Epigenetic Modifications During Sex Change Repress Gonadotropin Stimulation of *Cyp19a1a* in a Teleost Ricefield Eel (*Monopterus albus*)

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In vertebrates, cytochrome P450 aromatase, encoded by *cyp19a1*, converts androgens to estrogens and plays important roles in gonadal differentiation and development. The present study examines whether epigenetic mechanisms are involved in *cyp19a1a* expression and subsequent gonadal development in the hermaphroditic ricefield eel. The expression of the ricefield eel *cyp19a1a* was stimulated by gonadotropin via the cAMP pathway in the ovary but not the ovotestis or testis. The CpG within the cAMP response element (CRE) of the *cyp19a1a* promoter was hypermethylated in the ovotestis and testis compared with the ovary. The methylation levels of CpG sites around CRE in the distal region (region II) and around steroidogenic factor 1/adrenal 4 binding protein sites and TATA box in the proximal region (region I) were inversely correlated with *cyp19a1a* expression during the natural sex change from female to male. In vitro DNA methylation decreased the basal and forskolin-induced activities of *cyp19a1a* promoter. Chromatin immunoprecipitation assays indicated that histone 3 (Lys9) in both regions I and II of the *cyp19a1a* promoter were deacetylated and trimethylated in the testis, and in contrast to the ovary, phosphorylated CRE-binding protein failed to bind to these regions. Lastly, the DNA methylation inhibitor 5-aza-2′-deoxycytidine reversed the natural sex change of ricefield eels. These results suggested that epigenetic mechanisms involving DNA methylation and histone deacetylation and methylation may abrogate the stimulation of *cyp19a1a* by gonadotropins in a male-specific fashion. This may be a mechanism widely used to drive natural sex change in teleosts as well as gonadal differentiation in other vertebrates. (*Endocrinology* 154: 2881–2890, 2013)

In vertebrates, pituitary gonadotropins (LH and FSH) control the production of gonadal steroids such as estrogens and androgens. The ratios of gonadal steroid hormones produced are important to the orchestration of many physiological processes, including sexual differentiation and reproduction (1–4). The conversion of estrogens from androgens is catalyzed by an enzyme complex, aromatase, which consists of a flavoprotein (reduced nicotinamide adenine dinucleotide phosphate)-cytochrome P450 reductase and a cytochrome P450 aromatase, the latter of which is encoded by *Cyp19a1* gene (5). Studies on mammals show a decreased ability of FSH to stimulate *Cyp19a* in the mature testes (6, 7). Similarly, *cyp19a1a* gene expression in the teleost testis is unresponsive to stimulation by gonadotropins (8). These findings, in conjunction with the observation that elevated aromatase leads to male infertility (9), suggest that the inhibition of aromatase activity is critically required for the testis to achieve its functional capacity.

Epigenetic modification of genomic DNA and histones alters gene expression from the same genetic blueprint and are crucial for development and differentiation (10, 11). The dominant mechanisms for epigenetic regulation include DNA methylation and histone acetylation, which inhibit and stimulate gene transcription, respectively (11). Recent studies suggested that epigenetic mechanisms may regulate *Cyp19a1* expression in mammals including the human (12), sheep (13), buffalo (14), and rat (15). In skin

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Abbreviations: Ad4BP, adrenal 4 binding protein; ChIP, chromatin immunoprecipitation; CRE, cAMP response element; CREB, CRE-binding protein; EPE, ricefield eel pituitary extracts; hCG, human chorionic gonadotropin; Sf-1, steroidogenic factor 1.

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fibroblasts, one pathway for such regulation is by DNA methylation of the *CYP19* I.3/II promoter region to repress cAMP-stimulated aromatase activity (16). To date, whether this regulatory pathway is also involved in regulating *Cyp19a1* response to gonadotropin/cAMP signals in the gonad, particularly the testis, remains to be clarified.

The ricefield eel, *Monopterus albus*, is a protogynous hermaphrodite fish that changes sex naturally from a functional female to a functional male. *M albus* expresses 2 forms of *cyp19a1* genes: *cyp19a1a*, mainly in the gonad, and *cyp19a1b*, mainly in the brain. The continuous decrease of gonadal *cyp19a1a* mRNA levels occurs during the natural sex change and is thought to be a requisite for female-to-male transformation (17). However, mechanisms underlying this down-regulation in the ricefield eels are unclear. This study explores the possibility that methylation and acetylation state of the *cyp19a1a* promoter and its associated histones is key to the suppression of this gene and therefore successful sex change. Our data indicate that the DNA methylation of the *cyp19a1a* promoter is inversely correlated with ricefield eel *cyp19a1a* expression, and a repressive local chromatin status is established during sex change from female to male to down-regulate aromatase activity. In conjunction with the previously published data from mammals (14, 16), we suggest that epigenetic modifications of the *Cyp19a1* promoter may represent an important mechanism widely used by vertebrates to differentially alter gonadal *cyp19a1* gene expression in response to pituitary gonadotropins. As such, these mechanisms may contribute significantly to the recrudescence, maturation, and senescence of gonadal function in diverse vertebrates.

Materials and Methods

Hormones and reagents

Forskolin (F3917), SQ22,536 (S153), and human chorionic gonadotropin (hCG; CG10) were purchased from Sigma Chemical (St Louis, Missouri). For stock solutions, SQ22,536 and hCG were dissolved in sterile double-distilled water, and forskolin was dissolved in ethanol. The ricefield eel pituitary extracts (EPE) were prepared from mature female fish (gonadosomatic index >5%, presumably with high FSH and LH stores) according to the methods previously described (18). The protein concentrations of the pituitary extracts were quantified by Coomassie Brilliant Blue staining methods. All stocks were stored at −20°C and diluted to the desired concentrations with medium before use.

