Androgen dependent development of a modified anal fin, gonopodium, as a model to understand the mechanism of secondary sexual character expression in vertebrates

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Abstract **Male external genitalia show structural variations among species. Androgenic hormones are essential for the morphological specification of male type copulatory organs, while little is known about the developmental mechanisms of such secondary sexual characters. Western mosquitofish *Gambusia affinis* may offer a clue to the sexual differentiation researches, because they show a prominent masculine sexual character for appendage development, anal fin to gonopodium (GP) transition, and its formation could be induced in early juvenile fry by exogenously supplied androgens. We show that GP development is promoted by androgen dependent augmentation of *sonic hedgehog* (*Shh*) expression. Two AR cDNAs were cloned and identified as ARα and ARβ from western mosquitofish. Both *ARs* were predominantly expressed in the distal region of outgrowing anal fin rays. Exposure of fry to androgen caused anal fin outgrowth concomitant with the *Shh* induction in the distal anal fin ray epithelium. When AR signaling was inhibited by its antagonist flutamide in fry, the initial induction of the *Shh* was suppressed accompanying retarded anal fin outgrowth. Similar suppression of anal fin outgrowth was induced by treatment with cyclopamine, an inhibitor of Shh signaling. These observations indicate that androgen dependent *Shh* expression is required for anal fin outgrowth leading to the formation of a genital appendage, the GP in teleost fishes. Androgen-induced GP formation may provide insights into the expression mechanism regulating the specification of sexual features in vertebrates.**

Keywords: Copulatory organ; Gonopodium; Secondary sexual character; Androgen receptor; Sonic hedgehog; Teleost fish

1. Introduction

External sexual characters, which often appear in sexually differentiated reproductive organs, have evolved in each species presumably for survival and/or reproduction. In amniotes and also in some fishes, several types of copulatory organs have been developed for sperm transport. All elasmobranchs and holocephalans perform internal fertilization, and the medial border of the pelvic fin of male elasmobranchs is modified to form a tubular structure, termed the clasper. Most teleosts have external fertilization, but some species develop copulatory organs formed from an elongation of the anterior region of the anal fin, termed gonopodium (GP) [1–4] and its suspensorium of axial and appendicular support skeletal elements [5–7]. Such morphological diversification of copulatory organs has evolved as a phenotypic adaptation for developing external to internal fertilization under various environmental influences. The copulatory organ developments commonly involve a process of androgen dependent organogenesis as secondary sexual character. However, the molecular developmental mechanisms underlying androgenic functions are as yet largely unelucidated. Understanding the process of such sexually dimorphic expression, which is to understand the mechanisms of sex hormone dependent organogenesis underlying such reproductive diversity among species, is one of the central problems in biology.

The expression of masculine phenotypes is regulated by steroid hormones, particularly androgens such as testosterone and 5α-dihydrotestosterone (DHT) in mammals. Evidence supporting the crucial role of androgen is based on pharmacological and genetic data. Androgen antagonists [8,9] or inhibitors of its synthesis [10,11] interfere with testicular function and external genitalia development. Mutations in genes for the androgen receptor (AR) [12–14] or for androgen synthetic enzymes [15,16] result in abnormalities in male sexual differentiation and development. In fishes, male secondary sexual characters also appear as an elongation of the fin ray, kidney hypertrophy, increase in skin thickness, and an appearance of breeding colors [17] as well as in the development of copulatory organs. In some fishes, it is known that the androgens, especially 11-ketotestosterone (11KT), are present in higher levels in the blood plasma of mature males than in females and could stimulate male secondary sexual characters [18–20].

Western mosquitofish *Gambusia affinis*, a species of the family Poeciliidae, shows a prominent masculine sexual character for appendage development, the anal fin to GP transition (Fig. 1A and B) [1–3] and its appendicular supportive skeletal elements [5–7]. The GP serves to transfer sperm bundles into the urogenital sinus of the female (Fig. 1C) [7,21,22] and structural alteration of appendicular support is thought to be necessary for the development of a functional GP [23].

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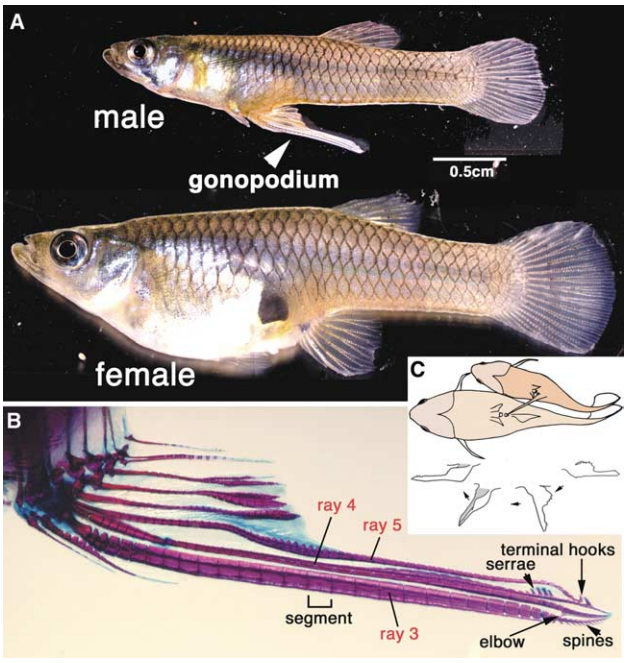

Fig. 1. Androgen dependent sexual dimorphism in western mosquitofish (*Gambusia affinis*). (A) Mature male and female. (B) Bone staining of gonopodium (GP). The distal portion of the GP is composed of the 3rd, 4th, and 5th fin rays and the distal tip is equipped with spines, serrae, an elbow, and hooks. The 3rd fin ray, as the axial center of rotation for the GP, is prominently thickened (33 mm TL, 1 year old, $n = 4$). (C) For copulation, the GP swings forward and sperm bundles, spermatozeugmata, are directly transported into the female urogenital sinus (the illustration from Fig. 43 in Rosen and Gordon 1953 and Fig. 5 in Peden 1972) [7,21,22].

