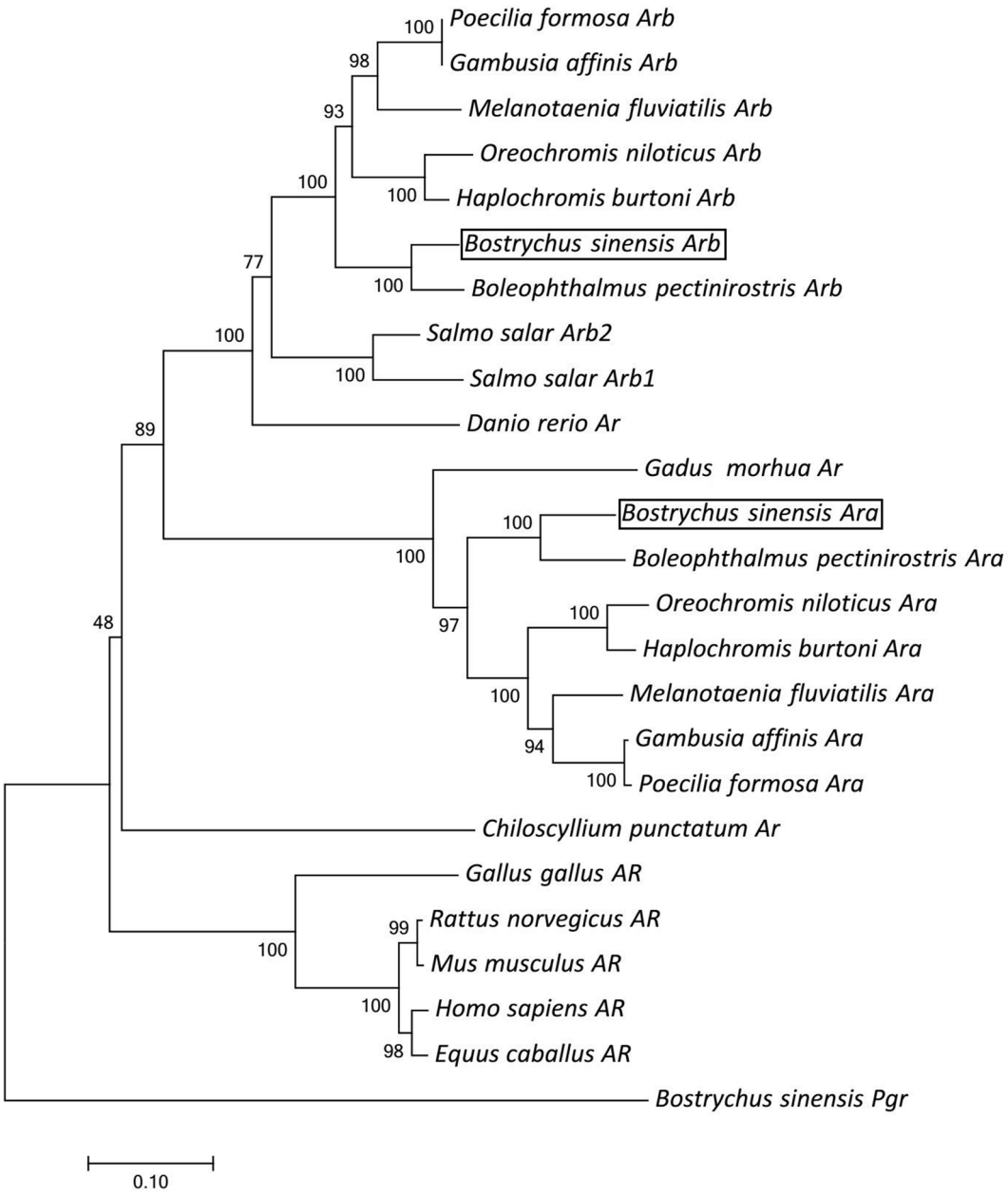

Fig. 2. Phylogenetic analysis of *B. sinensis* Arα and Arβ. Multiple species' amino acid sequences of Ars were aligned using Clustal W. GenBank accession numbers for sequence data analyzed are: *Bostrychus sinensis* Pgr, ALL41462.1; *Equus caballus* AR, NP_001157363.1; *Homo sapiens* AR, AAA51780.1; *Mus musculus* AR, NP_038504.1; *Rattus norvegicus* AR, NP_036634.1; *Gallus gallus* Ar, NP_001035179.1; *Chiloscyllium punctatum* Ar, BAI49423.1; *Poecilia formosa* Arα, ANN14184.1; *Poecilia formosa* Arβ, AKJ74871.1; *Gambusia affinis* Arα, BAD52085.1; *Gambusia affinis* Arβ, BAD52084.1; *Melanotaenia fluviatilis* Arα, AIZ00466.1; *Melanotaenia fluviatilis* Arβ, AIZ00467.1; *Haplochromis burtoni* Arα, NP_001273265.1; *Haplochromis burtoni* Arβ, NP_001273260.1; *Oreochromis niloticus* Arα, NP_001266542.1; *Oreochromis niloticus* Arβ, NP_001266544.1; *Boleophthalmus pectinirostris* Arα, XP_020778212.1; *Boleophthalmus pectinirostris* Arβ, XP_020789247.1; *Gadus morhua* Ar, ACN97554.1; *Danio rerio* Ar, EF153102.1.; *Salmo salar* Arb1, AIC76504.1; *Salmo salar* Arb2, AIC76505.1. The alignment was performed using MEGA 6.0 program and the Clustal W method. Then a phylogenetic tree was constructed using the neighbor-joining method with a bootstrap value of 1000 trials for each position.

density of OSNs for pheromone detection [39,40]. Besides the number of OSNs, the positions of OSNs within the olfactory epithelium change from deep to superficial throughout the year [41]. The androgen-induced reorganization of OSNs (density and position) might contribute to higher sensitivity to pheromones. Alternatively, androgens may directly bind to androgen receptors in the olfactory epithelium to modulate the expression of receptors for pheromones [20]. The finding in mice supports this hypothesis by showing that T modulates the expression of vomeronasal receptors, thus altering behavioral responses to pheromones [17]. In *B. sinensis*, mature fish displayed greater EOG