Animals and gonadal tissues

All procedures and investigations were reviewed and approved by the Center for Laboratory Animals of Sun Yat-sen University and were performed in accordance with the guiding principles for the care and use of laboratory animals. The ricefield eels used for in vivo studies (n = 30, body length 25–37 cm, and body weight 18–43.7 g) were provided by Dazhong Breeding Co Ltd (Jianyang, Sichuan, China), whereas those used for other studies (n = 150, body length 20–55 cm, and body weight 19–70 g) were purchased from a local dealer in Guangzhou, Guangdong, China. Fish were killed by decapitation, and the phenotypic sex and gonadal developmental stages were verified by histological examination. The transition from female to male was classified into 5 phases according to our previous work (17), namely female stage, early intersexual stage, middle intersexual stage, late intersexual stage, and male stage.

Total RNA isolation and quantification of *cyp19a1a* expression

Total RNA was isolated from frozen or cultured tissue fragments using TRIzol reagent (Invitrogen, Carlsbad, California). Real-time PCR analysis was established to determine the mRNA expression levels of ricefield eel *cyp19a1a* in the present study. Details are provided in Supplemental Materials and Methods, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

In vitro incubation of gonadal fragments

The gonads of the ricefield eels at different sexual stages, female, middle intersex, and male were dissected out. Approximately 50 mg of gonadal minces were incubated in 1 mL of L15 media (Sigma) containing 0.1 U/mL penicillin (Gibco, Gaithersburg, Maryland) and 0.1 μg/mL streptomycin (Gibco) in 24-well plates (Corning, Tewksbury, Massachusetts) at 25°C in a humidified incubator under 5% CO_2. The effects of hCG, EPE, forskolin, and SQ22,536 on gonadal *cyp19a1a* expression were examined. Details are provided in Supplemental Materials and Methods.

Determination of DNA methylation status by sodium bisulfite genomic sequencing

One microgram of genomic DNA was subjected to sodium bisulfite treatment with the CpGenome Fast DNA modification kit (Chemicon International, Temecula, California) according to the manufacturer's instructions. Details are provided in Supplemental Materials and Methods.

Cyp19a1a-Luc reporter vectors and in vitro methylation

The *cyp19a1a*-Luc reporter vector was generated by inserting a 1860-bp fragment containing the 5′ flanking region of the ricefield eel *cyp19a1a* gene into the pGL3 basic vector (Promega life Science, Madison, Wisconsin) between *Sac*I and *Xho*I sites. This fragment was amplified by PCR from ricefield eel genomic DNA with primers CYP19PF1 and CYP19PR1 (Supplemental Table 1). A reporter construct containing a mutated cAMP response element (CRE) site (from 5′-CTACGTCA-3′ to 5′-CTAATTCA-3′) in the *cyp19a1a* promoter was also generated, and details are provided in Supplemental Materials and Methods.

The in vitro methylation of the cyp19a1a-Luc reporter vector was performed with 5 U of M.SssI methylase (New England Biolabs, Inc, Beverly, Massachusetts) for each microgram of the vector in the presence of 160 mM S-adenosylmethionine at 37°C

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for 3 hours. The completion of CpG site methylation in the promoter region was confirmed by digestion with methylation-sensitive *Hha*I restriction enzyme followed by agarose electrophoresis. Fully methylated reporter vectors were used for transient transfection assays.

Cell culture, transient transfection, and dual-luciferase reporter assay

Both TM4 (mouse Sertoli cell line) cells and rat granulosa cells in primary culture were maintained at 37°C in a humidified incubator under 5% CO_2, with the former in DMEM/F12 (Gibco) containing 2.5% fetal bovine serum, 5% horse serum, and 1 mg/mL penicillin-streptomycin (Gibco), and the latter in DMEM/F12 (Gibco) containing 10% fetal bovine serum and 1 mg/mL penicillin-streptomycin (Gibco). The details for the isolation of rat granulosa cells for primary culture were described in Supplemental Materials and Methods. The rationale for choosing these 2 cell types was that TM4 cells conserve a large spectrum of functional features present in immature Sertoli cells, in which *Cyp19a1* expression is up-regulated by cAMP signals (19), as in granulsoa cells (20). The promoter of ricefield eel *cyp19a1a* contains a consensus CRE site; and thus, the cellular context of TM4 and rat granulose cells may be appropriate for examining the regulation of *cyp19a1a* promoter activities by cAMP signaling pathway.

Vectors for transfection were prepared from overnight bacteria cultures using PureLink HI-Pure plasmid DNA purification kit (Invitrogen) according to the manufacturer's protocol and transfected into primary cultures of rat granulosa cells or TM4 cells using Lipofectamine 2000 (Invitrogen). Reporter luciferase activities were assayed with a dual luciferase kit (Promega, Madison, Wisconsin). Details are provided in Supplemental Materials and Methods.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with an EZ ChIP assay kit (Merck Millipore, Billerica, Massachusetts) using the manufacturer's protocol with minor modifications for the tissues of ricefield eels. The sheared chromatins were immunoprecipitated with either 3 μg of specific antibody against phosphorylated CRE-binding protein (CREB; antiphospho-CREB; Merck Millipore) or with 3 μg of specific antibodies against histone H3K9 (antiacetylated histone H3K9, and antitrimethyl-histone H3K9; Merck Millipore) or with 1 μg of specific antibody against RNA polymerase II or with 1 μg of normal mouse IgG (as a negative control). Details are provided in Supplemental Materials and Methods.

