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A transcriptomic comparison of theca and granulosa cells in chicken and cattle follicles reveals *ESR2* as a potential regulator of *CYP19A1* expression in the theca cells of chicken follicles

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ABSTRACT

Previous studies have shown that theca and granulosa cell layers in follicles do not play the same roles in mammals and birds, especially regarding the synthesis of estrogen. The functions of these two cell types have been well characterized in cattle, but they remain unclear in chickens. To clarify this issue, a comparison of small yellow follicles (SYFs) in chickens and cattle at different follicular development stages was done by weighted gene co-expression network analysis (WGCNA). The modules obtained from WGCNA were used for further identification of the key genes associated with *CYP19A1* expression. Module preservation analysis showed high similarity between cow_D (the follicular phase before the LH surge) and chicken_SYF (small yellow follicle between 6 and 8 mm in diameter) datasets, and 10 top hub genes highly associated with *CYP19A1* expression in chicken SYFs were identified in each module. A comparison of the transcriptomes of theca and granulosa cells (TCs and GCs) between chicken SYFs and cattle follicles at the differentiation stage, as well as the aforementioned hub genes, revealed that *ESR2* is a potential regulator of *CYP19A1* expression in the theca cells of chicken SYFs. Furthermore, 197 cell-specific (179 in theca and 18 in granulosa) and 235 cell-biased expressed genes (196 in theca and 39 in granulosa) in chicken small yellow follicles were also identified by transcriptomic comparison of theca and granulosa cells.

1. Introduction

Estrogen is a steroid hormone and has extensive effects on female reproduction. The synthesis of steroid hormones in females mainly occurs in the somatic cells (theca and granulosa cells, hereinafter TCs and GCs) of ovary follicles during the reproductive period, but the location of hormone synthesis differs between mammals and birds. Like cattle and other mammals, the chicken ovarian follicle consists of one oocyte enclosed by two layers of somatic cells, TCs and GCs. The interactions between the two cells as well as between the cells and oocyte within the follicle play important roles in folliculogenesis, i.e., these two somatic cells specialize and cooperate with each other to support oocyte development until ovulation. In mammals, TCs de novo produce androgens and GCs exert estradiol conversion according to the "two-cell, two-gonadotropin" concept (Erickson et al., 1979; Edson et al., 2009). For an unknown reason, TCs produce testosterone and estradiol in birds based on the three-cell model of avian follicular steroidogenesis (Porter et al., 1989; Rodríguez-Maldonado et al., 1996; Lee et al., 1998). Aromatase, encoded by *CYP19A1*, is responsible for a key step in the biosynthesis of estrogens through aromatization of androgens. The aromatase enzyme can be detected in many tissues, including gonads. Several studies have detected the expression of *CYP19A1* in GCs in mammals and in TCs in chickens (Tilly et al., 1991; Lee and Bahr, 1994). In cattle follicular fluid, estradiol peaks at the differentiation stage after follicle selection, whereas in chickens, the *CYP19A1* expression reaches the highest level in small yellow follicles (Wang and Gong, 2017) at the selection stage. Currently, the mechanism of avian follicle selection and the functions of the two main cells in follicles (GCs and TCs) are still unclear.

The reproductive strategy of avian species involves several follicle development phases that occur in an orderly and progressive fashion (Johnson, 2015). The growth and development of chicken follicles initiates in small, slow-growing white follicles (SWF, 1–5 mm), followed by the selection of a single follicle into the pre-ovulatory hierarchy among a cohort of small yellow follicles (SYF, 6–8 mm), which finally become the 5–7 largest orderly arranged pre-ovulatory follicles (F7, F6, F5, … F2, F1, > 9 mm). Unlike in monotocous species, the mystery of follicle selection from a cohort of follicles in chickens remains unclear.

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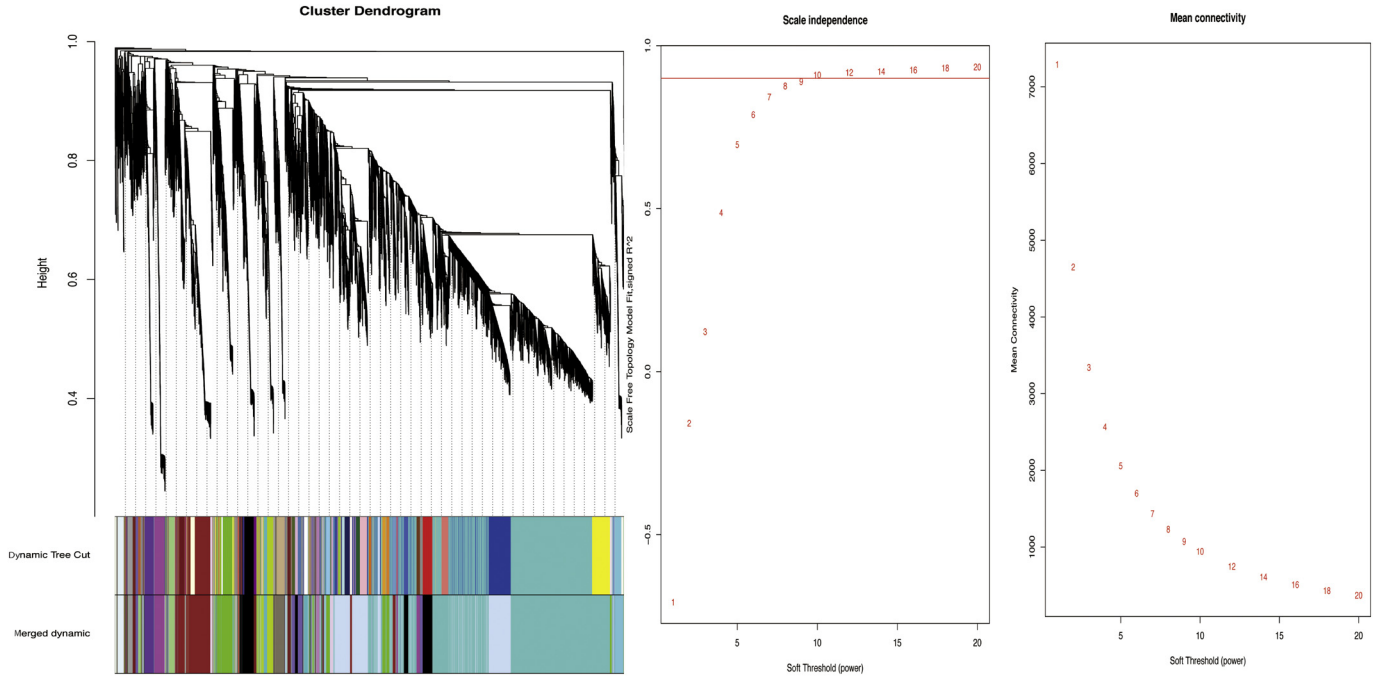

