***Dicer1*, *AGO3*, and *AGO4* microRNA machinery genes are differentially expressed in developing female reproductive organs and overexpressed in cancerous ovaries of chickens¹**

W. Jeong,^{*2} H. Bae,^{†2} W. Lim,[‡] and G. Song^{†3}

^{*}Department of Animal Resources Science, Dankook University, Cheonan 330-714, Republic of Korea; [†]Institute of Animal Molecular Biotechnology and Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Seoul, 02841, Republic of Korea; and [‡]Department of Biomedical Sciences, Catholic Kwandong University, Gangneung, 25601, Republic of Korea

ABSTRACT: MicroRNA (miRNA)-mediated gene silencing is a key mechanism regulating numerous biological processes such as development of organs and tumorigenesis. The expression of miRNA machinery genes linked to miRNA biogenesis and processing is finely regulated. Despite accumulating evidence for chicken miRNA in the female reproduction system, precise regulatory mechanisms are largely unknown. Therefore, the objective of this study was to determine changes in expression levels of miRNA machinery genes in developmental stages of the oviduct and ovarian carcinogenesis of laying hens. In the present study, differential expression of miRNA machinery genes during ovarian carcinogenesis was determined

using cancerous and normal ovaries collected from normal laying hens and hens with cancer. Our results showed that 3 miRNA machinery genes (*Dicer1*, *AGO3*, and *AGO4*) were differentially expressed as laying hens' reproductive organs developed. These genes were simultaneously upregulated in cancerous ovaries compared with those in normal ovaries. Their transcripts were abundantly localized in glandular epithelial cells of cancerous ovaries. Our results indicate that *Dicer1*, *AGO3*, and *AGO4* play critical roles in the development of reproductive organs and ovarian carcinogenesis in laying hens, suggesting that simultaneous overexpression of these genes might serve as a prognostic factor for ovarian cancer.

Key words: *AGO3*, *AGO4*, chicken, *Dicer1*, microRNA, ovarian cancer

© 2017 American Society of Animal Science. All rights reserved.

J. Anim. Sci. 2017.95:4857–4868
doi:10.2527/jas2017.1846

INTRODUCTION

MicroRNAs (**miRNA**) are short noncoding RNA molecules of 18 to 23 nucleotides in length. Their perfect or partial base pairing with specific target mRNA promotes cleavage of target mRNA, leading to retardation of post-transcriptional expression of target genes (Garzon et al., 2006; Kloosterman and Plasterk, 2006). For these actions of miRNA, precise miRNA need to be

biosynthesized and processed by several miRNA machinery factors such as *Dicer1*, *Drosha*, and *Argonaute* (**AGO**) family proteins. These miRNA machinery genes influence specific expression patterns, binding partners, and biochemical capabilities in different tissues under normal and disease condition (Huang et al., 2014). Dysregulation of oncogenes and/or tumor suppressor genes by inappropriate expression of miRNA could lead to the development of ovarian cancer.

Several previous studies have provided evidence for oviductal involvement in chicken ovarian cancer pathogenesis (Giles et al., 2004; Hawkrigde et al., 2010). Recently, we have reported that several miRNA regulate the expression of genes closely involved in chicken oviduct development and ovarian carcinogenesis (Lim and Song, 2015). Although chicken miRNA targeting several genes involved in oviduct development and ovarian carcinogenesis have

¹This research was supported by the Basic Science Research Program (No. NRF-2017R1D1A1B03031644) funded by the Ministry of Education, Science, and Technology, Republic of Korea. The authors have declared no conflict of interest.

²These authors contributed equally to this work.

³Corresponding author: ghsong@korea.ac.kr

Received June 20, 2017.

Accepted September 6, 2017.

been identified, the functional or regulatory aspect of most miRNA machinery genes in chickens is currently unclear. Therefore, the objectives of the present study were 1) to investigate differential and cell-specific expression of several chicken miRNA machinery genes during the development of reproductive organs; 2) to determine differential expression of *Dicer1*, *AGO3*, and *AGO4* that might be closely involved in chicken oviduct development in normal and cancerous ovaries; and 3) to demonstrate spatial expression of their transcripts in normal and cancerous ovaries from hens.

MATERIALS AND METHODS

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. White Leghorn chickens were exposed to a light regimen of 15 h light and 9 h dark. They were provided ad libitum access to feed and water and subjected to standard poultry husbandry guidelines.

Tissue Samples

Study One. Zinc (20 mg/kg) was added to the diet to effectively reduce feed intake and induce molting of laying hens as previously described (Creger and Scott, 1977; Berry and Brake, 1985). Briefly, hens ($n = 5$ per time point) in the molting group (normal feeding group, 6 and 12 d after onset of zinc feeding) and the recrudescence group (20, 25, 30, and 35 d after onset of zinc feeding and 8, 13, 18, and 23 d of normal feeding after cessation of egg production and removal from the high-zinc diet) were euthanized using 60 to 70% carbon dioxide prior to collecting the oviduct on each assigned day (Jeong et al., 2013).

Study Two. Treatment with diethylstilbestrol (DES; a synthetic estrogen agonist) and recovery of the oviduct were conducted as previously described (Lim et al., 2011; Song et al., 2011). A DES pellet (10 mg) was implanted into the abdominal region of 1-wk-old female chicks to release the hormone for 10 d. The DES pellet was removed from all chicks for 10 d. Then, a 10-mg daily dose was administered for 10 additional days. Control chicks were normally raised without any treatment for 37 d after hatching. Five 37-d-old control chicks and five 37-d-old DES-treated chicks were euthanized using 60 to 70% carbon dioxide to collect oviduct samples, which were cut into 5- to 7-mm pieces and frozen in liquid nitrogen. The remaining tissues were cut into 10- to 15-mm pieces and fixed in fresh 4% paraformaldehyde in PBS (pH 7.4). After 24 h of fixation, tissues were dehydrated with 70% ethanol for 24 h and embedded in Paraplast-Plus (Leica

Microsystems GmbH, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5 μ m thickness.

