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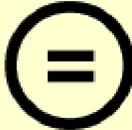
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**A Dissertation for the Degree of Doctor of Philosophy**

**Elucidation of SERPINB3, SERPINB11, GAL11,  
SPP1, and A2M as Prognostic Biomarkers for  
Epithelial-Derived Ovarian Cancer**

상피성 난소암의 예측 생체지표로서 SERPINB3, SERPINB11,  
GAL11, SPP1, A2M 유전자의 규명에 관한 연구

**August, 2013**

**By**

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## **ABSTRACT**

# **Elucidation of SERPINB3, SERPINB11, GAL11, SPP1, and A2M as Prognostic Biomarkers for Epithelial-Derived Ovarian Cancer**

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Ovarian cancer is the most fatal gynecological malignancy leading to cancer-related deaths in women worldwide. Diagnosis of ovarian cancer at an early stage when 90% of patients can be cured is very difficult due to lack of symptoms and early detection markers. Therefore, in most of patients, this disease is detected at an advanced stage (Stage III-IV) which results in a low survival rate (< 30%). More than 90% of ovarian carcinomas originate from ovarian surface epithelial cells; therefore, it is called epithelial-derived ovarian cancer (EOC). The risk of EOC increases with incessant ovulation involving rupture and repair of the surface epithelium of ovaries. For investigation with animal models of EOC, laying hens are the most relevant animal model because they spontaneously develop EOC as occurs in women through ovulating almost every day. As in women, EOC in the hen is age-related and grossly and histologically similar to that in women. Recently studies have shown that ovarian

cancer could arise from epithelium from the oviduct as oviduct-related genes are up-regulated in EOC of hens. Therefore, the objectives of this study were to determine: 1) the distribution and localization of developmentally-regulated genes in the oviduct including serpin peptidase inhibitor, clade B (ovalbumin), member 3 (SERPINB3), SERPINB11, gallicin 11 (GAL11), secreted phosphoprotein 1 (SPP1) and alpha 2 macroglobulin (A2M) in normal and cancerous ovaries of laying hens; 2) the expression pattern of target genes among normal and cancer cells of ovaries from laying hens and human ovarian cancer cell lines; and 3) the functional role of target genes in human EOC.

First, we investigated the functional role of SERPINB3 gene associated with programmed cell death and immune responses related to human EOC using the laying hen animal model. Of 136 laying hens studied, EOC was found in 10 (7.4%). *SERPINB3* mRNA was induced in cancerous, but not normal ovaries of laying hens ( $P < 0.01$ ), and it was abundant only in the glandular epithelium (GE) of cancerous ovaries. Further, several microRNAs, specifically *miR-101*, *miR-1668* and *miR-1681* were discovered to influence *SERPINB3* expression via its 3'-UTR which suggests that post-transcriptional regulation influences *SERPINB3* expression in chickens. SERPINB3 protein was localized predominantly to the nucleus of glandular epithelium in cancerous ovaries of laying hens, and it was also abundant in the nucleus of both chicken and human ovarian cancer cell lines. Of 109 human patients with EOC, 15 (13.8%), 66 (60.6%) and 28 (25.7%) had ovaries with weak, moderate and strong expression of SERPINB3 protein, respectively. Strong expression of SERPINB3 protein was a prognostic factor for platinum resistance (adjusted OR; odd

ratio, 5.94; 95% Confidence Limits, 1.21-29.15), and for poor progression-free survival (PFS; adjusted HR; hazard ratio, 2.07; 95% CI; confidence interval, 1.03-4.41).

Secondly, we identified SERPINB11 as a novel carcinogenesis-related gene in cancerous ovaries of laying hens that had similar characteristics to that detected in women with EOC. SERPINB11 was most abundant in the GE of endometrioid adenocarcinoma of cancerous, but not normal, ovaries of laying hens. In addition, bisulfite sequencing revealed that about 30% of -110 CpG sites are methylated in ovarian cancer cells, whereas those -110 CpG sites are demethylated in normal ovarian cells. On the other hand, in human ovarian cancer cells such as OVCAR-3, SKOV-3 and PA-1 cells, immunoreactive SERPINB11 protein was predominantly in the cytoplasm and had a similar expression pattern to that in ovarian cancer cells of laying hens.

The third candidate gene studied was GAL11 also known as avian beta-defensins (AvBDs). AvBDs are small cationic peptides having three cysteine disulphide bonds between their cysteine residues. They play essential roles in the innate immune system and also stimulate proliferation of epithelial cells and fibroblasts. Although we found the avian homolog of human beta-defensin 11 little is known about its expression in cancerous ovaries of laying hens. Results of this study determined that GAL11 is most abundant in the glandular epithelium of endometrioid-type ovarian tumors, but not normal ovaries of hens. In addition, *miRNA-1615* was discovered to influence GAL11 expression via its 3'-UTR which suggests post-

transcriptional regulation of GAL11 expression in chickens.

Fourth, we demonstrated expression of SPP1, a highly phosphorylated protein containing a polyaspartic acid sequence and a conserved RGD motif. SPP1 plays important roles in physiological processes such as inflammatory responses, calcification, organ development, immune cell function and carcinogenesis. Results of the present study indicate that *SPP1* mRNA and protein are significantly more abundant in GE of ovarian endometrioid carcinomas, but not in other cancerous and normal ovaries of hens. Further, *miRNA-140* was discovered to influence *SPP1* expression via its 3'-UTR which suggests post-transcriptional regulation of *SPP1* expression in chickens.

Finally, we identified A2M (also known as ovostatin) that has the unique feature of inactivating/inhibiting most known proteases including serine-, threonine-, cysteine-, aspartic- and metalloproteases. In this study, we determined expression patterns of *A2M* in normal and cancerous ovaries from laying hens. Expression of *A2M* was most abundant in GE of endometrioid adenocarcinomas of cancerous, but not normal ovaries of laying hens based on results from quantitative RT-PCR and *in situ* hybridization analyses.

Collectively, the present results indicate that expression of SERPINB3, SERPINB11, GAL11, SPP1 and A2M is clearly associated with the development of ovarian carcinogenesis. These results provide new insights into the prognostic biomarkers for epithelial-derived ovarian cancer to diagnose and to evaluate responses

to therapies for treatment of EOC in humans. Therefore, target genes, especially SERPINB3, may play an important role in ovarian carcinogenesis and be a novel biomarker for predicting platinum resistance and a poor prognosis for survival in patients with EOC.

**Keywords:** epithelial-derived ovarian cancer, SERPINB3, SERPINB11, GAL11, SPP1, A2M

**Student Number:** 2010-22859

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## LIST OF ABBREVIATION

<b>3'UTR</b>	3' untranslated region
<b>A2M</b>	Alpha 2 macroglobulin
<b>ACTB</b>	Beta-actin
<b>APES</b>	3-aminopropyltriethoxysilane
<b>AvBD-11</b>	Avian beta-defensin 11
<b>BCL2</b>	B-cell lymphoma 2
<b>BMP7</b>	Bone morphogenetic protein 7
<b>BSA</b>	Bovine serum albumin
<b>CA125</b>	Cancer antigen 125
<b>CASP</b>	Caspase
<b>CTS</b>	Cathepsin
<b>cDNA</b>	Complementary DNA
<b>CI</b>	Confidence interval
<b>COX</b>	Cyclooxygenase
<b>CYP19A1</b>	Cytochrome P450, family 19, subfamily A polypeptide 1
<b>DAPI</b>	4',6-diamidino-2-phenylindole
<b>DEPC</b>	Diethylpyrocarbonate
<b>DES</b>	Diethylstilbestrol
<b>DIG</b>	Digoxigenin
<b>DMEM</b>	Dulbelex and mab-3-related transcription factor 1
<b>DNA</b>	Deoxyribonucleic acid

<b>dNTP</b>	Doxyribonucleotid triphosphate
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>EGF</b>	Epidermal growth factor
<b>EGFR</b>	Epidermal growth factor receptor
<b>EOC</b>	Epithelial ovarian cancer
<b>ESR1</b>	Estrogen receptor alpha
<b>ESR2</b>	Estrogen receptor beta
<b>ERBB2</b>	Erythroblastic leukemia viral oncogene homolog2
<b>FACS</b>	Flow cytometry
<b>FE-SEM</b>	Field emission-scanning electron microscopy
<b>FIGO</b>	International Federation of Gynecologist
<b>FOXL2</b>	Forkhead transcription factor L 2
<b>FSH</b>	Follicle stimulating hormone
<b>FSHR</b>	Follicle stimulating hormone receptor
<b>GAPDH</b>	Glyceraldehyde-3-phosphate dehydrogenase
<b>GE</b>	Glandular epithelium
<b>GFP</b>	Green fluorescent protein
<b>HR</b>	Hazard ratio
<b>IHC</b>	Immunohistochemistry
<b>IgG</b>	Immunoglobulin G
<b>LE</b>	Luminal epithelium
<b>LH</b>	Luteinizing hormone
<b>LHCGR</b>	Luteinizing hormone/choriogonadotropin receptor

<b>LPM</b>	Lateral plate mesoderm
<b>miRNA</b>	MicroRNA
<b>mRNA</b>	Messenger RNA
<b>MUC16</b>	Mucin 16
<b>MAPK</b>	Mitogen-activated protein kinase
<b>OR</b>	Odds ratio
<b>OS</b>	Overall survival
<b>PBS</b>	Phosphate buffered saline
<b>PDGF</b>	Platelet-derived growth factor
<b>PFS</b>	Progression-free survival
<b>PI3K</b>	Phosphoinositide 3 kinase
<b>PITX2</b>	Paired-like homeodomain transcription factor 2
<b>RBP</b>	Retinol binding protein
<b>RNA</b>	Ribonucleic acid
<b>RT-PCR</b>	Reverse transcriptase-polymerase chain reaction
<b>qRT-PCR</b>	Quantitative reverse transcriptase-polymerase chain reaction
<b>SCCA1</b>	Squamous cell carcinoma antigen 1
<b>SCCA2</b>	Squamous cell carcinoma antigen 2
<b>SERPINB3</b>	Serpin peptidase inhibitor, clade B, member 3
<b>SERPINB11</b>	Serpin peptidase inhibitor, clade B, member 11
<b>SFK</b>	Src family kinase
<b>SHH</b>	Sonic hedgehog
<b>SMAD5</b>	SMAD family member 5

<b>SNCA</b>	Synuclein alpha
<b>SOX9</b>	Sry-box 9
<b>SPP1</b>	Secreted phosphoprotein 1
<b>SSC</b>	Standard saline citrate
<b>TMA</b>	Tissue microarray
<b>TOM1L1</b>	Target of myb 1 (chicken)-like 1
<b>tRNA</b>	Transfer RNA
<b>TTR</b>	Transthyretin
<b>WL</b>	White Leghorn
<b>ZEB1</b>	Zinc finger E-box binding homeobox 1

# **CHAPTER 1**

## **Literature Review**

# 1. Epithelial-Derived Ovarian Cancer

## 1.1. General Characteristics

Ovarian cancer is the most fatal gynecological carcinoma even though it is the 8<sup>th</sup> most commonly diagnosed cancer and the 7<sup>th</sup> leading cause of cancer-related deaths in women worldwide. It accounts for 3.8% of the total cancer cases and 4.2% of the cancer-related deaths in women (Jemal et al., 2011). Ovarian cancer is very difficult to diagnose at an early stage which leads to International Federation of Gynecologist (FIGO) Stages III-IV disease at the time of diagnosis in approximately 70% of patients with ovarian cancer. In addition, only 30% of patients with ovarian cancers might be expected to survive for five years (Goodman et al., 2003; Heintz et al., 2006). This is because of the lack of specific symptoms and specific biomarkers for early detection of ovarian cancer in women (Bast et al., 2002; Pepe et al., 2001). Therefore, to overcome these limitations, the experimental methods for the early detection of EOC and predictors for prognosis for treatment to increase survival of patients are increasingly required from various fields of study.

Ovarian cancer can be divided into three tumor types which are epithelial tumors, sex-cord stromal tumors and germ cell tumors (Kurman and Shih, 2008; Kurman et al., 2008). Among the rest, more than 90% of human malignant ovarian cancers are derived from the germinal epithelium of the ovary. The etiology of epithelial-derived ovarian cancer (EOC) is not well known. The likelihood of developing EOC is associated with several factors such as age, genetics, epigenetics,

hormones and others. Previous studies suggest that the major causative factor of EOC is incessant ovulation which contributes to increased risk for genetic aberrations to the ovarian surface epithelium in response to repeated rupture and repair of the epithelial surface of the ovary (Auersperg et al., 2001; Fathalla, 1971). According to this hypothesis, taking oral contraceptives for more than five years and multiparity can reduce the incidence of ovarian cancer by suppressing ovulation and controlling hormone levels (Hippisley-Cox and Coupland, 2012; Purdie et al., 2003).

Another cause for development of EOC is family history. There are three types of hereditary ovarian cancer syndromes: 1) hereditary site-specific ovarian cancer, 2) hereditary nonpolyposis colorectal cancer, and 3) hereditary breast-ovarian cancer (Auersperg et al., 1997). Breast cancer is particularly closely associated with ovarian cancers. Women with breast cancer or family histories of breast/ovarian cancer have a 50% increase in the incidence of ovarian cancer (Auersperg et al., 2001). There are two notable genes in this mechanism, breast cancer genes 1 (BRCA1) and BRCA2, which regulate DNA damage response. Mutations of BRCA1 and BRCA2 genes increase the probability of developing breast and ovarian cancers (Kote-Jarai and Eeles, 1999; Xu and Solomon, 1996). Moreover, there are various forms of regulation at the levels of molecular genetics and epigenetics in ovarian carcinogenesis such as point mutations, deletions, gene amplifications, translocations, suppression of gene expression by miRNAs, changes in DNA methylation and acetylation and gene silencing, all of which are key factors increasing the risk of cancer.

## 1.2. Classifications

Epithelial-derived ovarian cancer is classified as follows: serous, endometrioid, mucinous and clear cell tumors based on tumor cell morphology and histology (Kaku et al., 2003). Serous carcinoma is the most common of EOC with specific characteristics that include multiple cysts, solid areas, glands and parts of papillae. Malignant serous carcinomas account for approximately 30% of ovarian serous carcinomas and nearly 70% of all EOCs. Most serous carcinomas are large and form bilaterally (Chen et al., 2003; Cho and Shih Ie, 2009). In development of ovarian serous carcinoma, a few gene mutations have been identified. For example, mutations in tumor protein 53 gene is frequently associated with malignant serous carcinomas (Singer et al., 2005). And V-KI-RAS2 kirsten rat sarcoma viral oncogene homolog (KRAS) and V-RAF murine sarcoma viral oncogene homolog B1 (BRAF) gene mutations exist in the early grade serous carcinomas and they lead to activation of the mitogen activated protein kinase (MAPK) signaling pathway (Singer et al., 2003).

The next most common EOC is endometrioid carcinomas that make up 10-20% of all ovarian cancers. This type of cancer is composed of epithelial and stromal cells that resemble those of the endometrium. These tumors are associated with endometriosis due to genetic alterations and hyperplasia of the endometrium (Depriest et al., 1992; Fukunaga, 2008). In addition, endometrioid carcinomas have glands, solid masses or a fibrous consistency. The endometrioid carcinomas are related to various alterations in molecular genetics including the mutation of oncogenes, tumor suppressor genes and other genes associated with DNA repair. For example, activating

mutations of a key effectors of the wingless-type MMTY integration site family (WNT) signaling pathway and, catenin beta 1 (CTNNB1), as well as inactivating mutations of tumor suppressor gene, phosphatase and tensin homolog (PTEN), have been detected mainly in endometrioid carcinomas. Both of them are rare in the other types of ovarian cancers (Catasus et al., 2004; Wright et al., 1999).

The third most common EOC is the mucinous carcinomas which occur in a small percentage (3%) of primary ovarian carcinomas. Mucinous carcinomas are composed of papillae and solid areas, mucin-riched cytoplasm and large areas of necrosis and hemorrhage. Histologically, mucinous carcinomas are characterized with glands and cysts including abundant cytoplasmic mucins (Hart, 2005). The mechanism responsible for development of mucinous carcinomas has not been established; however mutations in the KRAS gene are commonly associated with mucinous ovarian tumors. This analysis indicates that KRAS mutations might be early events in the development of mucinous tumors (Cuatrecasas et al., 1997).

Clear cell carcinomas account for approximately 10% of EOC. Most ovarian clear cell carcinomas are malignant, as benign and borderline tumors are uncommon. Clear cell carcinomas are composed of clear cells that develop as tubular, papillary, solid or mixed types and hobnail cells which contain apical nuclei. Most of tumors are solid or cystic masses with one or more nodules protruding into the lumen (Cho and Shih Ie, 2009; Sugiyama et al., 2000; Tammela et al., 1998). In clear cell carcinomas of the ovary, the following genetic mutations have been found as follows: mutations of PIK3CA (20-25%), TP53 (8.3%), PTEN (8%) and BRAF (6.3%) (Campbell et al.,

2004; Mayr et al., 2006; Sato et al., 2000; Willner et al., 2007). In addition, these type of tumor are associated with over-expression of numerous genes such as HFF1 homeobox 1B (HNF-1B), SPP1, neuraminidase 3 (NEU3) and annexin A4 (Kajihara et al., 2010; Zorn et al., 2005).

## **2. Animal Models for Epithelial-Derived Ovarian Cancer**

### **2.1. Mammals**

The majority of women diagnosed at an advanced stage of EOC have a high probability of dying from the disease. EOC is associated with complex genetic and epigenetic alterations leading to ovarian cancer. Thus, it is very important to identify mechanism leading to initiation, promotion and progression of EOC. It is difficult to establish etiologies and pathogenesis of EOC in women; therefore, exploitation of animal models for EOC is essential.

Characteristics of the reproductive tract of large domestic animals, including bovine and porcine, are similar to humans. There are few reports on the development of EOC in livestock species as occurs in women. This low incidence may be due to multiple pregnancies, and longer periods of gestation and lactation making them unsuitable models for research on human ovarian cancer (Barua et al., 2009).

Primate models that develop non-spontaneous EOC are valuable to study

regulation of human EOC because their ovarian cancers have high similarity to human EOC in terms of anatomy, physiology, genetics and hormonal regulation of the reproductive organs (Connolly, 2009). In cynomolgus macaques taking oral contraceptives apoptosis was stimulated in the ovarian epithelium by progestins (Hankinson et al., 1992; Rodriguez et al., 2002). In addition, rhesus macaques were used to verify chemopreventive activity of therapeutic agents for treatment of ovarian cancer. The results revealed increased levels of the coenzymes NAD and FAD that lead to high redox potential and less hypoxia in the ovaries (Brewer et al., 2001a; Brewer et al., 2001b). Moreover proliferation of cells in EOC in rhesus macaque was stimulated to enter cell cycle arrest via induction of Rb, p53 and p21 by estrogen (Wright et al., 2003, 2005)

## **2.2. Rodents**

Genetically manipulated rodent models of each subtype of ovarian cancer have been used to improve knowledge of the etiologies and pathogenesis of EOC and confirm effects in preclinical tests of signal transduction inhibitors as potential therapeutic agents (Cho and Shih Ie, 2009; Stakleff and Von Gruenigen, 2003; Vanderhyden et al., 2003).

The rodent models for investigation of EOC were first established by genetically engineering changes in one or more oncogenes within mouse ovarian cells to generate mice with wild type or mutant genes (Trp53<sup>+/+</sup> or Trp53<sup>-/-</sup>) (Orsulic et al., 2002). These results showed that at least two oncogenes were needed to transform

ovarian surface epithelium and this occurred only in Trp53 null cells. Then, the novel technique for delivering recombinase directly to the ovarian surface epithelium cell layer was improved by Flesken-Niktin *et al* (2003) to inactivate the Trp53 and Rb genes. This study showed that injection of an adenoviral vector expressing Cre recombinase for conditional knockout of Trp53 and Rb alleles caused development of ovarian cancer (Flesken-Niktin *et al.*, 2003). Moreover, Connolly *et al* (2003) provided another approach by manipulating Mullerian inhibitory substance type 2 receptor promoter (MISIIR) to drive the simian virus 40T (SV40T) in transgenic mice. This technique resulted in EOC in 50% of the mice (Connolly *et al.*, 2003). However, the fact that EOC does not occur spontaneously in rodent models limits their clinical relevance (Barua *et al.*, 2009).

