ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF POULTRY SCIENCE

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International Journal of Poultry Science 9 (1): 88-93, 2010
ISSN 1682-8356
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Inducible Mis- and Over-Expression of PKCIW Regulates the Expression of AMH and P450arom in Chicken Embryo Fibroblast

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Abstract: Sex in birds is determined genetically as in mammals and a Z-linked gene DMRT1 is required for testis development. But it remains possible that the W chromosome contains a similarly critical factor for female development. The W-linked gene PKCIW was put forward as the seductive candidate for avian sex determination and/or sex differentiation. Mis-expression and over-expression of PKCIW were performed respectively in male and female chicken embryo fibroblast with tet-on inducible expression system in this study. The results of RT-PCR showed that with the PKCIW recombinant vector introducing, PKCIW mRNA expression was significantly improved in female chicken embryo fibroblast and was close to that of the female controls and in male chicken embryo fibroblast, in which normally there was no expression. Both mis- and over-expression of PKCIW led to up-regulated expression of P450arom and down-regulated expression of AMH, while large increase of P450arom in female and large decrease of AMH in male appeared. With mis- and over-expression of PKCIW, there was no significant change of FOXL2, SOX9, DMRT1 and ATP5A1W mRNA expression. These results showed the possible regulatory effect of PKCIW on AMH and P450arom in transcription level.

Key words: Chicken, sex determination, PKCIW, mis-expression, over-expression, tet-on

INTRODUCTION

Our knowledge on the molecular mechanism behind sex determination in mammals has been improved significantly since the discovery of the master testis-determining gene, SRY (Sex-determining Region of the Y chromosome) in human (Sinclair *et al*., 1990); however, it's puzzled that how sex is determined when it comes to birds, in which sex is determined genetically as in mammals (but with different sex chromosomes constitution: ZW female/ZZ male in birds and XX female/XY male in mammals). It has been proposed that avian sex determination depends either on a dominant ovary-determinant on W chromosome or a gene on Z chromosome with dosage effect (Graves and Shetty, 2001; Smith and Sinclair, 2004). Excitingly, in a recent paper, direct evidence is presented that DMRT1 is required for testis determination in chicken, possibly by a dosage effect (Smith *et al*., 2009b). But this still has not settle the debate on the mechanism of avian sex determination and it remains possible that the W chromosome contains a similarly critical factor for female development (Ellegren, 2009).

PKCIW, which is also known as ASW, HINTW and WPKCI, is a W-linked seductive candidate for avian sex determination. It encodes a functionally altered form of Protein Kinase C Inhibitor/interacting (PKCI), which belongs to the highly conserved, ubiquitous HIT (histidine triad) family of proteins (O'Neill *et al*., 2000). PKCIW lacks the critical HIT motif but it remains an α-helix region which is revolved in the dimerization of PKCI, while a bona fide HINT gene is present on the Z chromosome, called PKCIZ (Hori *et al*., 2000). Accordingly, Hori *et al*. (2000) proposed that PKCIW could form a heterodimer with PKCIZ and thereby inhibits the biological functions of PKCIZ in the female sex determination. The temporal and spatial expression patterns of PKCIW, in which it is strongly expressed in female chicken embryos, has also indicted its possible role in avian sex determination and/or differentiation.

To provide supporting evidence for the role of PKCIW in chicken sex determination and differentiation, mis-expression and over-expression of PKCIW was performed in chicken embryo fibroblast using the improved tetracycline (Tet)-on inducible expression system (Watanabe *et al*., 2007).

MATERIALS AND METHODS

Plasmids and constructs: Tet-on inducible expression vectors including pBI-EGFP and pCAGGS-rtTA2^{S}-M2 were generous gifts from Dr. Y. Takahashi (Watanabe *et al*., 2007). A full-length CDS fragment encoding PKCIW was cloned into blunt-ended Mlu I site of pBI-EGFP. Recombinant plasmid was confirmed by Mlu I enzyme digestion and colligated direction was identified by Bst XI enzyme digestion. The correct plasmid was verified by sequencing and then was designated pBI-EGFP-PKCIW.