Study Three. A total of 136 mature hens (88 over 36 mo of age and 48 over 24 mo of age) that had stopped laying eggs were euthanized to collect cancerous ovarian tissues. As controls, normal ($n = 5$) ovaries were also collected from egg-laying hens. The tumor stage of 10 hens with cancerous ovaries was evaluated according to characteristic features of chicken ovarian cancer (Barua et al., 2009; Lim et al., 2012). Epithelial ovarian cancers in chickens were classified based on cellular subtypes and patterns of cellular differentiation with reference to ovarian malignant tumor types in humans (Barua et al., 2009). Three hens were classified at stage III because ovarian tumor cells had metastasized to the gastrointestinal tract and liver surface with profuse ascites in the abdominal cavity. Five hens had tumor cells spread to distant organs, including the liver parenchyma, lung, gastrointestinal tract, and oviduct with profuse ascites. Therefore, they were classified as stage IV tumors. The other 2 hens had stage I disease, as their tumors were limited to their ovaries. Collected cancerous and normal ovaries containing follicles, glands, stromal cells, and blood vessels were frozen in liquid nitrogen or fixed in 4% paraformaldehyde for further analyses. Frozen tissue samples were cut into 5- to 7-mm pieces before they were frozen in liquid nitrogen. Other samples were cut into 10-mm pieces and fixed in 4% paraformaldehyde in PBS (pH 7.4). After 24 h of fixation, tissues were dehydrated in 70% ethanol for 24 h and embedded in Paraplast-Plus (Leica Microsystems GmbH). Paraffin-embedded tissues were sectioned at 5 μ m thickness for in situ hybridization.

Ribonucleic Acid Isolation

Total RNA was isolated from frozen tissues using Trizol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's recommendations. Quantity and quality of total RNA were determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

Quantitative Reverse Transcription-PCR Analysis

Complementary DNA was synthesized using total RNA extracted from each tissue and AccuPower RT PreMix (Bioneer Corp., Daejeon, Republic of Korea). Gene expression levels were measured using SYBR Green (Sigma-Aldrich Corp., St. Louis, MO) on a StepOnePlus Real-Time PCR System (Applied Biosystems Inc., Foster City, CA). The *GAPDH* gene was simultaneously analyzed as a control and used for normalization of loading. Target genes and *GAPDH* were analyzed in triplicate. Based on the sequence of target gene mRNA, a sense primer and antisense

primer were designed to amplify gene-specific products. For *GAPDH*, a sense primer (5'-ACA CAG AAG ACG GTG GAT GG-3') and an antisense primer (5'-GGC AGG TCA GGT CAA CAA CA-3') were used. Expression level of the target gene was determined using standard curves and threshold cycle (C_T) values. Their levels were normalized against the expression level of *GAPDH*. The PCR conditions were 95°C for 3 min followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s using a melting curve program (increasing the temperature from 55°C to 95°C at a rate of 0.5°C per 10 s) with continuous fluorescence measurement. ROX dye (Invitrogen Corp.) was used as a negative control for fluorescence measurement. Sequence-specific products were identified by generating melting curves. The C_T value represented the cycle number at which a fluorescent signal was statistically greater than the background. Relative gene expression was quantified using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

In Situ Hybridization Analysis

To obtain hybridization probes, PCR products were generated from cDNA with the same primers used for reverse transcription PCR (RT-PCR) analysis. The PCR products were gel purified and cloned into a TOPO vector (Invitrogen Corp.). After sequence verification, plasmids containing correct gene sequences were amplified with T7- and SP6-specific primers (T7: 5'-TGT AAT ACG ACT CAC TAT AGG G-3'; SP6: 5'-CTA TTT AGG TGA CAC TAT AGA AT-3'). Digoxigenin (DIG)-labeled RNA probes were then transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). Tissues were collected and fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μ m thickness, and placed on 3-aminopropyltriethoxysilane-treated (silanized) slides. These sections were then deparaffinized in xylene and rehydrated to diethylpyrocarbonate (DEPC)-treated water through a graded series of alcohol. These sections were treated with 1% Triton X-100 (Sigma-Aldrich Corp.) in PBS for 20 min and washed twice with DEPC-treated PBS. After that, they were digested with 5 μ g/mL proteinase K (Sigma-Aldrich Corp) in Tris-EDTA buffer (100 mM Tris-HCl and 50 mM EDTA, pH 8.0) at 37°C. Paraffin-embedded tissue sections were washed with DEPC-treated PBS twice (5 min each) and incubated in triethanolamine buffer (0.1 M triethanolamine) containing 0.25% (vol/vol) acetic anhydride. These sections were then incubated in a prehybridization mixture containing 50% formamide and 4x saline-sodium citrate for at least 10 min at room temperature. After prehybridization, sections were incubated with a hybridization mixture containing 40% formamide, 4x saline-sodium citrate, 10% dextran sulfate

sodium salt, 10 mM dithiothreitol, 1 mg/mL yeast tRNA, 1 mg/mL salmon sperm DNA, 0.02% Ficoll (Sigma-Aldrich Corp.), 0.02% polyvinylpyrrolidone, 0.2 mg/mL ribonuclease-free BSA, and denatured DIG-labeled complementary RNA probe overnight at 42°C in a humidified chamber. After hybridization, sections were washed with 2x saline-sodium citrate at 37°C for 15 min, 1x saline-sodium citrate at 37°C for 15 min in NaCl-Tris-EDTA buffer (10 mM Tris, 500 mM NaCl, and 1 mM EDTA) at 37°C for 30 min, and 0.1x NaCl-Tris-EDTA at 37°C for 30 min. After blocking with 2% normal sheep serum (Santa Cruz Biotechnology, Inc., Dallas, TX), sections were incubated with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche Applied Science) at 4°C overnight. The signal was visualized by exposure to a solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4 mM nitroblue tetrazolium, and 2 mM levamisole (Sigma-Aldrich Corp.).

