

1 Androgen-*dmrt1* positive feedback programs the rice field eel
2 (*Monopterus albus*) sex transdifferentiation

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17 Abstract

18 The rice field eel *Monopterus albus* is a hermaphroditic protogynous fish species that
 19 undergoes sex reversal from female to male. However, the potential mechanisms
 20 underlying the process of sex transformation are still unclear. We analyzed and
 21 compared the gene sequence of *M. albus dmrt1* 5' upstream region and its potential
 22 transcription factor binding sites with other known species and examined the *in vitro*
 23 effects of testosterone (T) on the expression levels of *dmrt1a* and *foxl2* in the ovotestis.
 24 Moreover, we cloned and analyzed the expression of genes encoding enzymes,
 25 11 β -hydroxylase (*11 β -h*) and 11 β -hydroxysteroid dehydrogenase (*11 β -hsd*), involved
 26 in the production of 11-ketotestosterone (11-KT). The results showed that, compared
 27 with other fish species, *M. albus dmrt1* 5' upstream region contained unique androgen
 28 response elements (AREs) with one on the sense strand and the other one on the
 29 antisense strand, indicating a crucial role for androgens in the transcriptional
 30 regulation of *dmrt1*. The expression of *dmrt1a* was induced but the expression of
 31 *foxl2* was inhibited by T manipulation *in vitro*, suggesting that blood androgen could
 32 activate the transcription of *dmrt1* in the ovotestis. Moreover, the expression levels of
 33 *11 β -h* and *11 β -hsd2* were predominantly expressed in testis, much less in ovotestis,
 34 and barely in ovary, suggesting the production of 11-KT during sex reversal.
 35 Androgens are synthesized in large amounts during sex reversal, leading to the
 36 promotion of *dmrt1* transcription, and thus, gonadal somatic cells transdifferentiation.
 37 Overall, androgen-*dmrt1* positive feedback programs the *M. albus* sex reversal.

38 **Key Words:** *Monopterus albus*; *Dmrt1*; *Foxl2*; *11 β -h*; *11 β -hsd*.

40 Introduction

41 Androgens in teleosts are essential for inducing male phenotype and male
42 gametogenesis, and female-to-male sex reversal in some species. Both testosterone (T)
43 and 11-ketotestosterone (11-KT) are detected in males, the latter being the potent
44 androgen responsible for testicular development (1). The regulation of enzymes
45 involved in the biosynthesis of 11-KT are critical for teleostean reproduction.
46 11β -hydroxylase (*11\beta-h*) and 11β -hydroxysteroid dehydrogenase (*11\beta-hsd*) are two
47 important steroidogenic substrates for the production of 11-KT (2, 3). During
48 spermatogenesis substantial changes in the expression level of *11\beta-h* are observed in
49 rainbow trout *Oncorhynchus mykiss* (4, 5), medaka *Oryzias latipes* (6), Atlantic
50 salmon (7), Nile tilapia *Oreochromis niloticus* (8, 9) and catfish *Clarias batrachus*
51 (10). Similarly, *11\beta-hsd* transcripts are present in the steroidogenic tissue of *O. mykiss*
52 and its transcriptional signals were observed in the Leydig cells of testes, in the thecal
53 cells of early vitellogenic ovarian follicles, and in the thecal and granulosa cells of
54 midvitellogenic and postovulatory follicles (2). Also, *11\beta-hsd2* is expressed in various
55 tissues of *O. niloticus*, with the highest expression level observed in the testis (3).

56 Many genes are known to be involved in gonadal differentiation in vertebrates.
57 *Dmrt1*, a gene that encodes a transcription factor with a DM-domain, is one of the
58 essential genes controlling testicular differentiation in mammals, birds, reptiles,
59 amphibians and fish (11-13). In *O. mykiss*, for example, *dmrt1* is expressed during
60 testicular differentiation but not during ovarian differentiation (14). *Dmy* is enough for
61 male development in *O. latipes* and loss of *dmy* in XY medaka causes male-to-female
62 sex reversal (15-17). *O. latipes* also has an autosomal copy of *dmrt1* which is
63 expressed in testis later than *dmy* but is essential for testis development (18, 19).
64 *Dmrt1* is not only associated with testis development, but also, may be crucial for the

65 ovary differentiation in zebrafish (20); however, Webster et al. (21) reported that
66 *dmrt1* is dispensable for ovary development but necessary for testis development by
67 regulating *amh* and *foxl2*. Wen et al. (22) observed that *dmrt1* expression was 70
68 times higher in the testis of olive flounder *Paralichthys olivaceus* than in the ovary.
69 Also, in European sea bass, the expression of *dmrt1* is increased in testis but
70 decreased in ovary (23).

