—Original Article—

Comparative analysis of temporal gene expression patterns in the developing ovary of the embryonic chicken

Minli YU[1], Yali XU[1], Defu YU[1], Debing YU[1] and Wenxing DU[1]

[1]*Department of Animal Genetics, Breeding and Reproduction, College of Animal Science and Technology, Nanjing Agricultural University, 210095 Jiangsu Province, PR China*

Abstract. Many genes participate in the process of ovarian germ cell development, while the combined action mechanisms of these molecular regulators still need clarification. The present study was focused on determination of differentially expressed genes and gene functions at four critical time points in chicken ovarian development. Comparative transcriptional profiling of ovaries from embryonic day 5.5 (E5.5), E12.5, E15.5 and E18.5 was performed using an Affymetrix GeneChip chicken genome microarray. Differential expression patterns for genes specifically depleted and enriched in each stage were identified. The results showed that most of the up- and downregulated genes were involved in the metabolism of retinoic acid (RA) and synthesis of hormones. Among them, a higher number of up- and downregulated genes in the E15.5 ovary were identified as being involved in steroid biosynthesis and retinol metabolism, respectively. To validate gene changes, expressions of twelve candidate genes related to germ cell development were examined by real-time PCR and found to be consistent with the of GeneChip data. Moreover, the immunostaining results suggested that ovarian development during different stages was regulated by different genes. Furthermore, a *Raldh2* knockdown chicken model was produced to investigate the fundamental role of *Raldh2* in meiosis initiation. It was found that meiosis occurred abnormally in *Raldh2* knockdown ovaries, but the inhibitory effect on meiosis was reversed by the addition of exogenous RA. This study offers insights into the profile of gene expression and mechanisms regulating ovarian development, especially the notable role of *Raldh2* in meiosis initiation in the chicken.
Key words: Chicken, Meiosis initiation, Microarray, Ovary development

The development of the ovary and formation of oogonia require serial regulation of specific genes and signaling pathways. Contrary to the case of the testis [1, 2], ovarian differentiation is not well characterized. In the chicken, what regulates meiosis and how ovarian germ cells adopt their specific fate during the embryonic stage are still unclear. The present study identified genes that are specifically depleted and enriched at critical stages during ovarian development. Thus, it provides a molecular explanation of meiosis onset and oocyte formation.

The ovarian germ cells enter meiosis during fetal life to initiate oogenesis. Indeed, the development of ovarian germ cells and meiosis initiation in chicken embryos are similar to those in mouse or human embryos, but they do have distinct features. In mammals, the primordial germ cells (PGCs) immediately enter into meiosis after settling in the genital ridge [3]. However, in the course of chicken embryonic ovarian development, there are several key time points when the germ cells experience transition from mitosis to meiosis. Germ cells in the chicken embryonic ovary divide by mitosis from the time they migrate and settle in the genital ridge until approximately E5.5, which is when the primary gonads are formed [4]. At this stage, the PGCs with strong proliferation activity can be identified by a PGC-specific marker (SSEA-1) [5]. Then at E8.5, ovary morphogenesis begins to develop the left ovary, while the right gonad fails to form a cortex and undergo regression gradually [6]. The germ cells in the E12.5 ovary enter the premeiotic prophase to prepare for meiosis initiation accompanied by the specialization of granulosa cells [7]. Then germ cells in the E15.5 ovary begin to enter meiosis in response to the induction of intrinsic RA [8]. By E18.5, the meiotic germ cells become increasingly widespread, and primary oocytes undergo meiotic arrest. Thus many changes occur in genes and pathways from the E5.5 to E15.5 stages. However, the exact understanding of the molecular mechanisms involved in the regulation of ovarian development in each stage, interaction of functional genes and their action on signaling pathways remains unclear. For instance, the special preparatory changes that occur in chicken PGCs before the initiation of meiosis remain undetermined.

Meiosis is a germ-cell-specific cell division process through which haploid gametes are produced for sexual reproduction [3]. Previous studies showed that RA can act as a meiosis-inducing factor in mouse gonads [3, 9]. During embryonic periods, RA is synthesized by the retinaldehyde dehydrogenase (RALDH2), which controls the entry of the germ cells into meiosis by inducing expression of the premeiotic gene *Stra8* (stimulated by retinoic acid gene 8) in females [3, 10]. In contrast, RA is degraded by the activity of CYP26B1 (the enzyme for RA catabolism), and meiotic initiation is inhibited in males [9].

In chicken, PGCs undergo a serial of epigenetic reprogramming steps before the initiation of meiosis [11]. Meiotic germ cells are first detected in the E15.5 chicken ovary when the expression of meiotic markers synaptonemal complex protein 3 (*Scp3*) and disrupted meiotic cDNA 1 homologue (*Dmc1*) is upregulated. Although several regulators, such as *Tet1* (tet methylcytosine dioxygenase 1) and *Piwil1* (piwi-like RNA-mediated gene silencing 1), have been reported to be crucial for meiosis [11], little is known about the expression of genes involved in meiotic regulation and the expression of them that contributes to normal meiosis. Studies of gonadal development in the chicken would provide insights into ovarian developmental pathways. In addition, some studies have undertaken screens to identify novel genes that are responsible for sexual dimorphism in the early embryonic gonads [12, 13]. These screens provide an important avenue for identifying new candidate genes involved in sex determination in the avian model and for discovery of new information concerning the fundamental signaling and hormonal pathways during gonadal development [13]. In this regard, the overall gene expression profiles obtained by microarray analysis would reveal the changes that occur in germ cells and lead to initiation of meiosis in the chicken.

The main objective of this study was to compare genes expressed in a stage-specific manner and the germ cell developmental pathways in the E12.5, E15.5 and E18.5 chicken ovaries with those in the E5.5 chicken ovaries. In order to reveal the real situation of the meiosis process in the chicken, an *in vivo Raldh2* knockdown model was made by LV-shRNA. The present study focused on the chicken ovary at E15.5, which is when the oocytes enter meiosis. Therefore, the study provided understanding about the initiation of meiosis and its key genes and pathways that regulate the meiosis process in the chicken ovary.

Materials and Methods

Experimental animals and sample collection

All animal experiments were approved by the Animal Care and Use Committee of Nanjing Agricultural University. Fertilized Hyline chicken (*Gallus Gallus*) eggs were incubated in an egg incubator (Victoria S.r.l., Guanzate, Italy) at 38.5 C and 60% humidity. A total of four test samples were used for this investigation: ovaries from E5.5, E12.5, E15.5 and E18.5 chickens, respectively. The gonads of E5.5 were carefully dissected from the embryonic mesonephros (N = 30) with a fine glass needle under a stereomicroscope (SZX16, Olympus, Tokyo, Japan). The left ovaries were retrieved from E12.5, E15.5 and E18.5 embryos (N = 10). Each sample was snap frozen in liquid nitrogen and stored at −80 C. We used a pooled design in order to obtain a sufficient amount of RNA to run an array.

Sexing of embryos by PCR

Gonads (E5.5, E12.5, E15.5 and E18.5) were collected according to phenotype sex as determined by morphology. ZZ and ZW genotypes were confirmed retrospectively by detecting *Xho I* repetition in the W chromosome as described previously [14]. The PCR reaction mixture consisted of 2.5 mM of each W chromosome primer and 0.5 mM of each 18S ribosomal RNA primer. PCR reactions were carried out in 20 μl of buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl,

2.5 mM MgCl$_2$) containing 2 mM DNA, 200 mM dNTPs, 1 U Taq DNA polymerase and 1 mM each of the four primers. The cycling parameters were as follows: 94 C × 5 min; 30 cycles of 94 C for 30 sec, 56 C for 30 sec and 72 C for 30 sec; and then 72 C for 5 min. PCR products were analyzed by 1.5% agarose gel electrophoresis. Only female (ZW) embryos showed the 168 bp W-linked *Xho1* fragment.