Statistical Analyses

Data for quantitative PCR were subjected to ANOVA according to the general linear model (PROC GLM) of the SAS program (SAS Inst. Inc., Cary, NC) to determine whether there was significant difference in gene expression level. Statistical significance was considered at $P < 0.05$. Data are presented as mean $\pm$ SEM unless stated otherwise.

RESULTS

Changes in Expression Pattern of MicroRNA Machinery Genes during Oviduct Regression and Recrudescence

To investigate the possible involvement of the miRNA-processing system in the development of chicken reproductive organs, differential and cell-specific expression of miRNA machinery genes including *Dicer1*, *Drosha*, *AGO1*, *AGO2*, *AGO3*, and *AGO4* in oviducts of hens at different days of regression and recrudescence phases was determined. Female reproductive organs of egg-laying hens include 1 functional ovary and the oviduct, consisting of 4 segments: the infundibulum (site of fertilization), the magnum (production and secretion of egg white components), the isthmus (secretion of the soft shell membrane components), and the shell gland (calcification of the soft shell membrane for egg shell formation; Leach et al., 1981; Lavelin et al., 2000; Wang et al., 2002). According to microarray analysis in our previous study, hundreds of genes are up- or downregulated in the magnum portion that undergoes the most dramatic changes depending on regression and recrudescence of the oviduct in the molting

Figure 1. Differential expression of microRNA machinery genes in chicken oviducts during regression and recrudescence periods. Relative expression of chicken (A) *Dicer1*, (B) *Drosha*, (C) *AGO1*, (D) *AGO2*, (E) *AGO3*, and (F) *AGO4* genes was analyzed by quantitative reverse transcription-PCR using cDNA templates from oviduct hens fed a normal commercial diet (Day 0), a high-zinc diet for regression of reproductive organs (Day 6 and Day 12), and a normal commercial diet after complete regression of reproductive organs for recrudescence (Days 20, 25, 30, and 35). These experiments were performed in triplicates and normalized to *GAPDH* gene expression. Each bar represents the mean $\pm$ SEM of 3 independent experiments. * $P < 0.05$; ** $P < 0.01$; † $P < 0.001$.

and postmolting processes (Jeong et al., 2013). In the present study, changes in expression levels of *Dicer1*, *Drosha*, *AGO1*, *AGO2*, *AGO3*, and *AGO4* mRNA in the oviduct of laying hens during induced molting were examined using quantitative RT-PCR analyses (Fig. 1). First, *Dicer1* mRNA levels were increased in the completely regressed oviduct (Day 12). They were then gradually decreased during the regression phase (from Day 20 to 35). Relative expression levels of *AGO3* and *AGO4* mRNA had contrasting patterns compared with those of *Dicer1*. *AGO3* and *AGO4* mRNA levels during

regression phase (at Day 6 and Day 12) were significantly decreased ($P < 0.001$ and $P < 0.01$, respectively) compared with those at Day 0 (control). They were then gradually increased during reproductive tissue remodeling. However, expression patterns of *Drosha*, *AGO1*, and *AGO2* mRNA showed irregular fluctuations during the regression and recrudescence phases.

Because *Dicer1*, *AGO3*, and *AGO4* genes showed significant changes in expression levels depending on the stage of oviduct regression or regeneration, cell-specific localization of transcripts of these genes in the

Figure 2. Cell specific expression of *Dicer1*, *AGO3*, and *AGO4* transcripts in chicken oviducts during regression and recrudesence periods. Localization of (A) *Dicer1*, (B) *AGO3*, and (C) *AGO4* transcripts in oviducts of hens on different days of the pre- and postmolting period were analyzed by in situ hybridization. GEC = glandular epithelial cell; LEC = luminal epithelial cell. The scale bar represents 200 μ m in the first and third horizontal panel and 50 μ m in the second horizontal panel.

magnum portion was evaluated using in situ hybridization analyses (Fig. 2). Consistent with quantitative RT-PCR results, *Dicer1* mRNA was abundantly expressed in glandular epithelial (GEC) and luminal epithelial (LEC) cells of the regressed oviduct on Day 12. It was faintly expressed in the GEC and LEC of the oviduct at Day 0 (control) and Day 6. However, no or very little expression of *Dicer1* mRNA was detected during the recrudesence period. Although mRNA of *AGO3* and *AGO4* were abundantly expressed in the GECs and LECs of the control and almost completely remodeled oviducts, *AGO3* or *AGO4* mRNA was not detectable in the regressed oviduct on Day 12 or Day 20.

Differential Expression of *Dicer1*, *AGO3*, and *AGO4* in Diethylstilbestrol-Stimulated Chick Oviducts

Next, we determined the effect of estrogen stimulation on the expression of *Dicer1*, *AGO3*, and *AGO4* that might be closely involved in the development of the chicken reproductive system using oviducts from DES-treated or nontreated chicks. Our previous study has revealed that DES treatment can induce dramatic growth and development of Day-37 chick oviducts, with global gene expression changes (Song et al., 2011). Results of quantitative RT-PCR analysis in the present study showed that the expression levels of *Dicer1* were decreased in DES-treated chick oviducts compared with those in nontreated oviducts of control chicks (Fig. 3A). Expression levels of *AGO3* mRNA in DES-treated chick oviducts were also decreased compared with those in nontreated control oviducts (Fig. 3B). However, expression levels of *AGO4* were significantly

increased in the magnum and shell gland of DES-treated chick oviducts compared with those in nontreated chick oviducts (Fig. 3C). Consistent with quantitative RT-PCR results, in situ hybridization analyses revealed that *Dicer1* and *AGO3* mRNA were the most abundant in the LECs and stroma of control oviducts. However, extremely weak or no expression of these mRNA was found in DES-treated oviducts (Fig. 4). On the other hand, *AGO4* transcripts were detected at lower abundance in the LECs of control oviducts, although they were predominantly abundant in the LECs and GECs of the shell gland of control oviducts. *AGO4* transcripts were also found at moderate levels in the LECs and GECs of the DES-treated magnum and isthmus.