### **2.3. Birds**

The laying hen is a valuable model for investigation of EOC because they develop EOC spontaneously at a high rate after producing eggs when more than two years of age. Similarly, natural menopause usually arises between 40- and 55-years of age in women when production of female steroid hormones, estrogen and progesterone, is decreasing with advancing age of their ovaries. Incessant ovulation in laying hens (almost every day) and women (once a month) is considered the major causative factor of EOC (Barua *et al.*, 2009; Damjanov, 1989; Fredrickson, 1987).

Prognostic molecular markers commonly expressed in human EOC are also detected in EOC of laying hens as hallmarks of ovarian cancer. First, ovarian cancers

in laying hens express ovarian cancer associated antigen (CA125, also known as MUC16) that is the most important biomarker of EOC in women (Jackson et al., 2007). Also, proliferation markers including proliferating cell nuclear antigen (PCNA) and cytokeratins, transforming growth factor alpha (TGFA), a proto-oncogene (ERBB2), a growth factor receptor (EGFR), a cell cycle inhibitor (p27) and oncofetal tumor markers (CEA, Lewis Y and Tag 72) are detected in both EOC of both women and laying hens (Rodriguez-Burford et al., 2001).

Ovarian carcinomas of the laying hen model presents histopathologically with serous, endometrioid, mucinous and clear cell carcinomas as occurs in women. Furthermore, the stages of ovarian cancer in laying hens are similar to that for EOC in women based on the following FIGO system classifications (Barua et al., 2009; Heintz et al., 2006). Stage I of EOC in laying hens indicates tumor growth limited to the ovary, firm nodules and little or no ascites. For stage II EOC in laying hens, ovarian tumors are larger and have metastasized to the oviduct with moderate ascites. Next, Stage III ovarian cancer in laying hens shows metastasis of the tumor to the pelvic organs, as well as peritoneal and abdominal organs including small and large intestine and mesentery and surface of the liver with copious ascites. Stage IV EOC in laying hens is characterized by severe metastasis to distant organs such as liver, lung and spleen with multiple solid tumors and copious ascites (Barua et al., 2009). Therefore, the laying hen is the only animal model that develops EOC spontaneously from surface epithelium of the ovaries at a incidence rate due to incessant ovulations and can be used for investigations to develop therapeutic agents for prevention and or treatment of EOC.

### **3. Estrogen Action in Female Reproductive System**

#### **3.1. Molecular Mechanisms of Estrogen**

Estrogen is the most important steroid hormone in the avian female reproductive tract as a primary sex hormone. It is an 18-carbon steroid molecule that includes an aromatic A ring with a phenolic hydroxyl group. Biosynthesis of estrogen is initiated from cholesterol that is metabolized by theca cells to produce androgens that are then converted to estrogens by granulosa cells that have the aromatase enzyme and secretion of estrogens is regulated by luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Hewitt et al., 2005; Hillier et al., 1994).

In general, estrogen plays crucial roles in the modification of several cell-types with respect to development and differentiation, altering expression of specific genes in a variety of organs, and regulation of various biological events including protection against apoptosis, osteoporosis, diabetes and Alzheimer's disease (Hewitt et al., 2005; Louet et al., 2004; Wise et al., 2005). For these biological actions, estrogen binds two classical nuclear receptors, estrogen alpha (ESR1) and beta (ESR2) (Nilsson et al., 2001).

Estrogen receptors have three functional domains, N-terminal domain (or A/B domain), DNA binding domain (C domain) and ligand-binding domain (E/F domain). These receptors activate and recruit various transcription factors that bind directly to estrogen response elements (ERE) in the 5'-flanking region of target genes

(Dougherty and Sanders, 2005; Hewitt et al., 2005; Nilsson et al., 2001). Moreover, indirect effects of the estrogen receptors resulting in gene transcription occur in response to interactions with SP1, AP1 and NF- $\kappa$ B proteins (Nilsson et al., 2001).

### **3.2. Effects of Estrogen in the Oviduct of Avian Models**

To demonstrate the biological functions and signaling pathways for estrogen, the oviduct of chicken, an oviparous animal, is a remarkable model for investigation of estrogen-related mechanisms (Dougherty and Sanders, 2005). Estrogen is well-known for induction of development and maintenance of the cell proliferation in the chicken oviduct. Physiologically, estrogens stimulate differentiation of three cell types in the oviduct of immature chicks as follows: 1) tubular glands cells produce major egg-white proteins called ovalbumin, ovotransferrin and ovomucoid, 2) goblet cells synthesize avidin in response to joint actions with progesterone and 3) ciliated cells provide for motility of, for example, sperm (Dougherty and Sanders, 2005; Palmiter and Wrenn, 1971b; Socher and Omalley, 1973). High concentrations of estrogen are involved in ovulation and oviposition of eggs in laying hens (Lague et al., 1975). In addition, estrogen modulates development of the preovulatory follicle and production of yolk proteins within liver that are transported to and incorporated into the yolk of the egg (Evans et al., 1988; Yoshimura et al., 1995) and calcium metabolism for egg shell formation and ovipositioning by interacting calcium-binding proteins (calbindins) and epithelial calcium channels (TRPVs) (Bar, 2009; Hincke et al., 2010).

Diethylstilbestrol (DES), a synthetic non-steroidal estrogen, binds directly to estrogen receptors to mimic effects of natural estrogens on development and functions of the chicken oviduct (Dougherty and Sanders, 2005; Seo et al., 2009; Song et al., 2011). Exposure of chicks to DES pellets placed subcutaneously in the abdominal region stimulates growth, development and differentiation of the immature chick oviduct to the adult form (Seo et al., 2009). In accordance with those results, DES treatment of chickens induced the oviduct to increase expression of egg white protein genes including those for ovalbumin, ovomucoid, lysozyme, hep21 (Oka and Schimke, 1969b; Song et al., 2011), SERPINB3 (Lim et al., 2012a) and SERPINB11 (Lim et al., 2011c) and development or oviposition-related genes such as A2M (Lim et al., 2011a), AHCYL1 (Jeong et al., 2012b), PTN (Lee et al., 2012a) and SPP1 (Lim et al., 2012d). However, continuous exposure of the immature chick oviduct to DES gives rise to physiological abnormalities in the reproductive organs.

### **3.3. Effects of Estrogen in the Ovarian Cancer**

Reproductive hormones, including gonadotropins and steroids hormones, affect the risk for development of ovarian cancer (Salehi et al., 2008). Estrogen, in particular, has long been implicated as a factor inducing ovarian cancer. For instance, menopausal women who have taken estrogen as hormone replacement therapy have an increased risk of ovarian cancer (Lacey et al., 2002) whereas women who have taken oral contraceptives for more than 5 years have a reduced incidence of ovarian cancer during premenopausal years (Schildkraut et al., 2002; Spillman et al., 2010)

High levels of estrogen can change immune response, phagocytic activity, growth factor levels and differentiation of cancer cells (Hrushesky et al., 1988). For example, estrogen increases angiogenesis that is one key feature of cancer development by promoting secretion of vascular endothelial growth factor (VEGF) and endothelial cell migration (Cullinan-Bove and Koos, 1993; Hyder et al., 2000). In addition, estrogen regulates expression of hepatocyte growth factor (HGF) (Liu et al., 1994) and epidermal growth factor (EGF), both of which activate proliferation of ovarian surface epithelial cells (Hsueh et al., 1981).

In animals, incessant exposure of the reproductive tract and mammary glands to estradiol induces development of papillary ovarian carcinomas in guinea pigs and rabbits that are similar to human benign serous carcinomas (Bai et al., 2000; Silva et al., 1998). Also, estrogen can increase proliferation of ovarian surface epithelial cells in ewes (Murdoch and Van Kirk, 2002).

Both ESR1 and ESR2 have been reported to be expressed in human ovarian cancers (Karlan et al., 1995). In the four subtypes of EOC, ESR1 was expressed abundantly in endometrioid carcinomas (100%) and detected in serous (97%) and mucinous (70%) carcinomas by immunohistochemical analysis. Moreover, expression of ESR1 was higher in malignant EOC than in ovaries with benign tumors and normal ovaries (Auersperg et al., 2001; Fujimura et al., 2001). In contrast, ESR2 is expressed in all types of EOC in sequence as follows: endometrioid, serous, clear cell, mucinous carcinomas (Fujimura et al., 2001; Ho, 2003).

On the other hand, the exact mechanisms of estrogen action are unknown regarding development of ovarian cancer. Therefore, advanced studies are required to verify the relationship between estrogenic activity and expression of its receptors and the etiology and pathogenesis of EOC.

## **4. Biological Roles of MicroRNAs in Development and Differentiation**

### **4.1. MicroRNAs in Epithelial-Derived Ovarian Cancer**

MicroRNAs (miRNAs) are small and non-coding single stranded RNAs. They consist of 18-23 nucleotides that are post-transcriptional regulators and transform cell fate through modulation of target-mRNA translation in various cells and tissues by binding partial sequences in the 3' untranslated region of target genes. In other words, miRNAs are known to control a variety of biological events such as growth, development, differentiation, oncogenesis, angiogenesis and cell cycle by regulating gene expression. They function through diverse mechanisms including inhibition of translation elongation and degradation of target mRNAs (Bartel, 2009a; Dang et al., 2013; Garzon et al., 2006a; Gregory et al., 2005b).

Mechanisms of oncogenesis are very complex with genetic and epigenetic processes changing expression of oncogenic and tumor suppressor genes via various mechanisms. An example of one epigenetic factor is miRNAs involved in the

initiation and progression of tumors through effects on oncogenes and tumor suppressor genes (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006). For example, the deletion and down-regulation of *miR-15a* and *miR-16-1* causes overexpression of BCL2 gene that is frequently shown to increase in level of expression in various human cancers through actions as an anti-apoptotic gene (Cimmino et al., 2006). In addition, *let-7* family members, first demonstrated to be onco-miRNAs, regulate the expression of the RAS oncogene that usually shows highly increased levels in lung cancer cells as compared to normal cells due to mutations in RAS genes (Johnson et al., 2005). So, transfection of *let-7* in lung cancer cells can protect from development of lung cancer or reduce tumor size if cells have RAS mutations (Esquela-Kerscher and Slack, 2006). Moreover, the MYC oncogene which regulates cell proliferation and apoptosis induces B-cell cancer through correlation with *miR-155* (Eis et al., 2005; Kluiver et al., 2005). Also collaboration between MYC oncogene and *miR-17-92* causes amplification of B-cell tumorigenesis (He et al., 2005). Furthermore, it is remarkable that several miRNAs (*miR-20*, *miR-92a*, *miR-93*, *miR-126*, *miR-132*, *miR-218* and *miR-221*) control intracellular signaling pathways downstream of vascular endothelial growth factors (VEGFs) that are remarkable regulators of vascular development and maintenance of carcinogenesis (Dang et al., 2013).

The miRNAs also regulate gene expression at post-transcription levels in EOC. Compared with normal ovaries, abnormal expression of miRNAs has been demonstrated in human EOC. For example, *miR-200a*, *miR-200b*, *miR-200c* and *miR-141*, *miR-429* are expressed in the epithelial phenotype of cancer cells by targeting

ZEB1 and ZEB2 that are E-cadherin repressor proteins and overexpressed in human endometrioid ovarian tumors (Iorio et al., 2007; Mateescu et al., 2011). In addition, the expression of *miR-21*, *miR-203* and *miR-205* is up-regulated in EOC as compared to normal ovaries of women and the abundance of these miRNAs increase considerably after treatment of 5-aza-2'-deoxycytidine to demethylate OVCAR3 cells. These results suggest that DNA hypomethylation might be involved in the mechanism for over-expression of oncogenic miRNAs (Iorio et al., 2007; Lou et al., 2010; Taylor and Gercel-Taylor, 2008). On the other hand, there are down-regulated miRNAs leading to an increase in cellular events. For instance, *miR-9*, *miR-15a*, *miR-22*, *miR-152* are suppressed in ovarian cancer cell lines and this repression is associated with increases invasion, migration and proliferation of the cancer cells (Bhattacharya et al., 2009; Guo et al., 2009; Li et al., 2010; Zhou et al., 2012).

In accordance with previous studies, the cancer-related miRNAs expressed aberrantly or mutated in various cancers might have crucial roles as modifiers of expression of oncogenes or tumor suppressor genes that regulate their target genes.

## **4.2. MicroRNAs in Oviduct**

For successful reproduction, regulation of development and differentiation of the female reproductive tract requires transcription, translation and post-transcriptional modifications of gene products (Nothnick, 2012). MicroRNAs function in post-transcriptional regulation of their target genes and influence growth, development and differentiation of various organs (Bartel, 2004). Therefore, the relationship between

miRNAs and maintenance of function of the female reproductive tract has been researched actively by many groups to understand and improve regulatory mechanisms affecting fertility.

Dicer (also known as Dicer1), ribonuclease III endonuclease, is considered an essential factor for the synthesis miRNAs. These miRNAs associate with the RNA-induced silencing complex (RISC) to regulate gene expression post-transcriptionally by binding of the miRNAs to 3'-UTR of complementary mRNA targets indicating perfect base-pairing (Cannell et al., 2008). In development of the female reproductive tract, Dicer plays an important role. Its function was originally verified by results indicating that loss of Dicer caused infertility in mutant female mice with a hypomorphic Dicer allele (Otsuka et al., 2008). By regulating Mullerian duct mesenchyme-derived tissues with the *Amhr2-Cre* allele (Nagaraja et al., 2008), Dicer-deficient female mice were found to have degenerated oocytes and oviductal cysts (Gonzalez and Behringer, 2009) and degenerated follicular granulosa cell (Lei et al., 2010). Dicer is regarded as being essential for maintenance of function of the reproductive tract of females.

However, there is limited information on expression, regulation and function of miRNAs in the human oviduct. Loss of Dicer expression and decreased expression of miRNAs indicated significant effects on oviductal phenotype and infertility and the results of these studies suggest that *miR-29a*, *miR-29b*, *miR-143* and *miR-145* might modulate development of the oviduct as key regulators of post-transcriptional events (Gonzalez and Behringer, 2009; Hong et al., 2008; Nagaraja et al., 2008).

In chickens, there are several miRNAs that may intimately regulate mechanisms for development and differentiation of the oviduct by modulating genes required for oviduct development (Jeong et al., 2012b; Lee et al., 2012a; Lim et al., 2012d; Lim et al., 2013). Some of these miRNAs are affected by estrogen as *miR-124a*, *miR-1615* and *miR-1669* are down-regulated in the chicken oviduct in response to estrogen whereas *miR-1710* and *miR-1782* are up-regulated during development of chicken oviduct treated with estrogen (Jeong et al., 2012b; Lim et al., 2013). Based on previous reports, miRNAs might have essential functions during morphogenesis of the chicken oviduct. On the other hand, few miRNAs have been investigated to determine their effects on modulating target genes in the oviduct so their biological mechanisms of action are unknown.

## **5. Candidate Biomarkers for Epithelial-Derived Ovarian Cancer**

### **5.1. Serpin Peptidase Inhibitor, Clade B, Member 3 (SERPINB3)**

SERPINB3, also known as squamous cell carcinoma 1 (SCCA1), was discovered originally in squamous cell carcinoma of the cervix (Kato and Torigoe, 1977). It belongs to the serpin superfamily of protease inhibitors related to apoptosis, immune response, blood coagulation, cell migration and invasiveness of cells (Gettins,

2002b; Suminami et al., 1991). SERPINB3 regulates programmed cell death through different biological process in diverse cancer types and over-expression of this gene is one characteristic of epithelial-derived cancerous cells. SERPINB3 decreases apoptosis mediated by carcinostatis substances and by TNFA -induced cell death by suppressing cytochrome c release from the mitochondria (Hashimoto et al., 2005; Kato, 1996). In addition, in apoptosis mechanisms, SERPINB3 is upstream of caspase-3, one of its molecular targets, which attenuates caspase-3 activity and apoptosis (Suminami et al., 2000). Moreover, SERPINB3 specifically modulates activity of c-Jun NH<sub>2</sub>-terminal kinase-1 (JNK-1) (Katagiri et al., 2006). Therefore, SERPINB3 might be regarded cellular factor with multiple functions related to apoptotic regulation in cancer development.

## **5.2. Serpin Peptidase Inhibitor, Clade B, Member 11 (SERPINB11)**

SERPINBs are one of group in the serpin superfamily of serine and cysteine proteinase inhibitors having crucial roles in various biological events such as blood coagulation, angiogenesis, inflammation and fibrinolysis (Askew et al., 2007). Most clade B serpin genes are intracellular proteins that primarily suppress target proteases whereas SERPINB5 and SERPINB11 are intracellular non-inhibitory proteins (Askew et al., 2007; Bird et al., 2001; Luo et al., 2007). SERPINB5 is a class II tumor suppressor gene called as maspin (mammary serine protease inhibitor). This gene was demonstrated to induce apoptosis of breast and prostate cancer cells (Luo et al., 2007; Zou et al., 1994). Moreover, methylation of the 5' flanking region of SERPINB5

causes gene silencing in colorectal, ovarian, skin and thyroid carcinomas (Bettstetter et al., 2005; Boltze et al., 2003; Khalkhali-Ellis, 2006). Unlike SERPINB5, SERPINB11 functions as an inhibitor of angiogenesis through repressing endothelial cell migration and controlling mitogenesis (Zhang et al., 2000). However, a functional role of SERPINB11 development cancer through interacting with other types of proteases is not known.

### **5.3. Gallicin 11 (GAL11)**

GAL11 (also known as beta-defensin 11; DEFB11) belongs to avian defensins that are members of the beta-defensin subfamily members that exhibit antimicrobial activity against microbes including gram-positive/-negative bacteria or fungi (Fukunaga, 2008; Harwig et al., 1994; van Dijk et al., 2008; Xiao et al., 2004). Avian beta defensin genes identified in chicken leukocytes can be subdivided into 14 classes (Abdel Mageed et al., 2009). Among them, GAL11 expression increases significantly in response to lipopolysaccharides (Mageed et al., 2008) and DES (Song et al., 2011) in chicken. On the other hand, there is no previous report on the relationship between GAL11 and cancer.

In mammals, there are several reports on identification of the role of beta-defensins in carcinogenesis. First of all, the low expression of human beta-defensin 1 (DEFB1) is involved in renal cell carcinomas, prostate cancer, basal cell carcinomas and oral squamous cell carcinomas as a tumor suppressor (Donald et al., 2003; Gambichler et al., 2006; Joly et al., 2009). And overexpression of DEFB3 increases

development of oral cancer through recruitment of macrophages via EGF that induces DEFB3 expression (Kesting et al., 2009). In addition, DEFB2 and DEFB3 function as proto-oncogenes in oral squamous cell carcinomas, whereas DEFB1 works as a tumor suppressor gene (Winter et al., 2011). With these results, it is possible to suggest that beta-defensins influence carcinogenesis through alteration of inflammation and cytokine production.

#### **5.4. Secreted Phosphoprotein 1 (SPP1)**

SPP1 (also called as osteopontin), is a highly phosphorylated integrin-binding ligand and N-linked glycoprotein originally isolated from bones of rats (Butler, 1989). This gene has crucial functions in a variety of physiological processes including cell to cell interactions, inflammatory responses, wound healing, calcification, morphogenesis of organs and tumorigenesis (Sodek et al., 2000). In blood, increases in SPP1 are associated with several types of cancers (Hotte et al., 2002; Le et al., 2003). Especially, in development of ovarian cancer, SPP1 expression increased abundantly as compared with normal ovaries. In addition, its expression was localized predominantly to serous carcinoma which is one of subtype of EOC (Kim et al., 2002). Results of clinical experiments with postoperative patients also indicated that SPP1 is a biomarker for not only detecting specific types of ovarian cancer, but also a marker for examination of responses to primary treatments for cancer in place or in addition to the use of CA125 as a biomarker for cancer.

#### **5.5. Alpha-2-Macroglobulin (A2M)**

The alpha 2 macroglobulins (A2M) function as protease inhibitors in serum of mammals and are able to bind a variety of cytokines and growth factors (Armstrong, 2006; Sottrup-Jensen, 1989; Tayade et al., 2005; Vanleuven et al., 1988). Proteases and their inhibitors take part in various biological events such as oncogenesis and metastasis because of their capacity to degrade extracellular matrix proteins (Lopez-Otin and Matrisian, 2007). Similar to other protease inhibitors, A2M is increased in plasma of women with inflammatory and neoplastic lesions of the ovary (Zbroja-Sontag, 1983). In addition, A2M increases in blood of laying hens more than 6 months prior to detection of advanced-stage EOC whereas A2M suppresses DNA synthesis in mouse ovarian tumor cells as a cytotoxic factor in serum (Hawkrige et al., 2010; Koo, 1983a, b). These results suggest that increased levels of A2M in plasma of laying hens develop in the late-stages of ovarian cancer as compared with its concentration in serum of normal laying hens (Hawkrige et al., 2010). According to various lines of evidence, A2M might be a novel biomarker for improvements in early detection of ovarian cancer.