Treatment of ricefield eels with DNA methylation inhibitor 5-aza-2′-deoxycytidine

The ricefield eels were treated with DNA methylation inhibitor 5-aza-2′-deoxycytidine (Sigma) via abdominal implantation, which was performed in Dazhong Breeding Co Ltd from July to August in 2010, when the natural sex change usually takes place. SILASTIC brand pellets (Dow Corning Corp, Midland, Michigan) of 1.5 mm × 1.5 mm × 30 mm with or without DNA methylation inhibitor were prepared by mixing an unpolymerized SILASTIC brand elastomer with curing agent (SILASTIC medical grade MDX4-4210; Dow Corning) at a ratio of 10:1, spreading the mixture onto an aluminum mold with parallel 1.5- × 1.5- × 30-mm cubic slots, and letting cure for 24 hours at room temperature to semitransparent silicon rubber strips.

Thirty ricefield eels of body length 25–37 cm and body weight 18–47.3 g were maintained in concrete tanks (4 × 2 × 2 m^3) under natural conditions and fed with fly larvae to satiety twice a day. After acclimatization for 1 week, the ricefield eels (10 individuals each treatment) were implanted in the intraperitoneal cavity with a control (no DNA methylation inhibitor) or solid SILASTIC brand pellet (Dow Corning) containing DNA methylation inhibitor (1 and 5 mg/kg^{-1} body weight). Biopsy examinations on gonadal tissues of individual animals were made on the day of implantation and 4 weeks after implantation according to the previously reported methods (21). During treatment the ricefield eels in both the control and DNA methylation inhibitor-treated groups took food normally, and the percentage relative growth rates were 0.396 ± 0.063 for the control and 0.304 ± 0.060 and 0.216 ± 0.042 for the lower and higher doses of DNA methylation inhibitor, respectively. Seven individuals in the control and 6 in each of the DNA methylation inhibitor groups survived the experimental procedure. The gonadal tissues obtained from biopsy operations were fixed in Bouin fluid, wax embedded, sectioned at 8 μm, and stained with hematoxylin and eosin for histological examination. The gonadal change was categorized as toward male if both structure and sexual status were switched from an earlier stage to a later one, as toward female if in an opposite direction or as unchanged if remained in similar stages. The mRNA levels of gonadal *cyp19a1a* were analyzed with real-time PCR as above.

Statistical analysis

All data except the number of fish with gonadal changes after in vivo treatment of 5-aza-2′-deoxycytidine are presented as means ± SEM. Differences among groups was determined by 1-way ANOVA followed by the Dunnett test (for comparing treatment groups with the control group) or Tukey multiple comparison test (for comparing all pairs of groups) or determined by Fisher's exact test as specified in figure legends using the SPSS software package (IBM SPSS, Armonk, New York). Significance was set at $P < .05$.

Results

Gonadotropins stimulate *cyp19a1a* expression in the ovary but not in the testis of ricefield eel in vitro

The effects of gonadotropins on *cyp19a1a* expression in the gonads of ricefield eels at different sexual stages were examined in vitro. Both hCG (Figure 1A) and ricefield EPE (Figure 1B) stimulated *cyp19a1a* expression dose dependently in the ovary but had no effects on the intersexual gonad (ovotestis) or testis. Similarly, forskolin, an adenyl cyclase activator that mimicks gonadotropin signaling by elevating cAMP, also dose dependently stimulated *cyp19a1a* expression in the ovary without affecting the intersexual gonad or testis (Figure 1C), whereas the production of cAMP in the in vitro-incubated ovotestis

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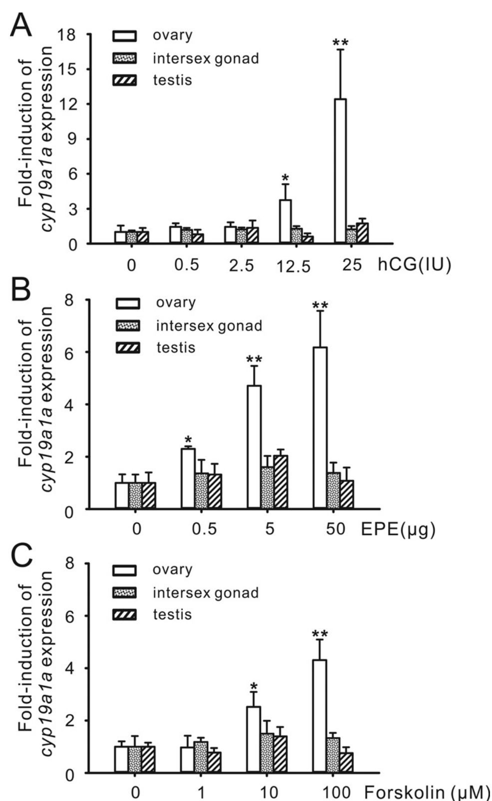

Figure 1. The stimulation of ricefield eel *cyp19a1a* expression by gonadotropins. Gonadal tissues were treated in vitro with hCG (A), EPE (B), and forskolin (C), respectively. The mRNA levels of *cyp19a1a* were determined by real-time RT-PCR and were presented as fold induction relative to the vehicle controls (water for hCG and EPE and ethanol for forskolin, which were designated as 0). Each bar represents mean ± SEM of triplicates. *, $P < .05$; **, $P < .01$ relative to vehicle control (1 way ANOVA followed by the Dunnett test).