Comparative Expression and Cell-Specific Localization of *Dicer1*, *AGO3*, and *AGO4* mRNA in Normal and Cancerous Ovaries of Hens

Because our results suggested that *Dicer1*, *AGO3*, and *AGO4* were closely involved in the growth and development of the chicken oviduct, we hypothesized that the expression pattern of these genes might differ between normal and cancerous tissues from chicken ovaries. To determine the expression pattern and cell-specific localization of *Dicer1*, *AGO3*, and *AGO4* mRNA in normal and cancerous ovaries from laying hens, quantitative RT-PCR and in situ hybridization analyses were performed. Results of quantitative RT-PCR showed a 1.8-fold increase of *Dicer1* mRNA in cancerous ovaries ($P < 0.05$) in comparison with normal ovaries of laying hens (Fig. 5A). Results of in situ hybridization demonstrated that *Dicer1* mRNA was

Figure 3. Differential expression of *Dicer1*, *AGO3*, and *AGO4* in normal and diethylstilbestrol (DES)-treated chick oviducts. Relative expression of chicken (A) *Dicer1*, (B) *AGO3*, and (C) *AGO4* genes was analyzed by quantitative reverse transcription-PCR using oviducts from DES-treated and nontreated Day-37 chicks. These experiments were performed in triplicate and normalized to *GAPDH* gene expression. IF = infundibulum; MG = magnum; IS = isthmus; SG = shell gland. Each bar represents the mean $\pm$ SEM of 3 independent experiments. * $P < 0.05$; ** $P < 0.01$; † $P < 0.001$.

predominantly detected in the GEC. It was detected in the LE and stromal cells of cancerous ovaries to a much lesser extent (Fig. 5B). There was no detectable *Dicer1* mRNA in normal ovaries. As shown in Fig. 6A, expression levels of *AGO3* mRNA were 2.1-fold ($P < 0.05$) greater in cancerous ovaries than those in normal ovaries. The *AGO3* mRNA was abundantly expressed in the stroma of cancerous ovaries, but not in normal ovaries (Fig. 6B). Quantitative PCR revealed that *AGO4*

mRNA was increased 2.9-fold ($P < 0.01$) in cancerous ovaries compared with that in normal ovaries of laying hens (Fig. 7A). The *AGO4* mRNA was predominantly localized to the GECs, with moderate expression in the LECs and stroma of cancerous ovaries. However, it was undetectable in normal ovaries (Fig. 7B). In the negative control, sense probe for *Dicer1*, *AGO3*, or *AGO4* failed to detect a signal. These results showed simultaneous upregulation of *Dicer1*, *AGO3*, and *AGO4* expression specifically in the GEC of cancerous ovaries of hens, suggesting that these genes might be useful biomarkers for ovarian cancer.

DISCUSSION

MicroRNA-mediated gene regulation is a crucial mechanism for morphogenesis, development, and function of female reproductive organs in chickens as in mammals. Although several chicken miRNA regulating several genes involved in oviduct development and ovarian carcinogenesis have been identified, expressional or functional aspects of most miRNA machinery genes regulating miRNA processing in chicken reproductive organs are currently unknown. In the present study, we demonstrated that the expression of miRNA machinery genes *Dicer1*, *AGO3*, and *AGO4* was differentially regulated during chicken oviduct development. Their levels were increased in the GE of cancerous ovaries compared with that in normal ovaries from laying hens.

As one miRNA machinery gene, *Dicer1* has many important roles in morphogenesis and function of reproductive organs. For example, *Dicer1*^{-/-} mice have an abnormal meiotic progression, which renders a significant reduction in testis mass and sperm number (Greenlee et al., 2012). In miRNA processing, an Ribonuclease III endonuclease *Dicer1* can cleave pre-miRNA into 21- to 25-nucleotide transiently double-stranded miRNA duplexes. These miRNA duplexes are then transferred to RNA-induced silencing complex containing AGO proteins AGO1, AGO2, AGO3, and AGO4 that unwind these miRNA duplexes to single-stranded small RNA. Within the AGO protein family, only AGO2 has catalytic activity to cleave target mRNA. It acts on both cleavage and noncleavage mechanisms, whereas AGO1, AGO3, and AGO4 are catalytically inactive. They solely rely on a noncleavage gene silencing mechanism (Meister et al., 2004; Yekta et al., 2004; Hauptmann and Meister, 2013). In the present study, we found that *Dicer1*, *AGO3*, and *AGO4* were differentially expressed in the magnum portion, which underwent the most dramatic morphological changes during oviductal regression and recrudescence accompanying the molting process. In response to feeding a high-zinc diet, dramatic regression of female reproductive organs

Figure 4. Cell specific expression of *Dicer1*, *AGO3*, and *AGO4* in normal and diethylstilbestrol (DES)-treated chick oviducts. Localization of (A) *Dicer1*, (B) *AGO3*, and (C) *AGO4* transcripts in oviducts of DES-treated and nontreated Day-37 chicks were analyzed by in situ hybridization. GEC = glandular epithelial cell; LEC = luminal epithelial cell. The scale bar represents 200 μ m in the first and third horizontal panel and 50 μ m in the second horizontal panel.