71 The rice field eel *Monopterus albus* is a hermaphroditic fish species that
72 undergoes sexual reversal from a functional female to a male (24). The *M. albus* is
73 emerging as a specific model for studying vertebrate sexual development due to its
74 small genome size and naturally occurring sex reversal (25). Yeung et al. (26, 27)
75 examined the effects of exogenous androgens on sex reversal and sex steroid profiles
76 in the female of *M. albus*. He et al. (28) observed the ovarian differentiation,
77 morphogenesis and expression of some gonadal development-related genes in *M.*
78 *albus*. Several genes related to sex determination and differentiation have been
79 identified in *M. albus*, including *cyp19a1a* (29), *sox9a* (30), *cyp17* (31), *sox17* (32),
80 *dmrt1* (33), *jnk1* (34), *foxl2* (35), miRNAs (36) and gonadal soma-derived factor (37).
81 We also investigated the transcription profiles of some genes involved in gonad
82 development and sex reversal in the *M. albus* (38, 39). Moreover, a
83 chromosome-scale assembly of *M. albus* genome is currently available (40). However,
84 the biology events and potential mechanisms underlying the process of
85 female-to-male sex reversal in this species are still unclear.

86 Huang et al. (33) reported that not only is *dmrt1* expressed specifically in the
87 gonads of *M. albus*, but its multiple isoforms are differentially co-expressed during
88 gonad transformation. Also, Sheng et al. (41) observed that the *dm* genes are involved
89 in the sexual differentiation of *M. albus*. However, the regulation of *dmrt1* in *M. albus*

90 during sex reversal remains largely unknown. As an important *cis*-acting element,
 91 core promoter plays pivotal role in the regulation of metazoan gene expression (42,
 92 43). We assumed that a) the 5' flanking region of *M. albus dmrt1* contains unique
 93 promoter motifs that regulates its transcription during sex reversal, b) there is no sex
 94 determination gene in *M. albus*, which sex transformation is an evolution process, c)
 95 and thus, the process of female-to-male sex reversal is controlled by endocrine
 96 regulation and sex hormones play a vital role during this process. To test this
 97 hypothesis, we analyzed and compared the gene sequence of *M. albus dmrt1* 5'
 98 upstream region and its potential transcription factor binding sites with other fish
 99 species and examined the *in vitro* effects of T on the expression of *dmrt1a* and *flox2* in
 100 the ovotestis. Moreover, we cloned and examined the expression patterns of genes
 101 encoding enzymes, *11 β -h* and *11 β -hsd2*, involved in the production of 11-KT in the
 102 testis, ovotestis and ovary tissue, so as to reveal the molecular mechanism of sex
 103 reversal in *M. albus*.

104 **Results**

105 ***Nucleotide sequence of dmrt1 5' upstream region***

106 The 5' flanking region of *M. albus dmrt1* was 1421 bp in size. *In silico* functional
 107 analysis showed the transcription binding sites for AP-1, Oct-1, Zen-1, USF, C/EBPa,
 108 GATAx, STATx, Foxd3, SRY, Dmrt3, Ftz, ERE, ARE and Sox family of transcription
 109 factors (Fig. 1). Specifically, in comparison with other known fish species, the
 110 sequence of *M. albus dmrt1* 5' upstream region contained two unique androgen
 111 response elements (AREs), with one on the sense strand (-638 bp ~ -648 bp) and the
 112 other one on the antisense strand (-903 bp ~ -917 bp) (Supplementary Table 2).

113 ***Histological change***

114 After 6 hours of tissue culture, cells began to migrate from the periphery of the

gonad. Growing tissue appeared after about 5-6 days and the cells were closely arranged and gradually sparse around tissue. There were three types of cells including spindle-shaped fibroblasts, elliptical nuclei; polygonal epithelioid cells; round germinal stem cells, mononuclear or multicellular. The number of cells increased dramatically forming a single layer within 5-6 days. The epithelioid cells and germinal stem cells began to vacuolate and were gradually apoptosis with the extension of culture time, and fibroblasts dominated after 11-12 days (Fig. 2A-D).

Effects of T on the expression levels of dmrt1a and foxl2

On day 6 and day 12, with increasing concentrations of T, the expression level of *foxl2* was significantly decreased ($p < 0.05$) (Fig. 3A) but the expression level of *dmrt1a* was significantly increased ($p < 0.05$) (Fig. 3B).