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High purity, endotoxin-free pBI-EGFP-PKCIW and pCAGGS-rtTA2^{S}-M2 DNA were prepared from DH5α cells with Endo-Free Plasmid Mini Kit (Omega).

Experimental design: The unsymmetrical expression characteristics of PKCIW in male and female chicken embryos aroused our interest in exploring its function on sex determination and/or sex differentiation. Four groups (each group with three repeats) were designed: The Mis-expression in Males (MM), the Male Controls (CM), the Over-expression in Females (OF) and the Female Controls (CF). The mRNA expression dynamics of both P450arom and AMH, which played important roles in the process of female and male sex differentiation respectively, were used to evaluate the effect of mis-expression and over-expression of PKCIW on chicken gonadal development. Meanwhile, the mRNA expression of FOXL2, SOX9, DMRT1 and ATP5A1W, which expressed sexual dimorphism were also detected to assess the position of PKCIW in the gene regulatory network of chicken sex determination.

Cell culture, transfection and sexing: Transfections of Chicken Embryo Fibroblasts (CEF) were prepared at 85%-95% confluency with 1.4 µg of pBI-EGFP-PKCIW and 0.7 µg of pCAGGS-rtTA2^{S}-M2 using 4 µL of Lipofectamine 2000 (Invitrogen) in 12-well dishes, followed by doxycycline (Dox) (sigma) administration (1 µg/ml) at 6 h. After 12 further hours, green fluorescent was observed and then the cells were harvested.
Parts of the tissues were collected while isolating chicken embryos to extract genomic DNA and identify the sex of embryos by an approach previously described (Feng *et al*., 2007).

Semi-quantitative RT-PCR: Total cellular RNA was extracted using Trizol (Invitrogen) and first-strand cDNA was synthesized using MMLV reverse transcriptase according to the manufacturer's protocol (MBI). Primers, annealing temperature, number of PCR cycle and expected product size for each gene are shown in Table 1. PCR reactions were carried out for each gene in 15 µl mixture and equal volume of PCR amplification products were subjected to electrophoresis in 1.8% agarose gel stained with ethidium bromide and visualized with a Bio-Rad imaging system. The mRNA expression of each gene was obtained using the Quantity-one software (BIO-RAD) and each target gene normalized to ß-actin, which was used as an internal control.

Statistical analysis: Data were expressed as mean±SE calculated from 3 replicates and were tested with T statistic in SPSS 13.0. Statistical significance was set at $p<0.05$.

RESULTS

Cell culture and GFP expression: Fibroblasts were appeared from the tissue block at 1 day after cultivation and bespreaded the bottom of culture flask at 3-4 days after adhering wall. Under the fluorescent microscope, green fluorescence was observed in cytoplasm and nucleus 12 h post transfection following adding Dox (Fig. 1). No green fluorescence could be observed if there was no Dox and both the cell morphology and growth rate had no significant change after the addition of Dox.

Mis- and over-expression of PKCIW: Figure 2 showed that PKCIW mRNA expression significantly increased in the over-expression in females as compared with that in the female controls in which PKCIW displayed background expression. And PKCIW mRNA expression in mis-expression in males was close to that of the

Table 1: Sequence of primers and conditions used for RT-PCR

Gene	F: forward primer; R: reverse primer	Annealing temperature (°C)	Number of PCR cycle	PCR product (bp)
PKCIW	F:TAGACGCGTCTGAGGGAGTGTTGTA	60	28	472
	R: ATCCGTACGCGTGCTGCAAC			
ATP5A1W	F: AGCGTGGTGCTTCATTTTGTG	60	30	325
	R:TTGGAGGCATGGATGTTTCTT			
P450arom	F: GCTTGGATTACAGTGCATTG	57	40	111
	R: CCAGGACCAGACAGGGCT			
FOXL2	F: AGAACAGCATCCGCCACAA	60	36	100
	R: GGGTCCAGCGTCCAGTAGT			
DMRT1	F: GTTCTGCATGTGGCGGGA	57	32	114
	R: CCTCTTCCTGGGCTTGCT			
AMH	F: CTCCCTCACCAACTACTCAACC	60	34	131
	R: TGCCAGTCCCCAAAATGCT			
SOX9	F: AAGTCGGTGAAGAACGGG	60	34	202
	R: GCTGAGCGTCCGTTTTGG			
β-actin	F: TGGGTATGGAGTCCTGTGGT	60	26	160
	R: AGGGCTGTGATCTCCTTCTG			