Figure 5. Differential and cell-specific expression of *Dicer1* gene in normal and cancerous ovaries from laying hens. (A) Relative expression of chicken *Dicer1* gene was analyzed by quantitative reverse transcription-PCR using cDNA templates from normal and cancerous chicken ovaries. These experiments were conducted in triplicate, and values were normalized to those of *GAPDH* gene expression. Each bar represents the mean ± SEM of 3 independent experiments. * $P < 0.05$. (B) Localization of *Dicer1* transcripts in normal and cancerous chicken ovaries was analyzed by in situ hybridization. Cross-sections of normal and cancerous ovaries of hens were hybridized with antisense or sense chicken *Dicer1* complementary RNA probes. GEC = glandular epithelial cell. The scale bar represents 200 μ m in the first and third horizontal panel and 50 μ m in the second horizontal panel.

occurs in hens within 12 d. These regressed reproductive organs are then gradually redeveloped by onset of feeding a normal diet (Scott and Creger, 1976; Shippee et al., 1979; Jeong et al., 2013). Interestingly, we observed that the expression of the *Dicer1* gene was reduced following regeneration of the regressed oviduct whereas the expression of *AGO3* and *AGO4* genes was increased during the recrudescence phase.

One possible explanation for these differential gene expression patterns of *Dicer1*, *AGO3*, and *AGO4* during oviduct development might be hormonal regulation of gene expression. Because dramatic remodeling of female reproductive organs is closely coupled with changes in estrogen concentrations, expression of miRNA machinery genes during molting and oviduct regeneration might be directly or indirectly affected by changes in estrogen levels following withdrawal (in the regression phase) and restart (in the recrudescence phase) of ovarian steroidogenesis (Hoshino et al., 1988; Jeong et al., 2013). Previously, we have reported that the expression of several miRNA targeting oviduct-related genes is regulated by estrogen stimulation (Lim and Song, 2015). Results of the present study showed that *Dicer1* and *AGO3* gene expression was decreased in exogenous estrogen (DES)-stimulated oviducts, whereas *AGO4* gene expression was increased in the shell gland of DES-treated chick oviducts. Estrogen regulates the expression of numerous estrogen-responsive genes that greatly contribute to the development and function of reproductive organs. Diethylstilbestrol is a synthetic nonsteroidal estrogen that can bind to estrogen receptor α (ESR1) and act similar to endogenous estrogen derived from ovaries (Kohler et al., 1969; Palmiter and Wrenn, 1971; Dougherty and Sanders, 2005). It stimulates massive growth and various histological changes of juvenile chick oviducts (Dougherty and Sanders, 2005) based on dynamic changes in gene expression (Song et al., 2011). Results of the current study showed that the expression of *Dicer1*, *AGO3*, and *AGO4* was changed in DES-induced developed oviducts, suggesting that expression of these genes might be directly or indirectly affected by estrogen in a tissue- and cell-specific manner. This could lead to large scale of genomic changes associated with the development and function of the chicken oviduct in response to hormonal action. However, the precise mechanism involved in the microprocessor activity of these genes in the oviduct needs to be intensively investigated in the future.

In previous studies, we have identified several chicken miRNA affecting expression of genes involved in the development and carcinogenesis of reproductive organs. For example, chicken *miR-1798*, *miR-1699*, *miR-223*, and *miR-1744* can inhibit post-transcriptional expression of cell cycle genes such as *CCND1*, *CCNE2*,

Figure 6. Differential and cell-specific expression of *AGO3* gene in normal and cancerous ovaries from laying hens. (A) Relative expression of chicken *AGO3* gene was analyzed by quantitative reverse transcription-PCR using cDNA templates from normal and cancerous chicken ovaries. These experiments were conducted in triplicate, and values were normalized to those of *GAPDH* gene expression. Each bar represents the mean $\pm$ SEM of 3 independent experiments. * $P < 0.05$. (B) Localization of *AGO3* transcripts in normal and cancerous chicken ovaries was analyzed by in situ hybridization. Cross-sections of normal and cancerous ovaries of hens were hybridized with antisense or sense chicken *AGO3* complementary RNA probes. GEC = glandular epithelial cell. The scale bar represents 200 μ m in the first and third horizontal panel and 50 μ m in the second horizontal panel.

Figure 7. Differential and cell-specific expression of *AGO4* gene in normal and cancerous ovaries from laying hens. (A) Relative expression of chicken *AGO4* gene was analyzed by quantitative reverse transcription-PCR using cDNA templates from normal and cancerous chicken ovaries. These experiments were conducted in triplicate, and values were normalized to those of *GAPDH* gene expression. Each bar represents the mean $\pm$ SEM of 3 independent experiments. ** $P < 0.01$. (B) Localization of *AGO4* transcripts in normal and cancerous chicken ovaries was analyzed by in situ hybridization. Cross-sections of normal and cancerous ovaries of hens were hybridized with antisense or sense chicken *AGO4* complementary RNA probes. GEC = glandular epithelial cell. The scale bar represents 200 μ m in the first and third horizontal panel and 50 μ m in the second horizontal panel.

CDK1, and *CDK3*, which are predominantly found in cancerous ovaries (Lee et al., 2012). These results indicate that dysfunction of the chicken miRNA machinery system can lead to dysregulation of miRNA targeting oncogenes and proto-oncogenes with the potential to initiate ovarian carcinogenesis. In fact, deregulation of miRNA is generally considered to be a prerequisite for the initiation of carcinogenesis. Altered expression of miRNA machinery genes has been found in a variety of tumors in human. Such alteration has been reported to be useful for predicting many diverse carcinomas, including ovarian cancer (Horikawa et al., 2008; Huang et al., 2014). Although accumulating evidence has implied that the chicken is the most relevant animal model to identify biomarkers for epithelial-derived ovarian cancer (Murdoch et al., 2005), how miRNA machinery genes are expressed and how they act on the initiation and progression of malignancies have not been reported in chickens. Based on previous reports and the present findings suggesting that *Dicer1*, *AGO3*, and *AGO4* are closely involved in development of chicken reproductive organs, their expressional changes are likely to contribute to the development and progression of ovarian cancer in laying hens. We found that expression levels of *Dicer1*, *AGO3*, and *AGO4* were increased in cancerous ovaries, especially in the GECs of cancerous ovaries. However, extremely weak or no expression of these mRNA was found in normal ovaries. In various cancerous tissues, a number of complex glandular architectures are commonly found. This is especially true for ovaries, because these glandular structures are mainly detected in endometrioid-type tumors with characteristics such as nuclear atypia, cribriform foci, and atresia of stromal follicles (Barua et al., 2009). Because the functional role of chicken miRNA machinery genes in ovarian cancer has not been previously reported, GECs cells in cancerous ovaries might undergo highly active processing of global miRNA in response to synchronous overexpression of several miRNA-processing factors, which likely contributes to the dysregulation of genes preventing carcinogenesis and controlling tumor cell-specific cell death.