Fig. 1. Visual representation of the Gene Co-Expression Network.
(A) Module clustering tree of chicken_SYF. Different colors in the module bar indicate different modules. (B) Selection of the soft-threshold powers of chicken_SYF. The power that fit the index curve and flattened out upon reaching approximately 0.9 for the first time was selected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A comparison of the follicular development of chickens with that of a well-studied species such as cattle may facilitate our understanding of chicken follicle selection. In this study, RNA sequencing was first performed on the TCs and GCs of SYFs in chickens before follicle selection; then the corresponding data on cattle were downloaded from the GEO database for a comparison to calibrate the time scale of follicular development between the two species through Weighted Gene Co-Expression Network Analysis (WGCNA). Afterwards, regulators of aromatase were investigated by comparing the transcriptomes in GCs and TCs between SYFs in chickens and follicles in cattle at the differentiation stage, focusing on the differences in the same type of cells between the two species (i.e., transcription factors highly expressed in GCs in cattle but not in chickens and transcription factors highly expressed in TCs in chickens but not in cattle). Furthermore, cell-specific and cell-biased gene expression in ovaries was also explored by comparing the transcriptomes of GCs and TCs in chickens to get rough insights into their roles in follicular development.

2. Material and methods

2.1. Ethical procedures

The experimental procedures used in this study met the guidelines of the Care and Use of Laboratory Animals of the Standing Committee of Hubei People's Congress ($5^{\#}$) and were approved by the Biological Studies Animal Care Committee of Hubei Province, P.R. China, and the Ethics Committee of Huazhong Agricultural University, P.R. China. All efforts were made to minimize animal suffering.

2.2. Animals, sample collection, and data preparation

Sexually matured hens of a local variety (Wuxin chicken) from Hubei province (25 wk. of age) were purchased from the chicken farm of Huazhong Agricultural University (Wuhan, China). Four hens were killed by cervical dislocation, and SYFs with diameters of 6–8 mm were obtained from each hen ovary, followed by separation of the granulosa cell layer and the theca cell layer, washing in 1 * PBS and storage in liquid nitrogen. A total of 8 samples were collected from 4 hens, with 2 samples (theca cell layer and granulosa cell layer) collected for each hen. For each ovary, 3 duplicate small yellow follicles were taken and pooled as a biological sample for further isolation of the granulosa cell layer and theca cell layer.

Total RNA was extracted from eight samples (including biological duplicates) for Illumina sequencing (Sequencing Mode: Paied-end, 2 * 150 bp). The sequenced dataset of chicken small yellow follicles was defined as chicken_SYF. RNA-seq profiles of theca and granulosa tissues in *Bos taurus* were downloaded from Gene Expression Omnibus (GEO) with the accession number GSE34317 (Walsh et al., 2012). Raw data from two stages were selected: Selection (cow_S) and Differentiation (cow_D) in lactating Holstein-Friesian cows ($n = 17$) for further construction of the co-expression network. After removing the adapter and low-quality reads, the remaining reads were mapped to the Ensembl reference genome (Gallus_gallus.Gallus_gallus5.0.dna.toplevel.fa for chicken and Bos_taurus.UMD3.1.dna_sm.toplevel.fa for cow) for each species by Hisat2 (version 2.0.5), and multiple mapped reads were removed to ensure that only unique reads were counted in further analyses. Gene expression levels were quantified by RPKM (reads per kilobases per million mapped reads). Raw counts and RPKM of the 2 species were calculated by Cufflinks (Version: 2.2.1; (Trapnell et al., 2012; Pertea et al., 2016).

2.3. Module formation and characterization

A total of 6626 genes in cow_D, 6694 in cow_S, and 4036 in chicken_SYF were removed to decrease noise in the initial module characterization. Modules were determined using a dynamic tree-cutting algorithm, with each determined weighted factor β (β above 0.9 was chosen as a power cutoff; Fig. 1, Figs. S1–S2). Then, the stage similarity in cattle to that of SFY in chicken was determined based on gene overlap by matching module identifiers in the chicken network to the most similar module in the cow_D and cow_S networks. In each network, Module Membership (MM) was calculated, and the top 30% of