Secondary sexual characters generally appear in puberty. The GP formation will offer an entry point for researches on sexual differentiation, because its development can be induced in early juvenile fry. The androgen inducibilities for development of both the GP and its appendicular support had been discussed in western mosquitofish. Its GP development is subdivided into a first stage of growth and segmentation and a subsequent stage of differentiation of terminal areas such as spines, hooks and serrae (Fig. 1B) [24]. Turner reported that the androgen treated females develop male type GP, suggesting the utility of administrating exogenous androgen to stimulate GP development [24,25]. Rosa-Molinar revealed that the developmental process of the sexually dimorphic anal fin appendicular support is organized in two phases, anteriolization of both the anal fin and its appendicular support, and androgen dependent elongation of the hemal spines of vertebrae 14–16 [6,7]. This work focused on early GP development of the anal fin outgrowth and segmentation stage to understand the molecular developmental mechanisms of androgen dependent expression of secondary sexual character.

In addition, the western mosquitofish has been noted as a species bio-indicator to screen substances with suspected endocrine activities, especially for androgenic and anti-androgenic chemicals, due to its marked sexual dimorphism. The reduction in size of the GP [26,27] and masculinization of females [28–30] has been reported in several countries.

Recently, it was reported that msxC gene expression is associated with growth of the sword and GP in swordtail fishes [4]. However, the molecular mechanism of androgen dependent GP development is largely unknown.

Understanding the molecular pathway of the androgen action during GP development may provide insights into the mechanisms controlling differentiation of sexual features. In this paper, analyses to elucidate structures, expression patterns and developmental functions of regulatory genes for western mosquitofish GP formation were performed. It was revealed that androgen dependent *Shh* expression was required for regulation of anal fin outgrowth leading to the formation of genital appendage, the GP in teleost fishes.

2. Materials and methods

2.1. Animals

Western mosquitofish (*Gambusia affinis*), kindly provided by Drs. Wakamatsu and Ozato of Nagoya University, were utilized for isolating and analyzing cDNAs. For gene expression analysis and histological analyses, feral-caught adults were obtained from a commercial source (Meito suien) and maintained at 28.5 ± 1 °C as a laboratory breeding colony. Sexual maturation was confirmed by the mating and fertile males were defined as adults. Spawned fry were transferred to a separate tank and treated with ethynyl testosterone (ET) for analyzing GP formation. The breeding conditions were described in the respective sections for each experiment.

2.2. Administration of androgen and androgen antagonist to fry

For the ethynyl testosterone (ET) treatments of fry, stock solutions of 16–320 μM ET (Sigma) were dissolved in dimethyl sulfoxide (DMSO) and frozen at −20 °C until use. Stock solutions were directly diluted in 23.5 ± 1 °C tank water containing the fry. Fry were exposed to 1.6–32 nM ET or 0.01% DMSO from day 1 after birth at a density of 20 fry per 200 ml. Fresh solutions of ET were added every 2 days. The concentration of ET to promote GP development in early juvenile fry was determined based on previous studies [24,25]. Experimental specimens were collected at day 2 to day 17 of treatment (day 3 to day 18 after birth), hereafter indicated by days of treatments.

In experiments with an AR-antagonist, flutamide was co-treated with ET at a concentration of 3.6 μM after 2 days of 3.2 nM ET-treatment at 23.5 ± 1 °C; with daily exchanges of solutions of ET and flutamide. The GP development of each treatment was monitored by counting the segments of the 3rd fin ray. Statistically significant differences of additional segmentation rates were tested using the Student's *t* test.

2.3. Cyclopamine treatment to fry

Fry were exposed to 10 μM cyclopamine (Funakoshi) with 3.2 nM ET starting at day 5 of ET treatment at 23.5 ± 1 °C, with daily exchange of solutions.

2.4. RNA isolation and RT-PCR analysis

Total RNA was extracted from each tissue by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method using Isogen (Nippon Gene).

To obtain the cDNA fragments for ARα, ARβ, Shh, and Patched1 (Ptc1), oligonucleotide primers were designed based on the amino acid sequences of the highly conserved central regions of these genes and then utilized to amplify western mosquitofish fry cDNAs by RT-PCR, respectively. RT-PCR was carried out using SuperScrip™ One-Step RT-PCR with PLATINUM Taq System (Invitrogen). The primers, which yielded a 294 bp fragment homologous to Nile tilapia ARα, a 711 bp fragment highly homologous to Nile tilapia ARβ, a 404 bp fragment homologous to zebrafish Shh, and a 306 bp fragment homologous to zebrafish Ptc1, were as follows; AR-gS1: RGARTTCCTSTGCATGAARG, AR-gA2: GGAAMDTGYACNGAGATBAT, AR-S3: CTCACCTGYGGHAGCTGCAA, AR-A2: GAAGAGNAGCARDGCYTTCA, Shh-S1: CTGACNCCTNTNGCCTACAAGCA, Shh-A3:ACCCAGTCRAANCCNGCYTCCAC, Ptc1-S2: TGYGCHVTSYTYCTSCTSAAYCC, Ptc1-A2: YTCDGANCCDGCVAGCATVAG, respectively. Amplified products of expected

sizes were subcloned into pGEM-T Easy Vector (Promega) and sequenced.

