

Full Length Research Paper

Differential expression of Forkhead box protein 2 between genders in chickens

Xi Wang^{1,2}, Hongxia Li³, Mohammad Abdul Hamid¹ and Xingbo Zhao^{1*}

¹College of Animal Science and Technology, National Engineering Laboratory for Animal Breeding, Key Laboratory of Animal Genetics, Breeding and Reproduction, China Agricultural University, Ministry of Agriculture, Beijing-100193, China.

²Institute of Animal Science and Veterinary Medicine, Shanxi Academy of Agricultural Science, Taiyuan-030031, China.

³Shanxi Population and Family Planning Science Research Institute, Taiyuan-030006, China.

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Forkhead box protein 2 (*FoxP2*), acts as a key gene in vocalization development involving language learning of humans and songbirds. In the present study, expression pattern was analyzed in different breeds and genders of chickens. Transcriptional levels tested by real-time polymerase chain reaction (PCR) showed that roosters had significantly higher mRNA expression than hens and capons, but the difference was not significant in different breeds. Protein expression levels tested by western blotting indicated they were similar with transcriptional test, and that roosters showed significant higher protein expression levels than hens and capons. These results suggest that mRNA and protein expression of *FoxP2* were sexually dimorphic. Therefore, *FoxP2* is a key gene in gender development, and probably regulates rooster crow.

Key words: Forkhead box protein 2, mRNA expression, protein expression, gender, chicken.

INTRODUCTION

Forkhead proteins are important transcriptional regulators that are involved in pattern formation during vertebrate development, as well as in tissue specific gene expression and tumorigenesis (Accili and Arden, 2004; Carlsson and Mahlapuu, 2002; Dirksen and Jamrich, 1992; Dirksen and Jamrich, 1995; El-Hodiri et al., 2001; Erickson, 2001; Kaufmann and Knochel, 1996; Lai et al., 2001b; Lai et al., 1990; Lehmann et al., 2003; Li and Vogt, 1993; Tseng et al., 2004). Forkhead box protein 2 (*FoxP2*), a member of Foxp subfamily of Fox genes, is a nervous transcription factor, and mainly expresses in the brain. Its mutations could cause some neuropsychiatric disorders, and it plays a role in autism or more common forms of language impairment in humans (Hurst et al., 1990; Lai et al., 2001a; Vargha-Khadem et al., 1998).

The language skills of men are different from women (Haas and Adelaide, 1979), and men are more likely to

have autism and some other mental disorders (Baron-Cohen, 2002). Similar to humans, songbirds have the ability for vocal learning that results in song production (Nottebohm, 1972). As known, *FoxP2* is necessary for correct vocal imitation in songbirds (Haesler et al., 2004). In many songbird species, this ability is a secondary sex characteristic of males and is absent in females (Tramontin et al., 2003). Similarly, cock crows but hen does not; although, this vocalization is not learnt in chickens.

The corpus striatum or "striped body" consists of the basal ganglia (basal nucleus) and the internal capsule. The basal ganglia are made up of neurons, so it is gray matter. The spatial expression of *FoxP2* in songbird's brain is very similar to humans with strong expression in the telencephalon, diencephalon, cerebellum, hindbrain, spinal cord, and retinal ganglion cells (Lai et al., 2003; Teramitsu et al., 2004). Some studies showed that striatal *FoxP2* is very important and is related to songbird sensorimotor learning (Haesler et al., 2007; Teramitsu et al., 2010); therefore, we procured the basal ganglia from chicken brain to investigate whether brain expression of

*Corresponding author. E-mail: zhxb@cau.edu.cn. Tel: +86-010-62733417. Fax: +86-010-62733417.

Table 1. *FoxP2* mRNA and protein expression in different chicken breeds and genders.

Breed	Gender	Short letter	Observed number	mRNA expression (Mean \pm SD) [†]	Protein expression (Mean \pm SD) [†]
White leghorns	Male	WL	6	348.78 \pm 191.15 ^{ab}	
	Capon	WC	6	128.25 \pm 25.26 ^c	5.25 \pm 0.69 ^a
White Plymouth Rock	Female	WF	6	155.81 \pm 58.83 ^{bc}	5.79 \pm 0.31 ^a
	Male	WM	6	401.98 \pm 283.29 ^a	7.01 \pm 1.24 ^b
Tibetan chicken	Male	T	6	252.50 \pm 149.07 ^{abc}	

SD, standard deviation; [†] Multiple comparisons among levels were performed by Dunnett's C of SAS8.0 and the significance level was set at $P < 0.05$. ^{a, b, c} Means within a column with no common superscript differ significantly ($P < 0.05$).