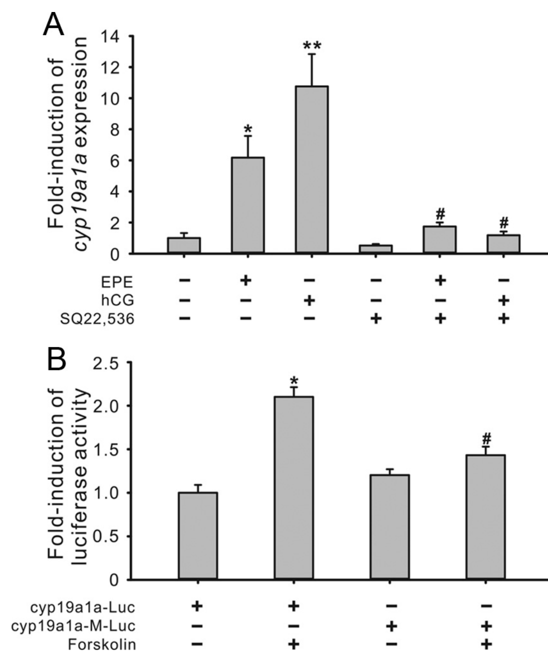

Figure 2. The involvement of cAMP signals in the stimulation of *cyp19a1a* expression by gonadotropins. A, SQ22,536 (10 μM), an adenylate cyclase-specific inhibitor, repressed the stimulation of *cyp19a1a* expression by hCG (500 IU/mL) and EPE (50 μg/mL) in the ovary in vitro. The mRNA levels of *cyp19a1a* were determined by real-time RT-PCR and were presented as fold induction relative to the vehicle control. Each bar represents mean ± SEM of triplicates. *, $P < .05$; **, $P < .01$ relative to vehicle control; #, $P < .05$ relative to EPE or hCG only without SQ22,536 (1 way ANOVA followed by the Tukey multiple comparison test). B, The CRE site mediated the stimulation of *cyp19a1a* promoter activities by forskolin in primary rat ovarian granulosa cells. Cyp19a1a-Luc, Promoter reporter with the wild type CRE; cyp19a1aM-Luc, promoter reporter with the mutated CRE. Data are expressed as fold changes relative to the control (cyp19a1a-Luc only). Each bar represents mean ± SEM of triplicates. *, $P < .05$ vs control; #, $P < .05$ vs cyp19a1a-Luc in response to forskolin (10 nM) (1 way ANOVA followed by the Tukey multiple comparison test).

and testis was significantly increased by the treatment of hCG, EPE, and forskolin just like in the ovary of ricefield eels (Supplemental Figure 1).

Gonadotropin stimulation of *cyp19a1a* expression in the ovary is mediated by cAMP signal pathway

To examine whether the stimulation of *cyp19a1a* mRNA expression by gonadotropins in the ovary was mediated by the cAMP signaling pathway, SQ22,536, a specific adenyl cyclase inhibitor, was included in the treatments by EPE and hCG. SQ22,536 (10 μM) effectively blocked the stimulation of *cyp19a1a* expression by hCG and EPE in the ovary, with approximately 81% inhibition for the former and 72% inhibition for the latter (Figure 2A).

Mutation of the CRE site of *cyp19a1a* 5′ flanking region (JQ797707; Supplemental Figure 2), from 5′-CTACGTCA-3′ to 5′-CTAATTCA-3′, significantly attenuated the stimulatory effects of forskolin on *cyp19a1a* promoter activities (~43% inhibition, Figure 2B), further supporting that the cAMP signaling pathway mediates the stimulation of *cyp19a1a* gene by gonadotropins.

The CRE site of 5′ flanking region of ricefield eel *cyp19a1a* gene is hypermethylated in the testis

Visual inspection of the 5′ flanking region of the ricefield eel *cyp19a1a* (Supplemental Figure 2) revealed that the CpG dinucleotides were not evenly distributed in the flanking region but formed 2 approximate clusters: 7 CpGs in the proximal promoter and exon 1 (−237 to +80; designated as region I) and 6 in the distal promoter (−1767 to −1606; designated as region II). The putative transcription factor binding sites as predicted by the web-based software transcription element search system (http://www.cbil.upenn.edu/tess) include 2 steroidogenic factor 1(Sf-1)/adrenal 4 binding protein sites (Ad4BP) binding sites (5′-CAAGGGCA-3′ and 5′-CAAGGTTA-3′) and 2 half-estrogen response element in region I and 1 CRE (5′-CTACGTCA-3′) containing a CpG dinucleotide and 2 half-ERE sites in region II. Deletion of region II significantly decreased the basal activities of *cyp19a1a* promoter (~31.3% decrease, Supplemental

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Figure 3A), and mutation of both Sf-1/Ad4BP binding sites totally blocked the activation of *cyp19a1a* promoter by Sf-1 (Supplemental Figure 3B), suggesting that both regions I and II are important for the regulation of *cyp19a1a* promoter activities.

The methylation status of each individual CpG dinucleotide in regions I and II of the *cyp19a1a* gene was determined by sodium bisulfite sequencing. The CpG dinucleotide within the CRE motif was methylated in all 10 clones from samples at the stages of middle intersex, late intersex, and male. This is in striking contrast to only 5 of 10 in the ovary (Figure 3).

The DNA methylation levels of the promoter regions of the *cyp19a1a* in the gonads increase and are inversely correlated with mRNA levels during sex change from female to male

In addition to the CpG dinucleotide within the CRE site, the other CpG sites in regions I and II were also progressively hypermethylated during the sex change from female to male, with the percentages of methylated CpG relative to total CpG dinucleotides increased from 50% to 85% within region I and from 56.7% to 100% within region II (Figure 3). Combined bisulfite restriction analysis was used to quantify these methylation changes, and results showed that the DNA methylation levels of both regions I and II in gonads were significantly increased during the sex change from female to male (Supplemental Figure 4, B and C), which was inversely correlated with mRNA levels of *cyp19a1a* in the gonads (Supplemental Figure 4D).