Total RNA isolation

The gonad tissues were suspended in TRIzol (Invitrogen), and total RNA was extracted and purified using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) by following the manufacturer's instructions. The quantity and quality of the RNA samples were checked using a NanoDrop ND-1000 Spectrophotometer (PEQLAB) and Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, US), respectively.

Microarray

Pooled RNA was generated from an equal amount of each RNA sample. Then four RNA pools for each stage were amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Agilent Technologies, Santa Clara, CA, US), by following the manufacturer's instructions. Labeled cRNA were purified with an RNeasy Mini Kit (QIAGEN). Each slide was hybridized with 1.65 μg Cy3-labeled cRNA using a Gene Expression Hybridization Kit in Hybridization Oven (Agilent Technologies, Santa Clara, CA, US), according to the manufacturer's instructions. After 17 hours of hybridization, slides were washed in staining dishes (Thermo Shandon, Waltham, MA, US) with a Gene Expression Wash Buffer Kit (Agilent Technologies), by following the manufacturer's instructions.

Microarray data analysis

Slides were scanned by an Agilent Microarray Scanner (Agilent Technologies) with the default settings (dye channel, green; scan resolution, 5 μm; PMT, 100%, 10%: 16 bit). Data were extracted by Feature Extraction software 10.7 (Agilent Technologies). The raw data were normalized by the Quantile algorithm in the GeneSpring 11.0 software. Gene expression was analyzed on an Affymetrix GeneChip Chicken Genome Array containing 38,535 probes. The data were grouped into those of the E5.5, E12.5, E15.5 and E18.5 chicken ovaries. Upregulated genes were identified by at least 2-fold changes. Gene ontology analysis was conducted at http://www.geneontology.org, and a pathway analysis was performed with the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg). A heat map was created using a clustering function algorithm.

Quantitative real-time polymerase chain reaction (QRT-PCR)

QRT-PCR was performed to examine the relative quantification of the expression levels of up- and downregulated genes involved in ovarian development at different stages. Twelve genes (*Bcl6*, *Amh*, *Bmpr1a*, *Cav1*, *Pgr*, *Piwil1*, *Smad5*, *Stra8*, *Scp3*, *Dmc1*, *Raldh2* and *Cyp26b1*) were selected and the primers are shown in Table 1. Real-time quantification of RNA targets was performed by using a SYBR Green RT-PCR kit (Takara, Otsu, Japan) on an ABI 7300 Fast Real-Time PCR System (Applied Biosystems) by following the manufacturer's instructions. The expression level of meiosis-related

Table 1. The primer sequences used in real-time RT-PCR

Genes	Accession no.	Primer sequences (5'–3')	Product length (bp)
Bcl6	NM_001012930	CTGTACCTGCAGATGGAGCA CTCTGCCTCGATAAGCCATC	154
Amh	NM_205030	TCCGTTACTCGGACCAACTC GAGCAATGCTAGGAGGCAAC	182
Bmpr1a	NM_205357	GGCAAATGGAGAGGTGAAAA TGAGGAGAGCCCTGTTGTCT	247
Cav1	NM_001105664	TGGAAGGCCAGTTTTACCAC GCCCATAGCCTCAAACAGAG	240
Pgr	NM_205262	TCTAACGCAAAGGCTGTCCT CACAAAGCAGTTGCCTCTCA	182
Piwil1	NM_001098852	CTGCTCTGGCCATAGTGACA CAGCAATTGACTGCTTTCCA	150
Smad5	NM_001014968	GCAACTACCACCATGGCTTT CCCCACCCCTTTACAAAACT	184
Dazl	NM_204218	CTGGGGAGCAAAGAA ACTACG CAAAGGTGTTCCTCAGACGGT	213
Scp3	XM_416330	CTGTATTTCAGCAGTGGGATG TGCGAAGTTCATTTTGTGC	225
Stra8	XM_416179	GTGAGGGACAGTGGAGGTAA CAGAAATGCCGCTTGTAAAT	166
Dmc1	XM_425477	GATGACAACAAGACGAGCAC CCCACCACCCAGAAGTT	190
Raldh2	NM_204995	GGGCAGTTCTTGCTACTATGGA TCTGCCCAACCAGCGTAAT	136
Cyp26b1	XM_426366	TTACCGACAAGGACGAGTTCA TCATTGTAGGAGGTCCATTTAGC	201
β-actin	NM_205518	ACGTCGCACTGGATTTCGAG TGTCAGCAATGCCAGGGTAC	282

genes was normalized to that of the Deleted in azoospermia-like (*Dazl*) gene, a marker of germ cells, and *β-actin* for the other genes. Results were analyzed using the delta-delta Ct method.

Knockdown of Raldh2 in the embryonic chicken

To knock down *Raldh2*, shRNA reagents were designed and packaged into a Lentivirus vector. Shell windowing at stage X was performed as described by Speksnijder and Ivarie [15]; 5 μl viruses (10^8 TU/ml) or 5 μl of the vehicle as a control was injected into the blastoderm with a syringe, the windows were then sealed with transparent tape, and the sealed eggs were further incubated at 38.5 C. Then RA solution (100 μl 0.5 μg/ml, dissolved in DMSO) was injected into the E12.5 embryo for further incubation. Until the end of E15.5, embryonic ovaries were harvested and analyzed for RT-PCR or immunohistochemistry.

BrdU incorporation

For BrdU incorporation into embryonic gonads, 100 μl of the BrdU solution (50 mg/ml, Sigma, St. Louis, MO, USA) was injected directly into the embryos. Subsequently, eggs were sealed with transparent tape and incubated for an additional 4 h at 38.5 C before harvesting the gonad samples. Then the cryosections were cut at a thickness of 10 μm to perform immunostaining. The sections were blocked

in TBS (containing 5% goat serum and 0.1% Triton X-100) for 30 min, following denaturation by addition of 2 N HCl at 37 C for 30 min and neutralization by addition of 0.1 M sodium tetraborate for 10 min. The slides were then incubated with a mouse anti-BrdU monoclonal antibody (1:200, DSHB, USA) as the primary antibody and subsequently with FITC-labeled goat anti-mouse IgG (1:1000, KPL, Gaithersburg, MD, USA) as the secondary antibody. The nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI), the samples were mounted, and images were acquired.