FoxP2 was different in different genders in chickens.

MATERIALS AND METHODS

Animals and tissue samples

All experimental chickens were 180 days old and were collected from chicken farm of China Agriculture University, including White Leghorn (male), White Plymouth Rock (capon), White Plymouth Rock (female), White Plymouth Rock (male) and Tibetan Chicken (male). We procured the basal ganglia and stored the tissue at -80°C .

Total RNA extraction and cDNA synthesis

Total RNA from the corpus striatum of chicken brain was extracted using Trizol reagent (Invitrogen) and chloroform according to manufacturer instructions. RNA was dissolved in diethylpyrocarbonate (DEPC) treated water, and stored at -80°C . Approximately, 1 μg total RNA was used to synthesize the first cDNA strand, using a MMLV-RT kit (Promega) according to the manufacturer protocol.

Real-time PCR

Real-time (RT) PCR was used to determine the expression of *FoxP2* in the corpus striatum of brain from different chicken breeds including Tibetan chicken (male), White Plymouth Rock (male, female and capon) and White Leghorn (male). Primers were designed to be of an appropriate length and had an optimal annealing temperature of 60°C . Holding house gene's primer, β -actin (F: 5'-GAAAATTGTGCGTGACATCG-3', R: 5'-CCTGAACCTCTCATTGCCA-3') were designed according to L08165.1 (GenBank) with product length of 150 bp. Target gene's primers (F: 5'-CGCAACGTCTCAAGCAATGA-3', R: 5'-GCCGTTGGTGTGTGGGAGTTT-3') were designed according to JN677529 (GenBank, which sequence had been submitted by our group), and product length was 161 bp. Real-time PCR was performed using the ABI Step One Plus Real-Time PCR Detection System (Applied Biosystems).

Antibody production and western blotting

Since commercially available primary antibodies are not suitable for chickens, we developed our own antibody against *FoxP2* in rabbit.

The *FoxP2* antibody was made against the 15-aa sequence SPELEDDREIEEPC in the N-terminal region of *FoxP2* according to JN677529. In order to ensure that the antibody is very specific to identify *FoxP2*, the peptides were chosen on the basis of its antigenicity (DNASTAR) and dissimilarity between family members *FoxP1* and *FoxP4*. Similarity of the amino acid sequence to other proteins was excluded by comparisons with family members *FoxP1* and *FoxP4*, as well as by protein blast (blastp) analysis (BLAST). The peptide was conjugated to keyhole limpet hemocyanin, and rabbits were immunized with the peptide and complete adjuvant (Sigma-Genosys). Antibody was purified on an affinity purification column with the peptide (Sigma-Genosys).

Approximately, 100 mg frozen specimens obtained from six males, six females and six capons of White Plymouth Rock chickens were crushed to powder using a liquid nitrogen-cooled mortar and were homogenized in 500 μl lysing buffer (7 M Urea, 2 M thiourea, 4% CHAPS, 65 mmol/L DDT). Homogenates were continually incubated in lysing buffer at 4°C for 1 h and subsequently centrifuged at 10000 g for 30 min at 4°C to obtain the supernatant. Protein levels were determined with the use of the detergent-compatible protein assay system (Bio-Rad Laboratories, Hercules, CA), and 40 μg of protein per lane was resolved by gel electrophoresis followed by their transfer to the polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were blocked with 3.5% bovine serum albumin (BSA) in Tris_HCl-buffered saline (TBS) for 12 h at 4°C . After been washed with TBS, the membranes were incubated for 1 h at 37°C with the antibodies for *FoxP2*. After washing, the membranes were incubated for 1 h at 37°C with horseradish peroxidase-conjugated goat anti-rabbit IgG which was secondary antibody (Kangwei, China). The immunoblots were detected with enhanced chemiluminescence reagents (Kangwei, China). β -Actin antibody was used as the internal standard from commercial antibody (Kangwei, China).