Molecular cloning of the full-length 11 β -h cDNA

The full length of 11 β -h cDNA sequence was 1812 bp with an open reading frame of 544 amino acids (Supplementary Fig. 2). The amino acid sequence contained without signal peptide cleavage site or transmembrane helix. Several conserved functional motifs were observed including steroid binding site, oxygen-binding region, Ozols' region, aromatic regions and heme-binding region (Supplementary Fig. 3). We compared the amino acid sequence of *M. albus* 11 β -h to that in other species and found 77% identity with *Dicentrarchus labrax*, 76% identity with *Micropogonias undulatus* and 75% identity with *Parajulis poecilepterus* and *Odontesthes bonariensis*. The phylogenetic tree analysis showed that the 11 β -h of *M. albus* and *Epinephelus coioides*, *P. poecilepterus*, *D. labrax*, *M. undulatus*, *O. bonariensis*, *O. latipes* and *O.*

137 *niloticus* were clustered together (Supplementary Fig. 4).

138 ***Expression of 11 β -H mRNA during sex reversal***

139 *11 β -h* was highly expressed in the testis, which was significantly higher than that
140 in the ovary and ovotestis ($p < 0.05$). Moreover, the expression level of *11 β -h* in
141 ovotestis was higher than in the ovary ($p < 0.05$) (Fig. 4).

142 ***Molecular cloning of the full-length 11 β -hsd2 cDNA***

143 The full length of *11 β -hsd2* cDNA sequence was 2267 bp with an open reading
144 frame of 407 amino acids (Supplementary Fig. 5). The amino acid sequence contained
145 without signal peptide cleavage site or transmembrane helix. Several conserved
146 functional motifs were found including NAD-binding domain, *11 β -hsd* conserved
147 sequence and catalytic site (Supplementary Fig. 6). We compared the amino acid
148 sequence of *M. albus 11 β -hsd2* to that in other species and found 83% identity with *O.*
149 *latipes*, 80% identity with *O. bonariensis* and 77% identity with *O. niloticus*. The
150 phylogenetic tree analysis showed that the *11 β -hsd2* of *M. albus* and *O. bonariensis*,
151 *O. latipes* and *O. niloticus* were clustered together (Supplementary Fig. 7).

152 ***Expression of 11 β -hsd2 mRNA during sex reversal***

153 *11 β -hsd2* was highly expressed in the testis, which was significantly higher than
154 that in the ovary and ovotestis ($p < 0.05$). Moreover, the expression level of *11 β -hsd2*
155 in ovotestis was significantly higher than in the ovary ($p < 0.05$) (Fig. 5).

156 **Discussion**

157 Promoters are, generally, located at the upstream of a transcription start site and
158 have a variety of regulatory motifs, such as the interaction of transcription factors

159 with their corresponding binding sites, which participate in gene regulation (44). In
 160 this study, analysis of the promoter region of *dmrt1* showed various transcription
 161 binding sites that potentially activated the transcription of *dmrt1*. Specifically, in
 162 comparison with the *dmrt1* 5' upstream region of other known fish species, only in the
 163 sequence of *M. albus*, there was one putative ARE on the sense strand (-638 bp ~ -648
 164 bp), indicating that AR (androgen receptor) was the specific transcription factor of
 165 *dmrt1* gene. Sex hormones play an important role in mediating physiological
 166 responses and developmental processes through their receptors across all vertebrates.
 167 Once androgen ligand binds to AR, the receptor becomes phosphorylated and
 168 translocates into the nucleus, in which it binds to ARE(s), and activates the
 169 transcription of *dmrt1* gene.

170 Steroids are known to play a crucial role in gonadal sex differentiation in many
 171 non-mammalian vertebrates, but also in the gonadal sex change of hermaphroditic
 172 teleosts. *In vitro* culture showed increased expression level of *dmrt1a* but decreased
 173 expression level of *foxl2* with increased T concentration and culture time, implying
 174 the role of androgen in the transcription of sex-related genes during sex reversal in *M.*
 175 *albus*. Similarly, a hormonal manipulation *in vitro* showed that 11-KT activated the
 176 Sertoli cells leading to the completion of spermatogenesis in Japanese eel *Anguilla*
 177 *japonica* (45). Also, Jo et al. (46) observed that the expression levels of *dmrt1* in
 178 ovary of *P. olivaceus* were significantly up-regulated by T treatment. Raghuveer et al.
 179 (47) observed that methyl testosterone treatment resulted in the initiation of testicular
 180 differentiation in juvenile catfish *Clarias gariepinus*, which is supported by specific