Immunofluorescence staining

Samples were fixed in 4% paraformaldehyde and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Tokyo, Japan). Cryosections were cut at a thickness of 10 μm to perform immunostaining on a freezing microtome (Leica Microsystems, Wetzlar, Germany). Three slides from different embryonic chicken ovaries were used for analysis of protein expression. After blockade in TBS (containing 5% goat serum and 0.1% Triton X-100) for 30 min, sections were incubated with antibodies directed against SSEA-1, BrdU (1:1000, DSHB, USA), SCP3 (1:500, BD Biosciences, San Jose, CA, USA) or γH2AX (1:1000, Abcam, Cambridge, UK) and then incubated with a PE or FITC goat anti-mouse IgG secondary antibody (1:1000, KPL, Gaithersburg, MD, USA). The nuclei were
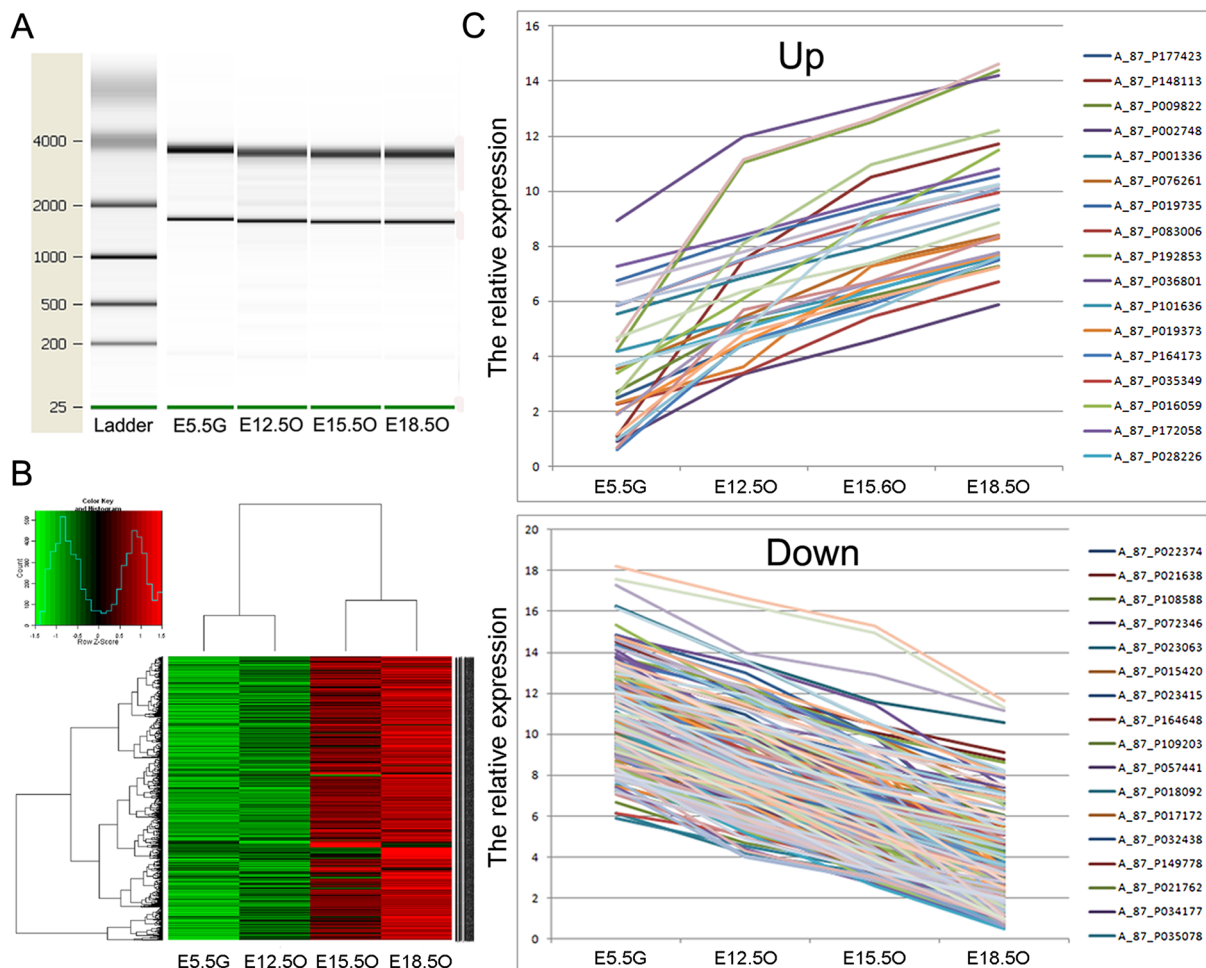
Fig. 1. Array analysis of gene expression profile. A: The test of RNA quality of the E5.5 gonad (G) and E12.5, E15.5 and E18.5 ovaries (O). B: Summary of the expression array analysis for E12.5, E15.5 and E18.5 chicken ovaries when compared with the E5.5 chicken ovary. The red and green colors indicate up- and downregulated genes, respectively, in the different embryonic stages. C: The significant differentially expressed genes in ovaries were up- or downregulated gradually from E5.5 to E18.5.

counterstained with DAPI, and the images were visualized using fluorescence microscopy (TCS SP5, Microsystems, Wetzlar, Germany).

Statistical analysis

The results are expressed as the means ± SD of at least three independent biological replicates. The images show representative results of experiments that were all repeated at least three times. Data were analyzed by ANOVA and Duncan's multiple range tests using the SAS 9.0 software. P < 0.05 was considered significantly different.

Results

Differentially expressed genes

Affymetrix microarray technology was used to analyze the gene expression profiles of chicken ovaries from the four groups. Firstly, the quantity and quality of each RNA sample were found to be sufficient for microarray (Fig. 1A). Based on the P values and fold-change cutoff,

transcripts that were significantly up or downregulated in the E12.5, E15.5 and E18.5 ovaries in comparison with the E5.5 ovaries were identified. Hierarchical clustering and global correlation analysis indicated that the samples were clearly separated by their stages (Fig. 1B). All up- and downregulated genes in the test samples were categorized into functional groups according to gene ontology. Within the E12.5 ovary, 4448 transcripts were significantly upregulated, and 4595 transcripts were downregulated. Similarly, 6184 and 5629 transcripts were up- and downregulated, respectively, in the E15.5 ovary. In the E18.5 ovary, 1139 transcripts were significantly upregulated and 1321 transcripts were downregulated (Fig. 1B). The differentially expressed genes were further examined in the test samples. In the E12.5 and E15.5 ovaries, 2756 and 3720 genes were upregulated, while 3173 and 4027 genes were downregulated, respectively (Fig. 1B). In the E18.5 ovary, 652 and 723 genes were up- and downregulated, respectively (Fig. 1B). Some significant genes were differentially expressed in the test samples (Fig. 1B).
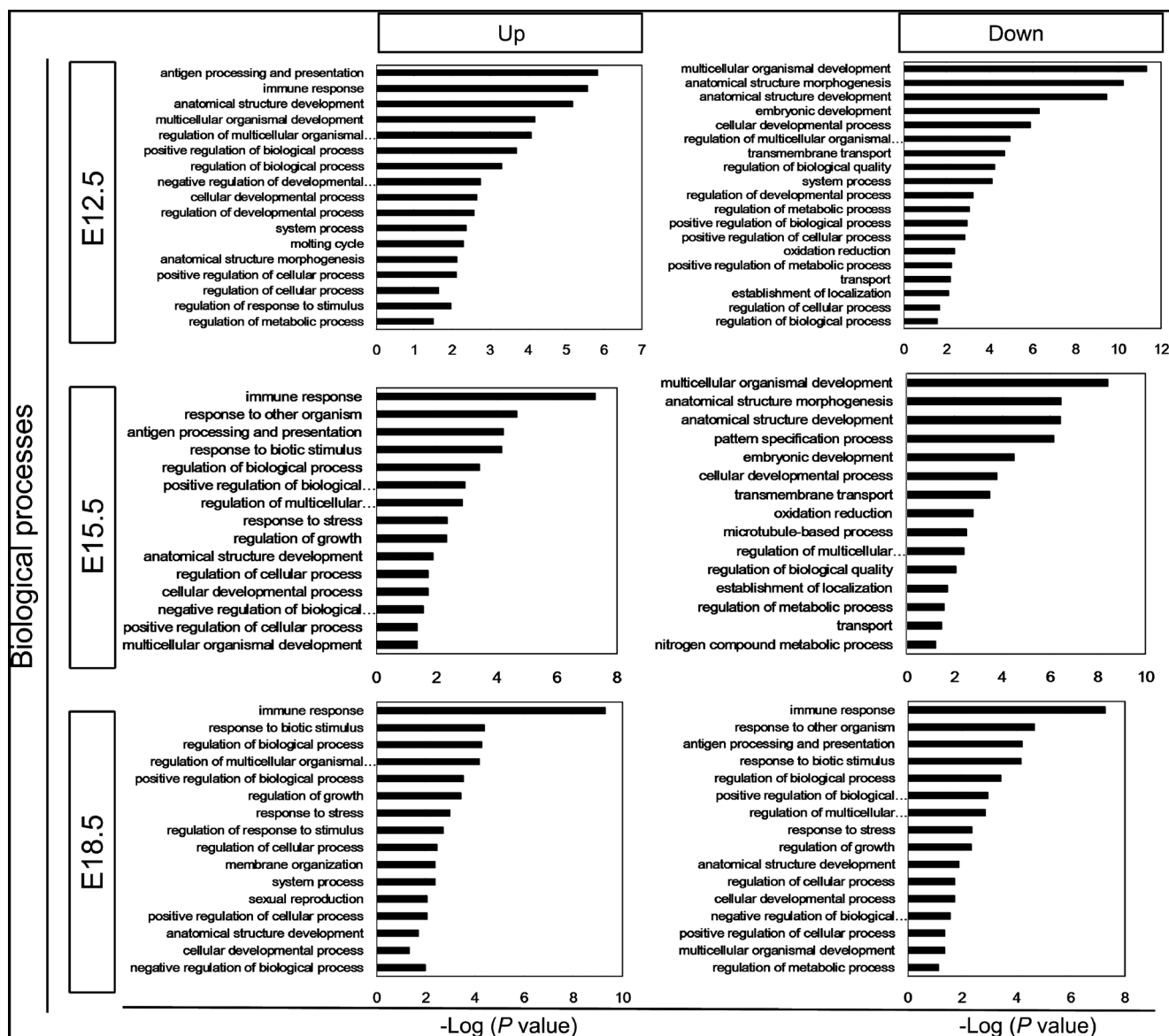
Fig. 2. Gene ontology. All of the up- and downregulated genes in E12.5, E15.5 and E18.5 chicken ovaries when compared with the E5.5 chicken ovaries were categorized into specific functional groups such as biological processes. The y-axis represents biological processes, and the x-axis represents the negative log of the P values of the enrichment of corresponding gene ontology terms.