Gray-scale value of western blot was used to measure its bands, and was analyzed by Quantity One 4.62 software. The mRNA expression levels gained from RT-PCR and the gray-scale value of protein were analyzed by one-way ANOVA in SAS 8.0. Multiple comparisons among levels were performed by Dunnett's C and the significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION

Our data show that there was no significant difference for *FoxP2* gene expression between different chicken breeds and gender ($p > 0.05$, Table 1), but the mRNA expression of *FoxP2* in both capons and females of White Plymouth Rock was lower than for males, whose expression was

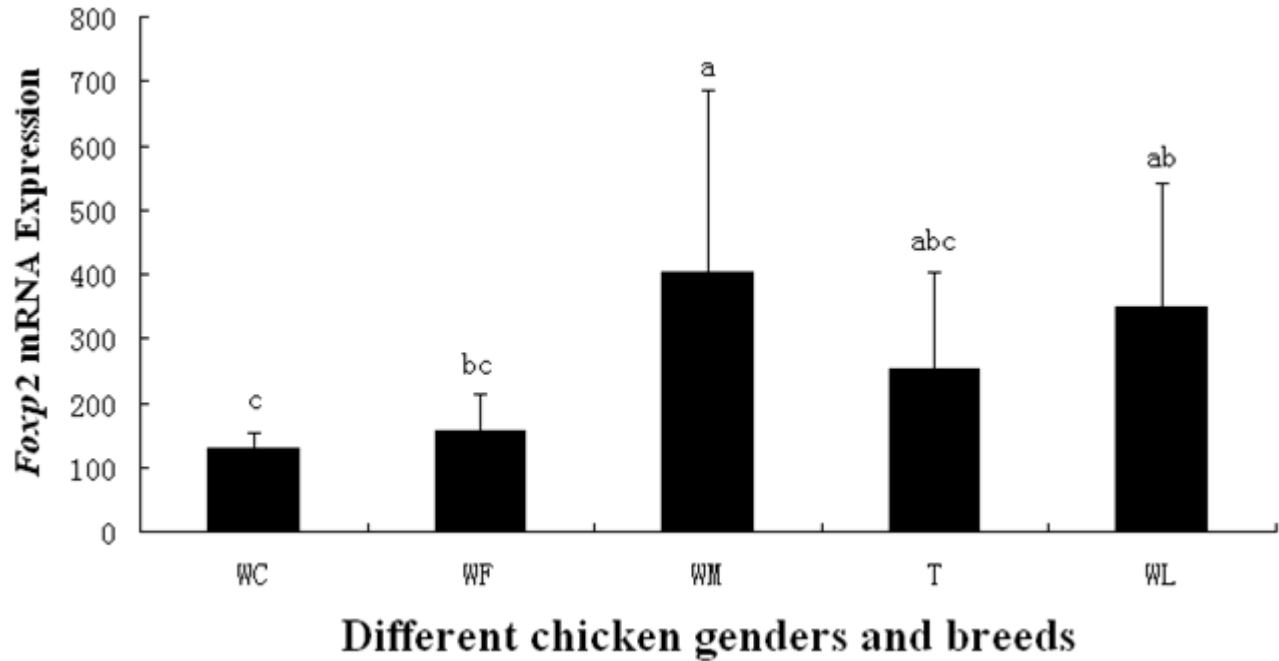


Figure 1. Column comparison of *FoxP2* mRNA expression in different chicken breeds and genders. *a*, *b* and *c*, means on the horizontal bars with no common superscript differ significantly ($P < 0.05$). WM, White Plymouth rock male; WF, white Plymouth rock female; WC, white Plymouth rock capon; T, Tibetan chicken (male); WL, White leghorns.