181 expression of two forms of *dmrt1*. The expression level of *dmrt1* is high in mature
182 testis of black porgy *Acanthopagrus schlegeli* during sex-reverse process (48).
183 Besides fish species, T-treated ovaries induce upregulated expression of *dmrt1* in the
184 ovotestis of *Rana rugosa* Frogs (49). Aoyama et al. (50) revealed that *dmrt1* was not
185 transcribed at any time during ovarian development but was expressed in the
186 female-to-male sex reversed gonad of amphibians. Hu et al. (35) also observed a high
187 level of *foxl2* expression in the ovary before sex reversal in *M. albus*, but its
188 transcripts decreased sharply when the gonad developed into the ovotestis and testis.
189 Overall, *dmrt1* is essential to maintain vertebrate testis determination (51). *Foxl2* is
190 required to prevent transdifferentiation of an adult ovary to a testis (52). We assumed
191 that the antagonism between *dmrt1* and *foxl2* might cause reprogramming gonad in *M.*
192 *albus* (53).

193 We further examined the expression of genes encoding key steroidogenic
194 enzymes during the process of sex reversal in *M. albus*. The expression of gonadal
195 *11 β -h* showed obvious sexual dimorphism, with high level in the testis and ovotestis,
196 indicating the vital role of this gene in testis development. Liu et al. (29) also reported
197 that *11 β -h* was markedly up-regulated at the onset of testicular development in *M.*
198 *albus*. Similarly, the expression level of *11 β -h* is comparatively low at the early
199 spermatogenesis and sharp increases during spermiogenesis, finally, reaches its
200 highest levels in Atlantic salmon (7). In *O. niloticus*, the expression levels of two
201 isoforms of *11 β -h* are detected in testis from 50 days after hatching (dah) onwards and
202 strongly expressed in sex reversed XX testis after fadrozole and tamoxifen treatment,

203 but completely inhibited in 17 β -estradiol induced XY ovary (9). In *C. batrachus*,
 204 *11 β -h* is expressed ubiquitously with high levels in testis and could be detected as
 205 early as at 0 dah as supported by high level of 11-KT in serum and testicular tissue
 206 during pre-spawning and spawning phases, which might facilitate the initiation and
 207 normal progression of spermatogenesis (10). The gonadal *11 β -hsd2* showed similar
 208 expression pattern with *11 β -h* in *M. albus*, indicating the vital role of these two genes
 209 in the female-to-male reversal. Similarly, *11 β -hsd2* is expressed in a wide variety of
 210 tissues in *O. niloticus*, with the highest expression in testis (3). Yu et al. (31) found
 211 that the expression levels of 17 α -hydroxylase, were dominantly expressed in testis,
 212 less in ovary, and the least in ovotestis, consistent with the sex reversal process of *M.*
 213 *albus*. Similarly, the expression levels of *11 β -h* and *11 β -hsd2* are predominantly
 214 expressed in testis, much less in ovotestis, and barely in ovary, consistent with a role
 215 in the production of 11-KT during sex reversal.

216 During female-to-male sex reversal, the expression level of *foxl2* is sharply
 217 decreased in *M. albus* (35). Also, the aromatase transcripts are decreased when gonad
 218 develops into the ovotestis and testis (29). As a result, synthesise of estrogen may
 219 decrease during sex reversal. Androgen is the substrate for the production of female
 220 hormone, the level of androgen may thus increase. In this study, T also showed a
 221 higher inhibitory effect on *foxl2* than positive impact on *dmrt1a*. In this regard, these
 222 results are accord with the withdrawal hypothesis of estrogen proposed by Nagahama
 223 (54). Moreover, serum T level in female *M. albus* reaches a peak two months after
 224 spawning and is significantly higher than the estrogen level (55). Therefore, we

suggested that the high level of androgen is the main driving factor for sex reversal in *M. albus*. However, the withdrawal of estrogen during sex reversal is passive, not active, due to the inhibitory action of androgen-*dmrt1a* on the aromatase-*foxl2*.

In conclusion, the gene sequence of *M. albus dmrt1* 5' upstream region contained two unique AREs, indicating that AR was the specific transcription factor of *dmrt1*. Also, the *dmrt1a* was positive regulated by T, suggesting that the blood androgen could promote the transcription of *dmrt1* during sex reversal. Moreover, high expression levels of *11β-h* and *11β-hsd2* were observed during female-to-male sex reversal, indicating the large production of 11-KT during this process. Overall, as shown in Fig. 6, androgens are synthesized in large amounts in *M. albus* during sex reversal, promoting the transcription of *dmrt1* via putative ARE(s), which in turn, induces ovarian somatic cells to transdifferentiate into testicular somatic cells.

Methods

Fish

The wild *M. albus* (body weight ~200 g) were collected from Hubei, China and transported to the Fish Breeding Laboratory, Shanghai Ocean University (Shanghai, China). After 30 days of acclimation, the animals were sacrificed by anesthesia with MS-222 and dissected on ice. A portion of the gonad was fixed in Bouin's fluid for histological assessment of the sexual status. The other samples were frozen in liquid nitrogen and stored at -80 °C. All experiments were performed with the approval from the Institutional Animal Care and Use Committee of Shanghai Ocean University.