Among them, 30 genes were gradually upregulated and 207 were downregulated from E5.5 to E18.5 stage (Fig. 1C).

Gene ontology analysis

To facilitate biological interpretation of the transcriptional profiles, we performed gene ontology (GO) and pathway analysis of the genes differentially expressed among four distinct stages. The results showed that the most abundant GO terms and pathways for differential genes were found in E5.5 to E18.5 ovaries. All of the up- and downregulated genes in E5.5, E12.5, E15.5 and E18.5 ovaries were categorized into three major functional groups, such as biological process (Fig. 2), molecular function and cellular

component according to gene ontology (data not shown). Each major group was further divided into several subcategories. In E12.5, E15.5 and E18.5 ovaries, most of the upregulated genes fell into the subcategories, regulation of biological and cellular processes, membrane, and protein binding in terms of biological processes, cellular component, and molecular functions, respectively (data not shown). Most of the downregulated genes fell into establishment of localization, intracellular part and regulation of transcription factor activity in the functional groups, respectively (data not shown). However, the number of genes that fell into each subcategory of functional groups varied in our test samples. In detail, the genes upregulated at E15.5 ovaries in comparison with E5.5 ovaries showed

Fig. 3. *Gallus Gallus* retinol metabolic pathway from KEGG. Differentially expressed genes in the E15.5 chicken ovary compared with E5.5 chicken ovary were identified. The colored boxes show differentially expressed genes in the process of retinol metabolism. The green box represents the upregulated genes. The blue box represents the downregulated genes. The black box represents genes that showed no change.

enrichment of in biological processes mainly related to regulation of biological and cellular processes, anatomical structure development and cellular developmental process (Fig. 2). Cellular component analysis showed that genes involved in the membrane and extracellular space were highly expressed (data not shown). Meanwhile, protein, ion binding and signal transducer activities were overrepresented in molecular functions (data not shown). In contrast, most of the genes downregulated at E12.5, E15.5 and E18.5 were involved in biological processes such as multicellular organism development, embryonic development, cellular developmental process, regulation of metabolic process and anatomical structure development (Fig. 2). GO enrichment analysis indicated that the largest proportion of upregulated genes was involved in biological and cellular process pathways, whereas genes involved in germ cell development were the most highly enriched (data not shown).

Comparative pathway analysis of up- and downregulated genes in ovaries at differential stages

All selected genes were tested to examine their involvement in biological and cellular process pathways by using the KEGG pathway database. KEGG pathways associated with differentially expressed genes among the four distinct stages are listed in Supplementary Table S1 (online only). GO analysis showed that the significantly enriched pathways of these upregulated genes were related to steroid biosynthesis ($P < 2 \times 10^{-3}$) and that downregulated genes were related

to retinol metabolic processes ($P < 8 \times 10^{-3}$). The results showed that the upregulated genes in E15.5 ovaries in comparison with E5.5 ovaries were involved in steroid biosynthesis, cell adhesion molecules (CAMs), cytokine-cytokine receptor interaction and ECM-receptor interaction, whereas the downregulated genes were related to regulation of glycine, serine and threonine metabolism, retinol metabolism and alanine metabolism. Additionally, upregulation of steroid biosynthesis and CAMs were observed in E12.5 ovaries in comparison with E18.5 ovaries (Supplementary Table 1). Furthermore, retinol metabolism and the PPAR signaling pathway were found to be downregulated following the developmental stage (Supplementary Table 1). Significantly up- and downregulated genes of retinol metabolism pathways were examined in E15.5 chicken ovaries compared with E5.5 chicken ovaries. The results showed that most of the differentially regulated genes were involved in the synthesis and catabolism of RA, which is thought to be essential for ovarian germ cell development (Fig. 3).

Identification of the role of E15.5-expressed genes in the meiosis process

All of the meiosis-related genes differentially expressed in E15.5 chicken ovaries compared with E5.5 chicken ovaries are shown in Table 2. In brief, hydroxysteroid (17-beta) dehydrogenase 7 (*Hsd17b7*), *Amh*, aldehyde dehydrogenase1 family, member L2 (*Aldh1l2*), *Piwil1*, and *Dazl* were upregulated in E15.5 chicken ovaries compared with

Table 2. Information of meiosis-related genes differentially expressed in the ovaries at E15.5 compared with E5.5.

