Androgen-\textit{dmrt1} positive feedback programs the rice field eel \textit{(Monopterus albus)} sex transdifferentiation

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Abstract

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

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

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

Many genes are known to be involved in gonadal differentiation in vertebrates. Dmrt1, a gene that encodes a transcription factor with a DM-domain, is one of the essential genes controlling testicular differentiation in mammals, birds, reptiles, amphibians and fish (11-13). In O. mykiss, for example, dmrt1 is expressed during testicular differentiation but not during ovarian differentiation (14). Dmy is enough for male development in O. latipes and loss of dmy in XY medaka causes male-to-female sex reversal (15-17). O. latipes also has an autosomal copy of dmrt1 which is expressed in testis later than dmy but is essential for testis development (18, 19). Dmrt1 is not only associated with testis development, but also, may be crucial for the
ovary differentiation in zebrafish (20); however, Webster et al. (21) reported that

dmrt1 is dispensable for ovary development but necessary for testis development by
regulating amh and foxl2. Wen et al. (22) observed that dmrt1 expression was 70
times higher in the testis of olive flounder Paralichthys olivaceus than in the ovary.
Also, in European sea bass, the expression of dmrt1 is increased in testis but
decreased in ovary (23).

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

Huang et al. (33) reported that not only is dmrt1 expressed specifically in the
gonads of M. albus, but its multiple isoforms are differentially co-expressed during
gonad transformation. Also, Sheng et al. (41) observed that the dm genes are involved
in the sexual differentiation of M. albus. However, the regulation of dmrt1 in M. albus
during sex reversal remains largely unknown. As an important cis-acting element, core promoter plays pivotal role in the regulation of metazoan gene expression (42, 43). We assumed that a) the 5' flanking region of M. albus dmrt1 contains unique promoter motifs that regulates its transcription during sex reversal, b) there is no sex determination gene in M. albus, which sex transformation is an evolution process, c) and thus, the process of female-to-male sex reversal is controlled by endocrine regulation and sex hormones play a vital role during this process. To test this hypothesis, we analyzed and compared the gene sequence of M. albus dmrt1 5' upstream region and its potential transcription factor binding sites with other fish species and examined the in vitro effects of T on the expression of dmrt1a and flox2 in the ovotestis. Moreover, we cloned and examined the expression patterns of genes encoding enzymes, 11β-h and 11β-hsd2, involved in the production of 11-KT in the testis, ovotestis and ovary tissue, so as to reveal the molecular mechanism of sex reversal in M. albus.

Results

Nucleotide sequence of dmrt1 5' upstream region

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

Histological change

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 11β-h cDNA*

The full length of 11β-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* 11β-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 11β-h of *M. albus* and *Epinephelus coioides*, *P. poecilepterus*, *D. labrax*, *M. undulatus*, *O. bonariensis*, *O. latipes* and *O.
nloticus were clustered together (Supplementary Fig. 4).

Expression of 11β-H mRNA during sex reversal

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

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

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

Expression of 11β-hsd2 mRNA during sex reversal

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

Discussion

Promoters are, generally, located at the upstream of a transcription start site and have a variety of regulatory motifs, such as the interaction of transcription factors
with their corresponding binding sites, which participate in gene regulation (44). In this study, analysis of the promoter region of *dmrt1* showed various transcription binding sites that potentially activated the transcription of *dmrt1*. Specifically, in comparison with the *dmrt1* 5′ upstream region of other known fish species, only in the sequence of *M. albus*, there was one putative ARE on the sense strand (-638 bp ~ -648 bp), indicating that AR (androgen receptor) was the specific transcription factor of *dmrt1* gene. Sex hormones play an important role in mediating physiological responses and developmental processes through their receptors across all vertebrates. Once androgen ligand binds to AR, the receptor becomes phosphorylated and translocates into the nucleus, in which it binds to ARE(s), and activates the transcription of *dmrt1* gene.