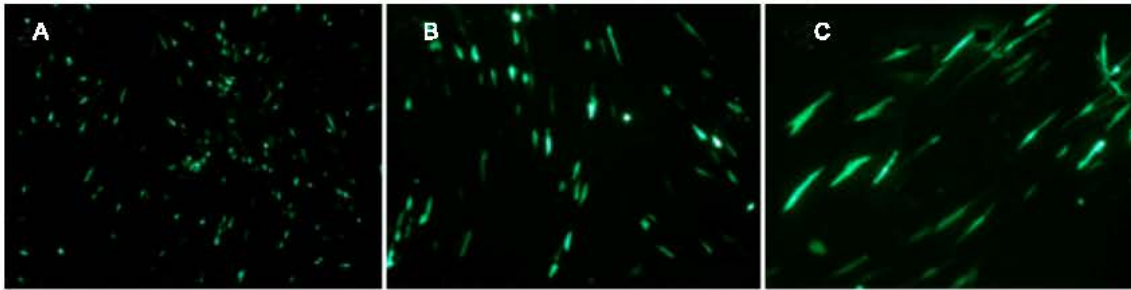


Fig. 1: GFP expressing in chicken embryo fibroblasts. Green fluorescence was observed in cytoplasm and nucleus of chicken embryo fibroblasts, A: x40, B: x100, C: x200

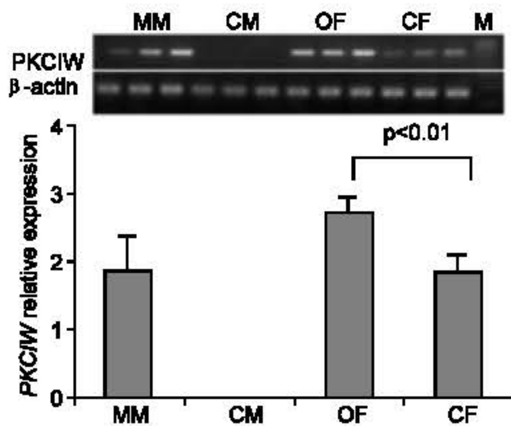
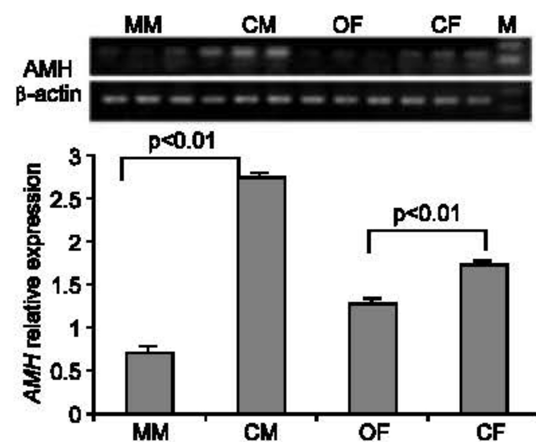


Fig. 2: PKCIW expression by Semi-quantitative RT-PCR. M-DNA Marker; MM-mis-expression in males; CM-male controls; OF-over-expression in females; CF-female controls. Upper and lower parts showed electrophoretogram of RT-PCR and corresponding graph, respectively

female controls, with no expression in the male controls as expected. The result showed that effective mis-expression of exogenous PKCIW was achieved in male chicken embryo fibroblast and over-expressed in female chicken embryo fibroblast.

Expressions of AMH and P450arom: AMH was expressed in every investigated group (Fig. 3) and showed highest expression in the male controls, lowest expression in the mis-expression in males. A significant reduction ($p<0.01$) in mis-expression in males was found compared with the male controls and AMH expression in over-expression in females was also lower than that of the female controls ($p<0.01$) but with a small decrease compared with that of in MM from CM. In Fig. 3, P450arom showed the highest expression in over-expression in females, which was significantly higher than that of the female controls ($p<0.01$), while there was no significant difference between mis-expression in males and male controls. No obvious difference was found between the male controls and female controls, but a very significant difference between the mis-expression in males and over-expression in females.