Isolation of 5' upstream region of dmrt1 and sequence analysis

247 Genomic DNA was isolated from gonad tissue by using manufacturer's protocol
248 (Qiagen, GmbH, Germany). The integrity of DNA was checked using 2% agarose gel
249 electrophoresis. Based on the DNA sequence of *M. albus dmrt1* obtained from NCBI
250 (Accession No: NW-018128265), the specific primers (Supplementary Table 1) were
251 designed to amplify the 5' upstream region of *dmrt1* gene. The JASPAR database and
252 associated tools (<http://jaspar.genereg.net>), Match (BioBase), AliBaba2.1 (Biobase)
253 and MOTIF (GenomeNet) were used to predict the transcription factor binding sites
254 (56-58).

255 ***Histology and light microscopy observation***

256 The dissected gonads were stored in 4% paraformaldehyde for 24 h. After
257 rinsing with flowing water, the gonads were dehydrated in a series of ethanol,
258 embedded in paraffin and cut by a microtome at 6 μ m thickness. After
259 hematoxylin-eosin dye, the stained sections were observed under an inverted
260 phase-contrast microscope (Olympus BX-53, Tokyo, Japan).

261 ***In vitro culture***

262 The ovotestis (Supplementary Fig. 1) was cut into $1 \times 1 \times 0.5$ mm³ small pieces,
263 washed three times with PBS $\times 1$, and then transferred to 24-well culture plates. The
264 control group was cultured in a medium containing 15% fetal bovine serum and 1%
265 penicillin/streptomycin. The treatment groups were cultured in a medium containing
266 additional 10 (low) or 100 ng/ml (high) of T. The gonadal tissues were cultured in
267 CO₂ incubator at 27 °C. One half of the medium was changed every other day. The
268 growth of cell was observed under an inverted microscope daily and sampled on day 6

269 and day 12 for *dmrt1a* and *folx2* expression analysis.

270 ***Total RNA extraction and cDNA synthesis***

271 Total RNA was extracted using Trizol method (Invitrogen, USA) according to
272 the manufacturer's instructions. The quality of total RNA was determined by using a
273 NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) measured at
274 260/280 nm, and the integrity was screened by 1.5% agarose gel electrophoresis.
275 The cDNA was synthesized by using a PrimeScript™ RT reagent Kit (Takara, China)
276 following the manufacturer's instructions. The obtained cDNA templates were stored
277 at -80 °C for gene cloning and qRT-PCR amplification.

278 ***Cloning the full-length cDNA of *11β-h* and *11β-hsd2* gene and sequence analysis***

279 The primers (Supplementary Table 1) were designed to amplify the internal
280 region of *11β-h* and *11β-hsd2* gene respectively by using Takara PCR Amplification
281 Kit (Takara, Japan). To obtain the full-length cDNA sequences, 3' and 5'
282 rapid-amplification of cDNA ends Polymerase Chain Reaction (RACE-PCR) was
283 carried out by using the SMART™ RACE cDNA Amplification Kit (Clontech, USA)
284 according to the manufacturer's instructions. The amplified PCR products were
285 excised by 1.5% agarose gel electrophoresis, and bands of expected size were
286 dissociated and purified by using a gel extraction kit (Omega, China). The PCR
287 products were directly ligated into PMD19-T simple vector (TaKaRa, China) and then
288 transformed into Escherichia coli BL21 competent cells (Transgen, China).

289 Prediction of the open reading frame on *11β-h* and *11β-hsd2* was performed by
290 using the BLAST Program of NCBI (<http://www.ncbi.nlm.nih.gov/blast>). Prediction

of the protein domains were carried out by using the SMART program (<http://www.smart.emble-heidelberg.de/>), InterPro (<http://www.ebi.ac.uk/interpro/>) and IMGT (<http://www.imgt.org/>). Prediction of signal peptide was performed by using the SignalP 4.0 (<http://www.cbs.dtu.dk/services/SignalP/>). Conserved motifs were identified by using Conserved Domain Search Service from NCBI. Multiple alignments of amino acid sequences were performed by using the ESPript (<http://multalin.toulouse.inra.fr/multalin/>). The phylogenetic neighbor-joining (NJ) tree was constructed by using the MEGA 6.0 program (59), and the reliability was assessed by 1000 bootstrap replications.