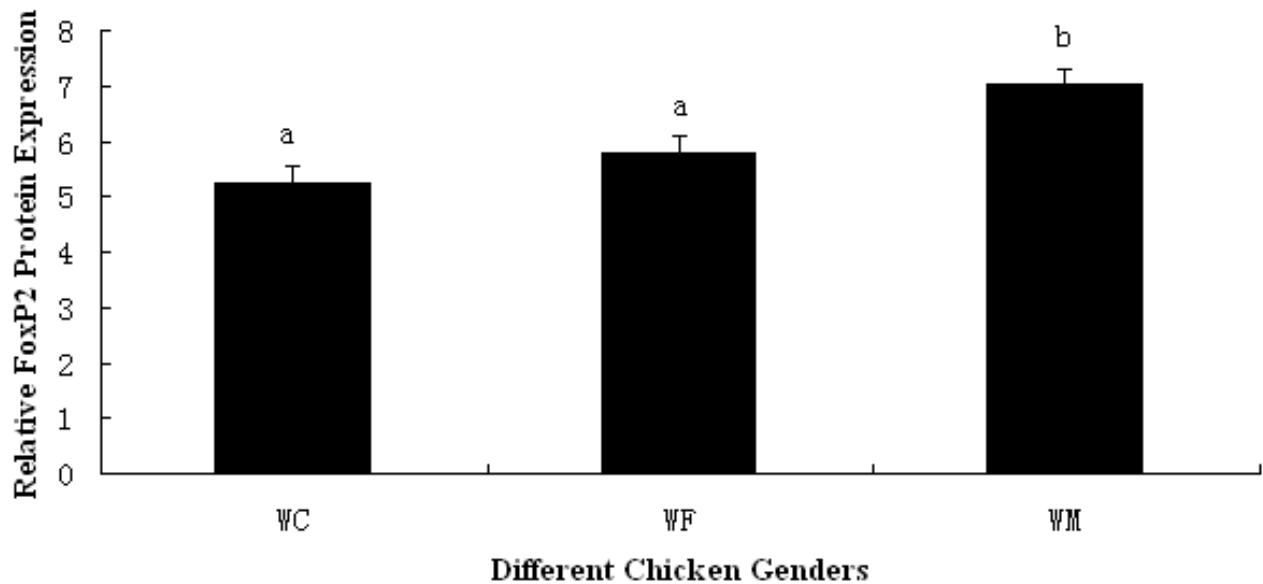


Figure 2. Column comparison of *FoxP2* protein expression in different chicken breeds. *a*, *b*, Means on the horizontal bars with no common superscript differ significantly ($P < 0.05$). WM, White Plymouth rock male; WF, white Plymouth rock female; WC, white Plymouth rock capon.

the highest among these breeds and gender (Figure 1). Protein expression of *FoxP2* in chickens was detected by western blotting, and the results were analyzed by Quality one 1.0 software. Our data show that protein

expression of male White Plymouth Rock is significantly higher than for capons and females ($p < 0.05$) (Table 1, Figures 2 and 3).

Moreover, *FoxP2* expression transiently increases at

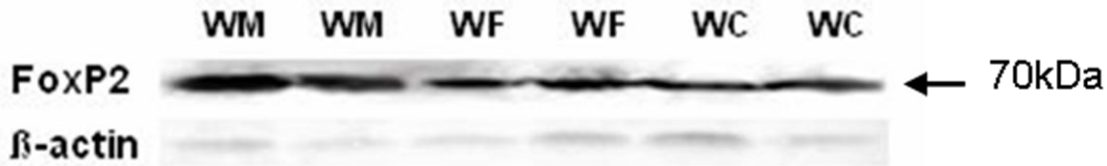


Figure 3. Western blotting for different genders of White Plymouth Rock chicken. WM, White Plymouth rock male; WF, white Plymouth rock female; WC, white Plymouth rock capon.

the time when young male zebra finches learn to sing in the basal ganglia song nucleus (Rochefort et al., 2007), which has close relation with normal song development (Scharff and Nottebohm, 1991; Sohrabji et al., 1990), and it was shown that local knockdown of *FoxP2* in this nucleus disrupts vocal learning (Haesler et al., 2007). Hamson et al. (2009) compared *FoxP2* expression of wild-type male rats with estrous or non-estrous female rats by the use of analyses of relative optical densities of *FoxP2* immunoreactivity, and they found significantly greater immune-labeling in the former than in the latter. This showed a gender difference in *FoxP2* expression in the brain of rat.

In this study, we showed that mRNA and protein expression of *FoxP2* were sexually dimorphic, and *FoxP2* was expressed at higher levels in males compared to females and capons. This may be related to the more diverse set of vocalizations in roosters, such as crowing that is absent in hens and capons, which would indicate that *FoxP2* might be involved in vocal production even in the absence of vocal learning. Although vocalization in chickens is not learned in contrast to songbirds, it is possible that *FoxP2* was associated with male secondary sex characteristics in chickens.

Furthermore, there is another speculation that *FoxP2* is related with autism that has sexually dimorphic characteristics. Gong et al. (2004) showed the *FoxP2* gene may be of interest in the genetic pathogenesis of autism in Chinese population. Hamson (2009) showed that men are more likely to have autism than women; so, we speculate that the reason may be that the expression of *FoxP2* is different between genders.

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