Steroids are known to play a crucial role in gonadal sex differentiation in many non-mammalian vertebrates, but also in the gonadal sex change of hermaphroditic teleosts. *In vitro* culture showed increased expression level of *dmrt1α* but decreased expression level of *foxl2* with increased T concentration and culture time, implying the role of androgen in the transcription of sex-related genes during sex reversal in *M. albus*. Similarly, a hormonal manipulation *in vitro* showed that 11-KT activated the Sertoli cells leading to the completion of spermatogenesis in Japanese eel *Anguilla japonica* (45). Also, Jo et al. (46) observed that the expression levels of *dmrt1* in ovary of *P. olivaceus* were significantly up-regulated by T treatment. Raghuveer et al. (47) observed that methyl testosterone treatment resulted in the initiation of testicular differentiation in juvenile catfish *Clarias gariepinus*, which is supported by specific
expression of two forms of *dmrt1*. The expression level of *dmrt1* is high in mature testis of black porgy *Acanthopagrus schlegeli* during sex-reverse process (48). Besides fish species, T-treated ovaries induce upregulated expression of *dmrt1* in the ovotestis of *Rana rugosa* Frogs (49). Aoyama et al. (50) revealed that *dmrt1* was not transcribed at any time during ovarian development but was expressed in the female-to-male sex reversed gonad of amphibians. Hu et al. (35) also observed a high level of *foxl2* expression in the ovary before sex reversal in *M. albus*, but its transcripts decreased sharply when the gonad developed into the ovotestis and testis. Overall, *dmrt1* is essential to maintain vertebrate testis determination (51). *Foxl2* is required to prevent transdifferentiation of an adult ovary to a testis (52). We assumed that the antagonism between *dmrt1* and *foxl2* might cause reprogramming gonad in *M. albus* (53).

We further examined the expression of genes encoding key steroidogenic enzymes during the process of sex reversal in *M. albus*. The expression of gonadal 11β-*h* showed obvious sexual dimorphism, with high level in the testis and ovotestis, indicating the vital role of this gene in testis development. Liu et al. (29) also reported that 11β-*h* was markedly up-regulated at the onset of testicular development in *M. albus*. Similarly, the expression level of 11β-*h* is comparatively low at the early spermatogenesis and sharp increases during spermiogenesis, finally, reaches its highest levels in Atlantic salmon (7). In *O. niloticus*, the expression levels of two isoforms of 11β-*h* are detected in testis from 50 days after hatching (dah) onwards and strongly expressed in sex reversed XX testis after fadrozole and tamoxifen treatment,
but completely inhibited in 17β-estradiol induced XY ovary (9). In *C. batrachus*,

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

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

In conclusion, the gene sequence of *M. albus* \textit{dmrt1} 5′ upstream region contained
two unique AREs, indicating that AR was the specific transcription factor of \textit{dmrt1}.

Also, the \textit{dmrt1a} was positive regulated by T, suggesting that the blood androgen
could promote the transcription of \textit{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 \textit{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 \textit{dmrt1} and sequence analysis**
Genomic DNA was isolated from gonad tissue by using manufacturer’s protocol (Qiagen, GmbH, Germany). The integrity of DNA was checked using 2% agarose gel electrophoresis. Based on the DNA sequence of *M. albus* *dmrt1* obtained from NCBI (Accession No: NW-018128265), the specific primers (Supplementary Table 1) were designed to amplify the 5' upstream region of *dmrt1* gene. The JASPAR database and associated tools (http://jaspar.genereg.net), Match (BioBase), AliBaba2.1 (Biobase) and MOTIF (GenomeNet) were used to predict the transcription factor binding sites (56-58).

**Histology and light microscopy observation**

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

**In vitro culture**

The ovotestis (Supplementary Fig. 1) was cut into 1 × 1 × 0.5 mm³ small pieces, washed three times with PBS × 1, and then transferred to 24-well culture plates. The control group was cultured in a medium containing 15% fetal bovine serum and 1% penicillin/streptomycin. The treatment groups were cultured in a medium containing additional 10 (low) or 100 ng/ml (high) of T. The gonadal tissues were cultured in CO₂ incubator at 27 °C. One half of the medium was changed every other day. The growth of cell was observed under an inverted microscope daily and sampled on day 6.
and day 12 for *dmrt1a* and *folx2* expression analysis.

**Total RNA extraction and cDNA synthesis**

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

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

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

Prediction of the open reading frame on *11β-h* and *11β-hsd2* was performed by 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.

**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 $2^{-\Delta\Delta\text{Ct}}$ method (60).

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

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

Competing interests

The authors declare that they have no competing interests.
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Figure captions

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

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

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

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

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

Fig. 6. The framework for clarifying the mechanism of *M. albus* sex transdifferentiation. Androgens are synthesized in large amounts in the ovotestis, which activates the transcription of *dmrt1* via putative AREs, resulting in biological effects, which in turn, induces ovarian somatic cells to transdifferentiate into testicular somatic cells. As such, a positive regulatory loop programs the *M. albus* sex reversal. On the other hand, androgens inhibit the expression of *foxl2* and its function.
Fig. 1