To analyze the expression levels of *ARs*, *Shh*, and *Ptc1*, oligonucleotide primers were designed based on the obtained sequences as follows; ARα-QS: GGAGAGGAAGAACAGCAGCCTG, ARα-QA: CACTTGCATACGTTGGTCGTTG, ARα-QS: CCTTGAGTCCATCGAGCCT, ARβ-QA: AGATGTCTCATCCGCATGC, Shh-QS: TGTGGCGGAGAAGACCCTG, Shh-QA: GAGCTTCACCCCAGGCCAT and Ptc1-QS: GACGGCTGGCATCATTGTGTT, Ptc1-QA: GGAGATTGCACCGTCAAGCACT. Subsequently, RT-PCR analysis was performed on total RNA from fins of fry and various tissues of adult males. Amplification of elongation factor 1α (EF1α) was performed in all experiments as a control.

2.5. Isolation of western mosquitofish full length AR cDNAs

Based on the sequence information of obtained PCR products, the 5′ and 3′ ends of western mosquitofish AR sequences were amplified using a rapid amplification of cDNA ends kit (GeneRacer™ kit, Invitrogen). Obtained cDNAs were subjected to 5′-and 3′-RACE with Platinum Taq DNA Polymerase High Fidelity (Invitrogen) using specific primers for western mosquitofish ARα or ARβ, ARα-GSP-F1 (AAGGAGCTGGACCGCTTAGCG), ARα-GSP-R1 (TCCAGTAGCTGCGTGAGCTGAA), ARβ-GSP-F1 (CTGGACAGGAAGCGCCAGAAGTA), ARβ-GSP-R2 (CCACCTCAGGCTCGATGGACTCA), and ARβ-GSP-R3 (CCGTCGAAGTGTGAGCGTACTGGAC) with the supplied adapter specific primers, GeneRace™ 5′ primer (CGACTGGAGCACGAGGACACTGA) and GeneRace™ 3′ primer (GCTGTCAACGATACGCTACGTAACG). Amplified products were subcloned and sequenced.

2.6. Whole mount in situ hybridization

The expression patterns of *ARs*, *Shh*, and *Ptc1* in western mosquitofish fry were analyzed by whole mount in situ hybridization, essentially as described [31]. The digoxigenin (DIG)-labeled probes consisted of antisense RNA corresponding to the obtained PCR fragments of ARα (294 bp), ARβ (711 bp), Shh (404 bp), and Ptc1 (306 bp). For sections, stained specimens were embedded in Technovit 8100 (Heraeus Kulzer GmbH) and sectioned at a thickness of 10 μm.

2.7. Histological and immunohistochemical analyses

Fry were fixed in 4% paraformaldehyde (PFA) in PBS at 4 °C, dehydrated in graded ethanol, embedded in paraffin and sectioned with 2.0 μm thickness. Serial sections were subjected to masson/trichrome stain or for immunohistochemical staining with a monoclonal antibody to proliferating cell nuclear antigen (PCNA: DAKO). For skeletal stains, Alcian Blue/Alizarin Red staining was performed as described [32]. For BrdU incorporation experiments, fry were incubated in 50 μg/ml BrdU-containing water at a density of 5 fry per 50 ml for 20 h. For detection of BrdU, fry were incubated in Carnoy's fixative overnight at 4 °C and analyzed as described [33]. The fluorescent calcium binding dyes, calcein and tetracycline, were used to label bone deposition. Calcein and tetracycline were administrated into the breeding water at 8 and 20 μg/ml, respectively. Fry were treated in the above water for 24 h before analysis.

2.8. Microinjection of DiI

A 0.25% stock solution of the lipophilic dye 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) was prepared in DMSO. This was diluted in 0.3 M sucrose to a working concentration of 0.025%. DiI was injected into the most distal segment of the 3rd fin ray of the anal fin at day 4 of the 32 nM ET-treatment and the labeled cells were examined at day 13 of ET-treatment.

2.9. Phylogenetic analysis

Phylogenetic analysis of AR genes was performed on the amino acid sequences (369 residues) of C-terminal regions mainly composed of the DNA binding and ligand binding domains (DBD and LBD). Estimation of molecular phylogeny was carried out by the neighbor-joining method [34] using the CLUSTAL W program [35]. Confidence in the phylogeny was assessed by bootstrap resampling of the data (×1000) [36].

3. Results

3.1. Histogenesis of androgen induced GP formation

During early juvenile stages in normal development, the structures of male and female anal fins are indistinguishable. The sexual dimorphism driven by androgenic hormones mediated by the testis becomes apparent in the anal fin at late juvenile stages [37]. Pioneering studies reported precocious GP induction in immature males and females by administration of synthetic androgens such as ethynyl testosterone (ET) and methyl testosterone (MT) [7,24,25]. Turner described that western mosquitofish females have the potential to respond to androgenic signals and androgen-treated females develop male type GP [24]. We examined the process of initial GP outgrowth using early juvenile males and females with androgen treatment to analyze the mechanisms of androgen dependent GP morphogenesis.

Administration of ET at 1.6–32 nM in water starting at 1 day after birth effectively induced early GP outgrowth as below. The GP outgrowth, evaluated by the number of segments of 3rd fin rays, was prominently enhanced in the ET treated groups compared with controls (Fig. 2B). The total body lengths of controls and ET-treated fry were indistinguishable at each examined day. The growth of anal fin rays was observed at day 4 of ET-treatment (approximate total body length (TL) 9 mm) as the elongation of the most distal segment (Fig. 2D (c)), with fin outgrowth becoming prominent following additional segmentation of fin rays at day 6 (9 mm TL, Fig. 2A and B). Further outgrowths of the 3rd, 4th, and 5th fin rays, which are prospective intromittent fin rays, were accelerated to 17 days (11 mm TL), while the growth of other anal fin rays was diminished (Fig. 2A and B).