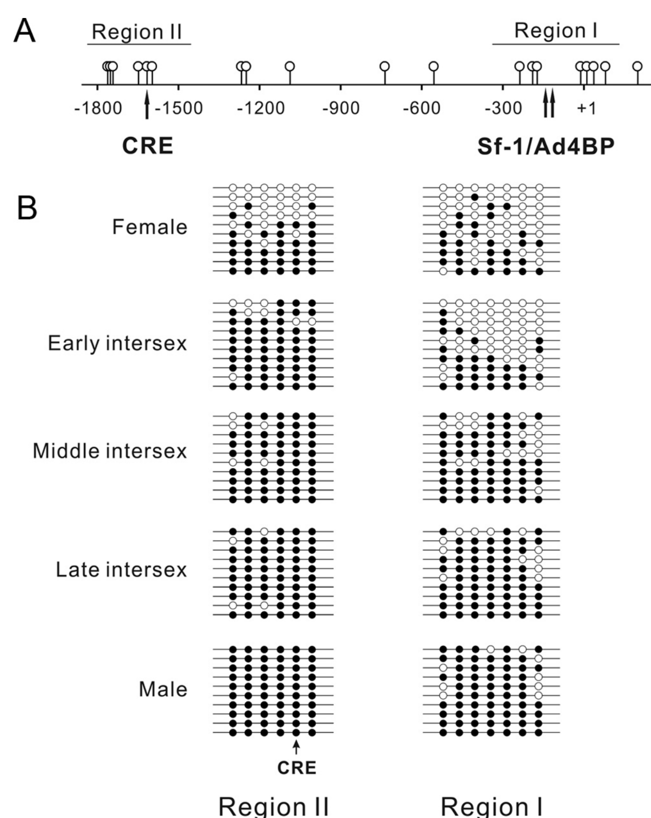

Figure 3. DNA methylation status of the individual CpG sites in regions I and II of *cyp19a1a* promoter as determined by sodium bisulfite sequencing. A, Schematic diagram of CpG dinucleotide (indicated by open circles) distribution and putative sites for Sf-1/Ad4BP and CRE in *cyp19a1a* promoter. B, Unmethylated and methylated CpGs were indicated by open and filled circles, respectively. At least 10 clones from each PCR product were subjected to nucleotide sequence analysis.

DNA methylation suppresses the basal and cAMP-stimulated *cyp19a1a* promoter activity in vitro

To assess the effects of DNA methylation on *cyp19a1a* promoter activity, in vitro methylated and unmethylated promoter reporter plasmids were transfected into primary rat granulosa cells and TM4 cells. The results showed that in vitro methylation decreased basal activities of the *cyp19a1a* promoter in both cell types (Figure 4). The stimulatory effects of forskolin on *cyp19a1a* promoter activity were significantly attenuated by DNA methylation in primary granulosa cells or TM4 cells, with about 68% inhibition for both cases (Figure 4).

Local chromatin of *cyp19a1a* promoter is inaccessible to phosphorylated CREB and repressive to transcription in the testis of ricefield eels

ChIP assays were carried out to further examine the epigenetic status of the *cyp19a1a* promoter in the ovary and testis of ricefield eels. Phosphorylated CREB binds to *cyp19a1a* promoter in the ovary but not testis (Figure 5), although phosphorylated CREB was present at similar levels in the testis and ovary of ricefield eels (Supplemental Figure 5). In the ovary, histone 3 (Lys9) in regions I and II of *cyp19a1a* promoter was acetylated and demethylated, whereas it was deacetylated and methylated in the testis, suggesting a repressive histone code in the latter. As expected, RNA polymerase was recruited to region I of *cyp19a1a* promoter in the ovary but not the testis.

DNA methylation inhibitor reverses the ontogenic gonadal development in ricefield eels

The possible involvement of DNA methylation in gonadal differentiation of the ricefield eel was examined by abdominal implantation of DNA methylation inhibitor 5-aza-2′-deoxycytidine. The sexual status of each fish was examined histologically before and after treatment (Figure 6A). A lower dose (1 mg/kg^{-1} body weight) of DNA methylation inhibitor resulted in gonadal development toward female in 2 of 6 fish, with the remaining 4 unchanged. A higher dose (5 mg/kg^{-1} body weight) resulted in gonadal development toward female in 4 of 6 fish, with the remaining 2 unchanged. In the control group, however, 5

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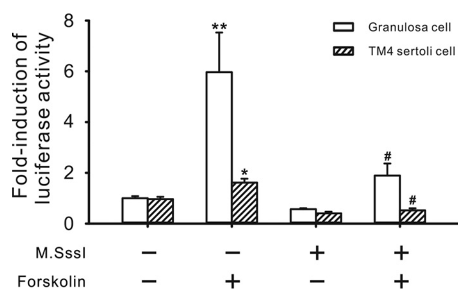

Figure 4. Effects of DNA methylation of *cyp19a1a* promoter on its basal and cAMP-stimulated activities. The reporter construct containing the *cyp19a1a* promoter was methylated in vitro by M. SssI, and methylated/unmethylated reporter constructs were transiently transfected to primary rat ovarian granulosa or TM4 cells, respectively. Luciferase activities were measured 48 hours after transfection, and forskolin (10 μM) was added 6 hours before cell collection. Each bar represents mean ± SEM of triplicates. *, $P < .05$; **, $P < .01$ relative to the unmethylated vector in the absence of forskolin; #, $P < .05$ relative to unmethylated vector in response to forskolin at 10 nM (1 way ANOVA followed by the Tukey multiple comparison test).

