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Novel genes and hormonal regulation for gonadal development during embryogenesis in chickens

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ABSTRACT

Asymmetrical gonadal morphogenesis is well known in female chickens in contrast to males where both gonads develop symmetrically. However, only a few genes have been reported to determine differential morphology between female and male gonads in chicken and their mechanisms of action are unclear. Therefore, we focused on three genes (TOM1L1, TTR, and ZEB1) that are related to cellular proliferation and embryonic development based on previous study indicating up- or down-regulated transcripts in the asymmetric female gonads between embryonic day 6 (E6) and E9 by microarray analyses. To define the validity of the gene expression pattern discovered, q-PCR and *in situ* hybridization analyses were performed. In the left female gonad between E6 and E9 the expression of *TOM1L1*, *TTR* and *ZEB1* increased at E9. On the other hand, *TOM1L1* and *TTR* increased significantly in both male gonads between E6 and E9. In addition, recombinant FSH and LH stimulated proliferation of gonadal cells and influenced expression of selected genes in chickens. This suggests that hormonal regulation is involved in growth and development in the embryonic gonad of chickens. Collectively, the results show differential gene expression between the left and right gonads in chicken embryos and that of is regulated by gonadotropin. These results provide novel insights into candidate genes regulating gonad development and differentiation.

1. Introduction

In vertebrates, left-right (L-R) asymmetric morphogenesis is important for development of various organs including the heart, liver, spleen and gut. The L-R asymmetric development is one of the orthogonal axes including anterior–posterior and dorsal–ventral axes for the body formation. It is conserved in vertebrates by genes encoding for various transcription factors and growth factors (Levin, 2005; Raya and Izpisua Belmonte, 2006). For example, Nodal is a member of the transforming growth factor-beta superfamily expressed in the left lateral plate mesoderm (LPM) (Hamada et al., 2002). At the node, an early asymmetric signal is generated and conveyed to the LPM through the function of Nodal signaling molecules in mammals. In addition, the homeobox gene *PITX2* is downstream of Nodal and cooperates with Nodal to develop the L-R axis (Liu et al., 2001; Patel et al., 1999). Asymmetric expression of PITX2 is involved in the process of organ morphogenesis such as heart, gut and lung (Campione et al., 1999).

In mammals, reproductive organs such as ovary and testis are positioned symmetrically in both sexes. Unlike mammals, bilateral development of the paired gonads is dependent on sex in chickens. Before sex determination, the gonads of chick embryos have no L-R asymmetrical morphology in either sex. The onset of sex specific differentiation becomes apparent histologically at embryonic day 6.5 (E6.5). Most female birds (ZW) have an asymmetric functional left ovary whereas the right gonad degenerates during embryonic development. In other words, only the left outer epithelial cell layer proliferates to form an ovary whereas the right cortex fails to develop into an ovary. On the other hand, testicular development in male (ZZ) chicks arises symmetrically and medulla cords thicken due to differentiation of Sertoli cells within the cords (Merchantlarios et al., 1984; Smith and Sinclair, 2004). Genes expressed asymmetrically during development of the gonads in chicks include *bone morphogenic protein 7 (BMP7)* (Hoshino et al., 2005), *paired-like homeodomain transcription factor 2 (PITX2)* (Rodriguez-Leon et al., 2008), *forkhead transcription factor L2 (FOXL2)* (Govoroun et al., 2004), and *estrogen receptor alpha (ESR1)* (Villalpando et al., 2000). For example, ESR1 is expressed in both sexes until E7. After that ESR1 is only expressed in the cortex of the left gonad of female chicken embryos and it is not expressed in either gonad in male chick embryos (Andrews et al., 1997; Nakabayashi et al., 1998). Misexpression of PITX2 in the left lateral plate mesoderm prevents development of the right gonad in

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female chick embryos and that gonad then degenerates (Rodriguez-Leon et al., 2008).

In the embryonic gonads of chickens, FSH and LH modulate the secretion of sex steroid hormones through their respective receptors FSHR and LHCGR. Both gonadotropins are known regulators of folliculogenesis and spermatogenesis (Gulyas et al., 1977; Thackray et al., 2010) with FSH inducing growth, development, and differentiation of granulosa cells in ovarian follicles of the ovary and Sertoli cells in the seminiferous tubules of the testis (Mendez-Herrera et al., 1998; Pedernera et al., 1999). LH is crucial for ovulation of the ovarian follicle and for stimulating secretion of testosterone by Leydig cells in the male testis (Casarini et al., 2011). In addition, FSH and LH stimulate germ cell proliferation in both sexes and modulate germ cell mitosis and meiosis during morphogenesis of embryonic gonads of chicks (He et al., 2013; Mendez et al., 2003).