GATA-1

ATTAGAATATCGTGACAAAAATTATTTTCAGTAATTCAACTCAAAATAGTGAAAACCTCAT

GTATTATATAAA

1343

ATTTCAGTACACACAGACTGAAAGTAGTTAAGCCGTTGGGTCTTTTTATTGTGATGATT

TTGGCCACAT

SOX5 SRY

-1274

TTAACAAAA CCCACCAAACAAAAAGAAAGCTAACCTAACAGCAACAGAAAAACGCT

GCGCAAATTAGA

-1205

GTTGGTCAGAAGTGATTTATTTAGCCATATTGAAAAATAAGCCTCTAGCAGAGTTAG

ACGCTATCTAA

Oct-1          Oct-1

-1136

AACGCATTAGCTGGTGGACGAATAAACATATCATTTTGCAATTTAAAGAAAAAGATGT

ATGGGGGTAAA

Stat3/Stat4     Oct-1

-1067

AAGGAGCGTGCTGCTGACTGTAACGTAAACGTGTACCCTCTCCAACTGCTCCCTCCA
AA TAC CGT ATT TG

Stat1:Stat2                GATA-1

-998

AATCAGG TAGACAT CTTTTTTCTTTTTCTTTGCTCGGACCAGATTTTTATTTGTAGGCCTCTCG

TTTTTCGATGT

GRE ARE*/SOX9 Sp1

-929

TTTCTGTCTCTCATTTGTGTGCAAGCCTTTTTTTAGCCTTTTTGGGCATTGTG

TCTCAGAAAGT

Oc1-1                        GR Stat6 USF

-860

TTGCTAAGTTTACGCAGAGCCAGC TAGAAACTAAGCTTGTTGCTTAGACAAACAT

GGTGCCCTTCATG

Pit-1a                        GATA-1

-791

GACTCCCTGAAAAGTTTTCTCTACTT TTTTCTACTATTCAAGTTTCTGTATTGTTA

TCCCATTATT

-722

TATTATCACCA CACATGCCAGACCA GATTGAAAAATTTGTAATTAACATTAAGAAGCTG

ATAAGCATT CT

SOX5/SOX9/SRY ARE          SRY
TTATGCTTGCACACACGGAGGAATTTGTTCTAGTATTTGAGCTTTTGCTAATTTTCAAAAGGAG
ACTTTTACTC

TBP TBP Oct-1

ATTGTAAGTATTTATGTGAGCTATTGTACTTTTTAGGACTTTATCGATTGGCACATT

ATAAGTAATA

Oct-1 GATA-1

ATTACTACAAAAATAGTTGTGTCTTTTTAGGTTTTACCTTTTTTCATACGAAATGTATGTA

TAGTCTACA

Oct-1

TTATTTTCTACTAAAAATTTTATAAAAAGTGCAGCCATGACCTCTGGTCTCCTCATCATA

TTAGGTCATGT

E2 SOX9

CCAGCTCAGGGAGATTTTTTTTTTTTTTCTGCGAACCTTTGTAGTTTTTCTTGTA

ACCGAGTATTT

Pit-1a C/EBPαp Oct-1

TCAACCTACACTACTGCTACTTTTATTTAATAGTTAGTAAGATGCAGTGTCTCCGCACT
584  GCAGCAGGTCA
585  ERE       GRE
586  -299
587  CTTGAAACACTGGGCTTTATGTAACATATTAAACATGGTTTACCAGTAATTTAGTGTA
588  AAACCAAATCA
589  GATA-1       Sp1       Oct-1
590  -170
591  GAGTGTAATAGAGAGACGCCACTGTCTGACAGCTTTCTCCCGTTTCAGCTCGTT
592  TGCTCCCAATGC
593  TBP   GATA-1       Sp1       Sp1
594  -101
595  AGTTTGAAAAAAGCCAGGATTTGGAAAAAATGCAATAGTAAGGCCGGGATGGGCG
596  [GAGACGGACAG 
597  Sp1       +1
598  -32
599  TGACCTTATAGCCCTCCACCCTGGCACCAAATAGCTCTAACACGGCTCTGCTCCATG
600  GACAGGTGTGGC
601  +38
602  AGTTGCAGCTAGTGGATGTTTTACTCCACTAGAACAGGAACGAGCGCA
603  AGCAGGTGCTGGACT
604
Fig. 2
**Fig. 3**

**A**

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**B**

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<td>0.05</td>
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<td>0.07</td>
</tr>
</tbody>
</table>
Fig. 4

![Bar chart showing relative mRNA expression levels for F, I, and M groups.](image-url)
Fig. 5

![Bar chart showing relative mRNA expression levels for different conditions F, I, M with significant difference indicated by stars.](image-url)
Fig. 6

Ovotestis

Positive regulatory loop

Testis

$Dmrt1$ (ARE)

Female somatic cells

Male somatic cells

Androgen (11-KT/T)

$Fosl2$