However, previous findings on human tumors are inconsistent with some findings in the present study. Although chicken *Dicer1* mRNA was significantly upregulated in cancerous ovaries of laying hens in the current study, human *Dicer1* was found to be downregulated in tumors of the lung (Karube et al., 2005), endometria (Torres et al., 2011), breast (Khoshnaw et al., 2012), and ovary (Pampalakis et al., 2010). A previous study has reported that downregulation of *Dicer1* is associated with advanced tumor stages and that patients with high *Dicer1* expression are correlated with increased survival (Merritt et al., 2008). However, the expression pattern of the *Dicer1* gene varies accord-

ing to tumor type, physiological conditions, and tumor progression. Contrary to the finding of downregulation of *Dicer1* in ovarian cancer, increased levels of *Dicer1* have been found in acute myeloid leukemia (Martin et al., 2009), smooth muscle neoplasm (Papachristou et al., 2012), and prostate cancer (Fu et al., 2010). Furthermore, Vaksman et al. (2012) have found that *Dicer1* is upregulated along tumor progression in serous ovarian carcinoma. One plausible explanation for such divergent expression patterns of *Dicer1* among tumors and tissues might be compensatory alterations of *Dicer1* and/or other miRNA machinery components of the RNA-interference cascade. In fact, the relative ratio of *Dicer1*, the AGO protein family, and other factors are important to maintain or change the balance of various miRNA under different physiological cellular conditions. For example, both over- and underexpression of *Dicer1* relative to normal conditions are associated with mucoepidermoid carcinomas (Chiosea et al., 2008). In addition, *Dicer1* is likely to exert paradoxical effects on carcinogenesis in women and laying hens as *Notch*, which acts as both an oncogene and tumor suppressor gene depending on tumor type (Zagouras et al., 1995; Reedijk et al., 2005; Kunnimalaiyaan et al., 2006; Wang et al., 2006; Kunnimalaiyaan and Chen, 2007; Sjölund et al., 2008). The controversial pattern of *Dicer1* expression in cancerous ovaries of human and chickens merits further study. Although *AGO2*, another component of the AGO family, is known to be upregulated in several types of human tumors including epithelial skin cancer (Sand et al., 2012), prostate cancer (Fu et al., 2010), and ovarian cancer (Vaksman et al., 2012), *AGO3* and *AGO4* have not yet been described in human ovarian cancer.

In conclusion, by showing that differential expression of several miRNA machinery genes including *Dicer1*, *AGO3*, and *AGO4* are closely related to estrogen-dependent development of the chicken female reproductive tract, we provide new insights into the role of these genes in development, regression, and recrudescence of chicken reproductive organs. Results of this study suggest that overexpression of *Dicer1*, *AGO3*, and *AGO4* is closely associated with ovarian carcinogenesis of laying hens. Although detailed regulatory and functional mechanisms whereby these miRNA machinery factors affect carcinogenesis and their expression in women with ovarian cancer remain to be precisely elucidated, results of the current study are expected to improve our understanding of miRNA biogenesis and processing systems in the development of chicken oviduct and ovarian carcinogenesis. Our study provides insights into overexpression of *Dicer1*, *AGO3*, and *AGO4* as a possible prognostic factor for diagnosis and prediction of ovarian cancer.