## **CHAPTER 2**

# **SERPINB3 in the Chicken Model of Ovarian Cancer: A Prognostic Factor for Platinum Resistance and Survival in Patients with Epithelial Ovarian Cancer**

## 1. Abstract

Serine protease inhibitors (SERPINs) appear to be ubiquitously expressed in a variety of species and play important roles in pivotal physiological processes such as angiogenesis, immune responses, blood coagulation and fibrinolysis. Of these, squamous cell carcinoma antigen 1 (SCCA1), also known as a SERPINB3, was first identified in squamous cell carcinoma tissue from the cervix of women. However, there is little known about the SERPINB3 expression in human epithelial ovarian cancer (EOC). Therefore, in the present study, we investigated the functional role of *SERPINB3* gene in human EOC using chickens, the most relevant animal model. In 136 chickens, EOC was found in 10 (7.4%). *SERPINB3* mRNA was induced in cancerous, but not normal ovaries of chickens ( $P < 0.01$ ), and it was abundant only in the glandular epithelium of cancerous ovaries of chickens. Further, several microRNAs, specifically *miR-101*, *miR-1668* and *miR-1681* were discovered to influence *SERPINB3* expression via its 3'-UTR which suggests that post-transcriptional regulation influences *SERPINB3* expression in chickens. SERPINB3 protein was localized predominantly to the glandular epithelium in cancerous ovaries of chickens, and it was abundant in the nucleus of both chicken and human ovarian cancer cell lines. In 109 human patients with EOC, 15 (13.8%), 66 (60.6%) and 28 (25.7%) patients showed weak, moderate and strong expression of SERPINB3 protein, respectively. Strong expression of SERPINB3 protein was a prognostic factor for platinum resistance (adjusted OR; odds ratio, 5.94; 95% Confidence Limits, 1.21–29.15), and for poor progression-free survival (PFS; adjusted HR; hazard ratio, 2.07; 95% CI; confidence interval, 1.03–4.41). Therefore, SERPINB3 may play an

important role in ovarian carcinogenesis and be a novel biomarker for predicting platinum resistance and a poor prognosis for survival in patients with EOC.

## 2. Introduction

Epithelial ovarian cancer (EOC) is the 7th leading cause of cancer-related deaths in women worldwide (Jemal et al., 2011; Suh et al., 2012). The clinical importance has grown to a greater extent because there are no relevant symptoms and no effective screening methods for early detection (Kim et al., 2009), which leads to International Federation of Gynecologist (FIGO) Stages III-IV disease at the time of diagnosis in over 75% of patients with EOC (Heintz et al., 2006). Although early-stage disease, well-differentiation, platinum sensitivity and optimal cytoreductive surgery have been suggested as favorable prognostic factors, but their value in prognosis has not been improved markedly (Pignata et al., 2011). Therefore, the early detection of EOC and prediction of prognosis for patient survival using specific biomarkers is increasingly recognized as a better approach to overcome these limitations. For this purpose, genetically manipulated rodent models have been developed to elucidate the etiology and pathogenesis of EOC. However, the artificial nature of the induced tumors in mice limits their clinical relevance (Barua et al., 2009; Stakleff and Von Gruenigen, 2003; Vanderhyden et al., 2003). Meanwhile, the laying hen is well known as the only animal that spontaneously develops tumors from ovarian surface epithelium, and the laying hen is a unique and suitable model to develop novel biomarkers and anti-cancer drugs for patients with EOC (Barua et al., 2009; Stammer et al., 2008; Vanderhyden et al., 2003).

Serpin peptidase inhibitor, clade B, member 3 (SERPINB3) is a member of the serpin superfamily of protease inhibitors involved in apoptosis, immune response,

blood coagulation, cell migration and invasiveness of cells (Gettins, 2002b; Suminami et al., 1991). It is also known as squamous cell carcinoma antigen 1 (SCCA1) first discovered in squamous cell carcinoma of the cervix (Kato and Torigoe, 1977). In humans, the gene for SERPINB3 is located on chromosome 18q21.3, and it inhibits papaine-like lysosomal cysteine proteases, cathepsin K (CTSK), CTSL and CTSS (Gettins, 2002b; Schick et al., 1998). Previously, we identified SERPINB3 in chickens and determined that it has moderate homology to its mammalian protein orthologue (approximately 36-47%). Avian SERPINB3 is expressed in the oviduct in response to estrogen in a tissue- and cell-specific manner (Lim et al., 2012a). Nevertheless, little is known about the expression and prognostic value of SERPINB3 in either chickens or humans with EOC.

MicroRNAs (miRNAs) are small and non-coding RNAs of 18-23 nucleotides in length. Those regulate gene expression post-transcriptionally and are also able to alter cell fate by controlling translation of target mRNAs in diverse tissues and cell types. Therefore, miRNAs play crucial roles in a various biological processes including vertebrate growth, development, differentiation and oncogenesis by regulating gene expression (Bartel, 2004; Garzon et al., 2006a; Gregory et al., 2005b). However, there are not published results of miRNA research related with SERPINB3 in chickens. In order to determine the role of SERPINB3 as a novel biomarker for EOC, we compared the distribution and localization of SERPINB3 between normal and cancerous ovaries of laying hens, and then we performed a miRNA target validation assay to investigate post-transcriptional regulation of SERPINB3 expression. After that we compared SERPINB3 expression among normal and cancer

cells of ovaries from laying hens, human EOC cell lines (OVCAR-3 and SKOV-3) and a human ovarian teratocarcinoma cell line (PA-1). Finally, we investigated the diagnostic and prognostic values of SERPINB3 expression in patients with EOC.

### **3. Materials and Methods**

#### ***Experimental animals and animal care and use***

The experimental use of chickens in the present study was approved by the Institute of Laboratory Animal Resources, Seoul National University. White Leghorn (WL) laying hens were managed according to approved standards for operation of the University Animal Farm, Seoul National University, Korea. All hens had free *ad libitum* access to feed and water.

#### ***Tissue samples in chicken model***

A total 136 laying hens (88 over 36 months and 48 over 24 months of age), which had stopped laying eggs, were euthanized for biopsy and collection of cancerous (n = 10) ovaries. As a control, normal (n= 5) ovaries were collected from egg-laying hens of similar age. We examined tumor stages in 10 hens with cancerous ovaries based on characteristic features of chicken ovarian cancer (Barua et al., 2009). Three hens had Stage III EOC as ovarian tumor cells had metastasized to the gastrointestinal tract and liver surface with profuse ascites in the abdominal cavity. In five hens, their tumors had metastasized to distant organs such as liver parenchyma, lung, gastrointestinal tract and oviduct with profuse ascites which is indicative of Stage IV EOC. The other two hens did not have tumors in any other organs; therefore, their ovarian tumors were classified as stage I EOC. Subsets of these samples were fixed in 4% paraformaldehyde for further analyses. After 24 h, fixed tissues were

changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Hens with EOC were classified based on their cellular subtypes and patterns of cellular differentiation with reference to human ovarian malignant tumor types (Barua et al., 2009).

### ***RNA isolation***

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

### ***Semi-quantitative RT-PCR analysis***

The expression of *SERPINB3* mRNA in normal and cancerous ovaries of hens was assessed using semi-quantitative RT-PCR as described previously (Song et al., 2007). Complementary DNA (cDNA) was synthesized from total cellular RNA (2 µg) using random hexamer (Invitrogen, Carlsbad, CA) and oligo (dT) primers and AccuPower<sup>®</sup> RT PreMix (Bioneer, Daejeon, Korea). The cDNA was diluted (1:10) in sterile water before use in PCR. After PCR, equal amounts of reaction product were analyzed using a 1% agarose gel, and PCR products were visualized using ethidium bromide staining.

### ***Quantitative RT-PCR analysis***

Gene expression levels were measured using SYBR<sup>®</sup> Green (Sigma, St. Louis, MO, USA) and a StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) (Song et al., 2006). The *GAPDH* gene was simultaneously analyzed as a control and used for normalization to account for variation in loading. Each target gene and *GAPDH* was analyzed in triplicate. ROX dye (Invitrogen) was used as a negative control for the fluorescence measurements. Sequence-specific products were identified by generating a melting curve in which the C<sub>T</sub> value represented the cycle number at which a fluorescent signal was statistically greater than background, and relative gene expression was quantified using the 2<sup>-ΔΔCT</sup> method (Livak and Schmittgen, 2001). For the control, the relative quantification of gene expression was normalized to the C<sub>T</sub> of the control ovary.

### ***In situ hybridization analysis***

For hybridization probes, PCR products were generated and were gel-extracted and then cloned into pGEM-T vector (Promega) as described previously (Ahn et al., 2010). After verification of the sequences, plasmids containing the correct gene sequences were amplified with T7- and SP6-specific primers then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). After hybridization and blocking, the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche). The signal was visualized by exposure to a solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4mM nitroblue tetrazolium, and 2

mM levamisole (Sigma).

### ***MicroRNA target validation assay***

The 3'-UTR of *SERPINB3* was cloned and confirmed by sequencing. The 3'-UTR was subcloned between the eGFP gene and the bovine growth hormone (bGH) poly-A tail in pcDNA3-eGFP (Clontech, Mountain View, CA) to generate the eGFP-miRNA target 3'-UTR (pcDNA-eGFP-3'-UTR) fusion constructs. In addition, mutants of *SERPINB3* 3'-UTR for each miRNA were generated by point mutation and then cloned into the same type of plasmids. For the dual fluorescence reporter assay, the fusion constructs containing the DsRed gene and either *miR-101*, *miR-1668* and *miR-1681* were designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miRNA). The pcDNA-eGFP-3'-UTR and pcDNA-DsRed-miRNA (4 µg) were co-transfected into 293FT cells using the calcium phosphate method. When the DsRed-miRNA is expressed and binds to the target site of the 3'-UTR downstream of the GFP transcript, green fluorescence intensity decreases due to degradation of the GFP transcript. At 48 h post-transfection, dual fluorescence was detected by fluorescence microscopy and calculated by FACSCalibur flow cytometry (BD Biosciences). For flow cytometry, the cells were fixed in 4% paraformaldehyde and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

### ***Cell culture***

A total of five cell lines, including three human ovarian epithelial cancer and

two chicken primary ovarian cells, were used in this study. Human ovarian cancer cell lines (OVCAR-3, SKOV-3, and PA-1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to supplier's directions. Two different chicken ovarian surface epithelial cells (normal and cancerous cells) were isolated and cultured as previously described with some modifications (Giles et al., 2006; Shepherd et al., 2006).

### ***Immunofluorescence microscopy for detection of SERPINB3 activation***

Ovarian cancer cells and normal ovarian cells obtained from laying hens, and three human ovarian cancer cell lines, OVCAR-3, SKOV-3, and PA-1, were examined for SERPINB3 expression patterns by immunofluorescence microscopy as described previously. Each type of cell was seeded onto Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY). After 24 h, cells were fixed with -20°C methanol and immunofluorescence staining was performed using an anti-human SERPINB3 monoclonal antibody (catalog number: ab55733; Abcam plc, Cambridge, UK). Cells were then incubated with Alexa Fluor 488 Rabbit anti-goat IgG secondary antibody (A21222, Invitrogen). Slides were overlaid with DAPI before images were captured using a Zeiss confocal microscope LSM710 (Carl Zeiss) fitted with a digital microscope camera AxioCam and Zen 2009 software.

### ***Human study population***

Clinical data were retrieved from a database of patients with EOC between

June 2003 and March 2009. Approval by the Institutional Review Board of Seoul National University Bundang Hospital was obtained in advance for the current study. The eligibility criteria were as follows for the patients: they were diagnosed with EOC; they were treated with maximal cytoreductive surgery followed by adjuvant taxane- and platinum-based chemotherapy if indicated; they had Eastern Cooperative Oncology Group performance status of 0–2; and they had no underlying disease affecting survival. Optimal cytoreductive surgery was defined as a residual tumor  $\leq 1$  cm, whereas suboptimal cytoreductive surgery was defined as a residual tumor  $> 1$  cm in maximal diameter. All patients except those with low-risk earlystage disease such as FIGO stage IA or IB with grade 1 or 2 disease received adjuvant chemotherapy using paclitaxel ( $175 \text{ mg/m}^2$ )/carboplatin (AUC 5.0) or paclitaxel ( $175 \text{ mg/m}^2$ )/cisplatin ( $75 \text{ mg/m}^2$ ) for 1-2 weeks after surgery, and the chemotherapy was repeated every 3 weeks for 6 cycles. Progression-free survival (PFS) was defined as the time elapsed from the date of completion of primary adjuvant chemotherapy to the date of clinically proven aggravation. Overall survival (OS) was calculated from the date of staging laparotomy to the date of cancer-related death or the end of the study. Platinum resistance was defined as the response to platinum-based chemotherapy with a minimum treatment-free interval of less than 6 months while platinum sensitivity was defined as the response to platinum-based chemotherapy with a minimum treatment-free interval of greater than or equal to 6 months.

### ***Immunohistochemistry***

The localization of SERPINB3 protein in normal and cancerous ovaries of hens was evaluated by immunohistochemistry (IHC) using an anti-human SERPINB3 monoclonal antibody at a final dilution of 1:500 (1 µg/ml), and antigen retrieval was performed using the boiling citrate method as described previously (Song et al., 2006). Negative controls included the substitution of the primary antibody with purified non-immune mouse IgG at the same final concentration. For IHC of human ovarian cancer tissues, representative core tissue sections (2 mm in diameter) were taken from paraffin blocks and arranged in new tissue microarray (TMA) blocks using trephine apparatus (Superbiochips Laboratories, Seoul, Korea). In cases with variable histologic features, the most representative area was selected for TMA construction. The IHC staining of human TMA samples was performed using similar methods for the laying hen model. After IHC, the results were assessed semi-quantitatively by one pathologist unaware of clinico-pathologic characteristics. All EOC tissues showed nuclear staining in most tumor cells, whereas no nuclear staining was observed in normal human ovarian tissues and the negative control where the primary antibody was substituted with purified non-immune mouse IgG at the same concentration. Thus, the staining intensity of tumor cells was graded as weak (1+), moderate (2+) or strong (3+).

### ***Statistical Analysis***

In order to investigate the role of SERPINB3 as a novel biomarker of ovarian carcinogenesis in the laying hen model and to predict clinical outcomes in patients with EOC, data were subjected to analysis of variance, Chi-squared and

Student's *t*-tests, Kaplan-Meier method with the log-rank test, logistic regression and Cox's proportional hazard analyses to determine odds ratio (OR), hazard ratio (HR), and 95% confidence interval (CI). Statistical analyses were performed using Excel (Microsoft, Redmond, WA, USA) and SPSS software (Version 19.0; SPSS Inc., Chicago, IL, USA). A probability value of  $P < 0.05$  was considered statistically significant.

## 4. Results

### *Expression and localization of SERPINB3 mRNA and protein in normal and cancerous ovaries of laying hens*

Comparisons of expression of *SERPINB3* mRNA between normal and cancerous ovaries of laying hens by RT-PCR revealed that it was expressed in only cancerous ovaries (Fig. 2-1A and 2-1B). Moreover, quantitative RT-PCR showed that *SERPINB3* mRNA was not induced only in ovaries with EOC ( $P < 0.01$ ; Fig. 2-1C). These results suggest that *SERPINB3* is a biomarker for EOC in laying hens.

*In situ* hybridization analysis demonstrated that *SERPINB3* mRNA was abundant in the glandular epithelium of cancerous ovaries of laying hens (Fig. 2-2A), but not the luminal epithelium, stroma, blood vessels or immune cells of cancerous ovaries. Consistent with these results, *SERPINB3* protein was detected predominantly in the nucleus of glandular epithelium in cancerous, but not normal ovaries of laying hens (Fig. 2-2B). These results indicate that *SERPINB3* is specifically expressed only in the glandular epithelium of cancerous ovaries of laying hens.

### *Post-transcriptional regulation of microRNAs affecting SERPINB3*

To investigate the possibility that *SERPINB3* expression is regulated at the post-transcriptional level by miRNAs, we performed a miRNA target validation assay. Analysis of potential miRNA binding sites within the 3'-UTR for *SERPINB3* using a

miRNA target prediction database (miRDB; <http://mirdb.org/miRDB/>) revealed three putative binding site for *miR-101*, *miR-1668* and *miR-1681* (Fig. 2-3A). Therefore, we determined if these three miRNAs influenced *SERPINB3* expression via its 3'-UTR. A fragment of the *SERPINB3* 3'-UTR harboring binding sites for the miRNAs was cloned downstream of the green fluorescent protein (GFP) reading frame, thereby creating a fluorescent reporter for function of the 3'-UTR region (Fig. 2-3B). After co-transfection of eGFP-*SERPINB3* 3'-UTR and DsRed-miRNA, the intensity of GFP expression and percentage of GFP-expressing cells were analyzed by fluorescence microscopy and FACS. As shown in Figures 2-3C and 2-3D, in the presence of *miR-101*, *miR-1668* and *miR-1681*, the intensity and percentage of GFP-expressing cells (58.7% in control vs. 46.7% in *miR-101*, 39.2% in *miR-1668*, 25.9% in *miR-1681*) decreased ( $P < 0.01$ ). These results indicate that these three miRNAs directly bind to the *SERPINB3* transcript and post-transcriptionally regulate *SERPINB3* gene expression.

#### ***Immunofluorescence detection of SERPINB3 protein in chicken and human ovarian cancer cells***

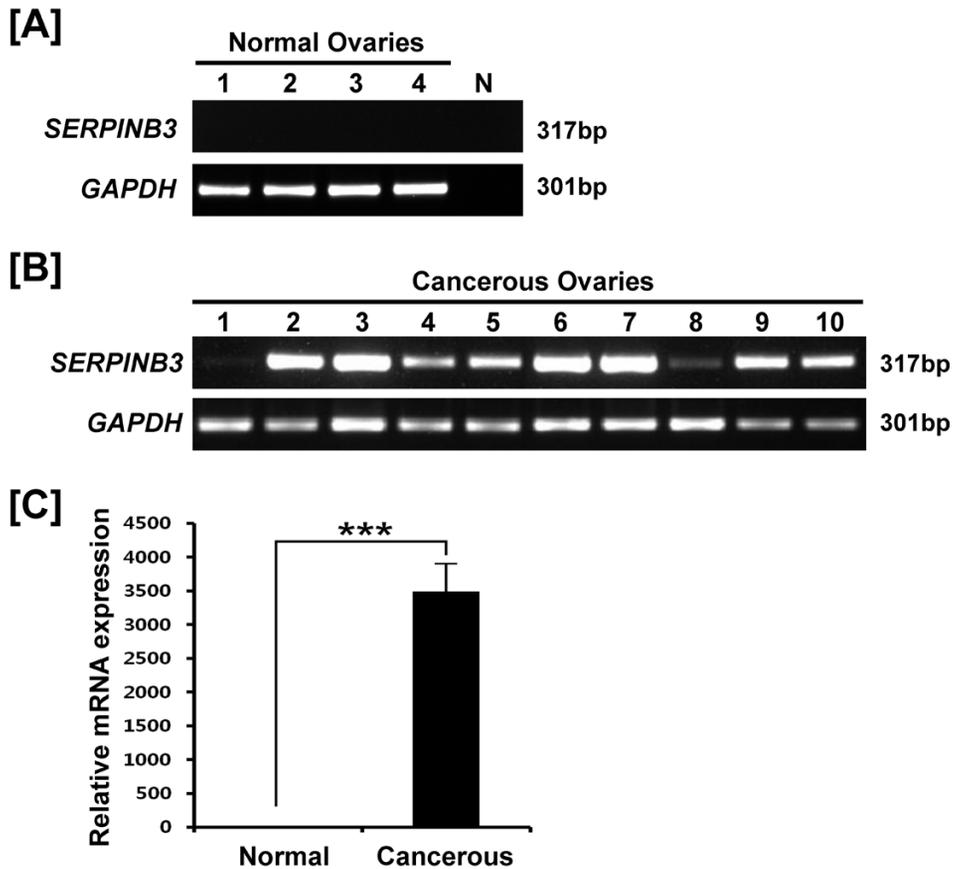
To compare the expression patterns of SERPINB3 protein between chicken and human ovarian cancer cells, we conducted immunofluorescence analysis. SERPINB3 protein was rarely detected in normal cells, but abundant in the nucleus of ovarian cancer cells of laying hens (Fig. 2-4A). Similarly, SERPINB3 protein was abundant in the nucleus of three human ovarian cancer cell lines, OVCAR-3, SKOV-3 and PA-1 cells (Fig. 2-4B).