300 ***Quantitative real-time PCR and expression analysis***

The expression levels of *dmrt1a*, *foxl2*, *11β-h* and *11β-hsd2* were quantified by real-time quantitative RT-PCR with specific primers (Supplementary Table 1), by using SYBR Premix Ex *Taq* II (Tli RNaseH Plus) Kit (Takara, Dalian, China) and CFX96TM real-time system (Bio-Rad, Hercules, CA, USA). At the end of the reactions, the credibility of the qRT-PCR was analyzed through melting curve. All samples were run in triplicate, and each assay was repeated three times. After finishing the program, the cycle threshold (Ct) value was automatically determined by the Bio-Rad CFX Manager software. The mRNA expression levels were calculated relative to β-actin using the $2^{-\Delta\Delta C_t}$ method (60).

310 ***Statistical analysis***

Raw data were assessed for the normality of distribution and the homogeneity of variance with the Kolmogorov-Smirnov test and Levene's test, respectively. The data

313 conformed to a normal distribution and were suitable for testing with analysis of
314 variance (ANOVA). The differences in mRNA expression levels of *dmrt1a*, *foxl2*,
315 *11β-h* and *11β-hsd2* between treatments were compared with one-way ANOVA at the
316 significance level of 0.05 ($p < 0.05$). Data analyses were performed using the software
317 SPSS for Windows (Release 20.0).

318

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322 **Author contributions**

323 B.W. and X.Q. designed the research and drafted the paper, L.P. conducted the
324 cell culture, J.G. isolated of 5' upstream region of *dmrt1*, H.W. conducted the gene
325 cloning, Q.W. conducted the gene expression.

326 **Competing interests**

327 The authors declare that they have no competing interests.

328

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495

496 Figure captions

497 Fig. 1. Nucleotide sequence of *M. albus dmrt1* 5' upstream region with its potential
498 transcription factor binding sites. The potential transcription binding sites are boxed
499 or underlined. (*) indicates ARE on the antisense strand. (+1) means transcription
500 start site.

501 Fig. 2. *In vitro* culture of ovotestis in *M. albus*. A: tissue culture after 6 hours; B:
502 tissue culture after 1 day; C: tissue culture after 6 days; D: tissue culture after 12 days.
503 E: epithelioid cells; F: fibroblast cells; G: germinal stem cells.

504 Fig. 3. Effects of T on the expression levels of (A) *foxl2* and (B) *dmrt1a* in the
505 ovotestis of *M. albus*. Different letters indicate significant difference between groups
506 within each time ($p < 0.05$).

507 Fig. 4. Expression level of *11β-h* during *M. albus* gonadal development. F, ovaries; I,
508 ovotestis; M, testis. (*) indicates significant difference with the former ($p < 0.05$).

509 Fig. 5. Expression level of *11β-hsd2* during *M. albus* gonadal development. F, ovaries;
510 I, ovotestis; M, testis. (*) indicates significant difference with the former ($p < 0.05$).

511 Fig. 6. The framework for clarifying the mechanism of *M. albus* sex
512 transdifferentiation. Androgens are synthesized in large amounts in the ovotestis,
513 which activates the transcription of *dmrt1* via putative AREs, resulting in biological
514 effects, which in turn, induces ovarian somatic cells to transdifferentiate into testicular
515 somatic cells. As such, a positive regulatory loop programs the *M. albus* sex reversal.
516 On the other hand, androgens inhibit the expression of *foxl2* and its function.