of 7 fish developed toward male, with the remaining 2 unchanged. The difference in the frequency of gonadal change between the control and both DNA methylation inhibitor groups was statistically significant ($P < .05$) as analyzed with a Fisher's exact test (Figure 6B). The gonadal *cyp19a1a* expression (Supplemental Figure 6) was significantly up-regulated in the group treated with the lower dose of inhibitor, although not in that with the higher dose. Similarly, in the in vitro-incubated testis fragments, the expression of *cyp19a1a* in response to hCG was significantly increased by treatment with 5-aza-2′-deoxycytidine at 25 μM but not at 50 μM (Supplemental Figure 7A), although the DNA methylation levels of both regions I and II of the *cyp19a1a* promoter were decreased at both doses (Supplemental Figure 7B).

The epigenetic regulation of gonadal *cyp19a1a* gene may be conserved

Comparison of gonadal *cyp19a1* promoters among vertebrates revealed 2 interesting conserved features, particularly in teleosts and *Xenopus laevis* (Figure 7): the presence of CRE motifs and/or Sf-1/Ad4BP binding sites and high densities of CpG dinucleotides around the Sf-1/Ad4BP binding site and TATA box in the proximal promoter region.

In the zebrafish, a teleost relatively distant from the ricefield eel, the proximal promoter region was significantly hypermethylated in the testis compared with the ovary (Supplemental Figure 8B). Furthermore, hCG was shown to stimulate the expression of *cyp19a1a* in the ovary but not the testis of zebrafish (Supplemental Figure 8C).

Discussion

The observation that gonadotropins stimulate ricefield eel *cyp19a1a* expression in the ovary but not the testis is in agreement with the in vivo-positive effects of hCG on the serum estradiol levels in females but not males (22, 23). Similar ovary-specific responses of *cyp19a1a* to hCG were also observed in zebrafish in our present study and in another teleost, the Atlantic croaker *Micropogonias undulatus* (8). These findings suggest that the suppression of *cyp19a1a* expression in males by reducing its transcriptional responsiveness to gonadotropins is a phenomenon conserved in multiple teleosts. Considering the powerful feminizing effects of estrogens, particularly in teleosts (4), and the continuous assault of pituitary gonadotropins and the presence of their cognate receptors and signaling molecules such as cAMP on/in testicular somatic cells, it is very important to repress the response of gonadal *cyp19a1a* to gonadotropin/cAMP

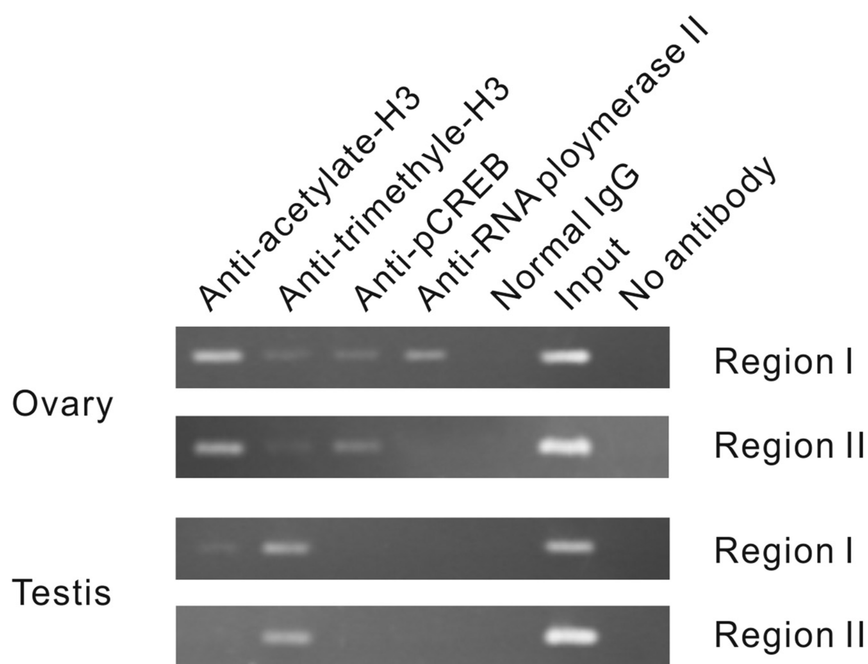

Figure 5. Binding of phospho-CREB to *cyp19a1a* promoter and its histone modification status in the gonads of ricefield eels. A ChIP was performed as described in *Materials and Methods*. The DNA fragments were immunoprecipitated using specific antibodies against acetyl-histone H3K9, trimethyl-histone H3K9, phospho-CREB, RNA polymerase II, or normal mouse IgG; PCR amplified using primer sets corresponding to region I or region II of ricefield *cyp19a1a* promoter; separated on 2.0% agarose gels; and visualized by staining with ethidium bromide.