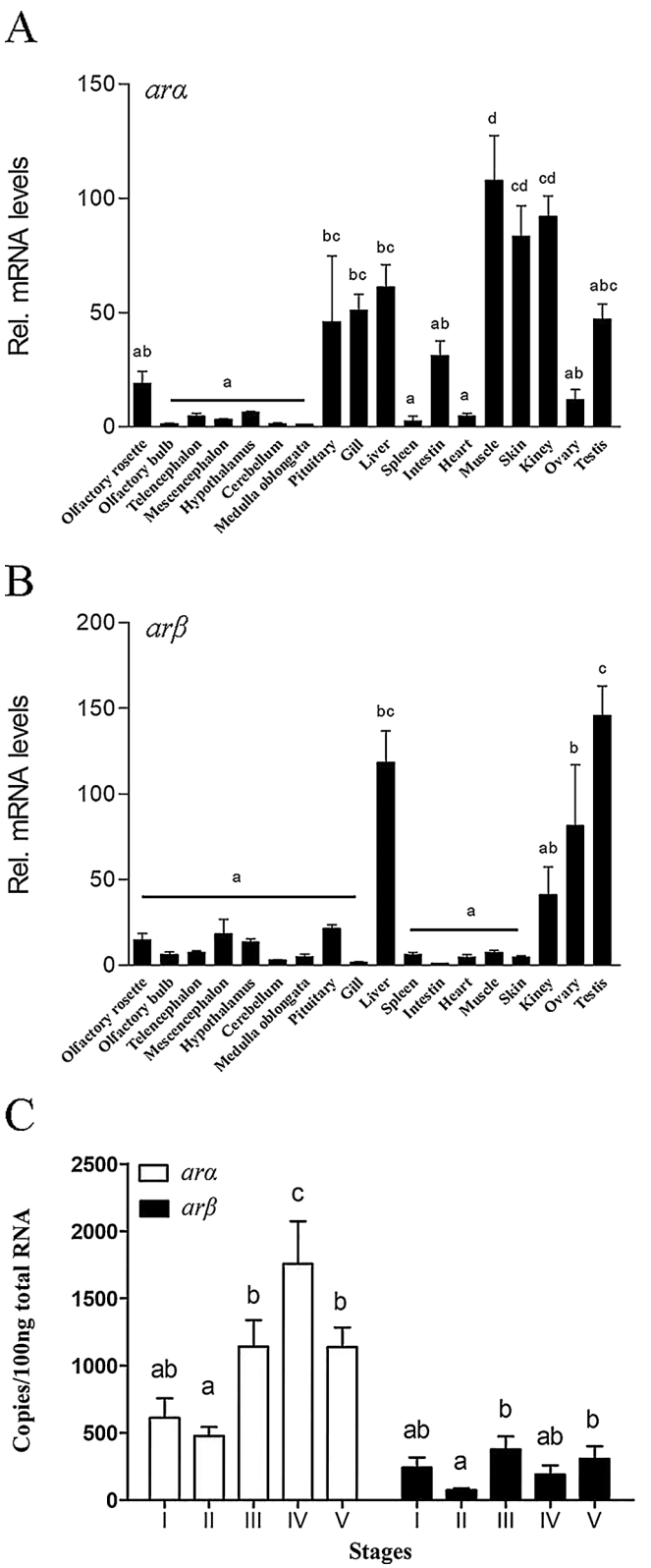

Fig. 3. Expression of *B. sinensis ar* transcripts. (A–B) Tissue specific expression patterns of *B. sinensis arα* (A) and *arβ* (B) transcripts. The levels of the respective mRNAs were determined using qPCR and normalized to the internal housekeeping gene (*β-actin*). Data are expressed as the mean ± SEM (n = 4). (C) Absolute quantity of *B. sinensis ar* transcripts in the olfactory rosette during spermatogenesis in male *B. sinensis*. Data are expressed as the mean ± SEM (n = 6). Bars marked with different letters are significantly different from each other ($p < 0.05$).

response to sex pheromone PGE_2 and higher olfactory *ep1* transcript levels than immature fish [30,31]. Moreover, Ep1 protein was found in the dendritic knobs of OSNs in the olfactory epithelium, indicating Ep1 is a candidate receptor for pheromone PGE_2. In the present study, we found that androgens up-regulated the expression of *ep1* in the olfactory rosette of *B. sinensis*, suggesting that androgens increase the olfactory sensitivity to pheromone PGE_2 by modulating the expression of *ep1*.

Since the AR agonist R1881 also increased the expression of *ep1*, we suppose that the effects of androgens are mediated by Ar in *B. sinensis*. As found in many other teleost species, two Ar subtypes (Arα and Arβ) were identified in *B. sinensis*. It is supposed that Arα and Arβ might perform different functions, because of their differential tissue expression pattern, their differential transactivation properties and the low similarity of DBDs [25]. Similarly, characteristics for *arα* and *arβ* were observed in *B. sinensis*. The *arα* was dominantly expressed in the gill, intestine, muscle and skin, while the *arβ* was dominantly expressed in liver, testis and ovary. The DBDs of *B. sinensis* Arα and Arβ shared only 69% sequence similarity, the predicted α-helixes of Arα and Arβ that interacts with ARE showed one amino acid difference (Supplemental Fig. 2C), and Arα showed higher transactivating capacity *via* AREs than Arβ (Supplemental Fig. 4). Furthermore, *B. sinensis* Arα but not Arβ displayed constitutive nuclear localization, which is similar to previous reports in Murray–Darling rainbowfish (*Melanotaenia fluviatilis*), mosquitofish (*Gambusia affinis*) and medaka (*Oryzias latipes*) Arα [24,25]. These results suggest that *B. sinensis* Arα and Arβ might have clear distinct biological functions.