517

518 Fig. 1

519 **GATA-1**

520 -1412

521 ATTAGAATATCGTGGACAAAAATTATTTTCAGTAATTCAACTCAAATAGTGAAACTCAT

522 GTATTATATAA

523 1343

524 ATTCAGTACACACAGACTGAAGTAGTTTAAGCCGTTGGGTCTTTTTATTGTGATGATT

525 TTGGCCACAT

526 **SOX5 SRY**

527 -1274

528 TTAACAAAACCCACCAACAACAAAGAAGCTAACCTAACAGCAACAGCAAAAACGCT

529 GCGCAAATTAGA

530 -1205

531 GTTGGTCAGAAGTGATTTATTTAGCCATATTTGAAAAATAAGCCTCTAGCAGAGTTAG

532 ACGCTATCTAA

533 **Oct-1**

Oct-1

534 -1136

535 AACGCATTTAGCTGTTTGAGCGAATAAACATATCATTTTGCAAATAAAGAAAAGATGT

536 ATGGGGTAAAA

537 **Stat3/Stat4**

Oct-1

538 -1067

539 AAGGAGCGTCTGCGGTAGACTAGTAACGTAACGTTACCTCTCCAACTGCTCCTCCA

540 AATACCGTATTTG

541 Stat1:Stat2 GATA-1

542 -998

543 AATCAGGTAGACATCCTTTTCTTTCTTGCTGGACCGATTTTATTTGTAGGCCTCTCG

544 TTTTTCGATGT

545 GRE ARE*/SOX9 Sp1

546 -929

547 TTTCTGTCTTCATCTTGTTTGTCACCGCCTTTTTTAGCCTTCTTTTGGGCATTGTG

548 TCTCAGAAAGT

549 Oct-1 GR Stat6 USF

550 -860

551 TTGCTAAGTTTACGCGAGAGCCAGCGTAGAACTAAACGTTGTTGCTAGACAACAAT

552 GGTGCCTTCATG

553 Pit-1a GATA-1

554 -791

555 GACTCCTCGAAAAGTTGTTCTCTACTTTTTCTACTATTTCAAGTTCTCTGGTTATTTAA

556 TCCCATTATT

557 GRE

558 -722

559 TATTATCACCACATGCCAGACCAGATTGAAAAATTTGGTAATTAACATTAAAGAACTG

560 ATAGCATCTTG

561 SOX5/SOX9/SRY ARE SRY

562 -653

563 TTATGCTTGCCACACACGGAGGAAATTTTGTTTCTAGTATTGCAGTACTTGAAAGCAGT

564 ACTTTTACTC

565 TBP TBP Oct-1

566 -584