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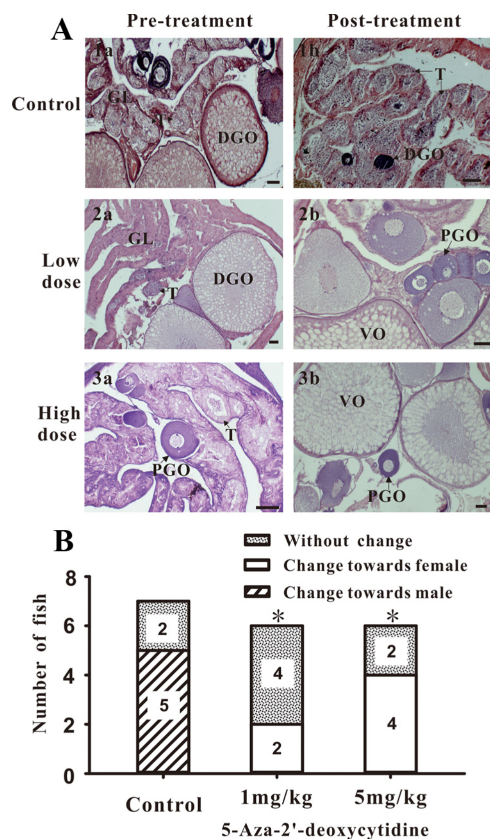

Figure 6. The representative gonadal histology (A) and numbers of fish in gonadal changes (B) in ricefield eels treated with DNA methylation inhibitor 5-aza-2′-deoxycytidine at a low (1 mg/kg) or high (5 mg/kg) dose. The ricefield eel (10 fish for each treatment) was implanted in the intraperitoneal cavity with a control (no DNA methylation inhibitor) or solid SILASTIC brand implant (Dow Corning) containing DNA methylation inhibitor. Biopsy examinations on gonadal tissues of fish were made on the day of implantation and 4 weeks after implantation. HE-stained gonadal sections of the same fish before (1a–3a) and after (1b–3b) treatment were shown in panel A. DGO, degenerating oocyte; GL, gonadal lamella; PGO, primary growth oocyte; T, testis lobule; VO, vitellogenic oocyte. *, Significant difference in the frequency of gonadal change as compared with the control ($P < .05$, Fisher's exact test).

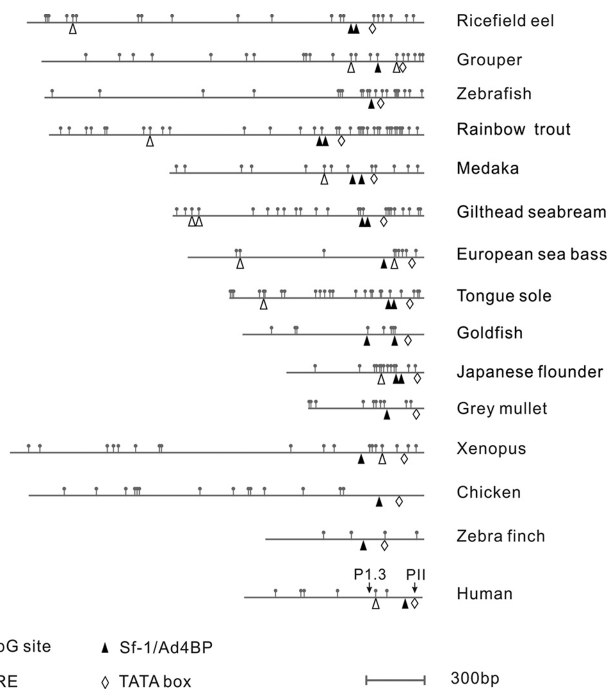

Figure 7. The schematic diagram of CpG sites and putative cis-acting elements in the 5′ flanking regions of gonadal *cyp19a1* genes in representative species of vertebrates. The 5′ flanking sequences of *cyp19a1* gene of different species were downloaded from *Entrez* (National Center for Biotechnology Information) and have the following accession numbers: grouper (EU239953); zebrafish (AF406757); rainbow trout (AB210815); medaka (D82969); gilthead seabream (AY779630); European sea bass (DQ177458); tongue sole (EF421177); goldfish (AF324895); Japanese flounder (AB303853); gray mullet (AY859426); Xenopus (AB179887); chicken (NC_006097); zebra finch (AF170273); human (16). The putative transcriptional binding sites were predicted based on the following motifs: Sf-1/Ad4BP (5′-YCAAGGYCR-3′) and CRE (5′-TGACGTCA-3′ or 5′-CGTCA-3′).

signals male specifically. However, the underlying mechanisms remain to be elucidated. Our present study in ricefield eels strongly suggested that DNA methylation and histone 3 (Lys9) deacetylation and trimethylation are largely responsible for abrogating the stimulatory effects of gonadotropin on testicular *cyp19a1a* gene expression in vivo. The epigenetic modifications of *cyp19a1a* may represent a crucial mechanism for establishing the sexually dimorphic expression of goandal *cyp19a1a* in teleosts and possibly other vertebrates as well.

As in mammals, the stimulation of *cyp19a1a* by gonadotropins in the ricefield eel ovary was mainly mediated by cAMP (20). However, the methylated CRE of the ricefield eel *cyp19a1a* promoter in the ovotestis and testis may directly block the binding of CREB (16), similar to the case of CTCF binding at the *H19/igf2* imprinting control region (11), thereby attenuating the stimulatory effects of gonadotropin/cAMP signals on *cyp19a1a*. In support of this, a ChIP assay revealed that the ricefield eel *cyp19a1a* promoter could bind phosphorylated CREB in the ovary but not in the testis, although phosphorylated CREB is also present in the testis at a similar level to that in the ovary as revealed by Western blot analysis. Furthermore, demethylating agent 5-aza-2′-deoxycytidine increased the expression of *cyp19a1a* in response to hCG in the in vitro-incubated testis fragments but decreased the DNA methylation levels of the *cyp19a1a* promoter, suggesting that the DNA methylation-mediated mechanism may interfere with the binding of phospho-CREB to *cyp19a1a* promoter in the testis of ricefield eels.