***SERPINB3 protein expression is associated with platinum resistance and survival in patients with epithelial ovarian cancer***

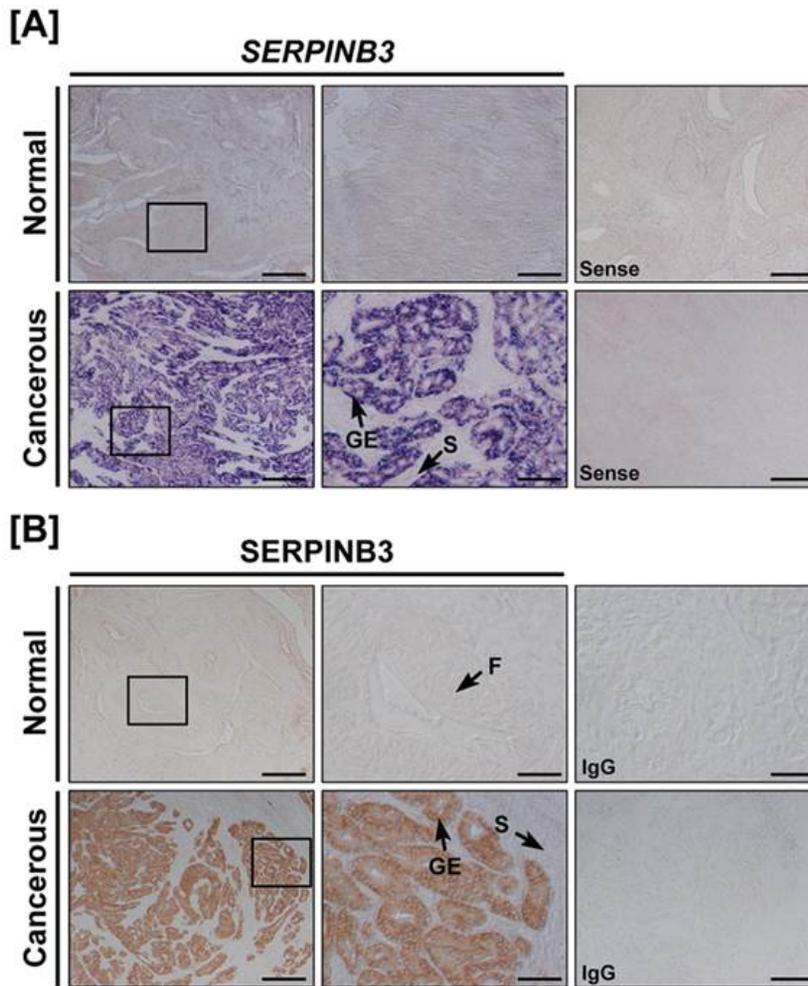
A total of 109 patients with a median age of 52 years (range, 23-82 years) were enrolled in the current study. Among all patients, 38 (34.9%) were in FIGO stage I, 20 (18.3%) in stage II, and 51 (46.8%) in stage III. Tumor grade was G1 in 21 (19.3%), G2 in 41 (37.6%) and G3 in 47 patients (43.1%). Histologically, 62 tumors (57%) were diagnosed with serous carcinoma, 17 (15.6%) with mucinous carcinoma, 12 (11%) with clear cell carcinoma, 9 (8.3%) with endometrioid carcinoma, 4 (3.6%) with undifferentiated carcinoma, and 3 (2.7%) with endometrioid and clear cell carcinoma, and 2 (1.8%) with serous and clear cell carcinoma. Optimal cytoreductive surgery was performed in 65 patients (59.6%), whereas 44 (40.4%) underwent suboptimal cytoreductive surgery. Ninety patients (82.6%) received adjuvant chemotherapy after surgery, and 84 (93.3%) received paclitaxel/carboplatin while paclitaxel/cisplatin was administered to 6 (6.7%) patients. Results of IHC analysis revealed that SERPINB3 protein was detected predominantly in glandular epithelium as for the laying hen model, and 15 (13.8%), 66 (60.6%) and 28 (25.7%) patients had weak, moderate and strong expression of SERPINB3 protein in ovarian glandular epithelium, respectively (Fig. 2-5).

The median time to follow-up was 69.2 months (range, 2-88 months), and 70 patients (77.8%) showed platinum sensitivity while 20 (22.2%) demonstrated platinum resistance. Strong expression of *SERPINB3* protein was more frequent in

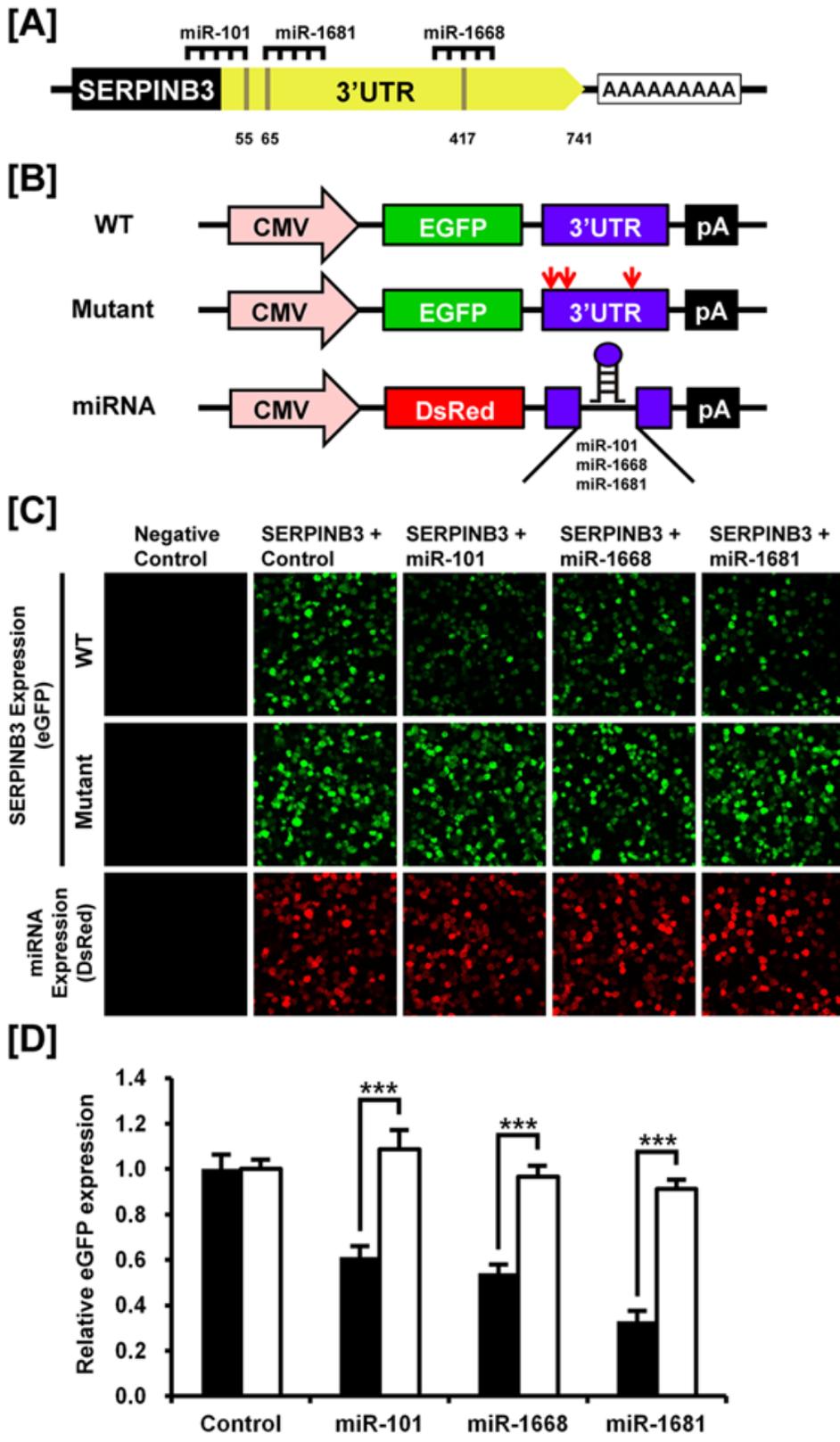
patients with platinum resistance (n=11, 55%) than in those with platinum sensitivity (n=17, 24.3%) ( $P < 0.01$ ). Strong expression of *SERPINB3* protein was also an independent prognostic factor for platinum resistance after adjustments using clinic-pathologic factors (adjusted OR, 5.94; 95% CI, 1.21-29.15) (Table 2-1). Strong expression of *SERPINB3* was associated with shorter PFS than weak or moderate expression (mean PFS were 25.7 vs. 47.8 months;  $P < 0.03$ ) in spite of no difference in OS (mean OS; overall survival, 49.5 vs. 60.9 months;  $P > 0.05$ ). Furthermore, *SERPINB3* was a poor prognostic factor for PFS using multivariate Cox's proportional hazard analysis (adjusted HR, 2.07; 95% CI, 1.03-4.41; Table 2-2), whereas there was no prognostic factor value for OS except suboptimal cytoreduction (adjusted HR, 6.83; 95% CI, 2.34-20.02 ).



**Figure 2-1. Expression and quantitation of *SERPINB3* mRNA in normal and cancerous ovaries of laying hens.** RT-PCR analysis was performed using cDNA templates from chicken *SERPINB3* and *GAPDH*-specific primers. [A] Lanes 1 to 4 show four different normal ovaries and N is negative control. [B] Lanes 1–10 show 10 different cancerous ovaries. [C] Quantitative RT-PCR analysis was performed using cDNA templates from normal and cancerous ovaries of laying hens (mean±SEM;  $P < 0.01$ ).



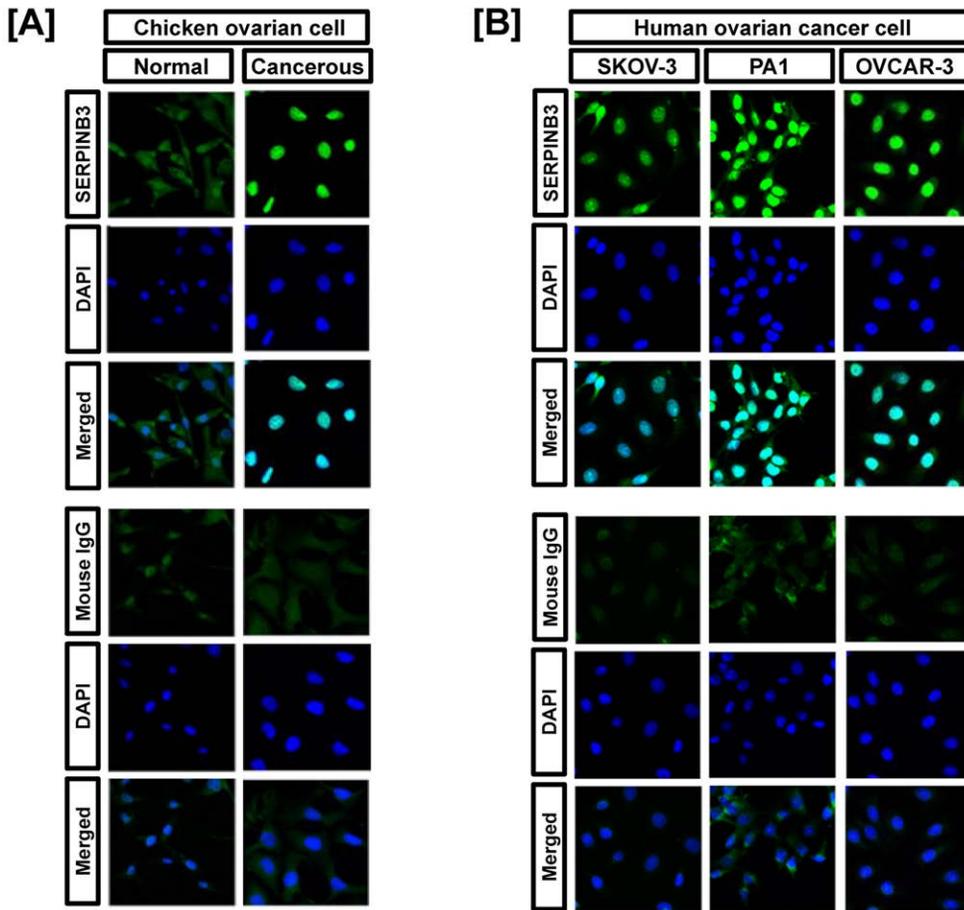
**Figure 2-2. Expression of *SERPINB3* mRNA and protein is unique to glandular epithelium of cancerous ovaries from laying hens.** [A] *In situ* hybridization analyses of *SERPINB3* mRNA. Cross-sections of normal and cancerous ovaries from laying hens were hybridized with sense or anti-sense chicken *SERPINB3* cRNA probes. [B] Immunohistochemical expression of *SERPINB3* protein: For negative control, the primary antibody was substituted with purified non-immune mouse IgG. F, follicle; GE, glandular epithelium; S, stroma; *Scale bar* represents 200  $\mu\text{m}$  (the first columnar panels and sense) or 50  $\mu\text{m}$  (the second columnar panels).



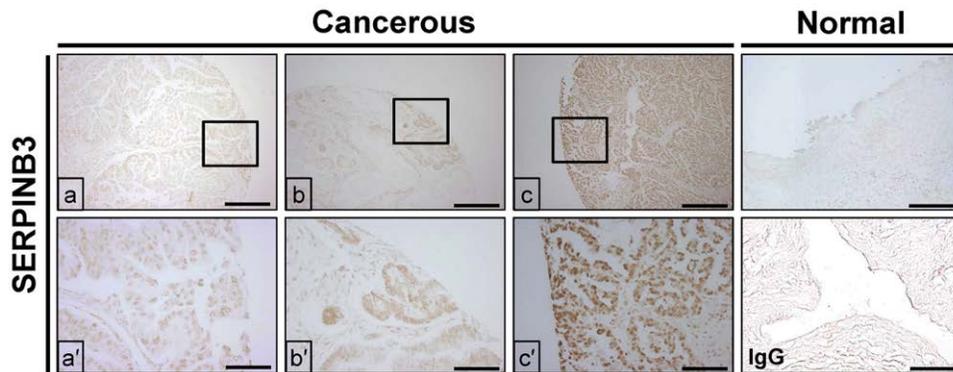
**Figure 2-3. *In vitro* target assay of microRNAs on the *SERPINB3* transcript. [A]**

Diagram of *miR-101*, *miR-1668* and *miR-1681* binding sites in *SERPINB3* 3'-UTR.

[B] Expression vector maps for eGFP with *SERPINB3* 3'-UTR and mutated *SERPINB3* 3'-UTR and Ds-Red with each miRNA. The wildtype (WT) and mutants of 3'-UTR of the *SERPINB3* transcript were subcloned between the eGFP gene and the polyA tail to generate the fusion construct of the GFP transcript following the miRNA target 3'-UTR (pcDNA-eGFP-3'-UTR) (top and middle panel) and the miRNA expression vector was designed to co-express DsRed and each miRNA (pcDNA-DsRed-miRNA) (bottom panel). [C] After co-transfection of pcDNA-eGFP-3'-UTR for the *SERPINB3* transcript and pcDNA-DsRed-miRNA for the *miR-101*, *miR-1668* and *miR-1681*, the fluorescence signals of GFP and DsRed were detected using fluorescent microscopy. [D] The rate of inhibition of eGFP expression from miRNA modulation was calculated by fluorescence-activated cell sorting (FACS). Solid bars represent WT of *SERPINB3* 3'-UTR and empty bars show the mutant of *SERPINB3* 3'-UTR for each miRNA. Error bars indicate the standard error of triplicate analyses. The asterisks denote statistically significant differences between WT vs. mutants (\*\*\*) ( $P < 0.001$ ).



**Figure 2-4. Immunofluorescence microscopy detected SERPINB3 protein in ovarian cancer cell lines of both women and laying hens.** [A] SERPINB3 protein was rarely detected in normal cells, but abundant in the nuclei of chicken ovarian cancer cells. [B] SERPINB3 protein was expressed in nuclei of human OVCAR-3, SKOV-3 and PA-1 cells. Cell nuclei were stained with DAPI (blue). All images were captured at 40× objective magnification.



**Figure 2-5. Immunohistochemistry was used to demonstrate expression of SERPINB3 protein in cancerous ovaries, but not in normal ovaries from women.** (a and a') Weak, (b and b') moderate and (c and c') strong expression of SERPINB3 protein was detected in women with epithelial ovarian cancer. There was either no expression or very weak expression of SERPINB3 in normal ovaries, whereas SERPINB3 protein was easily detectable in cancerous ovaries from women. The negative control used was mouse IgG instead of primary antibody. *Scale bar* represents 200  $\mu\text{m}$  (the first horizontal panels and IgG) or 50  $\mu\text{m}$  (the second horizontal panels).

**Table 2-1. Multivariate linear logistic regression analysis for factors affecting platinum resistance**

<b>Characteristics</b>	<b>Adjusted OR<sup>2</sup></b>	<b>95% CI<sup>3</sup></b>	<b>p value</b>
Age $\geq$ 52 years	0.93	0.25-3.41	0.91
FIGO stage III disease <sup>1</sup>	3.06	0.48-19.56	0.24
Grade 3 disease	4.36	1.14-16.71	0.03
Serous adenocarcinoma	0.49	0.11-2.12	0.34
Suboptimal cytoreduction	36.63	5.35-25.07	<0.01
Strong expression of <i>SERPINB3</i>	5.94	1.21-29.15	0.03

Abbreviation: FIGO, International Federation of Gynecology and Obstetrics; OR, odd ratio; CI, confidence interval.

**Table 2-2. Multivariate Cox’s proportional hazard analysis for poor prognostic factors affecting progression-free survival**

<b>Characteristics</b>	<b>Adjusted HR<sup>2</sup></b>	<b>95% CI<sup>3</sup></b>	<b>p value</b>
Age ≥52 years	1.16	0.62-2.17	0.63
FIGO stage III disease <sup>1</sup>	1.49	1.63-3.50	0.01
Serous adenocarcinoma	1.18	0.58-2.40	0.73
Grade 3 disease	1.34	0.76-2.52	0.85
Suboptimal cytoreduction	5.37	2.39-12.07	<0.01
Strong expression of <i>SERPINB3</i>	2.07	1.03-4.41	0.04

<sup>1</sup>International Federation of Gynecologist (FIGO) stages of epithelial ovarian cancer.

<sup>2</sup>Define HR

<sup>3</sup>95% Confidence interval statistic

## 5. Discussion

The laying hen is a well-known model for investigation of ovarian carcinogenesis and for development of anti-cancer agents for women because of the similarity in spontaneously arising carcinomas from epithelial ovarian cells (Rodriguez-Burford et al., 2001). Histology, metastasis and stages of EOC in laying hens are similar to those in humans which indicate the feasibility of using the laying hen model for investigating ovarian carcinogenesis (Barua et al., 2009). Furthermore, several biomarkers, including CA-125, are commonly expressed and used for detecting early-stage disease and monitoring therapeutic response in women with EOC and they are cross-reactive with biomarkers for EOC in laying hens (Jackson et al., 2007; Rodriguez-Burford et al., 2001).