Sample	Gene symbol	Chromosome No.	GenBank Accession No.	Gene or function
Up	SC4MOL	chr4	NM_001006438	sterol-C4-methyl oxidase-like
	HSD17B7	chr8	XM_001232165	hydroxysteroid (17-beta) dehydrogenase 7
	DHCR24	chr8	NM_001031288	24-dehydrocholesterol reductase
	ALDH1L2	chr1	XM_416314	aldehyde dehydrogenase1 family, member L2
	CYP17A1	chr6	NM_001001901	cytochrome P450, family 17, subfamily A, polypeptide 1
	FSHR	chr3	NM_205079	follicle stimulating hormone receptor
	STAR	chr22	NM_204686	steroidogenic acute regulator
	SPON2	chr4	BX931971	spondin 2, extracellular matrix protein
	AMH	chr28	NM_205030	anti-Mullerian hormone
	LHCGR	chr3	NM_204936	luteinizing hormone/choriogonadotropin receptor
	PIWIL1	chr15	NM_001098852	piwi-like 1 (Drosophila)
	SYCP3	chr1	XM_416330	synaptonemal complex protein 3
	DAZL	chr2	NM_204218	deleted in azoospermia-like
Down	CYP26A1	chr6	NM_001001129	cytochrome P450, family 26, subfamily A, polypeptide 1
	ALDH1A1	chrZ	NM_204577	aldehyde dehydrogenase 1 family, member A1
	CYP1A1	chr10	NM_205146	cytochrome P450, family 1, subfamily A, polypeptide 1
	ALDH1A2	chr10	NM_204995	aldehyde dehydrogenase 1 family, member A2
	CYP3A4	chr14	BX931257	cytochrome P450 3A80
	CYP26C1	chr6	XM_421678	cytochrome P450, family 26, subfamily C, polypeptide 1
	CYP26B1	chr4	XM_426366	cytochrome P450, family 26, subfamily B, polypeptide 1
	CYP3A4	chr14	BX931257	cytochrome P450 3A80
	ADH1B	chr4	NM_205092	alcohol dehydrogenase 1B (class I), beta polypeptide
	FGF8	chr6	NM_001012767	fibroblast growth factor 8 (androgen-induced)
	FOXA2	chrUnrandom	NM_204770	forkhead box A2
	KL	chr1	XM_417105	klotho
	HSD3B7	chr19	CR386199	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7
	ALDH8A1	chr3	XM_419732	aldehyde dehydrogenase 8 family, member A1

E5.5 chicken ovaries (Table 2). On the other hand, *Cyp26 (a1, b1, c1)*, cytochrome P450 3A80 (*Cyp3a4*), *Aldh1a1* and *Hsd3b7* were downregulated (Table 2). Additionally, *Stra8, Scp3* and *Dmc1*, reported to be meiosis-marker genes, were also identified as E18.5 upregulated genes (data not shown).

Then candidate KEGG was used to confirm the presence of our differentially expressed genes in E5.5, E12.5, E15.5 and E18.5 ovaries. We focused on genes expressed at E15.5, which are of major importance, in the subsequent experiments. In total, genes upregulated at E15.5, aldehyde dehydrogenase (*Aldh*), cytochrome P450, family 1, subfamily A (*Cyp1a*), and retinol dehydrogenase (*Rdh*), and genes downregulated at E15.5, cytochrome P450, family 26 (*Cyp26*) and cytochrome P450, family 3, subfamily A (*Cyp3a*), were specifically identified as being involved in retinol metabolism (Fig. 3).

Validation of differentially expressed genes by QRT-PCR

According to microarray analysis, several genes showed a similar pattern of expression in E12.5, E15.5 and E18.5 ovaries when compared with E5.5 ovaries. The expression of *Cav1* (caveolae protein 1), *Cyp8b1* and *Egr1* was upregulated gradually from E5.5 to E18.5 ($P < 0.01$), whereas *Aldh8a1* and *Cyp1a1*, related to RA metabolism, were downregulated gradually (Fig. 1C). Among the differentially expressed genes, *Bcl6* (B cell leukemia/lymphoma 6), *Amh* (anti-Mullerian hormone), *Bmpr1* (bone morphogenetic protein type I receptor), *Cav1*, *Pgr* (progesterone receptor), *Piwil1*, *Smad5* (SMAD family member 5), *Stra8, Scp3, Dmc1* and *Raldh2* showed significantly higher levels of expression and were involved in different stages of ovary development (Fig. 1C, Table 2). *Cyp26b1*, involved in RA metabolism, showed dramatically lower expression (Table 2). QRT-PCR was performed to further examine the relative quantification of gene expression levels. The E5.5 ovary was used as reference sample for the calculation of fold change difference. As expected, all 11 upregulated genes were detected at higher levels at E12.5, E15.5 and E18.5 than at E5.5, consistent with the microarray data (Fig. 4). In particular, *Amh* (> 813-fold), *Scp3* (> 89-fold), *Stra8* (> 20-fold), *Dmc1* (> 13-fold), *Bcl6* (> 10-fold), *Cav1* (> 53-fold), *Pgr* (> 25-fold), *Piwil1* (> 163-fold), *Smad5* (> 10-fold), *Bmpr1a* (> 3-fold) and *Raldh2* (> 34-fold) showed levels of expression that were several folds higher in the E15.5 ovary when compared with the E5.5 ovary (Fig. 4). In addition, only *Cyp26b1* showed 5-fold lower expression when compared with E5.5, leading to rapid accumulation of RA in the E15.5 ovary (Fig. 4).

Detection of germ cells in chicken ovaries at different stages

To confirm the stage-specific development of ovaries, several functional candidate proteins were selected for studying their expres-
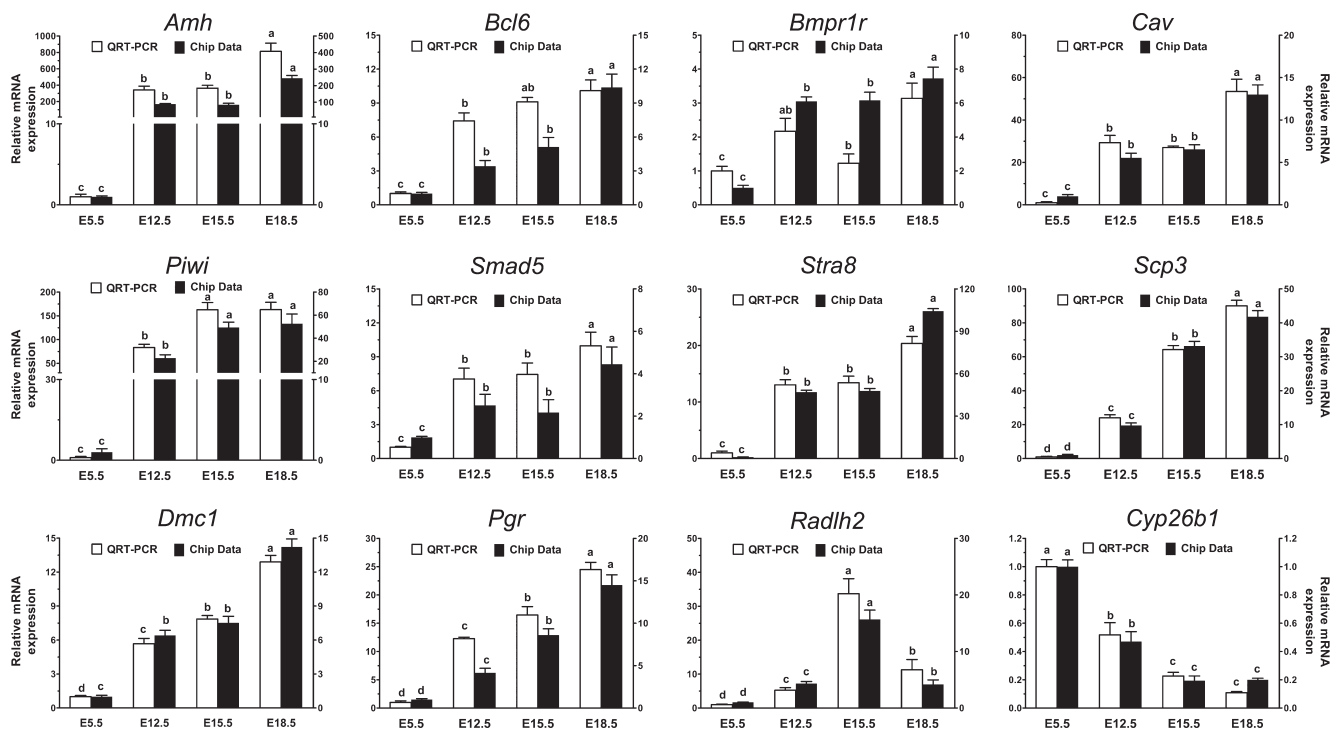
Fig. 4. Validation of the microarray results by QRT-PCR. The expression patterns of 12 candidate genes related to germ cell development were examined by QRT-PCR. The E5.5 ovary was used as a reference sample. The expression level of meiosis-related genes was normalized to that of *Dazl*, a marker of germ cells, and *β-actin* was used for the other genes. Results were analyzed using the delta-delta Ct method. Values are means ± SD of four independent experiments with triplicate embryos. Bars with different letters are statistically different (P < 0.05).

