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Androgens and androgen receptor signaling contribute to ovarian development in the chicken embryo

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ABSTRACT

Androgens and androgen receptor (AR) signaling play important roles throughout development. In the chicken, AR signaling is involved in reproduction; however, its specific role is unclear. We show that AR signaling is involved in the normal development of the female embryonic gonads. The AR mRNA level was detected in male and female embryonic gonads by quantitative RT-PCR, and its expression was higher in females than in males at all developmental stages examined. In female embryos, the AR localized to nuclei of cells in the left gonad. Although AR expression was low in the majority of the medulla, high expression was detected in cells of lacunae within the medulla. In addition, AR expression increased in cells of cortical cords within the cortex with the progression of development. AR expression in the right gonad was lower than that in left gonad throughout development. In the male gonad, the AR localized to the cytoplasm of cells in seminiferous tubules at all stages. Female AR knockdown (ARKD) embryos infected with a retrovirus expressing micro RNAs targeting the AR showed normal asymmetric gonads (development of the left and depression of the right gonads), whereas the number of lacunae decreased. Furthermore, there was a disruption in the structure of cortical cords. By contrast, the gonads of ARKD males developed normally during embryogenesis. These results indicate that androgens and AR signaling are essential for the development of lacunae and cortical cords in gonads of female embryos.

1. Introduction

The androgen receptor (AR) is a ligand-dependent transcription factor belonging to the steroid hormone receptor family, whose members contain an N-terminal transactivation domain, a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (Gelmann, 2002). The DBD and LBD are important for the transcriptional regulation of target genes, and the amino acid sequences of these domains are highly conserved across vertebrate species (Gelmann, 2002; Brinkmann et al., 1999). The homologue of AR in the chicken was cloned in 2006 (Katoh et al., 2006), and chicken AR was found to possess an open reading frame of 2,109 bp, encoding a protein of 703 amino acids that is highly homologous with the AR from other vertebrate species.

Androgens and AR signaling play crucial roles in reproductive development in males. For example, treatment of 33-day-old male chickens with dihydrotestosterone (DHT) and testosterone stimulates the growth of the comb, anterior pituitary, and testis (Zeller, 1970), indicating that appendicular and gonadal development requires androgens and AR signaling. However, the roles of androgens and the involvement of AR signaling in chicken gonadogenesis are not entirely known. Several embryonic tissues in male and female chickens express the AR, and expression is generally higher in the left ovary than in the right and left testes (Katoh et al., 2006). The AR localizes to nuclei of ovarian cells, whereas nuclear localization is not detected in cells of the testis, illustrating that androgens and AR signaling are important for ovarian, but not testicular, development in avians. However, the roles of androgens and AR signaling

Abbreviations: ACTB, actin, beta; AR, androgen receptor; ARKD, androgen receptor knockdown; ARKO, androgen receptor knockout; DBD, DNA-binding domain; DHT, dehydrotestosterone; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HE, hematoxylin and eosin; IHC, immunohistochemistry; LBD, ligand-binding domain; miRNA, micro RNA; qRT-PCR, quantitative real-time–polymerase chain reaction; RNAi, RNA interference; S.D., standard deviation; S.E.M., standard error of the mean; WT, wild-type.

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in gonadal development in chickens are not yet known.

AR knockout (ARKO) male mice generated by the cre-lox conditional knockout strategy have a female-like appearance and body weight (Yeh et al., 2002). The testes of male ARKO mice are 80% smaller and the serum testosterone concentration is lower than that in wild-type (WT) mice. Spermatogenesis is arrested at the pachytene spermatocyte stage in male ARKO mice. In addition, the number and size of adipocytes and the volume of cancellous bone are different between WT and ARKO mice. By contrast, *AR* expression is mainly observed in theca cells, the endometrium and endometrial glands of the uterus in the female rat (Hirai et al., 1994), granulosa cells and antral follicles, the theca interna, and stromal cells in the primate ovary (Weil et al., 1998). These observations clearly support the involvement of androgens and AR signaling in mammalian female reproduction. In addition, the average number of pups per litter in homozygous and heterozygous ARKO female mice is lower than that in WT female mice (Yeh et al., 2002). Hu et al. (2004) reported that sexually mature ARKO female mice exhibit a longer estrous cycle and reduced fertility, and a marked reduction in the number of corpora lutea in the ovary. Fewer oocytes and corpora lutea are produced in ARKO mice after superovulation. There is also extensive granulosa cell apoptosis in preovulatory follicles of ARKO mice, with a downregulation of p21 and progesterone receptor expression (Hu et al., 2004). Furthermore, the granulosa cell- and oocyte-specific ARKO mice generated by the cre-lox system exhibit premature ovarian failure and subfertility with a longer estrous cycle and fewer ovulated oocytes (Sen &Hammes, 2010). These findings support the involvement of androgens and AR signaling in female fertility.