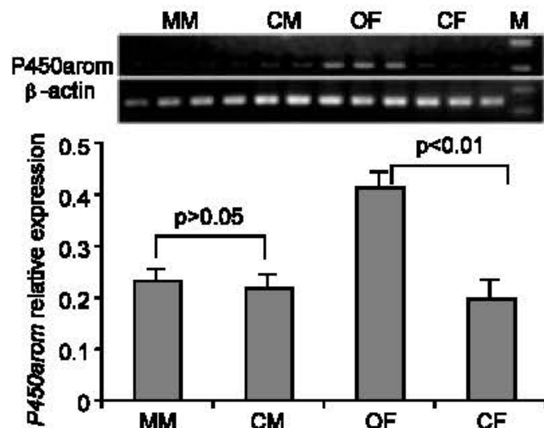


Fig. 3: AMH and P450arom expression by Semi-quantitative RT-PCR. M-DNA Marker; MM-mis-expression in males; CM-male controls; OF-over-expression in females; CF-female controls. Upper and lower parts showed electrophoretogram of RT-PCR and corresponding graph, respectively

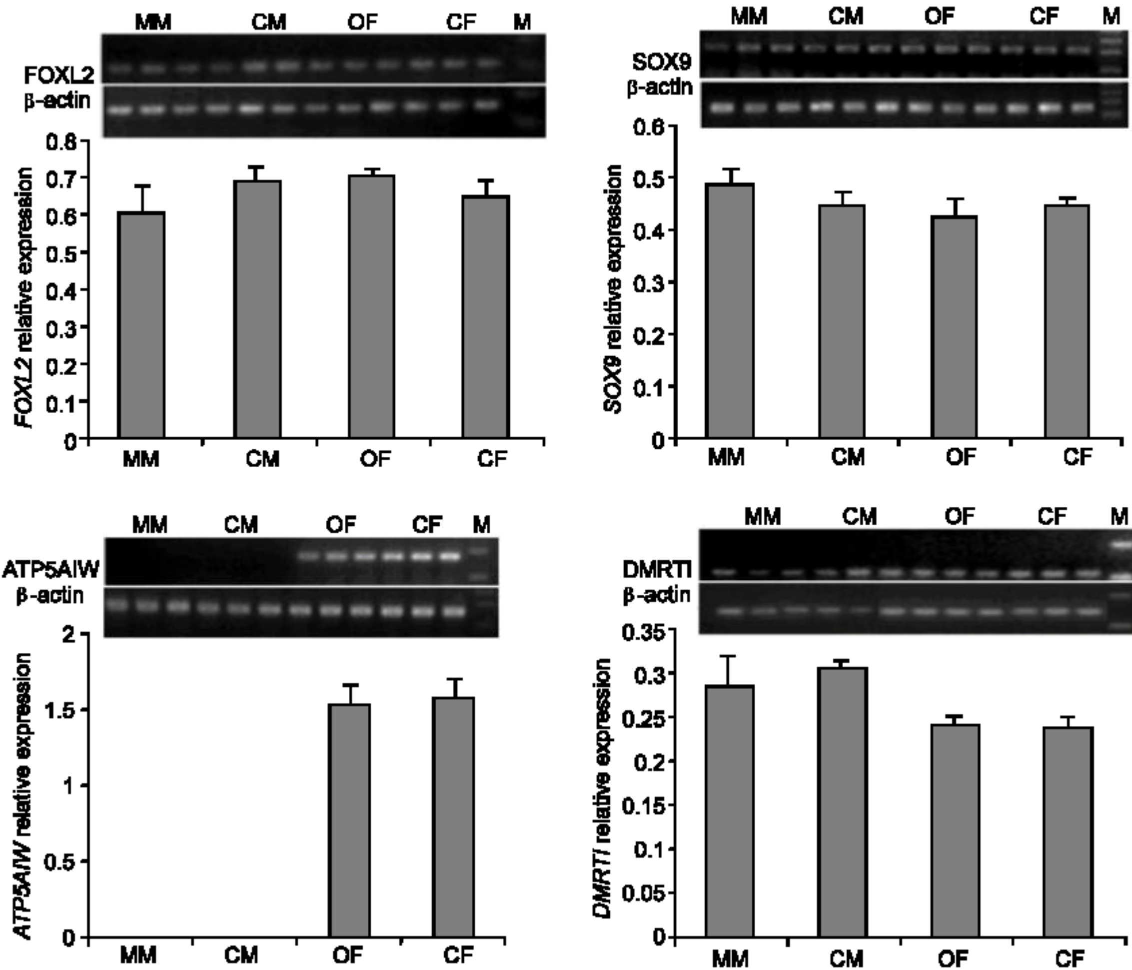


Fig. 4: FOXL2, SOX9, ATP5A1W and DMRT1 expression by semi-quantitative RT-PCR. M-DNA Marker; MM-mis-expression in males; CM-male controls; OF-over-expression in females; CF-female controls. Upper and lower parts showed electrophoretogram of RT-PCR and corresponding graph, respectively

Expressions of FOXL2, SOX9, ATP5A1W and DMRT1: The expressions of FOXL2, SOX9, ATP5A1W and DMRT1 in four groups were presented in Fig. 4. FOXL2 expression showed significant difference between the male controls and female controls, but no significant difference between other groups. SOX9 expression kept similar levels in each group. And DMRT1 exhibited higher expression in the male controls than female controls, mis-expression in males than over-expression in females, but no significant difference was found in four groups. Unlike other genes, ATP5A1W was expressed only in the female controls and the over-expression in females and no significant difference was observed between them.

DISCUSSION

In mammals, male sex determination initiated by the testis determinant SRY, together with genes required for spermatogenesis forms a cascade of gene expression within the testis development. Correspondingly, ovarian development is regulated by the other series of signal molecules such as WNT-4 and RSPO1 (Smith *et al.*, 2008). In birds, sex determination and differentiation is a complicated regulation process involved in multiple gene expression and interaction as in mammals. Several genes within the mammalian sex-determining cascade have been identified in birds within recent years. Of the genes involved in chicken gonadogenesis, some exhibit sexual dimorphism, such as DMRT1, AMH and SOX9, whereas PKCIW, P450arom, FOXL2 and ATP5A1W express only in female chicken gonads. Although the major sex determinant which will lead to the isolation