sion and localization using immunohistochemistry. During ovarian germ cell development in the chicken, there are two crucial events, the rapid proliferation of PGCs in the early embryonic stage and the process of meiosis in the late embryonic stage. Slices of ovarian tissue from E5.5 were stained with SSEA-1 (a specific marker of PGCs), and PGCs were detected as being generally distributed in medulla (Fig. 5A). In addition, it was found that the E5.5 ovary was composed of undifferentiated PGCs with strong proliferative activity based on the BrdU incorporation (Fig. 5A). Germ cells up to E12.5 had not initiated meiosis, there were no cells positive for γH2AX, which was specially expressed in germ cells positive for meiotic recombination, and γH2AX was shown to be initially expressed in very few germ cells in the developing ovarian cortex at E15.5 (Fig. 5B). Then positive cells gradually became widespread in the cortex at E18.5 (Fig. 5B). These results suggested that proliferation of ovarian germ cells occurred mainly in the early stage of ovarian development, while differentiation occurred in the late developmental stage. Furthermore, the identified proteins expressed in germ cell-specific manner demonstrated that the development of the ovary was regulated by various genes at different stages.

Confirmation the effect of Raldh2 on meiosis initiation in the chicken ovary

To further confirm the effect of *Raldh2* on meiosis initiation, the LV-shRNA specific to *Raldh2* was used to knock down its function. *Raldh2* knockdown chickens were produced by injecting a lentiviral

vector into stage X embryos. Then the expression of *Raldh2* and *Cyp26b1* was detected in manipulated chicken ovaries. The result showed that the expression of *Raldh2* was decreased sharply in E15.5 *Raldh2* knockdown ovaries, whereas the expression of *Cyp26b1* exhibited a relatively constant level (Fig. 6A). This caused the synthesis of RA to decrease and catabolism of RA remained, leading to a decrease in the accumulation of RA. The action of *Raldh2* on meiotic initiation was further confirmed by the detection of meiosis-related gene expression in *Raldh2* knockdown ovaries by QRT-PCR. It was found that *Raldh2* knockdown remarkably downregulated the mRNA expression levels of meiotic markers (*Stra8*, *Scp3* and *Dmc1*) in E15.5 chicken ovaries (Fig. 6B).

Knockdown of *Raldh2* prevented the appearance of meiotic cells, as the meiotic marker genes were hardly expressed, and SCP3 and γH2AX proteins were nearly undetectable in E15.5 *Raldh2* knockdown ovaries (Fig. 7). Germ cells did not enter meiosis in the *Raldh2* knockdown chickens, because *Raldh2* knockdown inhibited the synthesis of endogenous RA, resulting in an insufficient concentration of RA to support germ cells for meiosis initiation. However, the inhibitory effect on meiosis in the *Raldh2* knockdown ovary was reversed by the addition of exogenous RA (Fig. 7). Germ cells positive for SCP3 and γH2AX were indeed found in the cortex of E15.5 *Raldh2* knockdown ovaries treated with RA, though the proportion was lower than that of the control (Fig. 7). These results implied that meiosis had occurred abnormally in the *Raldh2* knockdown chicken. Together, these findings indicated that RA and its synthesis
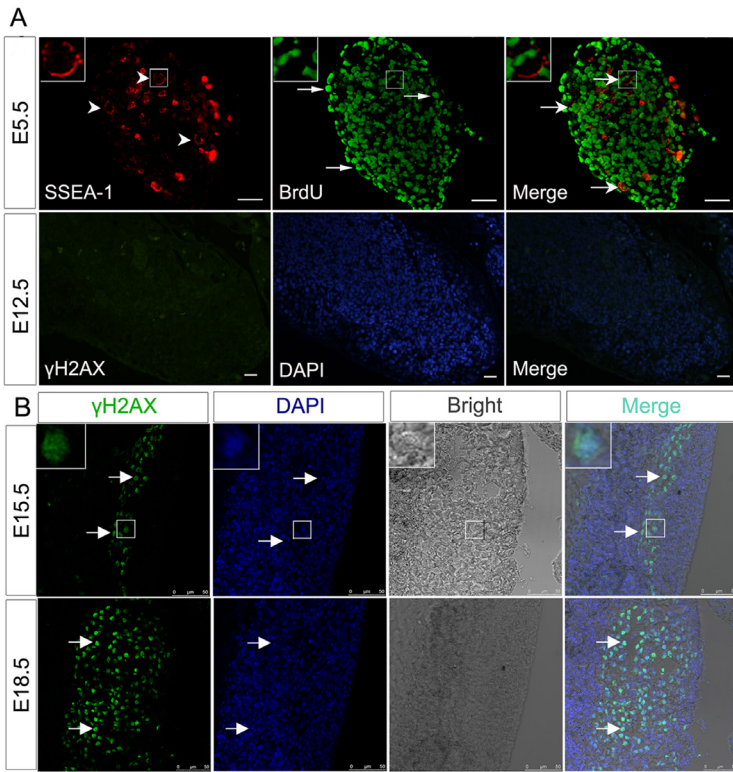
Fig. 5. The detection of germ cells in the embryonic chicken ovary. A: SSEA-1 and BrdU double staining in the E5.5 ovary and γH2AX staining in the E12.5 ovary. Arrowheads, SSEA-1+ PGCs; arrows, BrdU+ cells; arrows with larger arrow heads, SSEA-1+ and BrdU+ cells. Scale bar: 20 μm. B: Meiotic germ cells were identified by the detection of γH2AX staining in the E15.5 and E18.5 ovary. Arrows, germ cells positive for γH2AX; scale bar: 50 μm.
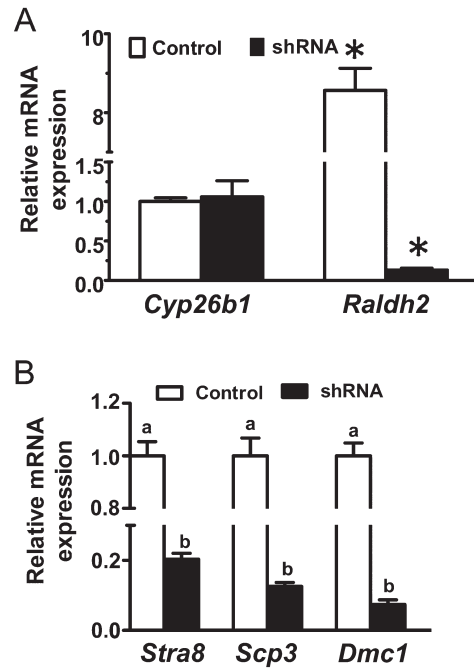
Fig. 6. Analysis of gene expression in the *Raldh2* knockdown chicken ovary. A: The detection of *Raldh2* and *Cyp26b1* expression in the ovary of E15.5 *Raldh2* knockdown chicken. Ovary samples from E15.5 chickens were collected and subjected to QRT-PCR for testing the expression of retinol synthesis (*Raldh2*) and metabolism (*Cyp26b1*) enzyme gene. An asterisk (*) means statistically different (P < 0.05). B: Analysis of meiosis-related gene expression in the E15.5 *Raldh2* knockdown chicken ovary by QRT-PCR. Values are means ± SD of four independent experiments with triplicate embryos. Bars with different letters are statistically different (P < 0.05).
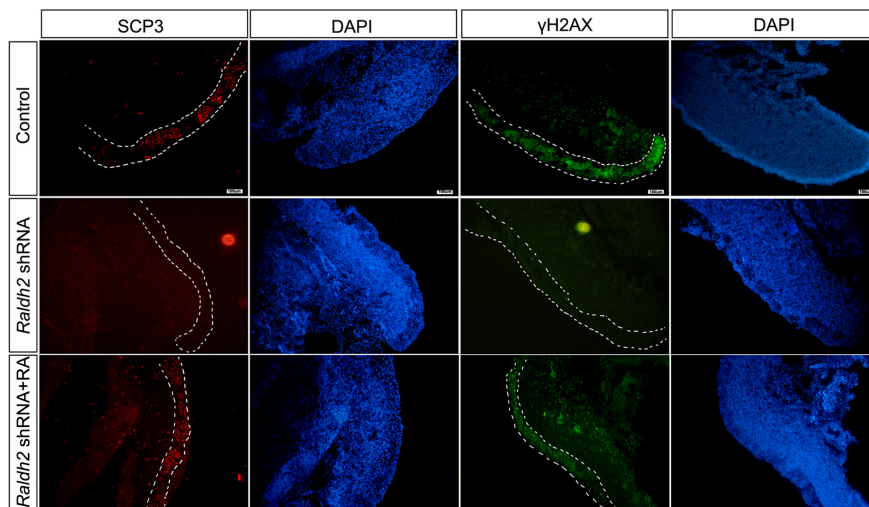
Fig. 7. The detection of germ cells that underwent meiosis in the *Raldh2* knockdown chicken ovary. To investigate the effect of *Raldh2* on meiosis initiation of chicken ovarian germ cells *in vivo*, LV-shRaldh2 was injected into the blastoderm. Then 100 μl of RA solution (0.5 μg/ml) was added to *Raldh2* knockdown chicken embryos at E12.5 for further incubation. Expressions of SCP3 and γH2AX protein were detected in the E15.5 chicken ovary by immunofluorescence. The nuclei were counterstained with DAPI. Scale bar: 100 μm.