We report that androgens and AR signaling contribute to ovarian development in chicken embryos. AR expression in female and male embryonic gonads was compared by quantitative RT-PCR (qRT-PCR) and immunohistochemistry (IHC). AR expression was higher in the female gonad than in the male gonad of chicken embryos at all developmental stages. Furthermore, the AR localized to nuclei of cells of lacunae in the medulla and cortical cords in the cortex of female gonads. Female ARKD embryos exhibited a decreased number of lacunae in both right and left gonads and disorganization of cortical cords in the left gonad. By contrast, no remarkable changes were observed in male gonads throughout development.

2. Materials and methods

2.1. Animals and ethics statement

Fertilized chicken eggs (*Gallus gallus domesticus*) were purchased from Takeuchi Hatchery (Nara, Japan). This study used the Hy-Line Maria chicken strain. Fertilized eggs were incubated at 37.8 °C. The numbers of eggs and embryos used are shown in Table S1. The sex of each embryo was checked by PCR genotyping using genomic DNA as the template (Fridolfsson and Ellegren, 1999).

All animal experiments described in this study were approved by the Institutional Animal Care and Use Committee of the National University Corporation Hokkaido University and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals, Hokkaido University. This study did not involve any human participants or specimens.

2.2. Quantitative real-time–polymerase chain reaction

For qRT-PCR, total RNA was extracted from gonads at embryonic day (E)7.5, E9.5, E11.5, E13.5, E15.5, E17.5, E19.5, E21.5. Total RNA was extracted using the RNeasy Kit (Invitrogen) according to the manufacturer's instructions. RNA was treated with DNase I and then reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) and an oligo(dT) primer. qRT-PCR was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems) and an ABI 7300 Fast Real-Time PCR System (Applied Biosystems). To achieve maximum efficiency in all reactions, the amplicon size was restricted to 90–101 bp (Table S2). The reactions were performed in triplicate using 96-well plates and a 10 μl reaction volume. The data were analyzed using the $^{\Delta\Delta}$Ct method, and the mRNA level of each target gene was normalized to that of beta actin (*ACTB*).

2.3. Construction of RNAi vectors

Gene-specific micro RNAs (miRNAs) targeting chicken *AR* and scrambled miRNAs (non-targeting, control) were designed by BLOCK-iT RNA interference (RNAi) Designer (Invitrogen) and siRNA Wizard v3.1 (Invitrogen) programs, respectively. We designed two miRNAs targeting two different regions of the *AR* mRNA and two different non-targeting controls (Table S2). Molecularly cloning of miRNA construct was performed as previous report (Das et al., 2006). The PCR program consisted of denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The reaction was terminated at 72 °C for 5 min. The PCR products were digested with *Nhe* I and *Mlu* I for the first site of pRFPRNAi.C, and *Mlu* I and *Sph* I for the second site of pRFPRNAi.C. Each fragment was cloned separately into pRFPRNAi.C. pRFPRNAi.C vectors containing double miRNAs were digested with *Not* I and *Cla* I, and cloned into RCASARNAi to which GFP was previously inserted. pRFPRNAi.C and RCASRNAi were provided by ARK-Genomics, The Roslin Institute (Das et al., 2006).

2.4. Preparation and injection of RCAS.A.miRNAs

Endotoxin-free proviral DNA was prepared using the PureYield™ Plasmid Miniprep Kit (Promega). The DNA was transfected into DF1 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were then transferred to 10 mm dishes (surface area, 78.5 cm^2) and cultured to subconfluence in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The active virus was concentrated from the pooled medium. Briefly, 100 ml of medium was centrifuged at 6,000 × *g* overnight. The supernatant was then carefully decanted, leaving a small pellet in 200 μl of medium. The resuspended viral solution was aliquoted and stored at −80 °C until use. The viral titer was determined as described previously (Smith et al., 2009; Nakata et al., 2013).

To examine the knockdown efficiency, we performed qRT-PCR in normal DF1 cells that endogenously express AR, DF1 cells transfected RCASA.miRNA.Sc for control, and DF1 cells transfected RCASA.miRNA.AR. The procedure was previously described.

Embryo injections were performed as described previously (Smith et al., 2009; Nakata et al., 2013) with minor modifications. Approximately 3 μl of concentrated virus containing 0.025% Fast Green tracking dye was injected into the subgerminal cavity of day 0 (Hamburger and Hamilton [HH] stage X, Hamburger and Hamilton, 1951) blastderms. For ARKD embryos, virus carrying RCASA.miRNA.AR was injected into 103 eggs (Table S1). For control embryos, virus carrying RCASA.miRNA.Sc was injected into 87 eggs (Table S1). The sex ratio of each group is shown in Table S1. The eggs were sealed and incubated until E8.5–12.5.

2.5. Immunohistochemistry and western blot analysis

For IHC, urogenital tissues from chicken embryos were fixed in formalin for 1 h at room temperature. Paraffin sections (thickness,