Gene Expression Profile of Estrogen Receptor Alpha and Beta in the Ovaries of Zi Geese (Anser cygnoides)

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Estrogens play a pivotal role in the development and maintenance of normal sexual and reproductive function (Heldring et al. 2007; Muriach et al. 2008). The biological actions of estrogens are manifested through two high-affinity estrogen receptors, estrogen receptor alpha (ERalpha) and estrogen receptor beta (ERbeta), which belong to a family of transcription factors, the nuclear receptor superfamily, and are expressed at different levels in target cells (Greene et al. 1986; Kupper et al. 1996; Gruber et al. 2004; Tenas-Sempere et al. 2004). With respect to the functions of estrogens, ERalpha and ERbeta have been chosen as candidate genes to study their relationship with reproductive traits in pigs (Goliasiova & Wolf 2004; Munoz et al. 2004; Spotter & Distl 2006), cattle (Szreda & Zwierzchowski 2007) and sheep (Bi et al. 2005). The expression of ERalpha and ERbeta in the ovary of pregnant swine has been examined, and the results suggest that estrogens act via both ERalpha and ERbeta in the regulation of ovarian function during pregnancy and are involved in the process of successful reproduction (Knapczyk et al. 2008).

In recent years, the role of ERalpha and ERbeta in the reproductive performance of poultry has been increasingly studied. The expression of ERalpha and ERbeta mRNA has been examined in the ovaries of chickens (Krust et al. 1986) and quail (Foidart et al. 1999; Ichikawa et al. 2003). The expression of ERalpha and ERbeta mRNA was detected in the ovarian stroma and in the white, yellowish, small yellow, granulosa and thecal layers of the walls of preovulatory follicles in the ovaries of laying hens (Hrabia et al. 2008). The change in expression of ERbeta mRNA in ovaries of preputial ducks has been elucidated (Ni et al. 2007).
Although remarkable progress has been made in understanding the impact of estrogens on ovarian function during ovarian development in poultry, via both ERalpha and ERbeta, studies of estrogen receptors in the ovaries of geese are rare. To our knowledge, the profile of ERalpha and ERbeta gene expression in the ovaries of developing and laying geese was not examined until the present study. Therefore, the purpose this study was to investigate the dynamic regulation of the expression of ERalpha and ERbeta mRNA in the ovaries of geese during the developmental and egg-laying stages.

**Material and Methods**

**Experimental design and animals**

Fifty-six female Zi geese (Anser cygnoides) were selected randomly from 100 geese in a local breeding farm and raised according to the standard program used at the farm. Geese were fed ad libitum with rice grain and were supplemented with green grass or water plants whenever possible during the experiment. Eight geese were killed at the age of 1 day and 1, 2, 3, 4, 5 and 8 months to obtain ovaries. The geese were sacrificed by electrical stunning followed by exsanguination. The ovaries were removed rapidly, frozen in liquid nitrogen, and then stored at -70°C until analysis.

**Total RNA extraction and reverse transcription PCR**

Following manufacturers’ instructions, total RNA was prepared from the ovaries of Zi geese with Trizol reagent (Invitrogen Corporation, Carlsbad, California, USA), and then stored at -70°C until analysis. cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Reverse transcriptase negative control reactions were performed to ensure the absence of genomic DNA contamination. The ovaries were removed rapidly, frozen in liquid nitrogen, and then stored at -70°C until analysis.

**Construction of ERalpha, ERbeta and GAPDH cDNA plasmid**

The recombinant plasmids containing ERalpha, ERbeta and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were termed pERalpha, pERbeta, and pGAPDH, respectively. The constructs were prepared from total RNA in the ovaries of Zi geese at 8 months of age, and the complementary double stranded cDNA fragments were subcloned into the pGEM-T Easy Vector System (Promega, Madison, Wisconsin, USA) as described (KANG et al. 2010).