In the olfactory rosette of *B. sinensis*, *arα* and *arβ* showed different expression levels and different expression patterns during spermatogenesis. The expression of *arα* transcripts was higher in mature than in immature males, which was similar to the expression change of *ep1* transcripts [31]. In mature male fish, the expression of *arα* transcripts is about 4 times higher than that of *arβ*. Importantly, activated Arα, but not Arβ, transactivated *ep1* promoter in HEK293T cell line. It is likely that Arα mediates androgen-induced up-regulation of *ep1* in the olfactory rosette of *B. sinensis*. Although it is supposed that teleost Arα and Arβ might perform different functions, no clear evidence has been shown to support this hypothesis so far. The present study is the first to show the different functions of Ars in olfaction. It is also worth mentioning that *arα* was widely distributed in the olfactory epithelium, and many Ep1-negative OSNs also expressed *arα*, suggesting that androgen signaling might also regulate the responsiveness to other pheromones in male *B. sinensis*.

In the present study, we found that the up-regulatory effects of androgens on *ep1* transcripts might be mediated by Arα in the olfactory rosette of *B. sinensis*. However, in mammals, there is no evidence of AR expression in either the vomeronasal organ or the sensory epithelium of main olfactory epithelium, suggesting the regulatory effects of androgens on olfactory receptors are not mediated by AR [42]. It is reported that Ar gene duplication which gave rise to the two different teleost Ars probably occurred during teleost-specific genome duplication [24]. The teleost Arβ is more closely related to AR from mammals, birds and reptiles, while Arα exhibits sequence divergence [23–25]. In the present study, we observed that Arα but not Arβ played a role in androgen-induced up-regulation of *ep1*. It is likely that the Arα which has been evolved in the teleost lineage might acquire a new function in olfaction, and this new function might not be present in higher vertebrates.

In summary, we report that 11-KT stimulates the expression of *ep1 via* Arα in the olfactory rosette of *B. sinensis*. This mechanism might explain the observations that, compared to immature fish, mature male *B. sinensis* have both greater EOG sensitivity to the sex pheromone PGE_2 and higher expression of *ep1* in their olfactory rosettes. To our knowledge, this is the first report describing the molecular mechanism by