of upstream regulatory factors and the identification of downstream components in the sex-determining pathway of birds has not been identified, AMH, P450arom, androgen and estrogen, are crucial factors in determining early embryonic development. It was proposed that the dividing point between upstream and downstream pathways is AMH in males and P450arom in females (Shimada, 2002). In the present

study, to evaluate the function of a putative dominant ovary-determinant, PKCIW, in chicken sex determination and differentiation, inducible mis-expression and over-expression was performed and the expression dynamics of these genes putatively involved in chicken gonadal development were detected.

AMH is a glycoprotein hormone produced and secreted by differentiating sertoli cells of the developing testis, and indeed is considered a marker of sertoli cell differentiation in mammals (Smith and Sinclair, 2004). Our observation that AMH in male controls is significantly higher than that in female controls is consistent with that described in male and female gonads. Both mis-expression and over-expression of PKCIW led to down-regulated expression of AMH with large decrease of AMH in male. It's presumed that PKCIW could down-regulate AMH expression in females and thereby participated fertilization progress in female chicken embryos, whereas high expression of AMH appeared in males without PKCIW.

Elbrecht and Smith (1992) demonstrated that in birds, estrogen is essential for ovarian differentiation, thus, P450arom plays a pivotal role as an early step for catalyzing the synthesis of estrogens from androgens. In this study, asymmetric change of P450arom expression with mis- or over-expression of PKCIW between males and females may be due to that the joint effect of endogenous and exogenous PKCIW could activate strong expression of P450arom mRNA in females, whereas certain male-liked factor(s) resistant to PKCIW mRNA expression, thereby lower increased P450arom expression was detected in males. The results suggest that PKCIW could possibly be effective on estrogens synthesis by regulating P450arom expression and then could act on ovarian differentiation in females.