Next, we performed histological analysis at day 10 of treatment (10 mm TL, Fig. 2C). Both proximo-distal (P-D) elongation and lateral expansion were observed in ET-treated anal fin rays compared with controls (Fig. 2C, compare b with a). The cells of the basal layer of the epidermis were positioned along the basement membrane (Fig. 2C (c)). Directly below the basement membrane of each side, lepidotrichia were formed in a P-D direction (Fig. 2C (c, e)). In the most distal part, actinotrichia composed of collagen fibrils developed as supportive elements in the rapidly elongating fin mesenchyme (Fig. 2C (c, f)) [38,39]. In a region forming actinotrichia (Fig. 2C (c)), mesenchymal cells condense prominently compared with those in a proximal region (Fig. 2C, compare f with e). These structural alterations were not observed in other fins. To gain an insight into mesenchymal cell status, cell proliferation analysis was performed. Proliferating cell nuclear antigen (PCNA) signals associated with cell proliferation were prominently observed in the distal condensed mesenchyme and the basal layer of the epidermis (Fig. 2C (d)), while such enhanced cell proliferation was not observed in other fins. Lepidotrichial ontogenesis, monitored by the fluorescence signals of calcein and tetracycline, was not observed in such distal proliferating mesenchymal regions (Fig. 2D (c, d)). These results indicate that the acceleration of cell proliferation preceded the lepidotrichial ontogenesis.

To examine the contribution of distal mesenchymal cells to anal fin outgrowth, the lipophilic dye DiI was injected into the distal proliferating mesenchyme at day 4 of ET-treatment (9 mm TL, Fig. 2D (c)). Migration of DiI-labeled cells along the proximo-distal axis of the fin ray was observed at day 13

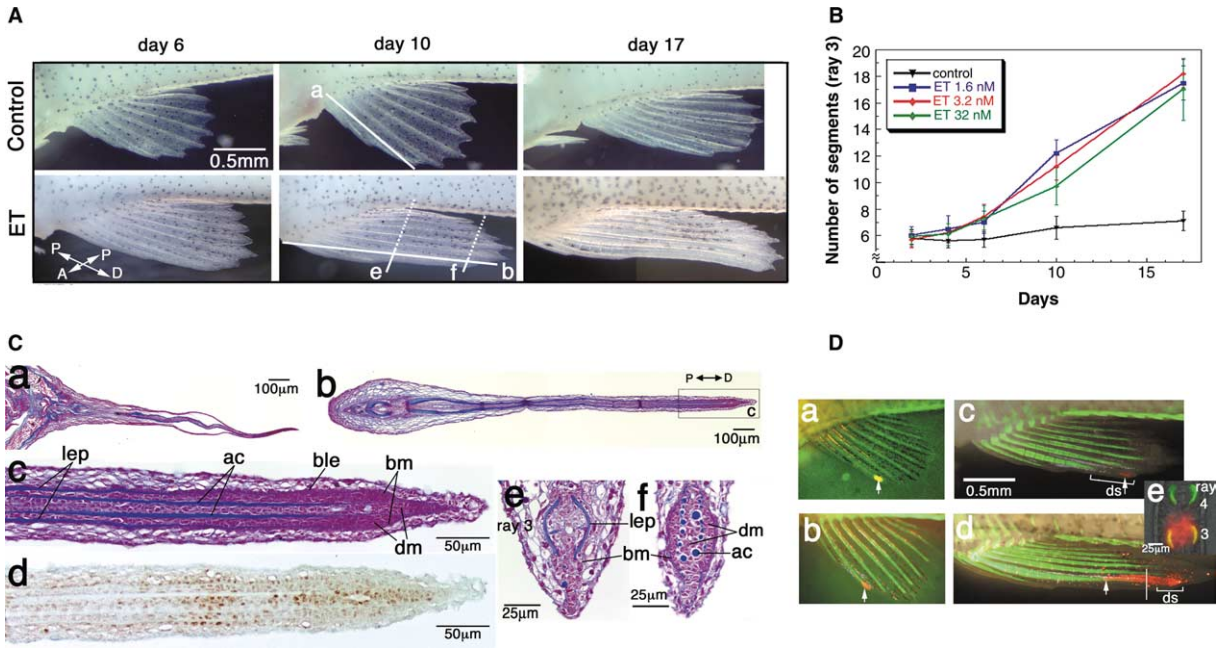

Fig. 2. Induction of GP formation in early juvenile fry by androgen treatment. (A) Anal fins of controls and ET (3.2 nM)-treated specimens at day 6 (9 mm TL) to day 17 (11 mm TL) of treatment. Generally similar GP formation was elicited in the range of 1.6–32 nM ET. White lines indicate the plane of sections shown in Fig. 2C. (B) Numbers of segments of 3rd fin rays. The anal fin outgrowth at 23.5 ± 1 °C was monitored by counting the segment number of 3rd fin rays ($n = 15$–39 for each point). Mean data are shown with standard deviation. Addition of new bone segments was stimulated after day 4 of ET-treatment. Statistically significant differences compared with controls were confirmed ($P < 0.05$). (C) Masson trichrome staining (a: control; b–f: ET-treated specimens) and PCNA staining (d: ET-treatment, 2.0 μm thickness) of anal fins at day 10 of treatment (10 mm TL, $n = 10$). ac, actinotrichia; ble, basal layer of the epidermis; bm, basement membrane; lep, lepidotrichia; dm, distal mesenchyme. (D) Migration of DiI-labeled cells (a, b: control; c–e: ET (32 nM)-treatment). DiI was injected into the distal mesenchyme adjacent to the calcein and tetracycline stained bone-depositing parts at day 4 of treatment (arrows in a and c, 9 mm TL, $n = 36$ for each group). BrdU-positive cells were observed at day 4 in the distal segment of ET-treated anal fins (data not shown). Injected fry were maintained for additional 9 days (b, d, 11 mm TL). Prominent migration of DiI-labeled cells along the proximo-distal orientation from the injection site (arrow) was observed in ET-treated outgrowing fins (d), compared with controls (b). White line indicates the plane of the section shown in (e, 10 μm thickness). ds, distal segment.