**qRT-PCR with SYBR Green I chemistry**

The qRT-PCR was performed on the first strand cDNA using the Line-Gene K Real-time PCR Detection System and software (Bioer Technology, Hangzhou, China) with SYBR® Premix Ex Taq™ (Takara Bio Inc., Dalian, China). Briefly, the 50 μl reaction consisted of 1 μl of cDNA, 25 μl of SYBR® Premix Ex Taq™ (2 × concentration), 2 μl of 20 μM of PCR forward primer and PCR reverse primer, and 22 μl of nuclease-free water. Thermal cycling was performed with an initial denaturation step of 10 s at 94°C, followed by 45 cycles of 5 s at 94°C, 56°C for 30 s, and then 72°C for 30 s, and then a final extension at 72°C for 10 min. Relative quantitation of gene expression was performed (see KANG et al. 2010).

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Accession number</th>
<th>Primer sequence (5’ - 3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
</table>
| ERalpha     | EF502052         | Forward: ACCCAACAGACCATATTAACGAA  
Reverse: CGCCAGACTAAGCCAATCATCAG          | 187 |
| ERbeta      | EF621308         | Forward: AATGCGGCTATGGAAGCTGGCATG  
Reverse: GGACTGACCGTGCTGAAGGAGAAT          | 163 |
| GAPDH       | DQ821717         | Forward: GCTGATGCTCCCAGTATGCTGAT  
Reverse: GTGTTGCAAGGGCATTTGCTGAC          | 86  |
Statistical analysis

Threshold and Ct (threshold cycle) values were determined automatically by the Line-Gene K Real-time PCR Detection software, using default parameters. The relative level of expression for ERalpha and ERbeta was calculated according to GAPDH (the normalizer) using the relative standard curve method. The relative level of expression was expressed as the mean of three or more means ±SD. The abundance of ERalpha and ERbeta in the ovaries of geese at 1 day of age was assigned a value of 1. All data were analyzed using SAS statistical software for Windows (SAS Institute Inc., Cary, NC, USA). The data were analyzed by ANOVA followed by Duncan’s test. Differences were considered to be significant at \( P < 0.05 \).

Results

The ratio 260 nm/280 nm of the RNA preparation was about 1.87. The integrity of 28S and 18S rRNA and the absence of genomic DNA were confirmed (proportionally 2:1, respectively) by agarose gel electrophoresis (data not shown).

The RT-PCR analysis showed the presence of ERalpha and ERbeta mRNAs in the examined ovaries during the developmental and laying stages. The products were 86, 187 and 163 bp for ERalpha, ERbeta and GAPDH mRNA (Fig. 1), respectively; the products corresponded to the approximate size for each as predicted, and their specificity was appropriate for qRT-PCR.

The PCR efficiencies (E) for the standard curves, when the plasmids pGAPDH, pERalpha and pERbeta were used as independent templates, are shown in Figure 2, in which the equations of

\[
\begin{align*}
E_{Ra\ lpha} & = -3.228x + 24.827 \\
R^2 & = 0.9988 \ E = 0.9726
\end{align*}
\]

\[
\begin{align*}
E_{Rbeta} & = -3.328x + 24.383 \\
R^2 & = 0.9995 \ E = 0.9975
\end{align*}
\]
the curves and the \( R^2 \) values are also presented. In all the standard curves of Ct versus DNA concentration, the \( R^2 \) values were more than 0.99, which indicated an excellent degree of colinearity between these parameters (PFAFFL 2001). In addition, the efficiency value (E) of amplification of ERalpha, ERbeta and GAPDH in the experiments was 0.9726, 0.9975 and 1.0423, respectively.