LITERATURE CITED

- Barua, A., P. Bitterman, J. S. Abramowicz, A. L. Dirks, J. M. Bahr, D. B. Hales, M. J. Bradaric, S. L. Edassery, J. Rotmensch, and J. L. Luborsky. 2009. Histopathology of ovarian tumors in laying hens: A preclinical model of human ovarian cancer. *Int. J. Gynecol. Cancer* 19:531–539. doi:10.1111/IGC.0b013e3181a41613
- Berry, W. D., and J. Brake. 1985. Comparison of parameters associated with molt induced by fasting, zinc, and low dietary-sodium in caged layers. *Poult. Sci.* 64:2027–2036. doi:10.3382/ps.0642027
- Chiose, S. I., E. L. Barnes, S. Y. Lai, A. M. Egloff, R. L. Sargent, J. L. Hunt, and R. R. Seethala. 2008. Mucoepidermoid carcinoma of upper aerodigestive tract: Clinicopathologic study of 78 cases with immunohistochemical analysis of Dicer expression. *Virchows Arch.* 452:629–635.
- Creger, C. R., and J. T. Scott. 1977. Dietary zinc as an effective resting agent for laying hen. *Poult. Sci.* 56:1706.
- Dougherty, D. C., and M. M. Sanders. 2005. Estrogen action: Revitalization of the chick oviduct model. *Trends Endocrinol. Metab.* 16:414–419.
- Fu, X., C. Xue, Y. Huang, Y. Xie, and Y. Li. 2010. The activity and expression of microRNAs in prostate cancers. *Mol. Biosyst.* 6:2561–2572. doi:10.1039/c0mb00100g
- Garzon, R., M. Fabbri, A. Cimmino, G. A. Calin, and C. M. Croce. 2006. MicroRNA expression and function in cancer. *Trends Mol. Med.* 12:580–587. doi:10.1016/j.molmed.2006.10.006
- Giles, J. R., H. L. Shivaprasad, and P. A. Johnson. 2004. Ovarian tumor expression of an oviductal protein in the hen: A model for human serous ovarian adenocarcinoma. *Gynecol. Oncol.* 95:530–533. doi:10.1016/j.ygyno.2004.07.061
- Greenlee, A. R., M. S. Shiao, E. Snyder, F. W. Buaas, T. Gu, T. M. Stearns, M. Sharma, E. P. Murchison, G. C. Puente, and R. E. Braun. 2012. Deregulated sex chromosome gene expression with male germ cell-specific loss of Dicer1. *PLoS One* 7:e46359. doi:10.1371/journal.pone.0046359
- Hauptmann, J., and G. Meister. 2013. Argonaute regulation: Two roads to the same destination. *Dev. Cell* 25:553–554. doi:10.1016/j.devcel.2013.06.009
- Hawkrige, A. M., R. B. Wysocky, J. N. Petite, K. E. Anderson, P. E. Mozdzia, O. J. Fletcher, J. M. Horowitz, and D. C. Muddiman. 2010. Measuring the intra-individual variability of the plasma proteome in the chicken model of spontaneous ovarian adenocarcinoma. *Anal. Bioanal. Chem.* 398:737–749. doi:10.1007/s00216-010-3979-y
- Horikawa, Y., C. G. Wood, H. Yang, H. Zhao, Y. Ye, J. Gu, J. Lin, T. Habuchi, and X. Wu. 2008. Single nucleotide polymorphisms of microRNA machinery genes modify the risk of renal cell carcinoma. *Clin. Cancer Res.* 14:7956–7962. doi:10.1158/1078-0432.CCR-08-1199
- Hoshino, S., M. Suzuki, T. Kakegawa, K. Imai, M. Wakita, Y. Kobayashi, and Y. Yamada. 1988. Changes in plasma thyroid hormones, luteinizing hormone (LH), estradiol, progesterone and corticosterone of laying hens during a forced molt. *Comp. Biochem. Physiol. A Comp. Physiol.* 90:355–359. doi:10.1016/0300-9629(88)91128-0
- Huang, J. T., J. Wang, V. Srivastava, S. Sen, and S. M. Liu. 2014. MicroRNA machinery genes as novel biomarkers for cancer. *Front. Oncol.* 4:113. doi:10.3389/fonc.2014.00113
- Jeong, W., W. Lim, S. E. Ahn, C. H. Lim, J. Y. Lee, S. M. Bae, J. Kim, F. W. Bazer, and G. Song. 2013. Recrudescence mechanisms and gene expression profile of the reproductive tracts from chickens during the molting period. *PLoS One* 8:e76784. doi:10.1371/journal.pone.0076784
- Karube, Y., H. Tanaka, H. Osada, S. Tomida, Y. Tatematsu, K. Yanagisawa, Y. Yatabe, J. Takamizawa, S. Miyoshi, T. Mitsudomi, and T. Takahashi. 2005. Reduced expression of Dicer associated with poor prognosis in lung cancer patients. *Cancer Sci.* 96:111–115. doi:10.1111/j.1349-7006.2005.00015.x
- Khoshnaw, S. M., E. A. Rakha, T. M. Abdel-Fatah, C. C. Nolan, Z. Hodi, D. R. Macmillan, I. O. Ellis, and A. R. Green. 2012. Loss of Dicer expression is associated with breast cancer progression and recurrence. *Breast Cancer Res. Treat.* 135:403–413. doi:10.1007/s10549-012-2169-3
- Kloosterman, W. P., and R. H. Plasterk. 2006. The diverse functions of microRNAs in animal development and disease. *Dev. Cell* 11:441–450. doi:10.1016/j.devcel.2006.09.009
- Kohler, P. O., P. M. Grimley, and B. W. O'Malley. 1969. Estrogen-induced cytodifferentiation of the ovalbumin-secreting glands of the chick oviduct. *J. Cell Biol.* 40:8–27. doi:10.1083/jcb.40.1.8
- Kunnimalaiyaan, M., and H. Chen. 2007. Tumor suppressor role of Notch-1 signaling in neuroendocrine tumors. *Oncologist* 12:535–542. doi:10.1634/theoncologist.12-5-535
- Kunnimalaiyaan, M., A. M. Vaccaro, M. A. Ndiaye, and H. Chen. 2006. Overexpression of the NOTCH1 intracellular domain inhibits cell proliferation and alters the neuroendocrine phenotype of medullary thyroid cancer cells. *J. Biol. Chem.* 281:39819–39830. doi:10.1074/jbc.M603578200
- Lavelin, I., N. Meiri, and M. Pines. 2000. New insight in eggshell formation. *Poult. Sci.* 79:1014–1017. doi:10.1093/ps/79.7.1014
- Leach, R. M., Jr., R. B. Rucker, and G. P. Van Dyke. 1981. Egg shell membrane protein: A nonelastin desmosine/isodesmosine-containing protein. *Arch. Biochem. Biophys.* 207:353–359. doi:10.1016/0003-9861(81)90042-4
- Lee, J. Y., W. Jeong, J. H. Kim, J. Kim, F. W. Bazer, J. Y. Han, and G. Song. 2012. Distinct expression pattern and post-transcriptional regulation of cell cycle genes in the glandular epithelia of avian ovarian carcinomas. *PLoS One* 7:e51592. doi:10.1371/journal.pone.0051592
- Lim, W., J. H. Kim, S. E. Ahn, W. Jeong, J. Kim, F. W. Bazer, J. Y. Han, and G. Song. 2011. Avian *SERPINB1* gene: Characteristics, tissue-specific expression, and regulation of expression by estrogen. *Biol. Reprod.* 85:1260–1268. doi:10.1095/biolreprod.111.093526
- Lim, W., J. H. Kim, S. E. Ahn, W. Jeong, J. Kim, F. W. Bazer, J. Y. Han, and G. Song. 2012. Avian *SERPINB1* gene: A marker for ovarian endometrioid cancer in chickens. *Exp. Biol. Med.* (Maywood) 237:150–159. doi:10.1258/ebm.2011.011250
- Lim, W., and G. Song. 2015. Differential expression of vitelline membrane outer layer protein 1: Hormonal regulation of expression in the oviduct and in ovarian carcinomas from laying hens. *Mol. Cell. Endocrinol.* 399:250–258. doi:10.1016/j.mce.2014.10.015
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 25:402–408. doi:10.1006/meth.2001.1262
- Martin, M. G., J. E. Payton, and D. C. Link. 2009. Dicer and outcomes in patients with acute myeloid leukemia (AML). *Leuk. Res.* 33:e127. doi:10.1016/j.leukres.2009.02.003
- Meister, G., M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng, and T. Tuschl. 2004. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol. Cell* 15:185–197. doi:10.1016/j.molcel.2004.07.007