In general, a number of complex glandular architectures are usually found in various carcinomas that arise in various organs such as stomach, bronchus, bladder, prostate, testis and ovary due to the ubiquitous nature of glands. Especially, in ovaries of both avian and mammalian species, these glandular structures are mainly in endometrioid-type tumors with several characteristics such as nuclear atypia, cribriform foci and atresia of stromal follicles. In addition, the glands of adenocarcinomas in women consist of a single layer of epithelial cells undergoing mitosis and sharp luminal margins (Barua et al., 2009). Our preliminary results also showed that the glandular architecture in primary ovarian epithelial carcinomas of laying hens is composed of a labyrinth of glands or lacelike papillary folding with large pleomorphic nuclei containing mitotic figures as previously reported (Barua et

al., 2009). The current study to find a novel prognostic biomarker for patients with EOC using the laying hen model identified high expression of *SERPINB3* gene in glandular epithelium of cancerous ovaries compared to that in normal ovaries from hens. The results suggest that *SERPINB3* may be associated with ovarian carcinogenesis through the activation of transcription factors or the inhibition of apoptosis. Our results also revealed that *SERPINB3* gene expression is post-transcriptionally regulated by several miRNAs critical to development of the chicken ovarian carcinogenesis. Furthermore, strong expression of *SERPINB3* protein was related to platinum resistance and shorter progression-free survival in patients with EOC. These findings support our hypothesis that *SERPINB3* plays a role in tumor development and proliferation from ovarian epithelial cells.

Although the functional role of *SERPINB3* gene in EOC biology is not known, results of the present study indicate clearly that *SERPINB3* is associated with glandular morphogenesis affecting ovarian carcinogenesis in both hens and women with EOC. In both women and hens, *SERPINB3* is expressed in the glandular epithelium, but there is little or no *SERPINB3* expression in other tissues and cells including stroma and blood vessels of the ovary. These findings indicate that *SERPINB3* protein may activate transcription factors or inhibit apoptosis leading to development of EOC in laying hens and women. In previous studies, *SERPINB3* was found to regulate programmed cell death by different mechanisms in various cancers and its over-expression was characteristic of cancerous cells of epithelial origin. In addition, *SERPINB3* attenuates apoptosis mediated by anti-cancer drugs for NK cells and by inhibiting cytochrome c release from the mitochondria (Kato, 1996; Suminami

et al., 2001; Vidalino et al., 2009). Moreover, glandular morphogenesis associated with SERPINB3 may contribute to epithelial-mesenchymal transition, which may deregulate the adhesion process to allow metastasis and increase the invasiveness potential of tumor cells in women (Quarta et al., 2010).

Strong expression of SERPINB3 was also associated with platinum resistance and shorter PFS in patients with EOC. The mechanism responsible for development of chemoresistance and a potential role for SERPINB3 has not been established in EOC. Nevertheless, dysfunctional permeabilization of lysosomes contributes to the development of chemoresistance in ovarian cancer cells (Castino et al., 2009), and SERPINB3 confers resistance to drug-induced apoptosis by inhibiting lysosomal cathepsin proteases in cancer cells (Suminami et al., 2000). Thus, SERPINB3 may contribute to platinum resistance following chemotherapy-induced lysosomal destabilization. Although strong expression of SERPINB3 was associated with shorter PFS, it is possible that platinum resistance associated with SERPINB3 leads to shorter PFS. This fact is supported by a previous study in which SERPINB3 expression was found to be associated with poor survival in patients with breast cancer (Collie-Duguid et al., 2012).

SERPINB3 or SCCA1 has been investigated in various types of squamous cell carcinoma (Chechlińska et al., 2010; Hsu et al., 2007; Nakashima et al., 2006; Shiiba et al., 2010), but it has only been suggested as a prognostic factor for breast cancer and lung adenocarcinoma (Collie-Duguid et al., 2012; Petty et al., 2006). To our knowledge, results of the current study are significant in being the first to establish

the likelihood of a functional role for SERPINB3 in EOC of laying hens. These results validate the laying hen as a model for research on human EOC. Further, the results strongly suggest that SERPINB3 has important functions in development of EOC in laying hens and that it is a novel biomarker for predicting platinum resistance and poor PFS in patients with EOC. Our hypothesis that SERPINB3 has a specific role in development of human EOC requires further evaluation in clinically prospective studies.

## **CHAPTER 3**

### **Avian SERPINB11 Gene: A Marker for Ovarian Endometrioid Cancer in Chickens**

## 1. Abstract

As serine and cysteine proteinase inhibitors, serpins, such as SERPINB5, cause ovarian, colorectal and pancreatic adenocarcinomas. We identified SERPINB11 as a novel estrogen-induced gene in chickens during oviduct development. The chicken is a unique animal model for research on human ovarian cancer, because it spontaneously develops epithelial cell-derived ovarian cancer as in women. Therefore, this study investigated the expression pattern, CpG methylation status, and miRNA regulation of the *SERPINB11* gene in normal and cancerous ovaries from chickens. Our results indicate that SERPINB11 is most abundant in the glandular epithelium of endometrioid adenocarcinoma of cancerous, but not normal, ovaries of hens. In addition, bisulfite sequencing revealed that about 30% of -110 CpG sites are methylated in ovarian cancer cells, whereas -110 CpG sites are demethylated in normal ovarian cells. Next, we determined whether *miR-1582* influences *SERPINB11* expression via its 3'-UTR and found that it does not directly target the 3'-UTR of *SERPINB11* mRNA. Therefore, it is unlikely that post-transcriptional regulation influences *SERPINB11* expression in the chicken ovary. On the other hand, in human ovarian cancer cells such as OVCAR-3, SKOV-3 and PA-1 cells, immunoreactive SERPINB11 protein was predominant in the cytoplasm and had a similar expression pattern to that in chicken ovarian cancer cells. Collectively, these results suggest that SERPINB11 is a biomarker for chicken ovarian endometrioid carcinoma that could be used for diagnosis and monitoring effects of therapies for the disease in women.

## 2. Introduction

Although ovarian cancer accounts for only 4% of cancer cases in women, it is the most lethal gynecological malignancy and the fifth leading cause of cancer-related deaths among women in the USA (Cvetkovic, 2003; Jemal et al., 2007; Wong and Auersperg, 2003). Ovarian cancer is rarely diagnosed at an early stage (Goodman et al., 2003) and over 75% of woman diagnosed are at an advanced stage of the disease, because it is generally asymptomatic (Bast et al., 2002) and there is no specific biomarker(s) for early detection (Barua et al., 2009; Pepe et al., 2001). There are three types of ovarian cancers: epithelial tumors, germ cell tumors and stromal tumors (Kurman and Shih, 2008; Kurman et al., 2008). More than 90% of human ovarian cancers are thought to arise from the germinal epithelium of the ovary (Auersperg et al., 2001). The rate of epithelial ovarian cancer is high because incessant ovulation causes genomic damage to the ovarian surface epithelium, increasing the possibility of gene mutations (Auersperg et al., 1998; Murdoch et al., 2005). Even though genetically manipulated rodent models have been used to elucidate some aspects of the etiologies and pathogenesis of ovarian cancer, the non-spontaneous nature of their ovarian cancer limits their clinical relevance (Barua et al., 2009; Stakleff and Von Gruenigen, 2003; Vanderhyden et al., 2003).

Chickens ovulate almost every day, whereas women ovulate only once each month. Epithelial-derived ovarian cancer in hens develops spontaneously at a high rate after they experience a severe depression in egg production when more than two years of age. Likewise, natural menopause in women usually occurs between 40 and

55 years of age when estrogen and progesterone production decreases progressively with advancing age of the ovaries. Given the prevalent hypothesis that the cause of ovarian cancer is incessant ovulation (Fathalla, 1971), the chicken is the only animal that spontaneously develops ovarian cancer of the surface epithelium of the ovaries at a high rate, as also occurs in women (Stammer et al., 2008). Therefore, the laying hen is a unique model for research on human ovarian cancer aimed at development of biomarkers and anticancer drugs for prevention, early diagnosis and therapies to treat the disease.

The serpin superfamily of serine/cysteine proteinase inhibitors is found in Eukarya, Eubacteria, Archaea and Poxviridae (Gettins, 2002a). Most serpins are plasma proteins that have important roles in pivotal physiological events including inflammation, blood coagulation and fibrinolysis (Askew et al., 2007). Unlike the majority of serpins that are inhibitors of proteases, several serpins, such as SERPINA7 and SERPINH1, are not known inhibitors of proteases, but are involved in hormone transport and protein folding, respectively (Askew et al., 2007; Dafforn et al., 2001; Zhou et al., 2006). There are 13 clade B serpin (SERPINB) genes based on in silico analysis of human genomic DNA (Silverman et al., 2004). The SERPINBs are predominantly intracellular proteins that mainly inhibit target proteases (Bird et al., 2001). However, there are two intracellular noninhibitory SERPINBs, SERPINB5 and SERPINB11 (Askew et al., 2007; Luo et al., 2007). SERPINB5 is a class II tumor suppressor gene also known as maspin (mammary serine protease inhibitor). It was identified in patients with breast cancer and found to promote apoptosis of invasive breast and prostate cancer cells (Luo et al., 2007; Zou et al., 1994). Additionally,

down-regulation of SERPINB5 caused ovarian, colorectal, pancreatic and endometrioid adenocarcinoma (Li et al., 2007). However, a functional role(s) for SERPINB11 in carcinogenesis is unknown.

Therefore, we investigated: (1) the expression of SERPINB11 in normal and cancerous ovaries from hens; (2) CpG methylation status of SERPINB11 between normal and cancerous ovarian cells in vitro; (3) SERPINB11 expression among chicken ovarian cells (normal and cancerous) compared with that in human epithelial-derived ovarian cancer cell lines, such as OVCAR-3 and SKOV-3, and human ovarian teratocarcinoma cell lines such as PA-1; and (4) whether SERPINB11 is regulated by post-transcriptional actions of specific microRNAs (miRNAs) using a miRNA target validation assay. We found that the laying hen is a unique model for the research on human ovarian cancer and that SERPINB11 has important functions in the development of epithelial ovarian cancer in hens, and should be useful for diagnosis and evaluation of therapies used to treat the disease.

### **3. Materials and Methods**

#### ***Experimental animals and animal care***

The experimental use of chickens in the present study was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5), Seoul, Korea. White Leghorn (WL) chickens were managed according to approved standards for operation of the University Animal Farm (Seoul National University, Seoul, Korea) for reproduction and embryo manipulation procedures, as well as standard operating protocols in our laboratory. All chickens had free access to feed and water for *ad libitum* consumption.

#### ***Tissue samples***

In this study, a total of 136 chickens (88 chickens over 36 months of age and 48 chickens over 24 months of age) which had completely stopped laying eggs were euthanized for biopsy and cancerous (n = 10) ovaries were collected. As a control, normal (n = 5) ovaries were also collected from egg-laying hens. We examined the tumor stage in 10 chickens with cancerous ovaries using characteristic features of chicken ovarian cancer (Barua et al., 2009). In three hens, ovarian tumor cells were classified as stage III as they had metastasized to the gastrointestinal tract and superficial surface of the liver with profuse ascites in the abdominal cavity (Table 1). In five hens, the tumors had metastasized to distant organs such as the liver parenchyma, lung, gastrointestinal tract and oviduct with profuse ascites and were

classified as stage IV tumors. The other two hens did not have tumors in any other organs and their ovarian tumors were classified as stage I. To include all cell types within the ovary, cross-sections were frozen or fixed in 4% paraformaldehyde for further analyses. Frozen tissue samples were cut into 5–7 mm pieces and frozen in liquid nitrogen. The other samples were cut into 10-mm pieces and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5  $\mu\text{m}$  and stained with hematoxylin and eosin (please see Supplemental Figure 1). Epithelial ovarian cancers in chickens were classified based on the cellular subtypes and patterns of cellular differentiation with reference to ovarian malignant tumor types in humans (Barua et al., 2009).

### ***Cell culture***

A total of five cell lines, including three human ovarian epithelial cancer and two chicken primary ovarian cells, were used in this study. Human ovarian cancer cell lines (OVCAR-3, SKOV-3 and PA-1) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured according to the supplier's directions. Two different chicken ovarian surface epithelial cells (normal and cancerous cells) were isolated and cultured as previously described (Giles et al., 2006; Shepherd et al., 2006) with some modifications. Briefly, small yellow follicles (> 8-12 mm) completely covered by the surface epithelial cells were received in a sterile vessel containing saline, washed and transferred to 5 mL prewarmed (37°C)

collagenase (1 mg/mL; Sigma-Aldrich Inc, St Louis, MO, USA) in a six-well tissue culture dish. To obtain the ovarian epithelial cells, the dish was moved to a 37°C incubator and swirled every 10 min. After a 30-min incubation period, the follicle surface tissue was scraped gently with sterile forceps, and 5 mL of the used collagenase was transferred into a sterile 15 mL conical centrifuge tube containing complete Dulbecco's modified Eagle's medium (DMEM)/F-12 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (HyClone, Waltham, MA, USA), 5% chicken serum (Sigma-Aldrich, Inc), 1% non-essential amino acids (Invitrogen), 1% sodium pyruvate (Invitrogen) and 1% antibiotics antimycotics (Abam; Gibco, Carlsbad, CA, USA) to inactivate the collagenase activity. The medium containing cells and ovarian tissue debris was filtered through the 0.45-µm filter. Diluted collagenase was washed by centrifugation at 1250 rpm for 10 min at room temperature. After removing the medium, a small pellet containing cells was re-suspended in 4 mL of complete DMEM/F-12 medium. Cells ( $4.0 \times 10^5$ ) were dispensed into each of the wells of a six-well tissue culture dish and incubated at 37°C, 5% CO<sub>2</sub>. The medium was changed every other day until the cells reached 70–80% confluency. All chicken primary ovarian epithelial cells were placed in short-term culture and expanded (two to three passages). The purity of these cell cultures was confirmed based on their expression of cytokeratin (epithelial cells) or vimentin (mesenchymal cells) proteins (Giles et al., 2006).

### ***RNA isolation***

Total cellular RNA was isolated from frozen tissues using Trizol reagent

(Invitrogen) according to the manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

### ***Semi-quantitative reverse transcription polymerase chain reaction analysis***

The expression of *SERPINB11* mRNA in normal and cancerous ovaries of chickens was assessed using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) as described previously (Song et al., 2007). The cDNA was synthesized from total cellular RNA (2 µg) using random hexamer (Invitrogen) and oligo (dT) primers and AccuPower<sup>®</sup> RT PreMix (Bioneer, Daejeon, Korea). The cDNA was diluted (1:10) in sterile water before use in PCR. For *SERPINB11*, the sense primer (5' CGG AGA CCT GAG CAT GTT GG 3') and antisense primer (5' TAT CAC CCC TGT GGA GCC TG 3') amplified a 337-bp product. For *GAPDH* (housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase), the sense primer (5' TGC CAA CCC CCA ATG TCT CTG TTG 3') and antisense primer (5' TCC TTG GAT GCC ATG TGG ACC AT 3') amplified a 301-bp product. The primers, PCR amplification and verification of their sequences were conducted as described previously (Song et al., 2007). PCR amplification was conducted using approximately 60 ng cDNA as follows: (1) 95°C for three minutes; (2) 95°C for 20 s, 60°C for 40 s (for *SERPINB11* and *GAPDH*) and 72°C for one minute for 33 cycles; and (3) 72°C for 10 min. After PCR, equal amounts of reaction product were analyzed using a 1% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted

from correctly sized bands under ultraviolet light using a Gel Doc<sup>TM</sup> XR+ system with Image Lab<sup>TM</sup> software (Bio-Rad, Hercules, CA, USA).

### ***Quantitative RT-PCR analysis***

Total RNA was extracted from each sample of normal and cancerous ovarian tissue from hens using TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands). Complementary DNA was synthesized using AccuPowerw RT PreMix (Bioneer). Gene expression levels were measured using SYBR<sup>®</sup> Green (Sigma-Aldrich) and a StepOnePlus<sup>TM</sup> Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The *GAPDH* gene was simultaneously analyzed as a control and used for normalization for variation in loading. Each target gene and *GAPDH* was analyzed in triplicate. Using the standard curve method, we determined the level of expression of the examined genes using the standard curves and C<sub>T</sub> values, and normalized them using *GAPDH* expression quantities. 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) and continuous fluorescence measurement. ROX dye (Invitrogen) was used as a negative control for the fluorescence measurements. Sequence-specific products were identified by generating a melting curve in which the C<sub>T</sub> value represented the cycle number at which a fluorescent signal was statistically greater than background, and relative gene expression was quantified using the 2<sup>-ΔΔCT</sup> method (Livak and Schmittgen, 2001). For the control, the relative quantification of gene expression was normalized to the C<sub>T</sub> of the control

oviduct.

### ***In situ hybridization analysis***

For hybridization probes, PCR products were generated and were gel-extracted and then cloned into pGEM-T vector (Promega) as described previously (Ahn et al., 2010). After verification of the sequences, plasmids containing the correct gene sequences were amplified with T7- and SP6-specific primers then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). After hybridization and blocking, the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche). The signal was visualized by exposure to a solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4mM nitroblue tetrazolium, and 2 mM levamisole (Sigma).

### ***Immunohistochemistry***

Immunocytochemical localization of SERPINB11 protein in normal and cancerous ovaries from chickens was performed as described previously (Song et al., 2006) and a goat anti-human SERPINB11 polyclonal antibody (Catalog number sc-85140; Santa Cruz Biotechnology, Inc) was used at a final dilution 1:100 (2 µg/mL). Antigen retrieval was performed using the boiling citrate method as described previously (Song et al., 2006). Negative controls included substitution of the primary antibody with purified non-immune goat IgG at the same final concentration.

### ***Prediction of transcription factor-binding cis-elements***

The presence of transcription factor-binding cis-elements within the SERPINB11 promoter region was predicted using a bioinformatics tool for orthologous sequences (<http://www.cbrc.jp/research/db/TFSEARCH.html>).

### ***Bisulfite sequencing***

DNA samples were prepared using an AccuPrep Genomic DNA Extraction Kit (Bioneer) and converted using MethylEasy<sup>TM</sup>Xceed (Human Genetic Signatures, North Ryde, NSW, Australia) according to the manufacturer's instructions. For amplifying the converted DNA, PCRs were performed with forward (5' TTA AAG TGT GTG TAT TTA TTA TT 3') and reverse (5' CCT AAA CAA ATT CTC TAA ATC CT 3') primers, which included the upstream region of the *SERPINB11* gene, as follows: 95°C for 1 min and 50 s, 35 cycles at 94°C for 20 s, 52°C for 20 s, 72°C for 30 s and 72°C for 10 min for the final synthesis. The PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced using an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems).

### ***Immunofluorescence microscopy for detection of SERPINB11 activation***

Ovarian cancer cells and normal ovarian cells obtained from laying hens, and three human ovarian cancer cell lines, including OVCAR-3, SKOV-3 and PA-1,

were examined for SERPINB11 protein expression patterns by immunofluorescence microscopy. Each type of cell was seeded onto Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA). After 24 h, cells were fixed with -20°C methanol, and immunofluorescence staining was performed using a goat anti-human SERPINB11 polyclonal antibody (Catalog number sc-85140; Santa Cruz Biotechnology, Inc). Cells were then incubated with Alexa Fluor 488 rabbit anti-goat IgG secondary antibody (A21222; Invitrogen). Slides were overlaid with 4',6-diamidino-2-phenylindole before images were captured. Images were captured using a Zeiss confocal microscope LSM710 (Carl Zeiss, Oberkochen, Germany) fitted with a digital microscope camera (AxioCam) using Zen 2009 software.

### ***MicroRNA target validation assay***

The 3'-UTRs of *SERPINB11* was cloned and confirmed by sequencing. The 3'-UTR was subcloned between the eGFP gene and the bovine growth hormone poly-A tail in pcDNA3-eGFP (Clontech, Mountain View, CA, USA) to generate the eGFP-miRNA target 3'-UTR (pcDNA-eGFP-3'-UTR) fusion constructs. For the dual fluorescence reporter assay, the fusion constructs containing the DsRed gene and *miR-1582* were designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miRNA). The pcDNA-eGFP-3'-UTR and pcDNA-DsRed-miRNA (3 µg) were co-transfected into 293FT cells using the calcium phosphate method. When the DsRed-miRNA is expressed and binds to the target site of the 3'-UTR downstream of the GFP transcript, green fluorescence intensity decreases due to degradation of the GFP transcript. At 48 h post-transfection, dual fluorescence was detected by

fluorescence microscopy and calculated by FACSCalibur flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). For flow cytometry, the cells were fixed in 4% paraformaldehyde and analyzed using the FlowJo software (Tree Star Inc, Ashland, OR, USA).

### *Statistical analyses*

Data presented for realtime PCR are expressed as mean $\pm$ SEM unless otherwise stated. Differences in the variances between normal and cancerous ovaries were analyzed using the *F* test, and differences between means were subjected to the Student's *t* test. Differences with a probability value of  $P < 0.05$  were considered statistically significant. Excel (Microsoft, Redmond, WA, USA) was used for statistical analyses.