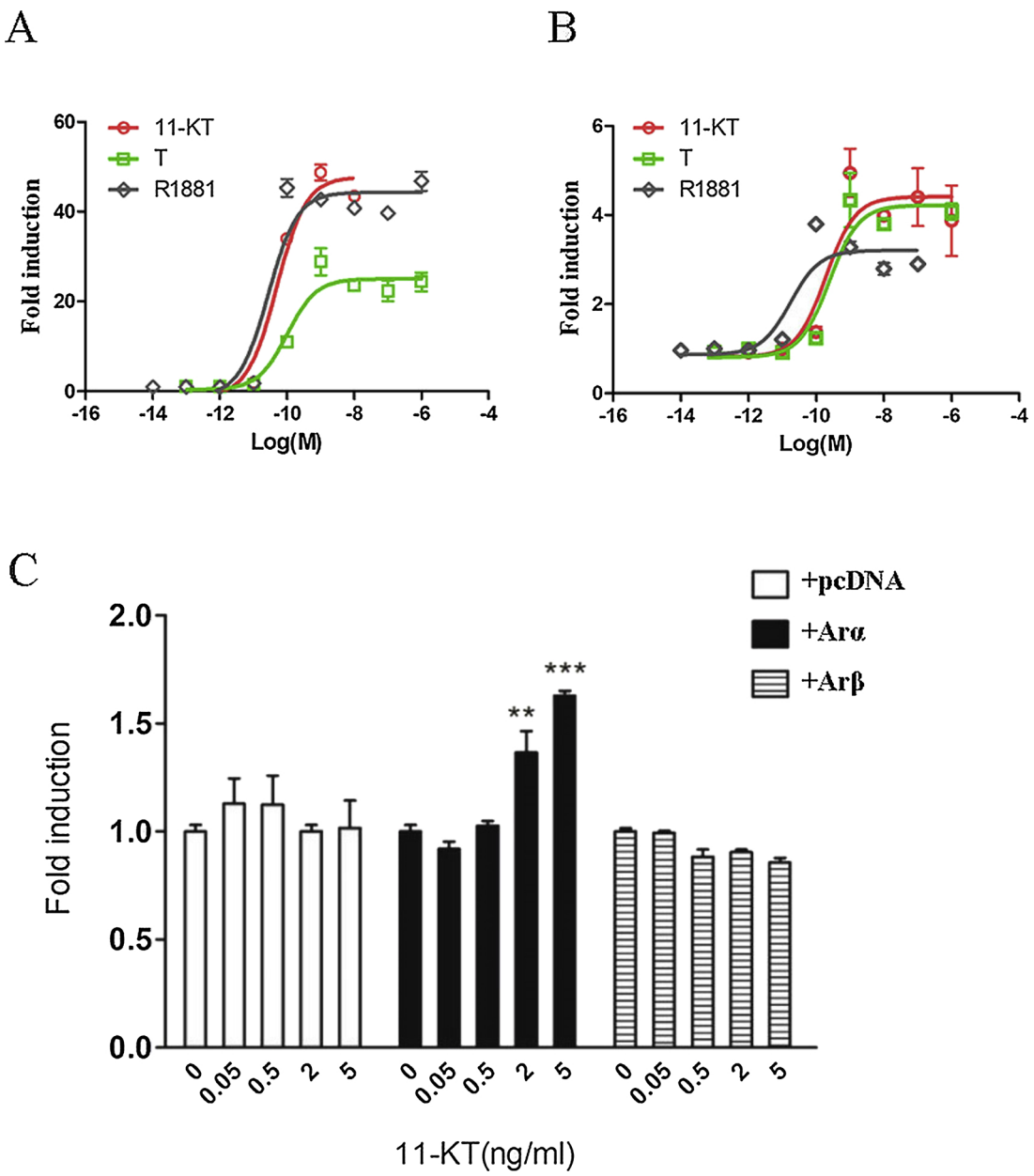

Fig. 4. Transactivation of the *B. sinensis* Arα and Arβ on MMTV and *ep1* promoter. *B. sinensis* Arα- or Arβ-transfected HEK293T cells (A and B respectively) were incubated with increasing concentrations (from 10 fM to 10 mM) of different steroids. Each point represents the mean ± S.E.M. (n = 3). Curves were generated using non-linear regression (GraphPad Prism 5). (C) The effects of 11-KT on the *ep1* promoter activities. The empty vector control (*i.e.* pcDNA), Arα- or Arβ-transfected cells were incubated with different concentrations of 11-KT for 24 h. Results are mean values ± S.E.M. (n = 4). ** $p < 0.01$; *** $p < 0.001$.

which androgens regulate the olfactory sensitivity to a prostaglandin sex pheromone in a teleost [30,31].

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.