concomitant with fin outgrowth (11 mm TL, Fig. 2D (d)). Analysis on the distribution of the DiI-labeled cells revealed strong labeling in mesenchymal cells in the ray and surrounding the bony part of the fin rays (Fig. 2D (e)). These results suggested that the distal mesenchyme contributed to the development of new bone segments. Hence, we performed gene expression analysis focusing on the distal region of the anal fin (see below: Fig. 4).

3.2. Isolation and characterization of western mosquitofish AR cDNAs

Responsiveness to androgens requires expression of functional AR, and its regulated expression is specific to cell types at several developmental stages. We first cloned two kinds of AR cDNAs, encoding 685 and 755 amino acids that contain the entire AR coding region, from western mosquitofish *Gambusia affinis*. The obtained AR cDNA sequences could be subdivided into several domains as defined by Krust et al. [40] (Fig. 3A). Alignment of the western mosquitofish ARs with other teleost ARs and tetrapod ARs illustrates that high similarity lies within the putative DNA binding (DBD) and the ligand binding (LBD) domains (Fig. 3A). As reported for all ARs, the N-terminal domain (NTD) sequences are divergent. Both western mosquitofish ARs contain motifs characteristic of a steroid hormone receptor family [41–44] within the putative DBD and LBD (Fig. 3B and C). In teleost fishes, two distinct subtypes of ARs have been identified from Nile tilapia (*Oreochromis niloticus*), Japanese eel (*Anguilla japonica*) [42,45] and Atlantic croaker (*Micropogonias undulatus*) [46]. In rainbow trout (*Oncorhynchus mykiss*), two isoforms of AR, probably derived from salmonid tetraploidy, were cloned [43].

A neighbor-joining tree based on comparison of the amino acid sequences in DBD and LBD suggested that the obtained western mosquitofish ARs were categorized into the two distinct clusters including Nile tilapia ARα or ARβ genes, which were therefore designated ARα (AB174849) and ARβ (AB099303), respectively (Fig. 3D). The deduced amino acid sequence of western mosquitofish ARβ is highly similar to those of the tetrapod ARs but much less to those of western mosquitofish ARα and Nile tilapia ARα (Fig. 3A). These results may indicate that the western mosquitofish ARβ is similar to tetrapod ARs based on sequence similarities. Branch length comparisons support this inference (Fig. 3D).

3.3. Expression of western mosquitofish AR mRNAs

There were slight differences in the tissue distributions of ARα and ARβ mRNAs. Both *ARs* were strongly expressed in adult fins. A significant level of *ARα* expression was also observed not only in adult testis and kidney but also in liver, while *ARβ* was expressed in testis and kidney (Fig. 3E). During androgen dependent outgrowth of the GP, western mosquitofish *ARβ* was predominantly expressed in the distal mesenchyme of anal fin rays (Fig. 4A and B: day 6, 9 mm TL; C: day 17, 11 mm TL), while the expression of *ARα* was observed in