In addition to CRE, Sf-1/Ad4BP (also known as Ftz-f1 in some species) could also activate the transcription of *Cyp19a1* and mediate the stimulatory effects of gonadotropin/cAMP signals (25-29). In contrast to the sexual dimorphic expression of gonadal *cyp19a1a* in teleosts, the

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expression of Ftz-f1 homologues in gonads did not show sexual dimorphism at the protein level in ricefield eels (Supplemental Figure 5) and at the mRNA levels in others (28-30). Interestingly, a relatively high density of CpG dinucleotides is present around the Sf-1/Ad4BP binding site in the proximal promoter (region I) of ricefield eel *cyp19a1a,* which were progressively hypermethylated during the sex change from female to male. A ChIP assay indicated that histone 3 in region I of the *cyp19a1a* promoter was apparently trimethylated and deacetylated at lysine 9 in the testis as compared with those in the ovary of ricefield eels. Methylation and deacetylation at histone 3 (Lys9) are known to cause transcription repression through chromosome condensation (31, 32). Thus, DNA hypermethylation and repressive chromatin structure of region I observed in the testis may render Sf-1/Ad4BP unable to assemble the transcription machinery and further suppress the stimulation of gonadotropin/cAMP signals on *cyp19a1a* in the testis.

The continuous decrease of *cyp19a1a* expression during the female to male sex change in ricefield eels is consistent with the reported decrease in serum 17β-estradiol during this process (33), and decreased expression of *cyp19a1a* was also observed in other protogynous hermaphroditic teleosts (34, 35). In teleosts, it is suggested that *cyp19a1a* up-regulation triggers ovarian differentiation, whereas its down-regulation induces testicular differentiation (36). Our present study showed that during the natural sex change from female to male of ricefield eels, the increase of DNA methylation level and repressive chromatin state of *cyp19a1a* promoter were inversely correlated with the *cyp19a1a* transcript levels. Importantly, in vivo treatment of ricefield eels with DNA methylation inhibitor 5-aza-2′-deoxycytidine reversed the characteristic gonadal remodeling during the sex change by enhancing the regeneration of new primary growth oocytes in the ovotestis, and gonadal *cyp19a1a* expression was induced significantly by the treatment of DNA methylation inhibitor at a lower dose. The reason that the higher dosage of DNA methylation inhibitor did not increase *cyp19a1a* expression in the gonad in vivo is not known yet.

A similar phenomenon was also observed in the expression of *cyp19a1a* in response to hCG in the in vitro-incubated testis after treatment with the DNA methylation inhibitor. It is considered that 5-aza-2′-deoxycytidine causes demethylation at low doses but has cytotoxic effects at high doses (37). The relative growth rates were slightly decreased in ricefield eels treated with the DNA methylation inhibitor, suggesting the DNA methylation inhibitor may have some slight cytotoxic effects on the ricefield eels. What these cytotoxic effects are and whether they are involved in the down-regulation of gonadal *cyp19a1a* expression remain to be investigated. However, a higher dose of 5-aza-2′-deoxycytidine was even more effective in reversing the gonadal development of the sex-changing ricefield eels than a lower does, suggesting that in addition to the activation of *cyp19a1a*, 5-aza-2′-deoxycytidine may promote the ovarian differentiation in the sex-changing ricefield eels via other mediators in the gonadal differentiation signaling pathways, especially at higher doses. Thus, results of our present study suggest that the epigenetic mechanisms, most likely through the regulation of gonadal *cyp19a1a*, and possibly other mediators of gonadal differentiation as well, may be crucial to initiate natural sex change and maintain male gonadal state in ricefield eels.

In addition to the ricefield eel, DNA hypermethylation of the proximal promoters of *cyp19a1a* genes was also observed in the testis of other teleosts including the zebrafish (Supplemental Figure 8), medaka (38), and European sea bass (24). In the European sea bass, a species with sex determination controlled by both genetic and temperature effects, Navarro-Martin et al (39) showed for the first time that an increased temperature during a critical period in early development was able to increase promoter DNA methylation and prevent gonadal expression of *cyp19a1a* and suggested that *cyp19a1a* promoter methylation is most likely part of the long-sought-after mechanism connecting temperature and environmental sex determination in vertebrates. Phylogenetic analysis of proximal promoters of *Cyp19a1* genes in the present study as well as by Navarro-Martin et al (39) showed a striking similarity across species in relation to the positions of Sf-1/Ad4BP binding site and CpG dinucleotides, especially among teleosts. Furthermore, in vitro DNA methylation blocked the response of the ricefield eel *cyp19a1a* promoter construct to cAMP signals and the European sea bass *cyp19a1a* promoter construct to Sf-1 (39). These lines of evidence suggest that the general DNA architecture that allows the epigenetic mechanisms to suppress the activation of *cyp19a1a* promoter by Sf-1/Ad4BP and CREB is highly conserved. This architecture is likely used by diverse teleosts to control sexual differentiation and gonadal maturation.

In conclusion, we demonstrated herein that the gonadotropin stimulation of *cyp19a1a* promoter activities is down-regulated in a male-specific fashion by epigenetic changes that include DNA methylation and histone modifications, leading to chromatin remodeling (Supplemental Figure 9). The epigenetic control of *cyp19a1a* gene expression could play an important role in the gonadal differentiation of teleosts and possibly other lower vertebrates. The mechanisms for establishing the cell/tissue-

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specific DNA methylation pattern and histone code of *cyp19a1a* promoters are worth further study.

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