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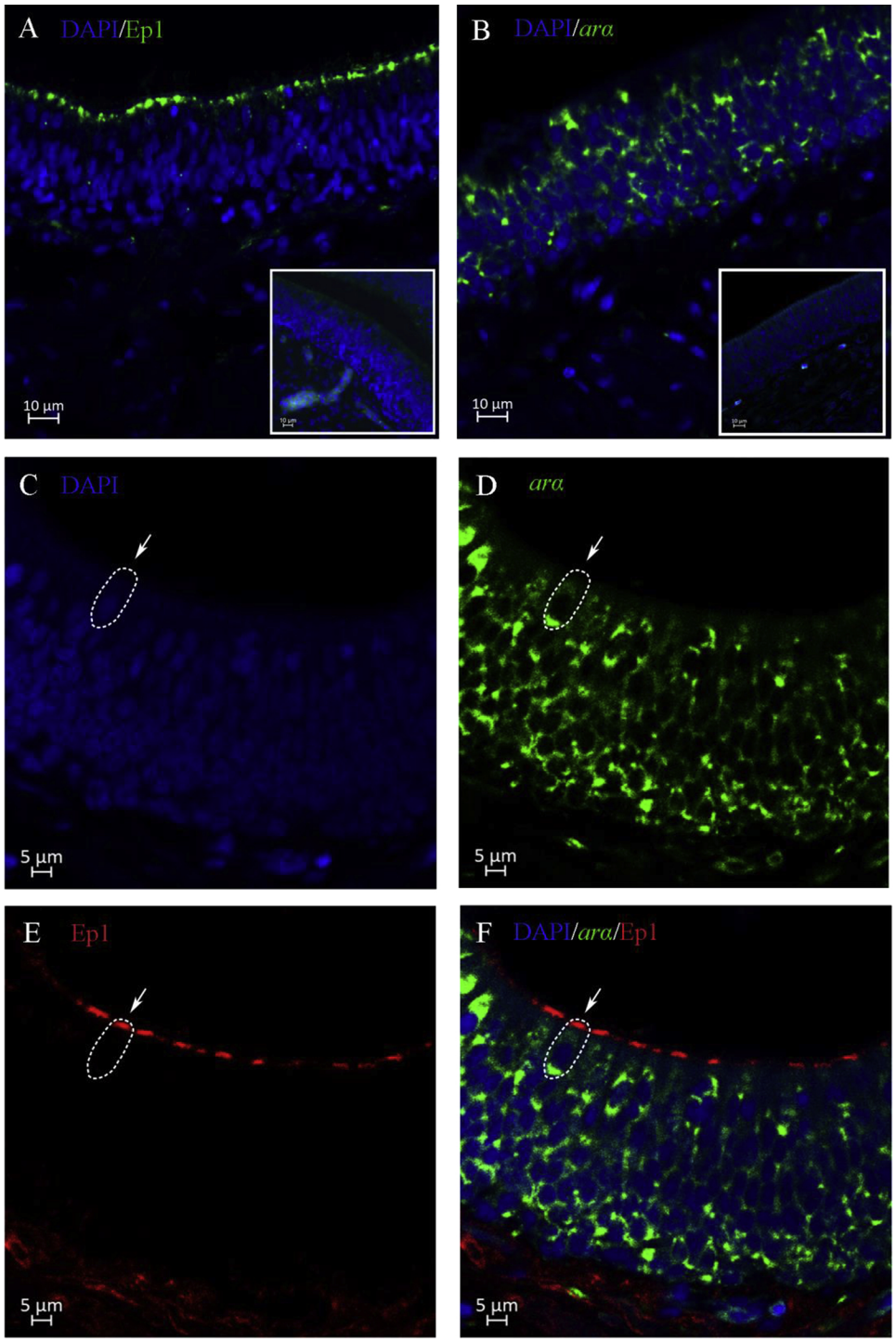

Fig. 5. Cellular localization analyses of Ep1 and *arα* in the olfactory rosette of *B. sinensis* using confocal microscope imaging. (A) Ep1 immunostaining of the olfactory rosette. The inset in panel A shows negative control (pre-immune mouse serum was used to replace the primary antibody). (B) Localizations of *arα* mRNA detected using *in situ* hybridization. The inset in panel B shows negative control using sense riboprobe. (C–F) Ep1 (red) and *arα* mRNA (green) double-colored fluorescent localizations. White arrows indicate colocalizations of Ep1 and *arα* mRNA signals within one cell; note that Ep1 signal was present in the dendritic knobs, while *arα* mRNA signal was present in the cytoplasm.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.jsbmb.2019.01.010.

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