567 ATTGTAGTATTTTTATGGTGAGCTATTTGCTACTTTTAGGAACTTATCGATTGCACATT

568 ATAAGTAATA

569 Oct-1 GATA-1

570 -515

571 ATTTACTACAAAATAGTTGTGTCTTTTAGGTTTACTTTTTCATCACTGAAGTATGTGA

572 TAGTCTCACA

573 Oct-1

574 -466

575 GTTTATTTCTACTAAATTTTATAAAAGTGCAGCCATGACCTCTGGGTTCCCTCATCATA

576 TTAGGTCATGT

577 E2 SOX9

578 -377

579 CCAGCTCAGTGGAGATTAAAAAATCTCGTGGCACTTTGTAGTTTTCCTTGTA

580 ACCGAGTATTT

581 Pit-1a C/EBPalp Oct-1

582 -308

583 TCAACCTACACTTACTGTGCTACTTTTAGTTAAGTAAAGATTCCGGTGCTTCCGCCACT

584 GCAGCAGGTCA

585 ERE GRE

586 -239

587 CTTGAAAACTGGGCTTTATGTAACTATAAACATGTTTTACCAGTAATTTAGTGTGA

588 AAACCAAATCA

589 GATA-1 Sp1 Oct-1

590 -170

591 GAGTGTAATAGAGAGACGCCACTGTCCTGACAGCTTTCTCCCCGTTTCCAGCTCGTT

592 TGCTCCCAATGC

593 TBP GATA-1 Sp1 Sp1

594 -101

595 AGTTTGGAAAAAAGCCCAGGATTGGGAAAAATGCAATAGTAAGGCGGGGATGGGCG

596 GAGACGGACAG

597 Sp1 +1

598 -32

599 TGACCTTATAGCCTCCACCCTGGCACCAATAAGCTCTAACCAGCCTTGTGCTCCATG

600 GACAGGTTTGGC

601 +38

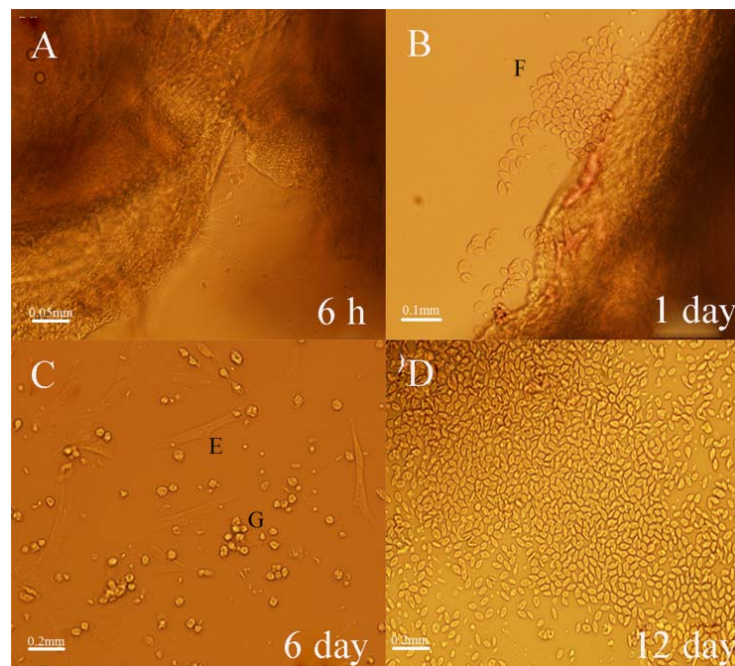
602 AGTTGGCAGCATAGTTGTATGGTTTTACTTCCACTATGAACAAGGACAAGCAGCGCA

603 AGCAGGTGCTGGACT

604

605 Fig. 2

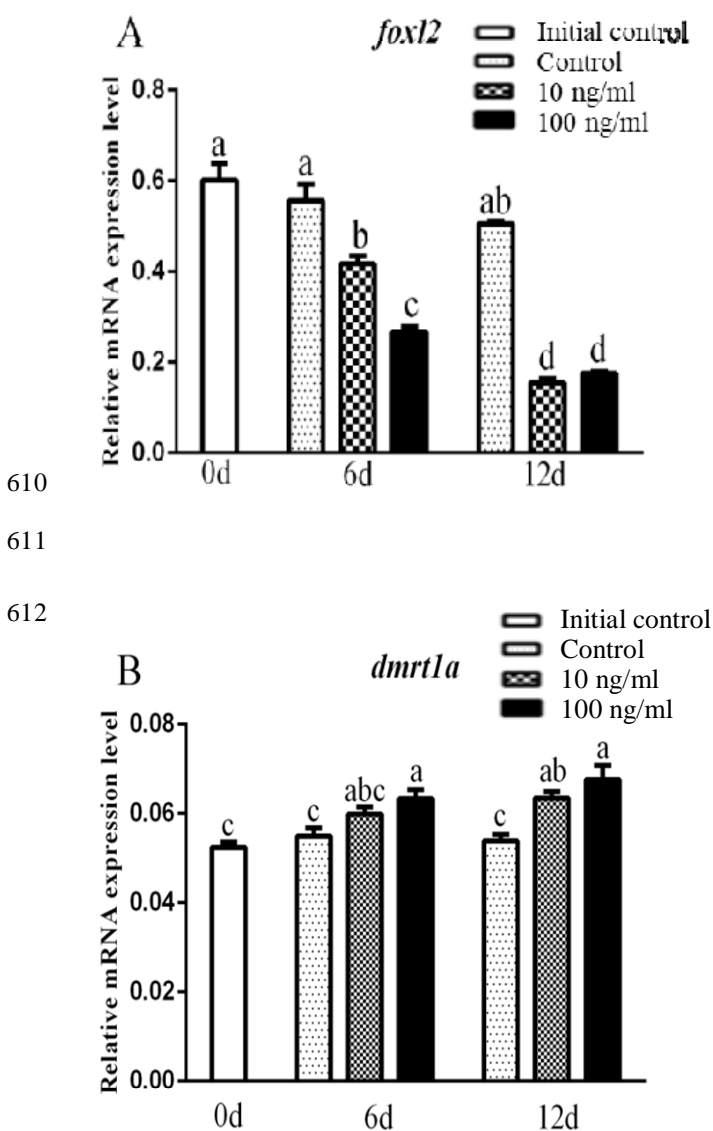
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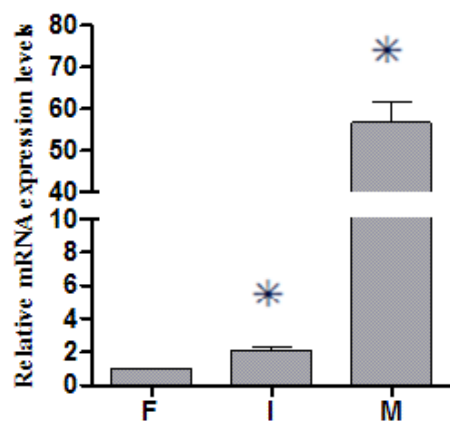
607

608

609 Fig. 3



613 Fig. 4

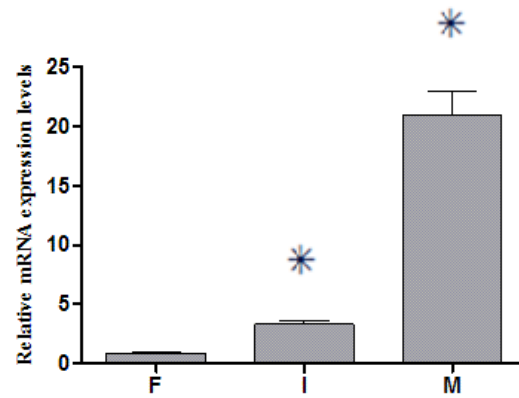


614

615

616 Fig. 5

617



618

619

Fig. 6