There are a few genes which have been identified as regulators for asymmetric development of chicken embryonic gonads. Nevertheless, understanding how gonadal development occurs asymmetrically is unclear. Since the chicken genomic sequence is known, high-throughput analyses using chicken DNA microarrays provide valuable insight into changes in gene expression related to various biological processes. Therefore, the objectives of present study were to: (1) investigate cell- and tissue-specific expression of three selected genes considered to be involved in the development of gonads of female and male chick embryos during embryogenesis based on microarray analyses between E6 and E9; and (2) determine whether FSH and LH regulate expression of the target genes. Collectively, results of this study identified asymmetrically expressed genes that likely affect gonadal morphogenesis between left and right gonads during embryogenesis and how expression of those genes is regulated by gonadotropins during gonadal morphogenesis.

2. Materials and methods

2.1. Experimental animals and animal care

The experimental use of chickens for this study was approved by the Animal Care and Use Committee of Korea University. All chickens were exposed to a light regimen of 15 h light and 9 h dark with *ad libitum* access to feed and water, and subjected to standard poultry husbandry guidelines.

2.2. Sex determination

Freshly laid eggs were incubated with intermittent rocking at 37 °C under 60–70% relative humidity. Sex was determined on embryonic day E2.5. Approximately 0.2 μl of embryonic blood was collected from the dorsal aorta, diluted in 15 μl of 1× phosphate buffered saline (PBS, pH 7.4), and boiled at 95 °C for 10 min to prepare the DNA template for PCR. Each 20 μl PCR reaction contained 2 μl of DNA template, 2 μl of PCR buffer, 1.6 μl of 2.5 mM dNTP mixture, 10 pmol each of forward and reverse primers of chicken W chromosome (F: 5′-CTA TGC CTA CCA CAT TCC TAT TTG C-3′ and R: 5′-AGC TGG ACT TCA GAC CAT CTT CT-3′), and 1 unit of Taq DNA polymerase. The thermal conditions for 35 cycles were 95 °C for 30 s, 66 °C for 30 s, and 72 °C for 30 s. Female sex was identified based on the strong bands detected in the agarose gel after separation of PCR products by gel electrophoresis.

2.3. Tissue samples

Embryonic gonads from 30 male and 30 female embryos were collected separately at E6 and E9 in a 1.5 ml tube containing diethylpyrocarbonate treated PBS (DEPC-PBS). Then we centrifuged the sample at 1080*g* for 5 min to allow collection of each gonad from the bottom of tubes. After removal of the DEPC-PBS, the gonads were stored at −80 °C until RNA was extracted. Also we collected whole embryos ($n = 10$) and fixed them in freshly prepared 4% paraformaldehyde in PBS (pH 7.4). After 24 h, embryos fixed in 4% paraformaldehyde were changed to 70% ethanol for 24 h and then dehydrated in a graded series of increasing concentrations of ethanol. Then, embryos were incubated in xylene for 3 h and embedded in Paraplast-Plus (Leica Biosystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5 μm.

2.4. Cell culture

Embryonic gonads ($n = 50$) from female chicks were obtained on day 6 and cultured with Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (Invitrogen), 2% chicken serum (Sigma, St. Louis, MO, USA), 1× nucleosides (Millipore, Temecula, CA, USA), 2 mM L-glutamine, 1× nonessential amino acids, β-mercaptoethanol, 10 mM sodium pyruvate and 1× antibiotic-antimycotic (Invitrogen). Cells were cultured in a CO_2 incubator maintained at 37 °C with an atmosphere of 5% CO_2 in air and 60–70% relative humidity. The cultured gonadal cells were subcultured at 3- to 4-day intervals by dissociating cell colonies using Accutase (Millipore, Temecula, CA, USA).

2.5. Hormonal treatment of gonadal cells

Monolayers of gonadal cells from day 6 female embryos were seeded and grown in culture medium to 80% confluence in 100-mm culture dishes. Cells were serum starved for 24 h, and then treated with recombinant FSH (100 nM; catalog number: F8174; Sigma, St. Louis, MO, USA), LH (100 nM; catalog number: L5269; Sigma, St. Louis, MO, USA) or FSH plus LH for 0, 6, 12 or 24 h. Based on preliminary dose–response experiments, the concentration of each recombinant hormone was selected for use in all experiments in the present study. This design was replicated in three independent experiments.

2.6. RNA isolation

Total cellular RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

2.7. Quantitative RT-PCR analysis

Total RNA was extracted from each left and right gonads of embryonic day 6 and 9 of both sexes using TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized using a Superscript® III First-Strand Synthesis System (Invitrogen). Gene expression levels were measured using SYBR® Green (Sigma, St. Louis, MO, USA) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The *glyceraldehydes 3-phosphate dehydrogenase (GAPDH)* gene was analyzed simultaneously as a control and used for normalization of data. *GAPDH* expression is assumed to be most stable among other housekeeping genes and it is used commonly for normalizing for variations in loading. Each target gene and *GAPDH* were analyzed in triplicate. Using the standard curve method, we determined expression of the examined genes using the standard curves and Ct values, and normalized them using *GAPDH* expression. The PCR conditions were 95 °C for 3 min, followed by 40 cycles at 95 °C for 20 s, 60 °C for 40 s, and 72 °C for 1 min using a