SOX9, encoding a member of the SOX family of transcription factors (including SRY), is structurally conserved in mammals and birds (Kent *et al.*, 1996). It's already known that SOX9 acts as an activator to AMH expression with SF1 in mammals (De Santa Barbara *et al.*, 1998). However, the founding in chicken suggests that SOX9 is not required for the initiation of AMH transcription as has been observed in mammals (Oreal *et al.*, 1998). In our investigation, no significant change of SOX9 expression appeared as AMH expression decreased in both males and females. The possible explanation is that SOX9 and AMH are in different sex-determining regulatory pathway, or SOX9 is probably not involved in this pathway in chicken.

DMRT1, a highly conserved gene encoding proteins with a unique DNA binding motif called the DM domain, is required for testis determination in chicken (Smith *et al.*, 2009b). However, there is no significant change of DMRT1 expression with PKCIW mis-expression and over-expression. Considering the temporal and spatial dynamics of DMRT1 and PKCIW, it may be speculated that PKCIW is not in the same sex regulatory pathway while DMRT1 is required for male determination.

FOXL2 is a winged helix/forkhead transcription factor gene involved in ovarian development. Govoroun *et al.* (2004) found that the spatial and temporal dynamics of FOXL2 and P450arom expression in chicken were in parallel and subsequent studies suggested that FOXL2 lie upstream of P450arom in avian sex determination (Hudson *et al.*, 2005). In the present study, P450arom expression was significantly increased whereas there was no obvious change of FOXL2 expression. It's presumed that an unknown factor besides FOXL2 also could act upstream of P450arom and regulate the expression of P450arom, which therefore, indicate that P450arom expression is up-regulated by the factor activated by the over-expression of PKCIW.

ATP5A1W, a W-linked gene, which had been identified from the female-minus-male subtracted cDNA macroarray (Yamada *et al.*, 2004), was also isolated from forward and reverse subtracted cDNA library between females and males in our laboratory (E3.5-E6.0) (Feng *et al.*, 2009). Our results showed that ATP5A1W was expressed only in females and no significant change of ATP5A1W expression was found as PKCIW over-expression. De Kloet (2001) suggested that ATP5A1W is not required for the survival or fertility of the African grey parrot. The results supported that ATP5A1W is probably not involved in the sex-determining pathway.

As described above, PKCIW could regulate AMH and P450arom mRNA expression, whereas the expression of FOXL2, SOX9, DMRT1 and ATP5A1W showed no obvious correlation with PKCIW at transcription level. The *in vitro* studies have demonstrated that PKCIW which formed a heterodimer with PKCIZ could inhibit the biological function of PKCIZ, supporting the predicted role of PKCIW in chicken sex determination (Pace and Brenner, 2003; Moriyama *et al.*, 2006). However, Smith *et al.* (2009a) reported that normal protein expression pattern of P450arom in females and AMH in males were found after infected with RCASBP virus carrying PKCIW. Therefore, it is presumed that there is a microRNA, perhaps, which play a role at post-transcriptional level and (or) translation level of AMH and P450arom, since significant mRNA expression of P450arom and AMH was detected but no in protein expression level with over-expression and mis-expression of PKCIW.

In summary, we have demonstrated the possible regulatory effect of PKCIW on AMH and P450arom in transcription level by transfecting PKCIW to chicken embryo fibroblast using tet-on inducible expression system. Further studies can be performed on the determination of whether PKCIW could regulate the mRNA expression of P450arom and AMH in chicken embryos through loss of function by *in ovo* electroporation in the chicken embryos and whether a critical factor (a microRNA or siRNA, perhaps) could regulate the protein expression of P450arom and AMH,

which will be of great value in clarifying avian sex-determining mechanism and regulatory network of gene related to avian sex determination and differentiation.

ACKNOWLEDGEMENTS

We are grateful to Dr. Y. Takahashi (Nara Institute of Science and Technology, Japan) for the kind gifts of pBI-EGFP and pCAGGS-rtTA2 S -M2. This work was supported in part by the National Natural Science Foundation of China (30671494), Doctoral Program Foundation of Institutions of Higher Education of China (20070504040) and the National Natural Science Foundation for Young Scholar of China (30800781).

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