## 4. Results

### *Differential expression of SERPINB11 in normal and cancerous ovaries of hens*

We previously reported expression of cysteine protease cathepsins in ovarian tissue from hens with ovarian cancer (Ahn et al., 2010). Based on the morphological and immunohistochemical differences between normal and cancerous ovaries from hens (Table 3-1 and Fig. 3-6), we hypothesized that expression patterns for SERPINB11 would differ between normal and cancerous ovarian tissues from hens. Based on RT-PCR analysis, *SERPINB11* mRNA was predominantly found in endometrioid carcinoma, but there was little or no expression in serous, mucinous, or clear cell carcinomas and normal ovaries (Fig. 3-1A and 3-1B). Further, the expression level of *SERPINB11* mRNA was greater ( $P < 0.01$ ) in cancerous ovaries from hens (Fig. 1C).

### *Localization of SERPINB11 mRNA and protein in cancerous ovaries of hens*

Cell-specific expression of *SERPINB11* mRNA and protein was determined using *in situ* hybridization analysis and immunohistochemistry. There was abundant *SERPINB11* mRNA localized predominantly in the glandular epithelium of cancerous ovaries with endometrioid carcinoma, but not in the luminal epithelium, stroma or blood vessels (Fig. 3-2). Consistent with this result, immunoreactive SERPINB11 protein was detected in the glandular epithelium of cancerous ovaries, but not in any other cell types of the same tissues (Fig. 3-3). However, SERPINB11 protein was

detectable at low abundance around developing follicles and the glandular epithelium of normal ovaries.

***Comparison of CpG methylation status in the upstream of SERPINB11 gene between normal and cancerous ovarian cells in hens***

The effect of CpG demethylation in the upstream region of a gene increases its transcriptional activity. Moreover, CpG methylation is closely associated with the development of cancers. We therefore investigated methylation patterns in the promoter region of the *SERPINB11* gene in normal ovarian epithelia and cancerous ovarian epithelia. Both normal and cancerous ovarian epithelial cells were extracted and cultured in vitro as previously reported (Giles et al., 2004). Bisulfite sequencing results showed that CpG sites at -54, -329 and -346 CpG from the transcriptional start site maintained methylation status in both cells. However, about 30% of the CpG site at -110 was methylated in ovarian cancer cells, whereas no CpG sites at -110 were methylated in normal ovarian epithelial cells (Fig. 3-4).

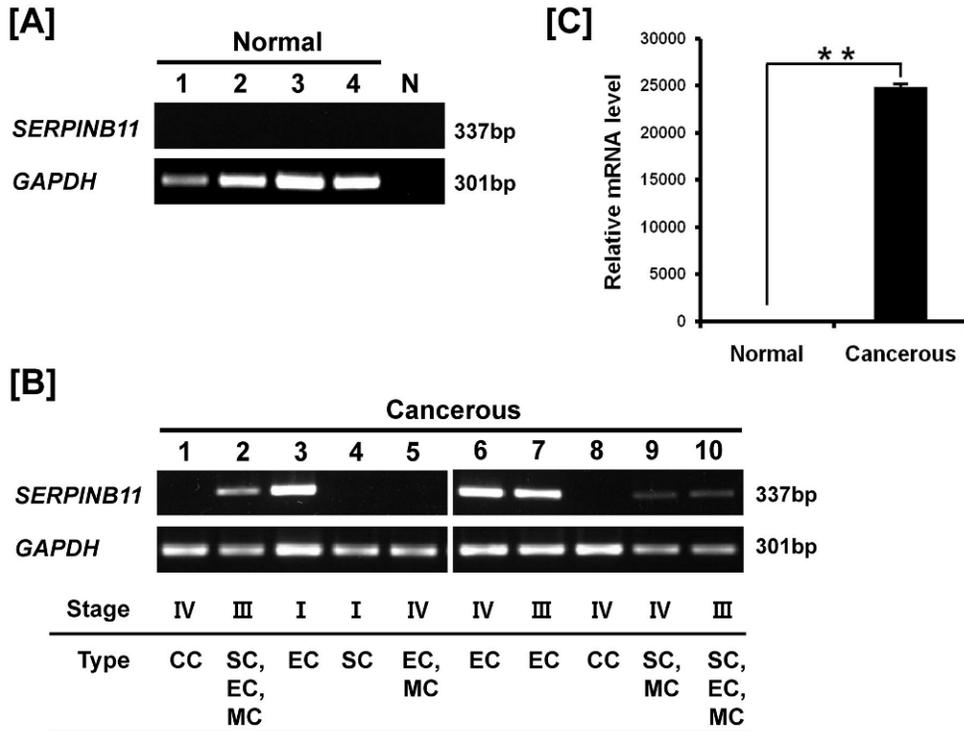
***Post-transcriptional action of miRNAs on SERPINB11***

Based on the possibility that *SERPINB11* expression is regulated at the post-transcriptional level by miRNAs, we performed a miRNA target validation assay. Analysis of potential miRNA binding sites within the 3'-UTR for *SERPINB11* using a miRNA target prediction database (miRDB; <http://mirdb.org/miRDB/>) revealed one putative binding site for *miR-1582*. Therefore, we examined whether *miR-1582*

influenced *SERPINB11* expression via its 3'-UTR. A fragment of the *SERPINB11* 3'-UTR harboring the *miR-1582* binding site was cloned downstream of the green fluorescent protein reading frame, thereby creating a fluorescent reporter for the function of the 3'-UTR region. In the presence of *miR-1582*, there was no significant decrease in green fluorescence from the reporter containing the *SERPINB11* 3'-UTR compared with the control. This result indicates that *miR-1582* does not directly target the 3'-UTR of *SERPINB11* mRNA (please see Fig. 3-7). Thus, post-transcriptional regulation of *SERPINB11* gene expression is highly unlikely.

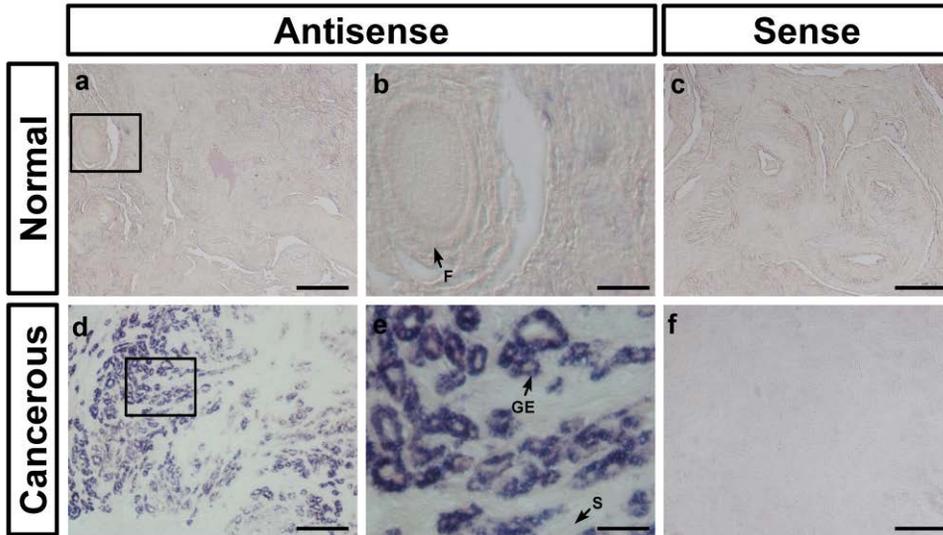
***Immunofluorescence detection of SERPINB11 protein in human immortalized ovarian carcinoma cells***

To compare expression patterns of *SERPINB11* protein between chicken and human ovarian cancer cells, two types of chicken cells (ovarian cancer cells and normal ovarian epithelial cells) and three human ovarian cancer cell lines (OVCAR-3, SKOV-3 and PA-1 cells) were cultured, fixed and then subjected to immunofluorescent microscopy. As illustrated in Figure 3-5, immunoreactive *SERPINB11* protein was relatively abundant in the cytoplasm of chicken ovarian cancer cells, but detectable at a lower level in the cytoplasm of normal ovarian epithelial cells. Similarly, *SERPINB11* was found in the cytoplasmic compartment of SKOV-3 cells and at lesser extent in PA-1 cells, but it was not detectable in OVCAR-3 cells.

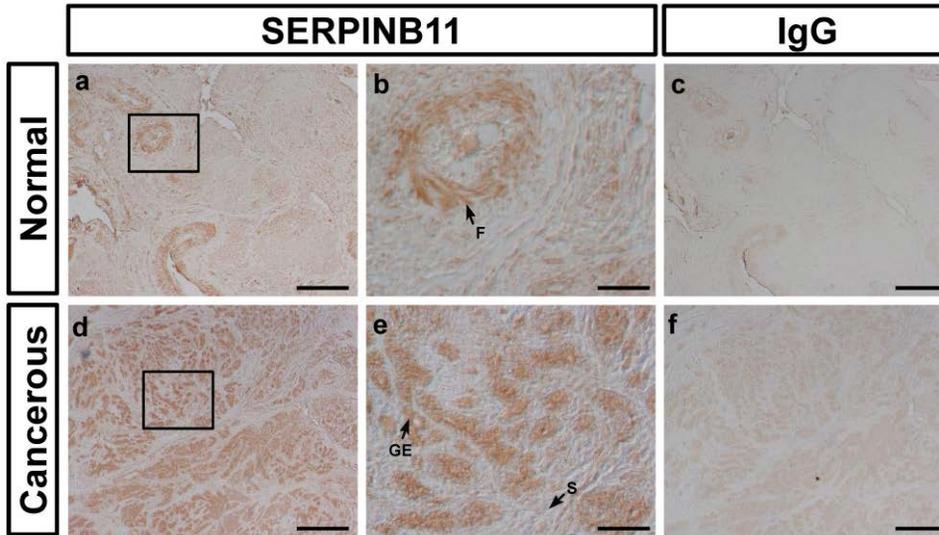


**Figure 3-1. Expression and quantitation of *SERPINB11* mRNA levels in normal and cancerous ovaries from hens.** [A and B] Reverse transcription polymerase chain reaction (PCR) analyses were performed using cDNA templates from normal and cancerous ovaries of chickens with chicken *SERPINB11* and *GAPDH*-specific primers. Lanes 1–4 show four different normal ovaries and N is negative control [A]. Lanes 1–10 in [B] show 10 different cancerous ovaries. *SERPINB11* mRNA was predominantly found in endometrioid carcinoma, but there was little or no expression in serous, mucinous or clear cell carcinomas or normal ovaries. [C] The quantitative realtime PCR analysis was performed using cDNA templates from normal and cancerous ovaries of chickens (mean±SEM;  $P < 0.01$ ). Please see Materials and methods for a complete description of the methods. [B] Lane 1, clear cell carcinoma

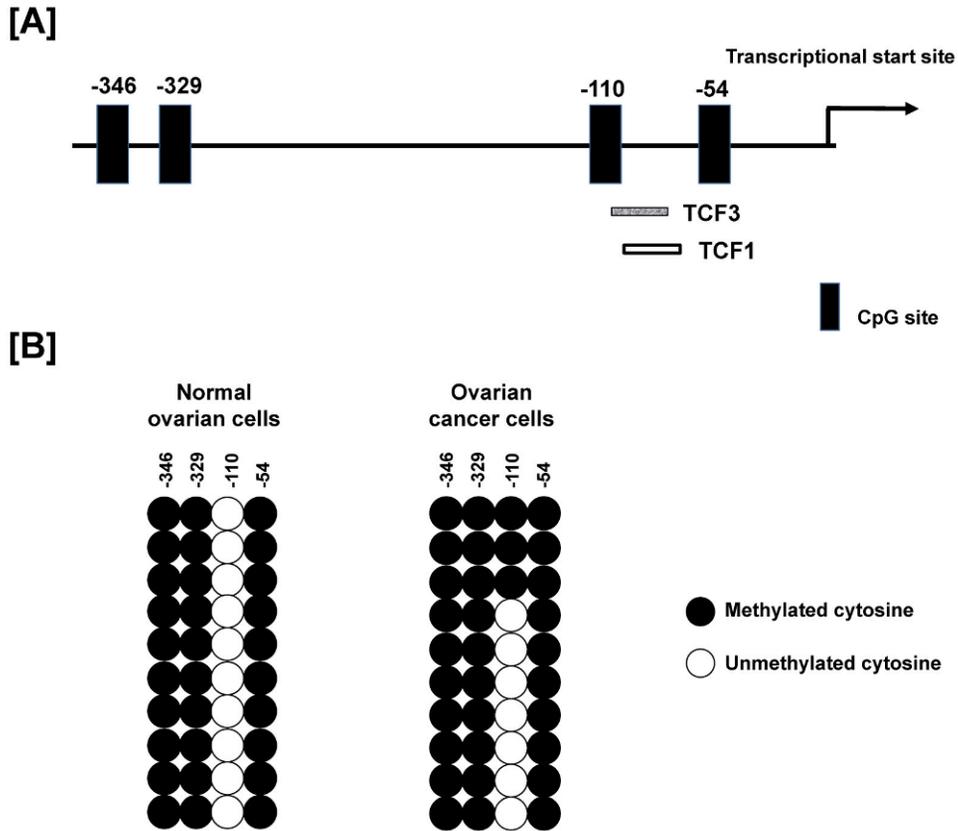
(stage IV); lane 2, endometrioid/serous/mucinous carcinoma (stage III); lane 3, endometrioid carcinoma (stage I); lane 4, serous carcinoma (stage I); lane 5, mucinous/endometrioid carcinoma (stage IV); lane 6, endometrioid carcinoma (stage IV); lane 7, endometrioid carcinoma (stage III); lane 8, clear cell carcinoma (stage IV); lane 9, serous/mucinous carcinoma (stage IV); and lane 10, serous/mucinous/endometrioid carcinoma (stage III)



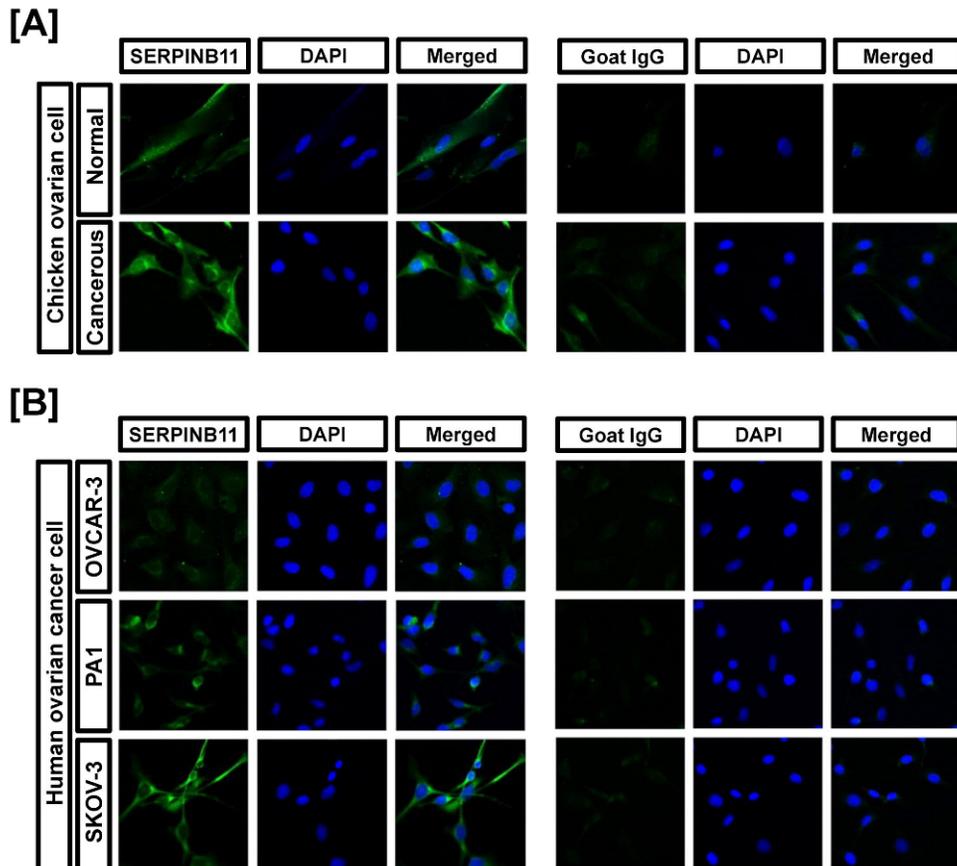
**Figure 3-2. *In situ* hybridization analyses of *SERPINB11* mRNAs in normal and endometrioid cancerous ovaries of hens.** Cross-sections of normal and cancerous ovaries of hens were hybridized with antisense or sense chicken *SERPINB11* cRNA probes. Please see Materials and methods for a complete description of the methods. F, follicle; GE, glandular epithelium; S, stroma. *Scale bar* represents 200  $\mu\text{m}$  (a, c, d and f ) or 50  $\mu\text{m}$  (b and e)



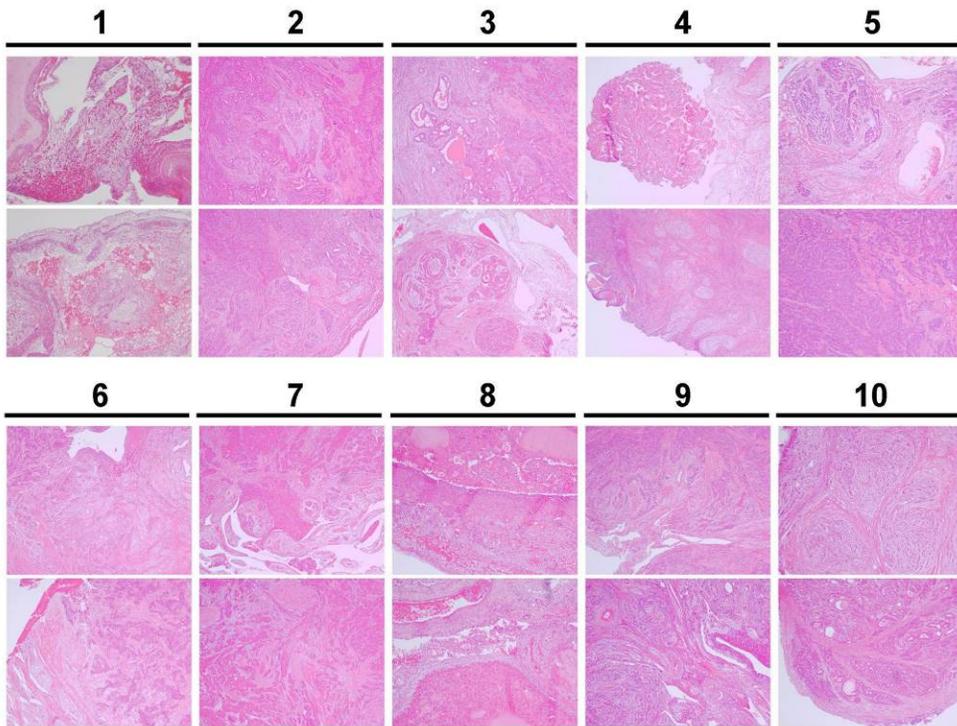
**Figure 3-3. Immunoreactive SERPINB11 protein in normal and endometrioid cancerous ovaries of hens.** For the IgG control, normal goat IgG was substituted for the primary antibody. Sections were not counterstained. Please see Materials and methods for a complete description of the methods. F, follicle; GE, glandular epithelium; S, stroma. *Scale bar* represents 200  $\mu\text{m}$  (a, c, d and f ) or 50  $\mu\text{m}$  (b and e)



**Figure 3-4. Bisulfite sequencing of CpG sites in the upstream region of the *SERPINB11* gene.** [A] Schematic of the four CpG sites in the promoter region of the *SERPINB11* gene is indicated by the heavy black vertical lines. The numbers on the line indicate positions relative to the transcription start site. [B] The CpG methylation status in the upstream region of the *SERPINB11* gene was analyzed in normal ovarian cells and ovarian cancer cells of hens by bisulfate sequencing. Each circle indicates a CpG site in the primary sequence, and each line of circles represents analysis of a single cloned allele. Closed and open circles are methylated and unmethylated CpGs, respectively