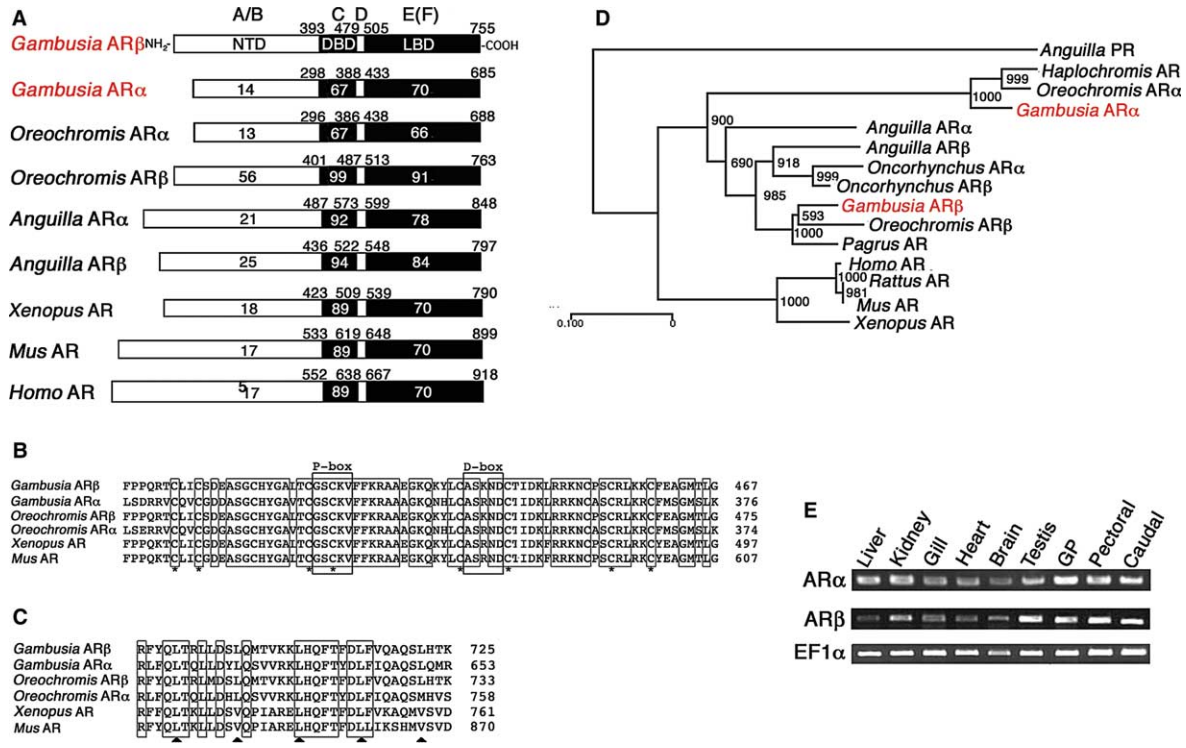

Fig. 3. Characterization of western mosquitofish AR cDNAs. (A) Structural comparison of western mosquitofish ARs with ARs of other species. The numbers above each box refer to the position of amino acids in the putative DNA binding (DBD) and the ligand binding (LBD) domains. The numbers within each box indicate the homology of each domain by percentage relative to western mosquitofish ARβ. (B, C) Comparisons of deduced amino acid sequences constituting zinc finger motifs in the DBD (B) and the putative leucine zipper motif in the LBD (C). Sequence identities are shown in boxes. The cysteine residues for the two zinc finger motifs are shown by asterisks. The individual leucines of the putative leucine zipper structure in the LBD are marked by black arrowheads. (D) A neighbor-joining tree based on comparisons of amino acid sequences. The C-terminal regions (369 residues) mainly composed by the DBD and LBD were used for the above analysis, excluding positions with a gap. Eel PR (AB032075) was used as an outgroup. Branched length is proportional to the number of amino acid substitutions and the scale bar indicates 0.1 amino acid substitution in the sequence. The numbers indicate the relative robustness of each node as assessed by boot strap analysis (1000 replications). *Anguilla japonica*, Japanese eel ARα (AB023960) and ARβ (AB025361). *Oncorhynchus mykiss*, rainbow trout ARα (AB012095) and ARβ (AB012096). *Pagrus major*, red seabream AR (AB017158), *Oreochromis niloticus*, Nile tilapia ARα (AB045211) and ARβ (AB045212), *Haplochromis burtoni* AR (AF121257), *Xenopus laevis* AR (U67129), *Mus musculus* AR (M37890), *Rattus norvegicus* AR (M20133), and *Homo sapiens* AR (M23263). (E) Expression of AR mRNA in adult male tissues. The *ARα* and *ARβ* expressions were detected by RT-PCR. Total RNA was prepared from liver, kidney, gill, heart, brain, testis, GP, pectoral fin, and caudal fin of male western mosquitofish ($n = 5$). EF1α was used as a control.

both epithelial and mesenchymal regions of the anal fin (Fig. 4I and J, day 6). In such *ARs* expressing distal anal fin rays, PCNA signals associating cell proliferation were prominently observed. Therefore, the distal region of the anal fin was predicted to be sensitive to the actions of androgen. However, RT-PCR analysis revealed that both *AR* expressions were observed not only in the developing GP but also in other fins (Fig. 4M, day 3 to day 10, $n = 20$ fins for each group). These results indicated that anal fin specific outgrowth appeared not solely attributable to *AR* expression levels. Hence, we examined the developmental process of GP in relation with possible down-stream genes to understand the mechanisms underlying the androgen dependent anal fin outgrowth.

3.4. *Shh and Ptc1 expressions correlate with androgen dependent fin outgrowth*

The GP mainly comprises the dermal skeleton of fin rays, lepidotrichia. The signaling molecule, sonic hedgehog (Shh), participates in developing or regenerating fins [32,47,48] and is thought to regulate proliferation and/or differentiation of scleroblasts. Shh signaling is also known to be required for morphogenesis of the penis [49] as well as the initial outgrowth of the genital tubercle (GT) and cloaca [50].

We, thus, isolated cDNAs for western mosquitofish Shh and Shh receptor Ptc1, and examined their spatial and temporal expression patterns. *Shh* was expressed clearly in two lateral domains of the basal layer of the epidermis adjacent to the proliferating distal mesenchyme (Fig. 4E, day 6, 9 mm TL). At the onset of anal fin outgrowth, *Shh* expression was first observed notably in the prospective intromittent fin rays (Fig. 4D arrowheads, day 6) and a significant level of *Shh* expression was sustained specifically in the outgrowing anal fin rays during GP development (Fig. 4F, day 17, 11 mm TL). Such enhancement of *Shh* expression by androgen was observed by RT-PCR in anal fins from day 3 of ET-treatment (Fig. 4M). Ptc1 is a target of Shh signaling and an increase of *Ptc1* expression is considered as an indicator of Shh signaling [51,52]. The *Ptc1* expression was detected in the distal epithelium and the mesenchyme adjacent to the *Shh* expressing region of ET-treated anal fin rays (Fig. 4G and H, day 6, 9 mm TL) following *Shh* expression (Fig. 4M). Such a close association between *Shh* expression and androgen-induced anal fin outgrowth suggests the possibility that the specific elongation of anal fin rays is supported by the activation of down-stream genes, including *Shh* expression under the influence of androgen action. To address this question, we