enzyme (RALDH2) are required for induction of meiosis onset during embryonic ovarian development in the chicken.

Discussion

A limited number of studies have demonstrated the interaction of capital genes for normal function of germ cells in vertebrate species. Early embryonic germ cells undergo rapid cell divisions until they enter mitotic arrest. A series of changes take place in ovarian germ cells in the later embryonic stage. Recently, it has been revealed that RA can act as a meiosis-inducing factor in the mouse gonad [3]. Previous studies also demonstrated that a balance between RA synthesis and degradation in the developing gonads was required for appropriate control of meiotic initiation [10]. However, the genes and pathways involved in RA metabolism need to be identified in avian PGCs or germ cells. We hypothesized from the present analysis that de novo synthesis of RA increased the expression of genes involved in meiosis-inducing factors, which resulted in the formation of oocytes in the chicken ovary.

Previously, gene functions for gonadal development have been reported with limited success [16], because the regulation of a gene does not always correlate with a specific function; instead, networks might cooperate together to determine certain characteristics. Therefore, microarray analysis can act as an alternative approach for discovery of the relationships of gene functions [13, 17]. In the present study, microarray analysis was conducted to identify significant genes in E12.5, E15.5 and E18.5 chicken ovaries in comparison with E5.5 chicken ovaries. The present data extended the observations by characterizing the transcriptome of different stage ovaries and analyzing the transition of mitosis to meiosis. The ovary has a stage-specific expression profile with a distinct transcriptome in the four stages. Additionally, gene ontology and genes involved in meiosis-related pathways were analyzed in test samples. The great value of this study was the discovery of the genes and gene-specific pathways that were differentially expressed during different stages of ovary development.

The analysis also shed light on the involvement of important biological processes such as steroid biosynthesis, retinol metabolism, and cell adhesion molecules in general and in particular for ovarian development. In addition to the important role of steroid hormones in regulating germ cell development, RA generated by sequential oxidation of vitamin A through RALDH, a key molecule in the germ cell development processes, also has important effects on both germ cell proliferation and meiosis initiation [18–20]. A large number of regulating genes were found to be involved in retinol metabolism and meiotic progression. Based on the average fold change, *Aldh1l2* and *Hsd17b7* showed higher expression levels at E15.5. It can be concluded that retinol metabolism might be involved in the generation of RA, and then also act directly in germ cell development. Analysis of gene expression revealed that the biological processes associated with RA metabolism and the steroid biosynthesis pathway played important roles in the regulation of germ cell development.

From the GO annotations at all time points, it was deduced that hormones have important effects on ovarian cell proliferation and differentiation. This is likely to be influenced by signal transduction, thereby leading to the onset of meiosis and preparation to form

primordial follicles in the chicken. The analysis also suggested that RA-induced meiosis at E15.5 was primarily associated with multiple steroid biosynthesis and retinal metabolic pathways. The transforming growth factor-β (TGF-β) family and its signaling pathway are related to various developmental processes including gonadogenesis caused by activation of the intracellular SMAD signaling factors. In this study, *Smad5* was found to be significantly upregulated in the developing embryonic ovary. Numerous different ligands in the TGF-β family are found in the developing gonads and in maturing follicles, including BMPs, AMH, activin, inhibin and FST [21]. These molecules may play important roles in regulating ovarian development. It was reported that BMP signaling has a considerable effect on maintenance of follicles in an undifferentiated state prior to follicle selection in the adult chicken ovary [22]. *Bmp7* has been shown to express in the early developing gonads, and it becomes restricted to the cortex of the ovary by E8 [23]. However, the exact function of BMPs has not yet been demonstrated; therefore, it is supposed that BMP signaling may also play a role in the developing chicken ovary. In addition, it has been reported that *Amh* is also expressed at a low level in the developing ovary [24, 25]. Moreover, in females, *Amh* may also play a crucial role in follicle maturation, because its expression continues to be upregulated in follicular and pre-granulosa cells in the left ovarian cortex in hens [21]. However, the role of *Amh* in follicle formation and development in embryonic stages has not been elucidated. Taken together, it is clear that TGF-β signaling plays an essential role in ovarian development in the chicken. Moreover, due to the large number of signaling ligands that can activate or repress the signaling pathways, further studies are required to explore the mechanism of coordinated cellular signaling during ovary differentiation and meiosis onset. Furthermore, research on the intracellular effectors that are essential for downstream signaling must be carried out.