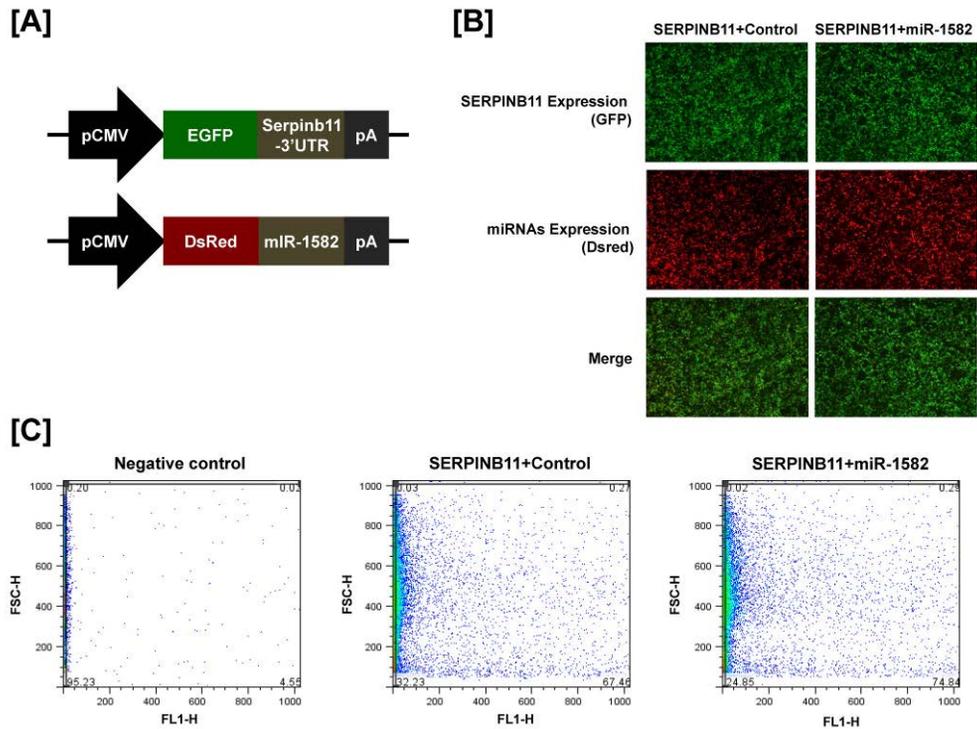


**Figure 3-5. Differential expression of SERPINB11 protein in normal and cancerous ovarian cells from hens [A] and human immortalized ovarian carcinoma cells [B].** [A] SERPINB11 protein was barely detectable in normal cells, but abundant in the cytoplasm of cancer cells. [B] SERPINB11 was expressed in SKOV-3 and PA-1 cells, but not in OVCAR-3 cells. Cell nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole) (blue). All images were captured at  $\times 40$  objective magnification

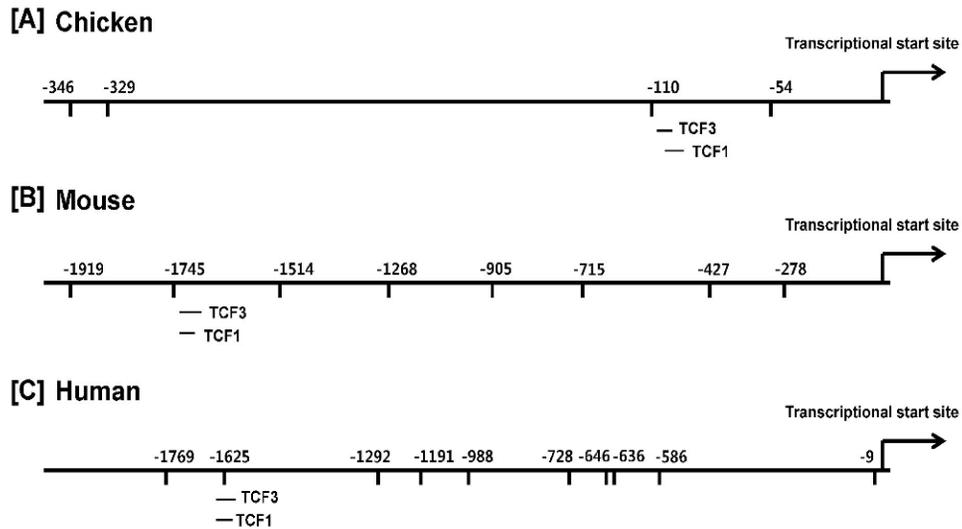


**Figure 3-6. Histological types of ovarian cancers in chickens used in this study.**

Briefly, clear cell carcinoma showed vacuolated cells consisting of nuclear atypia, serous carcinoma was a solid mass of cells with nuclear atypia, endometrioid carcinoma had many glands and mucinous carcinoma was differentiated around the stromal region. Each image shows two different regions within each type of carcinoma. 1, clear cell carcinoma; 2, serous/endometrioid/mucinous carcinoma; 3, endometrioid carcinoma; 4, serous carcinoma; 5, endometrioid/mucinous carcinoma; 6, endometrioid carcinoma; 7, endometrioid carcinoma; 8, clear cell carcinoma; 9, serous/mucinous carcinoma; 10, serous/endometrioid/mucinous carcinoma



**Figure 3-7. *In vitro* target assay of *SERPINB11* and *miR-1582*.** [A] Expression vector maps for eGFP with *SERPINB11* 3'-UTR and Ds-Red with *miR-1582*. The 3'-UTR of the *SERPINB11* transcript was subcloned between the eGFP gene and the polyA tail to generate the fusion construct of the GFP transcript following the miRNA target 3'-UTR (pcDNA-eGFP-3'-UTR) (upper panel) and miRNA expression vector was designed to co-express DsRed and miRNA (pcDNA-DsRed-miRNA) (lower panel). [B] After co-transfection of pcDNAeGFP 3'-UTR for the *SERPINB11* transcript and pcDNA-Ds-Red-miRNA for the *miR-1582*, the fluorescence signals of GFP and DsRed were detected using fluorescence microscopy and fluorescence-activated cell sorting [C]



**Figure 3-8. Comparison of the sequences around CpG regions of *SERPINB11* genes of chicken, mouse and human.** The 5' flanking region (about 2 kb) of mouse and human *SERPINB11* was compared with that of chicken *SERPINB11* to identify differences in sequences around each CpG site among those species. There are 8 and 10 CpG sites in mouse and human, respectively. In addition, the position of the -110 CpG site is located only within putative binding elements for transcription factor 3 (TCF3; E2A immunoglobulin enhancer-binding factors E12/E47) and transcription factor 1 (TCF1) encoding hepatocyte nuclear factor 1 alpha, which are highly conserved among species

**Table 3-1. Lesions, stages and type of chicken ovarian cancer**

ID	Lesions			Stage	Cancer type
	Ascites	Metastasis	Metastatic organ		
1	No	Yes	Peritoneum, small/large intestine, oviduct	IV	Clear cell carcinoma
2	Yes	Yes	peritoneum, small intestine, liver	III	Serous/Endometrioid/Mucinous carcinoma
3	No	No	-	I	Endometrioid carcinoma
4	No	No	-	I	Serous carcinoma
5	Yes	Yes	peritoneum, small/large intestine	IV	Endometrioid/Mucinous carcinoma
6	Yes	Yes	peritoneum, oviduct, small intestine	IV	Endometrioid carcinoma
7	Yes	Yes	peritoneum, liver	III	Endometrioid carcinoma
8	No	Yes	peritoneum, small/large intestine, oviduct	IV	Clear cell carcinoma
9	Yes	Yes	peritoneum, liver, oviduct, stomach	IV	Serous/Mucinous carcinoma
10	Yes	Yes	peritoneum, liver, intestine, oviduct	III	Serous/Endometrioid/Mucinous carcinoma

## 5. Discussion

Results of the present study are the first to identify a high level of expression of the *SERPINB11* gene in ovarian endometrioid carcinoma compared with normal ovaries of hens and the methylation status of CpG sites in the promoter region of *SERPINB11* in the surface epithelial cells of cancerous ovaries. Further, our results indicated that *miR-1582* does not interact with sites in the 3'-UTR of the *SERPINB11* gene to influence post-transcriptional regulation of its expression in chickens. These results support our hypothesis that SERPINB11 is a critical regulator for growth and developmental aspects of epithelial cells of the ovaries of hens as they transition from a normal to a cancerous state during carcinogenesis in the ovaries of laying hens.

We reported that lysosomal cysteine cathepsin B (CTSB), CTSC and CTSS genes are highly expressed in the cancerous ovaries of hens (Ahn et al., 2010). These and other proteases, such as metalloproteinase, serine, threonine, cysteine and aspartic proteases, occur naturally in all tissues to affect protein catabolism by hydrolysis of the specific peptide bonds (Lopez-Otin and Matrisian, 2007; Puente et al., 2003). Indeed, proteases play fundamental roles in a wide variety of biological events including cancer progression and metastasis due to their capacity to degrade extracellular matrix proteins (Lopez-Otin and Matrisian, 2007). Protease inhibitors can be classified based on the protease they block or by their inhibitory mechanism. Of these, serpins (serine protease inhibitors) are the largest and most broadly distributed superfamily of protease inhibitors, and they play pivotal roles in many important physiological cascades such as inflammation and blood coagulation

pathways (Irving et al., 2000; Law et al., 2006). At present, there are two intracellular non-inhibitory SERPINBs, i.e. SERPINB5 and -11 in mammals, but a functional role(s) for SERPINB11 in cancer biology has not been established. Therefore, in the present study, we used the unique chicken ovarian cancer model (Ahn et al., 2010; Barua et al., 2009; Giles et al., 2004), as it is the only animal that spontaneously develops ovarian cancer from the ovarian epithelia with a high incidence as occurs in women. As illustrated in Figures 3-2 and 3-3, results of the present study show that SERPINB11 is expressed predominantly in the glandular epithelium of cancerous ovaries of chickens. Unlike SERPINB5, SERPINB11 is an effective inhibitor of angiogenesis by inhibiting migration of endothelial cells and limiting mitogenesis and tube formation (Zhang et al., 2000). In laying hens, SERPINB11 may be involved in gland morphogenesis and angiogenesis. Alternatively, it is possible that SERPINB11 interacts reciprocally with other types of proteases in cancerous ovaries of chickens as it exhibits some inhibitory activity against several cysteine protease cathepsins (Askew et al., 2007).

In cancer cells, a variety of genes are either up-regulated or down-regulated by an epigenetic mechanism such as DNA methylation and histone modification (methylation and/or deacetylation) (Suzuki et al., 2011). The epigenetic regulatory mechanism mainly involves carcinogenesis cascades that, for instance, increase the rate of tumor growth by inactivation of tumor suppressor genes, and established oncogenes by hypermethylation of promoter regions of genes and gene silencing (Khalkhali-Ellis, 2006; Laird and Jaenisch, 1994). Indeed, the methylation of the promoter region of the *SERPINB5* gene leads to gene silencing in various cancers,

such as thyroid, colorectal and skin cancers (Bettstetter et al., 2005; Boltze et al., 2003; Khalkhali-Ellis, 2006; Reis et al., 2002). In the present study, our bisulfite sequencing results revealed that about 30% of -110 CpG sites were methylated in ovarian cancer cells, but not in normal ovarian epithelial cells (Fig. 3-4). This difference in methylation status between normal and cancerous ovarian cells is likely important in the development of cancer phenotypes. Expression of SERPINB11 may be epigenetically regulated, and its cell-type specific expression closely associated with DNA methylation. The results of this study also showed that the position of the -110 CpG site is located within putative binding elements for transcription factor 3 (TCF3; E2A immunoglobulin enhancer-binding factors E12/E47) and transcription factor 1 (TCF1) encoding hepatocyte nuclear factor 1 alpha. Furthermore, we compared the 5' flanking region (about 2 kb) of mouse and human SERPINB11 with that of chicken SERPINB11 to identify differences in sequences around CpG sites among these species. We identified eight and ten CpG sites in mouse and human, respectively. In addition, the position of the -110 CpG site is located only within putative binding elements for TCF3 and TCF1, which are highly conserved in each of the three species (Fig. 3-8). In humans, TCF3 is related to development of acute lymphoblastic leukemia (Barber et al., 2007), whereas TCF1 is mutated in 50% of liver cell adenomas and its mutation causes endometrial carcinomas (Rebouissou et al., 2004). Further research is required for elucidation of the relationship between SERPINB11 and TCF signaling cascades in cancerous ovaries of hens.

MicroRNAs (miRNAs), non-coding RNAs of 18–23 nucleotides in length, regulate gene expression and are capable of defining and altering cell fate by inducing

or inhibiting translation or cleavage of their target mRNAs through base pairing at partially or fully complementary sites (Garzon et al., 2006a). By regulating gene expression, miRNAs affect the function of a number of cellular processes in development, differentiation and oncogenesis (Bartel, 2004; Gregory et al., 2005b; Lu et al., 2005). About 500–1000 miRNAs are present in the mammalian genome, and up to 30% of human mRNAs are thought to be under the regulation of one or more miRNAs, suggesting functions of miRNAs in post-transcriptional regulation (Aravin and Tuschl, 2005; Lewis et al., 2005). In the present study, analysis of potential miRNA binding sites within 3'-UTR for SERPINB11 using the miRNA target prediction database (miRDB; <http://mirdb.org/miRDB/>) revealed one putative binding site for *miR-1582*. Thus, we investigated the effect of *miR-1582* on *SERPINB11* gene expression via its 3'-UTR, but *miR-1582* did not directly target the 3'-UTR of *SERPINB11* mRNA (Fig. 3-7). These results indicate that post-transcriptional regulation does not likely influence *SERPINB11* expression and that an alternative mechanism(s) is involved in regulation of its expression. Clearly, further research on the mechanism for post-transcriptional regulation of *SERPINB11* expression is needed.

In this study, we compared expression patterns of SERPINB11 protein in chicken and human ovarian cancer cells. Immunoreactive SERPINB11 protein was localized predominately to the cytoplasmic compartment in chicken cancer cells, as well as human SKOV-3 cells and, to a lesser extent, in human PA-1 cells. However, it was not detected in human OVCAR-3 cells (Fig. 3-5). This result indicates that chicken and human SERPINB11, a member of clade-B serpins characteristically

detected in cytoplasmic compartment (Silverman et al., 2001; Silverman et al., 2004), may be involved in carcinogenesis although it is not known how expression of SERPINB11 proteins is regulated by epigenetic programming.

Collectively, results of the present study demonstrate that *SERPINB11* gene expression is dramatically increased in hens with progressive endometrioid adenocarcinoma of the ovary. Therefore, our study provides new insights into the use of SERPINB11 as a biomarker for chicken ovarian endometrioid carcinoma and its use as a biomarker for diagnosis and response to therapies for treatment of the disease in humans. Our future research will examine the precise role(s) and biological target of SERPINB11 both chicken and human ovarian surface epithelial cell cancers.

## **CHAPTER 4**

# **Expression and Regulation of Gallicin 11 in the Oviduct in Response to Estrogen and in Ovarian Tumors of Chickens**

## 1. Abstract

Gallicins (GALs), also known as avian beta-defensins (AvBDs), are small cationic peptides having three cysteine disulfide bonds between their cysteine residues. They play essential roles in the innate immune system as well as stimulate proliferation of epithelial cells and fibroblasts. Although we found the avian homolog of human beta-defensin 11 to be highly expressed in chicks treated with the diethylstilbestrol (DES, a synthetic estrogen agonist), little is known about the hormonal and transcriptional regulation of GAL11 in the chicken oviduct and its expression in cancerous ovaries of chickens. Results of this study of young chicks revealed that DES induced *GAL11* mRNA and protein in the oviduct, specifically luminal and glandular epithelial cells. In addition, *microRNA-1615* was discovered to influence *GAL11* expression via its 3'-UTR which suggests post-transcriptional regulation of *GAL11* expression in chickens. Furthermore, we compared the expression patterns of the *GAL11* gene in normal and cancerous ovaries from laying hens which are models for human epithelial ovarian cancer. Our results demonstrated that GAL11 is most abundant in the glandular epithelium of endometrioid-type ovarian tumors, but not normal ovaries of laying hens. Collectively, these results suggest that GAL11 is an estrogen-induced gene during oviduct development and that it may be used as a biomarker for diagnosis of ovarian cancer and for monitoring effects of therapeutics on progression of ovarian carcinogenesis.

## 2. Introduction

The laying hen is one of the best animal models for studies of oviduct growth and development and ovarian tumorigenesis. Indeed many researchers use the chicken oviduct due to its sensitive response to sex steroids and rapid growth and differentiation (Dougherty and Sanders, 2005). Estrogen, as a sex hormone affecting the oviduct, induces epithelial cells within the chick oviduct to mature into tubular gland cells through cell proliferation and cytodifferentiation and to induce expression of egg-white protein genes such as ovalbumin (Palmiter and Wrenn, 1971b; Socher and Omalley, 1973). In addition, laying hens are the most relevant animal model to identify biomarkers for epithelial ovarian cancer. It is believed that incessant laying of eggs (ovulation) in hens increases the possibility of genetic mutations and damage in the ovarian surface epithelium that leads to epithelial ovarian cancer as occurs in women (Murdoch et al., 2005). Furthermore, several biomarkers such as ovarian carcinoma antigen 125 (CA125; also known as mucin 16, MUC16), epidermal growth factor receptor (EGFR), and V-ERB-B2 avian erythroblastic leukemia viral oncogene homolog 2 (ERBB-2) for human ovarian cancer (Anderson et al., 2010; Jackson et al., 2007; Johnson, 2009; Rodriguez-Burford et al., 2001) are highly expressed in similar patterns to those for chicken ovarian tumors (Hakim et al., 2009).

Defensins are small host defense peptides that play a pivotal role in innate immunity (Klotman and Chang, 2006). They consist of conserved cysteine-rich cationic domains and beta-pleated sheet structures connected by three intra-disulfide bonds (Ganz, 2003). In mammals, they are divided into three subgroups, alpha-, beta-

and theta-defensins, depending on the arrangement of disulfide bonds among cysteine residues (Ganz, 2003; Klotman and Chang, 2006). Avian defensins, known as gallinacins (GALs) (Harwig et al., 1994), were originally purified from chicken leukocytes and classified as members of the beta-defensin subfamily involved in antimicrobial activity against microorganisms such as Gram-positive/-negative bacteria and fungi (van Dijk et al., 2008; Xiao et al., 2004). At present, 14 classes of avian beta-defensin (AvBD) genes have been identified in the chicken (Abdel Mageed et al., 2009) of which 11 have been detected in the chicken oviduct and five of those GAL mRNAs increase in response to lipopolysaccharides (Mageed et al., 2008). Of interest, we found the avian homolog of human *beta-defensin 11* transcript to be highly expressed in chicks treated with the DES (Song et al., 2011). However, there is little known about the hormonal regulation of GAL11 in the chicken oviduct or its expression in cancerous ovaries of laying hens. Thus we focused on this gene in the present study.

The objectives of this study were to: (1) determine if estrogen regulates expression of *GAL11* during oviduct development in chicks; (2) determine whether expression of *GAL11* is regulated by post-transcriptional actions of specific microRNAs and (3) compare expression of *GAL11* in normal and cancerous ovaries from laying hens. Results of the present study indicate that *GAL11* is an estrogen-stimulated gene during development of the chicken oviduct and that it may be an initial biomarker gene for epithelial ovarian carcinogenesis in laying hens that may be used in research into the etiology of epithelial ovarian cancer in laying hens and perhaps in women.

### 3. Materials and Methods

#### *Experimental animals and animal care*

The experimental use of chickens for this study was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). All White Leghorn (WL) chickens were exposed to a light regimen of 15 h light and 9 h dark, *ad libitum* access to feed and water, and standard management practices for laying hens.

#### *Tissue samples*

**Study one**, Following euthanasia of mature WL hens, tissue samples were collected from brain, heart, liver, kidney, small intestine, gizzard, ovary, oviduct and testis of 1- to 2-year-old males (n = 3) and females (n = 3) (Lim et al., 2012d).

**Study two**, Female chicks identified by PCR analysis using W chromosome-specific primer sets (Lee et al., 2009) were treated with DES and to oviducts recovered (n = 5) as reported previously (Song et al., 2011).

**Study three**, A total of 136 laying hens (88 hens over 36 months of age and 48 hens over 24 months of age), which had completely stopped laying eggs, were euthanized for biopsy and identification of cancerous (n = 10) ovaries for study, along with normal (n = 5) ovaries also collected from laying hens. We examined tumor stage in 10 hens with cancerous ovaries based on characteristic features of chicken ovarian cancers (Barua et al., 2009).