The qRT-PCR results showed that, in the ovaries of Zi geese, the expression of ERalpha and ERbeta mRNA was greater at 1 to 5 months and 8 months compared with 1 day (Figs 3 and 4). In particular, the expression of ERalpha and ERbeta mRNA at 8 months was \( 2.47 \pm 0.23 \) fold and \( 29.07 \pm 1.25 \) fold greater than at 1 day, respectively (\( P<0.05 \)). The level of expression of ERalpha mRNA in the ovaries of the geese fluctuated and increased as the geese aged. The relative expression of ERalpha mRNA was not significantly different at 1, 2, 3 and 4 months of age (1.50 ± 0.11, 1.46 ± 0.12, 1.35 ± 0.10 and 1.39 ± 0.14 fold, respectively). The expression of ERalpha mRNA was significantly greater at 8 months compared with 5 months (\( P<0.05 \)), and the relative levels of expression were \( 1.86 \pm 0.17 \) and \( 2.47 \pm 0.23 \), respectively (Fig. 3). The level of expression of ERbeta mRNA...
in the ovaries of geese was not significantly different between 1 day and 1 month of age (P>0.05). However, the level of expression of ERbeta in the ovaries of geese at 2 to 5 months and at 8 months (1.96 ± 0.13, 2.58 ± 0.08, 2.08 ± 0.05, 3.25 ± 0.11 and 29.07 ± 1.25 fold, respectively) was significantly greater than at 1 day of age (P<0.05). The relative expression of ERbeta mRNA in the ovaries of geese was not significantly different at 1, 2 and 4 months (P>0.05), and between 3 and 5 months (P<0.05). The expression of ERbeta mRNA was significantly greater at of that observed.

Discussion

Estrogens play important role in the ovarian function of poultry. The primary mechanism of action of estrogen is via binding to and modulation of the activity of ERalpha and ERbeta, which are ligand-dependent nuclear transcription factors expressed at high levels in female tissues that are critical for reproduction (e.g. the ovary). However, the expression profile of these receptors in the ovaries of developing and laying geese was not examined until the present study. Quantitative PCR has become a standard method for the measurement of gene expression by evaluating the amount of mRNA produced (ONG & IRVINE 2002). Therefore, in the present work, the level of expression of estrogen receptor mRNA in the ovaries of developing and laying Zi geese were investigated by qRT-PCR.

In the present study, the level of expression of ERalpha mRNA increased and fluctuated from the newborn to the egg-laying stage; furthermore differences in expression of ERalpha mRNA were not significant during the first 4 months of age. These results suggest that ERalpha remained stable and then increased during the developmental stage because it plays a pivotal role in the development of the ovaries of the female and in sexual maturity of geese (DRUMMOND et al. 1999; MUNOZ et al. 2007). Although the change in the level of expression of ERbeta was similar to that of ERalpha, the increment of the expression of ERbeta from 2 to 8 months was greater than that of ERalpha (KANG et al. 2009). Data from mice with knockout of the estrogen receptor gene have confirmed that ERbeta is essential for the early development of ovarian follicles (COUSE et al. 1997). The results of this experiment suggest that the physiological roles of estrogens in the ovary are mediated more by ERbeta than by ERalpha during early postnatal development (YING et al. 2000; NILSSON et al. 2001; KOWALSKI et al. 2002; NI et al. 2007).

Interestingly, the level of expression of ERalpha and ERbeta mRNA in the ovaries of geese at 5 and 8 months was much higher than in the ovaries of newborn geese, and the relative expression of both ERalpha and ERbeta at 8 months was the highest measured, especially for ERbeta. These findings indicate that both ERalpha and ERbeta play important roles in the maintenance of ovarian function and in the process of successful reproduction (DRUMMOND et al. 2002; KNAPCZYK et al. 2008). In particular, it is more noticeable during the egg-laying stage. ERbeta is critical for granulose cell differentiation and the ovulatory response to gonadotropins (COUSE et al. 2005). ERbeta is also the predominant form of estrogen receptor in the ovary (JEFFERSON et al. 2002; COUSE & KORACH 2004). Therefore, it is understandable that the level of ERbeta mRNA was much greater than that of ERalpha mRNA from 2 to 8 months of age.

In summary, the results of the current study establish that ERalpha and ERbeta mRNAs are expressed and exhibit changes in the ovaries of Zi geese during the developmental and egg-laying stages. These findings demonstrate that the expression of both ERalpha and ERbeta mRNA in the geese ovaries was fluctuant, and that it increased from the neonatal to laying stage. These results support the further possibility that both ERalpha and ERbeta mediate ovarian function and the process of egg laying, and that ERbeta may play a more important role in mediating the response of the ovary to estrogen during the developmental and egg-laying stages.

References