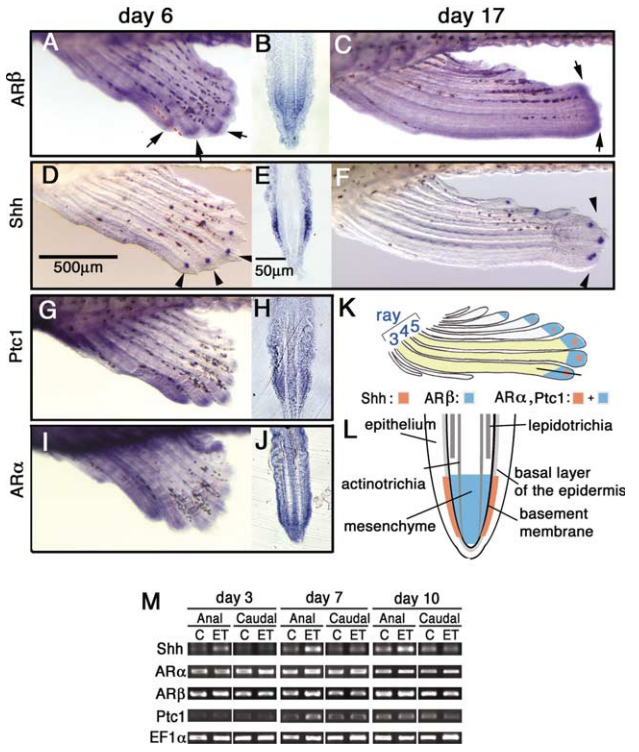

Fig. 4. Gene expression analysis of ARα (I, J), ARβ (A–C), Shh (D–F), and Ptc1 (G, H) during ET (32 nM)-induced GP outgrowth in early juvenile fry (days of treatments indicated, A, D, G, I: 9 mm TL, $n = 48$–103; C, F: 11 mm TL, $n = 20$). Arrows and arrowheads indicate *ARβ* and *Shh* expressions in the prospective intromittent fin rays, respectively. B, E, H, and J are the longitudinal sections of 3rd fin rays of A, D, G, and I, respectively (10 μm thickness). The plane of the sections is indicated as a red dotted line in (A). (M) RT-PCR for *Shh*, *ARα* and *ARβ*, and *Ptc1* in anal fins and caudal fins at days 3, 7, and 10 of treatment (days 3 and 7: 9 mm TL; day 10: 10 mm TL, $n = 20$ fins for each group). The ARα and ARβ genes were expressed in both fins. The *Shh* and *Ptc1* expressions were prominently induced in anal fins by the ET (32 nM)-treatment. (K, L) Schematic representation of developing GP, in which anterior 3rd to 5th fin rays are prospective intromittent fin rays. The black line in (K) indicates the plane of the sections shown in (L).

examined the effects of an AR-antagonist, flutamide, during GP development.

3.5. Inhibition of the initial induction of Shh and GP development by flutamide

Flutamide was co-administered with ET from day 2 of ET treatment (thereafter indicated as ET/F), which was before the appearance of the obvious induction of *Shh*, to examine its effect on the initial induction of *Shh*. ET/F treatments retarded anal fin outgrowth compared with the ET-treated group at day 9 (10 mm TL, Fig. 5A and B). There were no significant differences of the body length between ET-treated and ET/F-treated groups. The *Shh* expression was increased by the ET-treatment compared with controls at day 5 (9 mm TL), whereas the level of such *Shh* induction was comparatively low in the ET/F-treatments (Fig. 5D, $n = 20$ fins for each group). A decreased level of *Shh* induction by flutamide was also confirmed by in situ hybridization analysis (Fig. 5C, day 5, 9 mm TL). When flutamide was treated with ET from day 1, *Shh* induction was almost completely suppressed (data not shown). These results indicate that the initial induction of *Shh* expression is under the influence of androgen actions in the developing GP.

In mouse penile development, it is known that the Shh cascade is critical to postnatal penile morphogenesis related to male sexual function [49], though it is not clear whether *Shh* expression is under the influence of androgen action. The *Shh* expression during GP development indicates the possibility that some shared molecular mechanism might lie in the developmental processes of histologically different types of copulatory organ.

To examine the effects of androgen actions on cell proliferation in developing GP, we performed BrdU incorporation studies. We observed that the distribution of proliferating cells concentrated at the distal region of the developing GP at day 5 of treatment (9 mm TL, Fig. 5E, compare b with a), while a decreased number of BrdU-positive cells were distributed in ET/F treatments (Fig. 5E, compare c with b). These results imply that AR-signaling may regulate cell proliferation contributing to the increase of new bone segments in developing GP fin rays.

To further test the role of Shh in developing GP, we administrated cyclopamine, a steroidal alkaloid that blocks hedgehog signaling, with ET from day 5 of ET-treatment. There were no significant differences in the body length of each experimental group. Inhibition of Shh signaling by cyclopamine almost completely suppressed cell proliferation in distal mesenchyme at day 7 of treatment (9 mm TL, Fig. 5E, compare e with d) and the addition of new bone segments at day 9 (10 mm TL, Fig. 5B). Taken together, these observations indicate that anal fin specific enhancement of *Shh* expression by androgen was required for cell proliferation contributing to anal fin outgrowth, leading to the formation of genital appendage, the GP in teleost fishes.

4. Discussion

To address the role of AR signaling in anal fin to GP transition, we isolated two kinds of AR cDNAs, ARα and ARβ, and Shh and Ptc1 cDNAs from western mosquitofish. The ARβ might be more closely related with tetrapod ARs based on sequence similarities.

We next characterized the ARs and other regulatory gene expressions during the androgen dependent growth and segmentation stage of GP development. Our gene expression analysis revealed that both *ARs* were expressed in the distal portion of the anal fin rays and *Shh* expression was closely associated with androgen-induced anal fin outgrowth. Functional antagonism to AR signaling by flutamide treatment showed retarded cell proliferation in distal anal fin regions as observed in cyclopamine treated anal fins, accompanied by a reduced level of the *Shh* induction. These results appear to imply that AR-signaling with a relay to Shh signaling regulates cell proliferation that contributes to the development of new bone segments in developing GP fin rays.