A comprehensive understanding of organogenesis requires system-level knowledge of transcriptional network dynamics underlying germ cell development. By performing a microarray analysis on four stages of ovaries in the embryonic chicken, the genes and gene-specific pathways that were up- and downregulated in the course of ovarian development were discovered. The regulation of interaction between pathways was also discussed with emphasis on the meiosis process. This study provided a gene expression profile obtained during the processes of ovarian development in the embryonic chicken, but more importantly, it characterized the stage-specific genes at the level of the transcriptome. Moreover, by examining the system as a whole, new insights into mechanisms by which ovarian germ cells adopt their specific fates at different stages were obtained. In fact, we are just beginning to understand the real details of the intrinsic factors that regulate the development of the embryonic ovary and signaling pathways that are activated and the gene expression changes that occur in germ cells in response to these factors.

In conclusion, we categorized functional genes and extracted important biological pathways underlying ovarian development in the embryonic chicken. Pathway analysis suggested that genes with differential expression levels were mainly involved in steroid biosynthesis and retinol metabolism. Testing of 12 candidate genes related to germ cell development based on microarray data showed that the meiosis process was potentially regulated by these differentially

expressed genes. Evidence from the *in vivo Raldh2* knockdown chicken model in this study validated a crucial role of *Raldh2* in regulating meiosis entry. The microarray analysis provided abundant data that will lead us to further basic studies on ovarian development, especially studies on processes associated with meiosis.

Acknowledgments

References

1. **Duan J, Shao F, Shao Y, Li J, Ling Y, Teng K, Li H, Wu C.** Androgen inhibits abdominal fat accumulation and negatively regulates the PCK1 gene in male chickens. *PLoS ONE* 2013; **8**: e59636. [Medline] [CrossRef]

2. **Silva C, Wood JR, Salvador L, Zhang Z, Kostetskii I, Williams CJ, Strauss JF 3rd.** Expression profile of male germ cell-associated genes in mouse embryonic stem cell cultures treated with all-trans retinoic acid and testosterone. *Mol Reprod Dev* 2009; **76**: 11–21. [Medline] [CrossRef]

3. **Bowles J, Koopman P.** Retinoic acid, meiosis and germ cell fate in mammals. *Development* 2007; **134**: 3401–3411. [Medline] [CrossRef]

4. **Rengaraj D, Lee BR, Choi JW, Lee SI, Seo HW, Kim TH, Choi HJ, Song G, Han JY.** Gene pathways and cell cycle-related genes in cultured avian primordial germ cells. *Poult Sci* 2012; **91**: 3167–3177. [Medline] [CrossRef]

5. **Motono M, Ohashi T, Nishijima K, Iijima S.** Analysis of chicken primordial germ cells. *Cytotechnology* 2008; **57**: 199–205. [Medline] [CrossRef]

6. **Rodríguez-León J, Rodríguez Esteban C, Martí M, Santiago-Josefat B, Dubova I, Rubiralta X, Izpisúa Belmonte JC.** Pitx2 regulates gonad morphogenesis. *Proc Natl Acad Sci USA* 2008; **105**: 11242–11247. [Medline] [CrossRef]

7. **Ottolenghi C, Colombino M, Crisponi L, Cao A, Forabosco A, Schlessinger D, Uda M.** Transcriptional control of ovarian development in somatic cells. *Semin Reprod Med* 2007; **25**: 252–263. [Medline] [CrossRef]

8. **Yu M, Yu P, Leghari IH, Ge C, Mi Y, Zhang C.** RALDH2, the enzyme for retinoic acid synthesis, mediates meiosis initiation in germ cells of the female embryonic chickens. *Amino Acids* 2013; **44**: 405–412. [Medline] [CrossRef]

9. **Baltus AE, Menke DB, Hu YC, Goodheart ML, Carpenter AE, de Rooij DG, Page DC.** In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nat Genet* 2006; **38**: 1430–1434. [Medline] [CrossRef]

10. **Anderson EL, Baltus AE, Roepers-Gajadien HL, Hassold TJ, de Rooij DG, van Pelt AM, Page DC.** Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proc Natl Acad Sci USA* 2008; **105**: 14976–14980. [Medline] [CrossRef]

11. **Vincent JJ, Huang Y, Chen PY, Feng S, Calvopiña JH, Nee K, Lee SA, Le T, Yoon AJ, Faull K, Fan G, Rao A, Jacobsen SE, Pellegrini M, Clark AT.** Stage-specific roles for tet1 and tet2 in DNA demethylation in primordial germ cells. *Cell Stem Cell* 2013; **12**: 470–478. [Medline] [CrossRef]

12. **Chen H, Palmer JS, Thiagarajan RD, Dinger ME, Lesieur E, Chiu H, Schulz A, Spiller C, Grimmond SM, Little MH, Koopman P, Wilhelm D.** Identification of novel markers of mouse fetal ovary development. *PLoS ONE* 2012; **7**: e41683. [Medline]

13. **Gheyas AA, Burt DW.** Microarray resources for genetic and genomic studies in chicken: a review. *Genesis* 2013; **51**: 337–356. [Medline] [CrossRef]

14. **Clinton M, Haines L, Belloir B, McBride D.** Sexing chick embryos: a rapid and simple protocol. *Br Poult Sci* 2001; **42**: 134–138. [Medline] [CrossRef]

15. **Speksnijder G, Ivarie R.** A modified method of shell windowing for producing somatic or germline chimeras in fertilized chicken eggs. *Poult Sci* 2000; **79**: 1430–1433. [Medline] [CrossRef]

16. **Singh R, Sinclair KD.** Metabolomics: approaches to assessing oocyte and embryo quality. *Theriogenology* 2007; **68**(Suppl 1): S56–S62. [Medline] [CrossRef]

17. **Wang SH, Cheng CY, Tang PC, Chen CF, Chen HH, Lee YP, Huang SY.** Differential gene expressions in testes of L2 strain Taiwan country chicken in response to acute heat stress. *Theriogenology* 2013; **79**: 374–382: e1–e7. [Medline] [CrossRef]

18. **Duester G.** Retinoic acid synthesis and signaling during early organogenesis. *Cell* 2008; **134**: 921–931. [Medline] [CrossRef]

19. **Smith CA, Roeszler KN, Bowles J, Koopman P, Sinclair AH.** Onset of meiosis in the chicken embryo; evidence of a role for retinoic acid. *BMC Dev Biol* 2008; **8**: 85. [Medline] [CrossRef]

20. **Rengaraj D, Lee BR, Jang HJ, Kim YM, Han JY.** Comparative metabolic pathway analysis with special reference to nucleotide metabolism-related genes in chicken primordial germ cells. *Theriogenology* 2013; **79**: 28–39. [Medline] [CrossRef]

21. **Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA, Themmen AP.** Anti-Müllerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. *Endocrinology* 2001; **142**: 4891–4899. [Medline]

22. **Webb R, Campbell BK.** Development of the dominant follicle: mechanisms of selection and maintenance of oocyte quality. *Soc Reprod Fertil Suppl* 2007; **64**: 141–163. [Medline]

23. **Carré GA, Couty I, Hennequet-Antier C, Govoroun MS.** Gene expression profiling reveals new potential players of gonad differentiation in the chicken embryo. *PLoS ONE* 2011; **6**: e23959. [Medline] [CrossRef]

24. **Ayers KL, Sinclair AH, Smith CA.** The molecular genetics of ovarian differentiation in the avian model. *Sex Dev* 2013; **7**: 80–94. [Medline] [CrossRef]

25. **Mazaud S, Oréal E, Guigon CJ, Carré-Eusèbe D, Magre S.** Lhx9 expression during gonadal morphogenesis as related to the state of cell differentiation. *Gene Expr Patterns* 2002; **2**: 373–377. [Medline] [CrossRef]