- Merritt, W. M., Y. G. Lin, L. Y. Han, A. A. Kamat, W. A. Spannuth, R. Schmandt, D. Urbauer, L. A. Pennacchio, J. F. Cheng, A. M. Nick, M. T. Deavers, A. Mourad-Zeidan, H. Wang, P. Mueller, M. E. Lenburg, J. W. Gray, S. Mok, M. J. Birrer, G. Lopez-Berestein, R. L. Coleman, M. Bar-Eli, and A. K. Sood. 2008. Dicer, Drosha, and outcomes in patients with ovarian cancer. *N. Engl. J. Med.* 359:2641–2650. doi:10.1056/NEJMoa0803785
- Murdoch, W. J., E. A. Van Kirk, and B. M. Alexander. 2005. DNA damages in ovarian surface epithelial cells of ovulatory hens. *Exp. Biol. Med.* (Maywood) 230:429–433. doi:10.1177/15353702-0323006-11
- Palmiter, R. D., and J. T. Wrenn. 1971. Interaction of estrogen and progesterone in chick oviduct development. 3. Tubular gland cell cytodifferentiation. *J. Cell Biol.* 50:598–615. doi:10.1083/jcb.50.3.598
- Pampalakis, G., E. P. Diamandis, D. Katsaros, and G. Sotiropoulou. 2010. Down-regulation of Dicer expression in ovarian cancer tissues. *Clin. Biochem.* 43:324–327. doi:10.1016/j.clinbiochem.2009.09.014
- Papachristou, D. J., E. Sklirou, D. Corradi, C. Grassani, V. Kontogeorgakos, and U. N. Rao. 2012. Immunohistochemical analysis of the endoribonucleases Drosha, Dicer and Ago2 in smooth muscle tumours of soft tissues. *Histopathology* 60:E28–E36. doi:10.1111/j.1365-2559.2012.04192.x
- Reedijk, M., S. Odorcic, L. Chang, H. Zhang, N. Miller, D. R. McCready, G. Lockwood, and S. E. Egan. 2005. High-level co-expression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Res.* 65:8530–8537. doi:10.1158/0008-5472.CAN-05-1069
- Sand, M., M. Skrygan, D. Georgas, C. Arenz, T. Gambichler, D. Sand, P. Altmeyer, and F. G. Bechara. 2012. Expression levels of the microRNA maturing microprocessor complex component DGCR8 and the RNA-induced silencing complex (RISC) components argonaute-1, argonaute-2, PACT, TARBP1, and TARBP2 in epithelial skin cancer. *Mol. Carcinog.* 51:916–922. doi:10.1002/mc.20861
- Scott, J. T., and C. R. Creger. 1976. The use of zinc as an effective molting agent in laying hens. *Poult. Sci.* 55:2089. (Abstr.)
- Shippee, R. L., P. E. Stake, U. Koehn, J. L. Lambert, and R. W. Simmons III. 1979. High dietary zinc or magnesium as forced-resting agents for laying hens. *Poult. Sci.* 58:949–954. doi:10.3382/ps.0580949
- Sjölund, J., M. Johansson, S. Manna, C. Norin, A. Pietras, S. Beckman, E. Nilsson, B. Ljungberg, and H. Axelson. 2008. Suppression of renal cell carcinoma growth by inhibition of Notch signaling in vitro and in vivo. *J. Clin. Invest.* 118:217–228. doi:10.1172/JCI32086
- Song, G., H. W. Seo, J. W. Choi, D. Rengaraj, T. M. Kim, B. R. Lee, Y. M. Kim, T. W. Yun, J. W. Jeong, and J. Y. Han. 2011. Discovery of candidate genes and pathways regulating oviduct development in chickens. *Biol. Reprod.* 85:306–314. doi:10.1095/biol-reprod.110.089227
- Torres, A., K. Torres, T. Paszkowski, B. Jodłowska-Jędrych, T. Radomański, A. Książek, and R. Maciejewski. 2011. Major regulators of microRNAs biogenesis Dicer and Drosha are down-regulated in endometrial cancer. *Tumour Biol.* 32:769–776. doi:10.1007/s13277-011-0179-0
- Vaksman, O., T. E. Hetland, C. G. Trope, R. Reich, and B. Davidson. 2012. Argonaute, Dicer, and Drosha are up-regulated along tumor progression in serous ovarian carcinoma. *Hum. Pathol.* 43:2062–2069. doi:10.1016/j.humpath.2012.02.016
- Wang, X., B. C. Ford, C. A. Praul, and R. M. Leach Jr. 2002. Collagen X expression in oviduct tissue during the different stages of the egg laying cycle. *Poult. Sci.* 81:805–808. doi:10.1093/ps/81.6.805
- Wang, Z., Y. Zhang, Y. Li, S. Banerjee, J. Liao, and F. H. Sarkar. 2006. Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells. *Mol. Cancer Ther.* 5:483–493. doi:10.1158/1535-7163.MCT-05-0299
- Yekta, S., I. H. Shih, and D. P. Bartel. 2004. MicroRNA-directed cleavage of *HOXB8* mRNA. *Science* 304:594–596. doi:10.1126/science.1097434
- Zagouras, P., S. Stifani, C. M. Blaumueller, M. L. Carcangiu, and S. Artavanis-Tsakonas. 1995. Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc. Natl. Acad. Sci. USA* 92:6414–6418. doi:10.1073/pnas.92.14.6414