Shh is expressed in epithelia at numerous sites of epithelial-mesenchymal interactions in vertebrates [53,54]. Prostate development is unique in that it requires the presence of DHT [55]. In response to DHT stimulation, *Shh* is expressed in the urogenital sinus epithelium that forms the nascent prostate

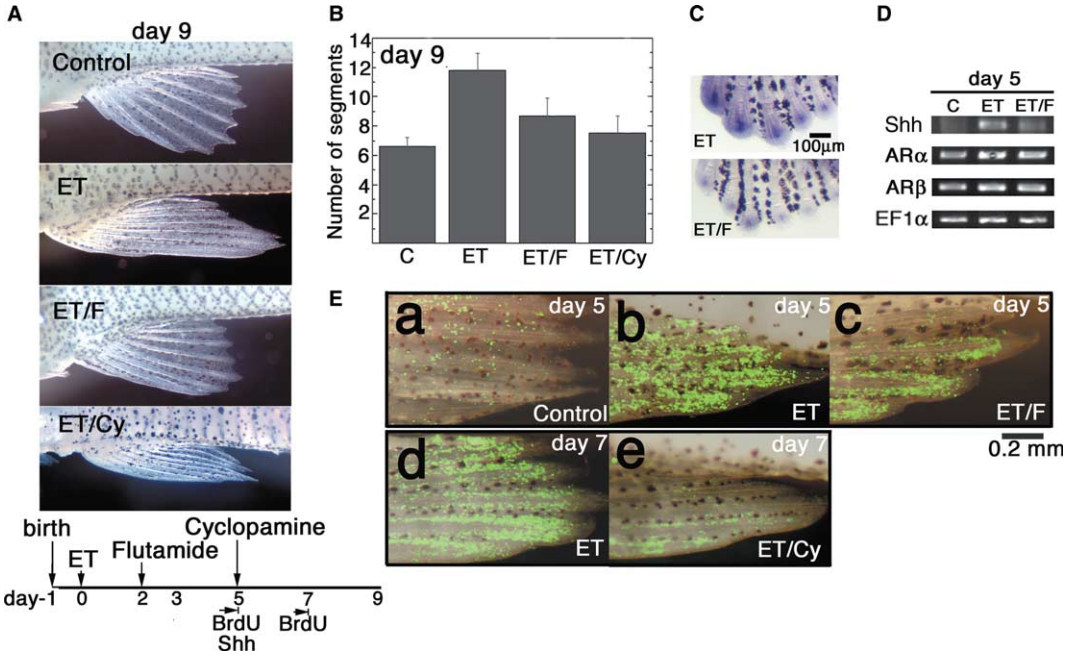

Fig. 5. Inhibition of GP development by flutamide or cyclopamine. (A) Anal fin outgrowth was monitored at day 9 of treatment (10 mm TL). Fry were exposed to flutamide with ET after 2 days of ET-treatment (indicated as ET/F). Cyclopamine was co-administrated with ET from the 5th day of ET-treatment (indicated as ET/Cy). (B) Numbers of segments of the 3rd fin rays were counted at day 9 to monitor the growth of the anal fin rays after various treatments (means ± standard deviation, 10 mm TL, $n = 15$–28). Both ET/F and ET/Cy groups show the reduced levels of segmentation compared with ET-treated group ($P < 0.05$). (C) *Shh* expression in anal fins at day 5 (9 mm TL) of ET (3.2 nM)-treatment and of flutamide (3.6 μM) co-treatment from day 2 ($n = 10$ for each group). (D) RT-PCR for *Shh* and *ARs* at day 5 of treatments. Total RNA was isolated from the anal fins at day 5, 3 days after the flutamide co-treatment (9 mm TL, $n = 20$ fins for each group). Note the inhibitory effects of flutamide on *Shh* expression in developing GP. (E) BrdU incorporation in distal anal fins after 5 days in controls (a), with ET (3.2 nM) (b), with flutamide (3.6 μM) and ET (c) (9 mm TL, $n = 20$ for each group). The incorporation was also monitored at day 7 of ET (d), with cyclopamine (10 μM) and ET (e) (9 mm TL, $n = 15$ for each group). The fry were incubated in BrdU-containing water for 20 hours and its incorporation was detected immunologically (by Alexa Flour 488).

bud [56]. The molecular mechanism of androgen dependent *Shh* expression is not yet elucidated.

During GP development, *Shh* was expressed in the basal layer of the epidermis, and *ARβ* expression was observed predominantly in the mesenchyme. Although functional characterization is required to elucidate which or both ARs are functional for GP development, our findings indicate possibilities including the indirect regulation of *Shh* expression by a putative paracrine mechanism whose signaling is mediated through androgen action from mesenchyme to epithelia. Other possibilities could also exist from our current data and further investigation of factors that mediate androgen signaling for epithelial expression of *Shh* via mesenchymal *AR* should bring a better understanding of the actions of sex steroids. GP development might offer a good model to investigate potential cross talks of growth factors and steroid hormone-signaling pathways during the expression of secondary sexual characters.

Recently, Thornton proposed that the AR was generated by gene duplication in the jawed vertebrate lineage, after the lamprey-gnathostome divergence [57]. The neighbor-joining tree obtained here suggests that the duplication event that gave rise to two different teleost ARs probably occurred in the actinopterygian lineage leading to teleosts after the divergence of actinopterygii and tetrapods. Such *AR* expression in teleost fins might provide novel informations on the role of AR in sexually dimorphic development. It may possibly pertain to morphological diversification as the secondary sexual characters in highly differentiated teleost fins.

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