### ***RNA isolation***

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

### ***Semi-quantitative RT-PCR analyses***

The expression of *GALI1* mRNA in normal and cancerous ovaries of laying hens was assessed using semi-quantitative RT-PCR as described previously (Song et al., 2007). Complementary DNA (cDNA) was synthesized from total cellular RNA (2 µg) using random hexamer (Invitrogen, Carlsbad, CA) and oligo (dT) primers and AccuPower<sup>®</sup> RT PreMix (Bioneer, Daejeon, Korea). The cDNA was diluted (1:10) in sterile water before use in PCR. After PCR, equal amounts of reaction product were analyzed using a 1% agarose gel, and PCR products were visualized using ethidium bromide staining.

### ***Quantitative RT-PCR analyses***

Gene expression levels were measured using SYBR<sup>®</sup> Green (Sigma, St. Louis, MO, USA) and a StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) (Song et al., 2006). The *GAPDH* gene was simultaneously

analyzed as a control and used for normalization to account for variation in loading. Each target gene and *GAPDH* was analyzed in triplicate. ROX dye (Invitrogen) was used as a negative control for the fluorescence measurements. Sequence-specific products were identified by generating a melting curve in which the  $C_T$  value represented the cycle number at which a fluorescent signal was statistically greater than background, and relative gene expression was quantified using the  $2^{-\Delta\Delta C_T}$  method (Livak and Schmittgen, 2001). For the control, the relative quantification of gene expression was normalized to the  $C_T$  of the control ovary.

### ***In situ hybridization analysis***

Location of mRNA in sections (5  $\mu\text{m}$ ) of chicken oviducts and ovaries was determined by non-radioactive *in situ* hybridization analysis as described previously (Lim et al., 2012d). After verification of the sequences, plasmids containing the correct gene sequences were amplified with T7- and SP6-specific primers and then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). After hybridization and blocking, the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche). 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).

### ***Immunohistochemistry***

Antibodies to GAL11 were obtained following immunization of rabbits with a KLH-conjugated synthetic peptide and validated by immunocytochemical localization of GAL11 protein in the chicken oviduct and ovaries as described previously (Abdel Mageed et al., 2009; Song et al., 2006).

### ***MicroRNA target validation assay***

The 3'-UTR of *GAL11* was cloned and confirmed by sequencing and then it was subcloned into pcDNA3eGFP (Clontech, Mountain View, CA) to generate the eGFP-miRNA target 3'-UTR (pcDNA-eGFP-3'-UTR) fusion constructs as described previously (Lee et al., 2011). For the dual fluorescence reporter assay, the fusion construct containing the DsRed gene and *miR-1615* was designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miRNA). At 48 h post-transfection, dual fluorescence was detected by fluorescence microscopy and calculated by FACSCalibur flow cytometry (BD Biosciences).

### ***Statistical analyses***

Data presented for real-time PCR are expressed as mean $\pm$ SEM unless otherwise stated. Differences in the variances between normal and cancerous ovaries were analyzed using the *F* test, and differences between means were subjected to the Student's *t* test. Differences with a probability value of  $P < 0.05$  were considered statistically significant. Excel (Microsoft, Redmond, WA, USA) was used for statistical analyses.

## 4. Results and Discussion

### *Multiple sequence alignment, pairwise comparisons, and phylogenetic analysis*

The *GAL11* gene spans 2,619 bp on chicken chromosome 3 and consists of four exons (Xiao et al., 2004). *GAL11* mRNA has 652 bp encoding a protein with 104 amino acid residues (Supplementary Fig. 4-1A). The amino acid sequence of chicken GAL11 was compared to those of six other species. The pair-wise comparisons of GAL11 orthologs revealed that chicken GAL11 protein is well conserved relative to other avian GAL11 proteins, but not with mammalian beta defensin 11 proteins (38.0-93.3%, Table 4-1). The phylogenetic tree constructed by the neighbor-joining method using Geneious software is presented in Supplementary Fig. 4-1B. The mouse and rat *beta-defensin 11* genes cluster together and form a larger cluster with cattle. However, human and avian *beta-defensin 11* are in a separate branch. Chicken and turkey *beta-defensin 11* genes cluster together and are closer to zebra finch than to other species. These results indicate that chicken *GAL11* diverged from mammalian *beta-defensin 11* at a very early stage in evolution.

### *GAL11 mRNA expression in various organs from chickens*

Tissue-specific expression of *GAL11* mRNA in brain, heart, liver, kidney, gizzard, small intestine, ovary, oviduct and testis of 1- to 2-year-old males (n = 3) and females (n = 3) was determined using RT-PCR analyses. Results indicated high levels of expression of *GAL11* mRNA in oviducts of female chickens (Fig. 4-1A and 4-1B),

and lower expression in kidney and brain from females and kidney from males as reported previously (Xiao et al., 2004). However, expression of *GAL11* mRNA was not detected in other organs analyzed regardless of sex. Based on this oviduct-specific abundant expression *GAL11* and data from our previous differential gene profiling of the chicken oviduct treated with DES (Song et al., 2011), we focused on GAL11 in the chicken oviduct.

### ***Localization of chicken GAL11 mRNA and protein in the chicken Oviduct***

The oviduct of egg-laying hens includes the infundibulum (site of fertilization), magnum (production of egg-white proteins), isthmus (formation of the shell membrane), shell gland (formation of the egg shell) and vagina. RT-PCR analysis revealed that *GAL11* mRNA was most abundant in the magnum, but it was also expressed in the isthmus. Little or no *GAL11* mRNA was found in the infundibulum and shell gland (Fig. 4-1C). *In situ* hybridization analysis was used to determine cell-specific localization of *GAL11* mRNA in the chicken oviduct. As illustrated in Figure 4-1D, *GAL11* mRNA was most abundant in the glandular epithelium (GE) of the magnum, and it was also expressed, at lower abundance, in the GE of the isthmus. Little mRNA was detected in the infundibulum and shell gland. Results of immunohistochemical analysis (Fig. 4-1E) were consistent with results from *in situ* hybridization analyses in that GAL11 protein was abundant in the GE and LE of the magnum and isthmus. It was also found, to a lesser extent, in LE of the shell gland. In fact, of these segments of the chicken oviduct, the fully differentiated tubular gland cells of the magnum synthesize and secrete egg-white proteins such as

ovalbumin, ovo-mucoid, ovotransferrin and avidin during egg formation, while the shell gland is involved with calcification of the egg membranes prior to oviposition (the expulsion of the complete egg from the vagina) (Kohler et al., 1968). Therefore, these results indicate that the magnum is the most estrogen-responsive portion of the chicken oviduct and it controls the quality of the produced egg. These results suggest that a functional role(s) of GAL11 as a component of egg-white proteins to provide antimicrobial activities to protect the chicken embryo from pathogenic microorganisms (Gong et al., 2010; Herve-Grepinet et al., 2010; Yang et al., 2002).

#### ***Effects of DES on expression of GAL11 mRNA and protein in the chicken oviduct***

In chickens, estrogen stimulates proliferation and cytodifferentiation of immature oviductal epithelial cells to mature tubular gland cells, as well as induction of expression of egg white protein genes during oviduct development (Palmiter and Wrenn, 1971b; Socher and Omalley, 1973). Indeed, the administration of exogenous estrogen to neonatal chicks results in an 8-fold increase in wet weight of the magnum within 3 days. Consistent with these results, we reported that exogenous DES affects growth, development and differentiation of the chicken oviduct (Seo et al., 2009) and discovered candidate genes and pathways regulating oviduct development in chickens (Song et al., 2011). Cell-specific expression of GAL11 in the oviductal segments of mature hens suggests regulation by estrogen during development of the chick oviduct. Therefore, we examined the effects of DES on GAL11 expression in the chick oviduct. As shown in Figure 4-2A and 4-2B, semi-quantitative RT-PCR and quantitative PCR analyses revealed that DES induced a 43-fold increase ( $P < 0.001$ ) in oviductal *GAL11*

mRNA as compared to control chicks (Fig. 4-2C). In addition, DES stimulated 162- ( $P < 0.001$ ) and 17-fold increases ( $P < 0.001$ ) in *GAL11* mRNA in the magnum and isthmus, respectively (Fig. 4-2D). *In situ* hybridization analyses revealed that *GAL11* mRNA was expressed specifically in GE of the magnum and isthmus of chick oviducts treated with DES (Fig. 4-2E). Consistent with results from *in situ* hybridization analyses, immunoreactive GAL11 protein was detected predominantly in GE and LE of magnum and isthmus of oviducts treated with DES. Little or no *GAL11* mRNA and protein were detected in the infundibulum and shell gland of DES-treated chicks or in any segment of the oviducts from control chicks (Fig. 4-2F).

#### ***Post-transcriptional action of miRNAs on GAL11***

As post-transcriptional regulators, microRNAs (miRNAs) play pivotal roles in a wide variety of biological events including vertebrate growth, development and differentiation (Bartel, 2009a). Based on the possibility that GAL11 expression is regulated at the post-transcriptional level by miRNAs, we performed a miRNA target validation assay (Fig. 4-3). Analysis of potential miRNA binding sites within the 3'-UTR for *GAL11* using a miRNA target prediction database (<http://mirdb.org/miRDB>) revealed only one putative binding site for *miR-1615*. Therefore, we examined whether *miR-1615* influenced *GAL11* expression via its 3'-UTR. A fragment of the *GAL11* 3'-UTR harboring the *miR-1615* binding site was cloned downstream of the green fluorescent protein reading frame, thereby creating a fluorescent reporter for the function of the 3'-UTR region. After co-transfection of eGFP- *GAL11* 3'-UTR and DsRed-miRNA, the intensity of GFP expression and percentage of GFP-expressing

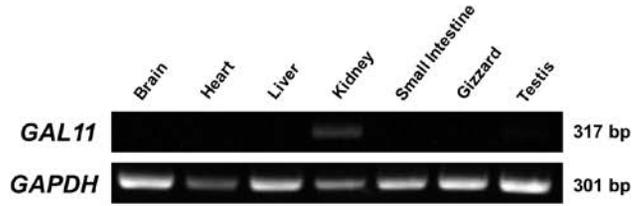
cells were analyzed by fluorescence microscopy and FACS. As shown in Figure 4-3C and 4-3D, in the presence of *miR-1615*, the intensity and percentage of GFP-expressing cells (43.6% in control vs. 30.4% in *miR-1615*) decreased ( $P < 0.01$ ). These results indicate that *miR-1615* binds directly to the *GAL11* transcript and post-transcriptionally regulates *GAL11* gene expression. Further, results from miRNA-specific quantitative PCR analyses revealed that DES induced a 1.57-fold increase ( $P < 0.05$ ) in expression of *miR-1615* in the oviduct as compared to control chicks. According to results of a miRNA-target search using the TargetScan program, *miR-1615* has the potential to regulate genes involved in various physiological processes, post-translation events and a variety of cellular metabolic processes, such as expression of estrogen receptor alpha (ESR1), apoptosis facilitator (BCL2), cell cycle genes and oncogenes. Therefore, we propose that *miR-1615* regulates expression of other DES-regulated genes which could be closely related to the regulation of *GAL11* gene expression and chicken oviduct development and differentiation; however, this requires further investigation.

### ***Differential expression of GAL11 in normal and cancerous ovaries of hens***

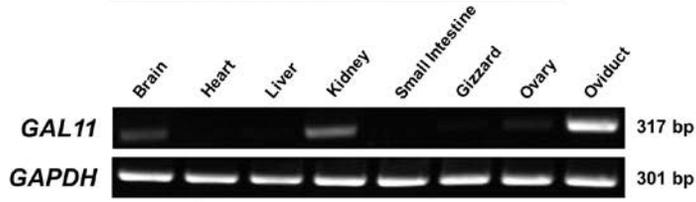
In our previous reports, several estrogen-stimulated genes, such as cathepsin B (*CTSB*) (Ahn et al., 2010), serpin peptidase inhibitor, clade B, member 11 (*SERPINB11*) (Lim et al., 2012f) and alpha 2 macroglobulin (*A2M*) (Lim et al., 2011a) in the chicken oviduct, are also expressed predominantly in GE of ovaries from laying hens with ovarian adenocarcinoma. Thus, we hypothesized that expression patterns for *GAL11* would differ between normal and cancerous ovarian tissues from laying

hens. Results of RT-PCR analysis revealed that *GAL11* mRNA was found in all carcinomas, but there was little or no expression in normal ovaries of laying hens (Fig. 4-4A and 4-4B). Further, the expression level of *GAL11* mRNA was greater ( $P < 0.01$ ) in cancerous ovaries from laying hens (Fig. 4-4C). Cell-specific expression of *GAL11* mRNA and protein was determined using *in situ* hybridization analysis and immunohistochemistry. There was abundant *GAL11* mRNA localized predominantly in GE of cancerous ovaries, but not in LE, stromal cells or blood vessels (Fig. 4-4D). Similarly, immunoreactive GAL11 protein was detected in GE of cancerous ovaries, but not in any other ovarian cell type (Fig. 4-4E). GAL11 protein was also detectable at low abundance in the stromal cells of cancerous ovaries. These results indicate that GAL11 may play a role in epithelial cell morphogenesis during development in chicken ovarian cancer and that its expression could be used for diagnosis of and monitoring effects of therapies for the disease.

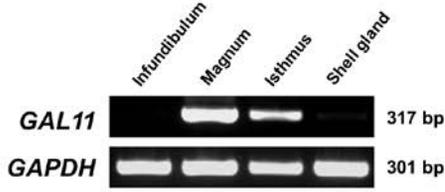
[A]



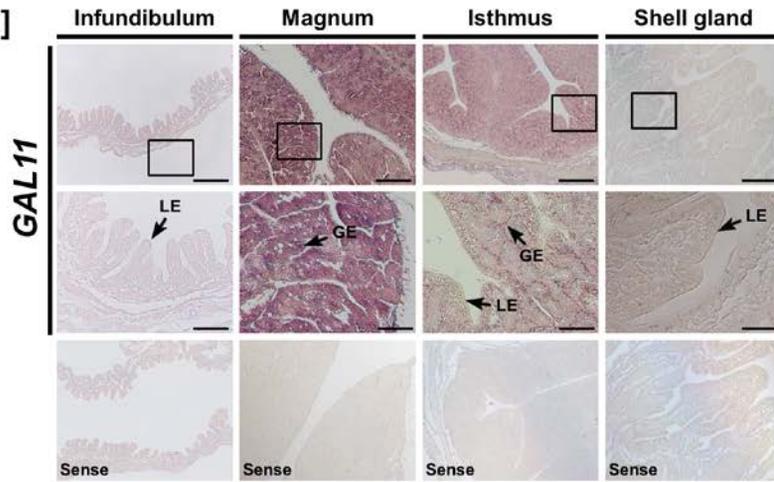
[B]



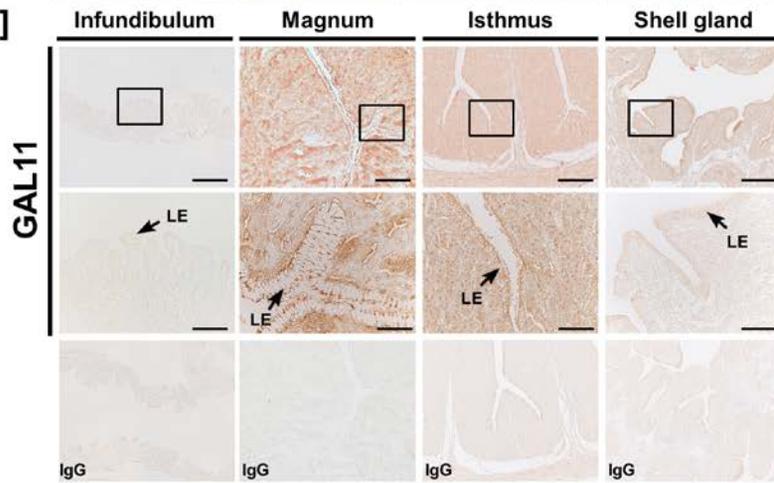
[C]



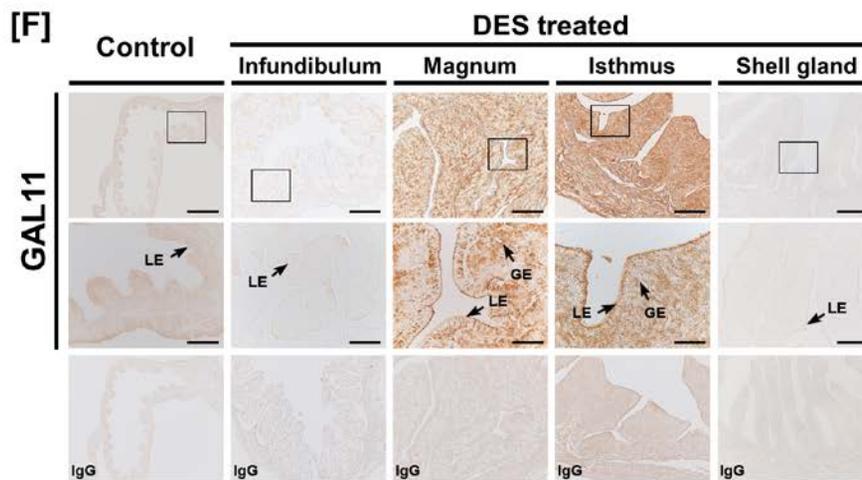
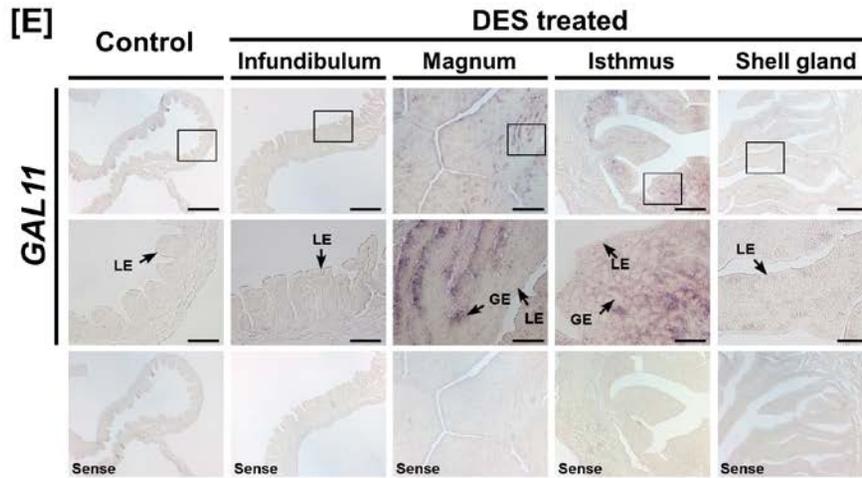
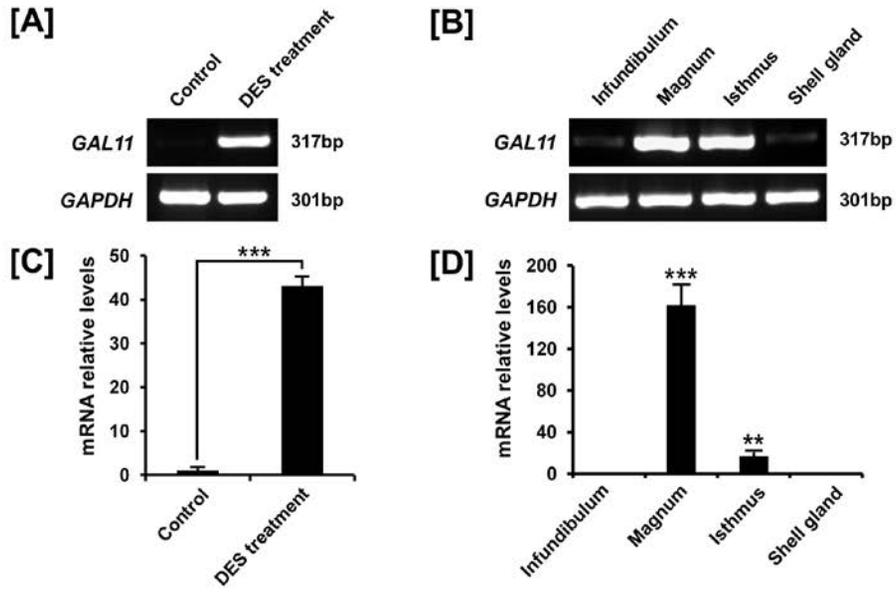
[D]



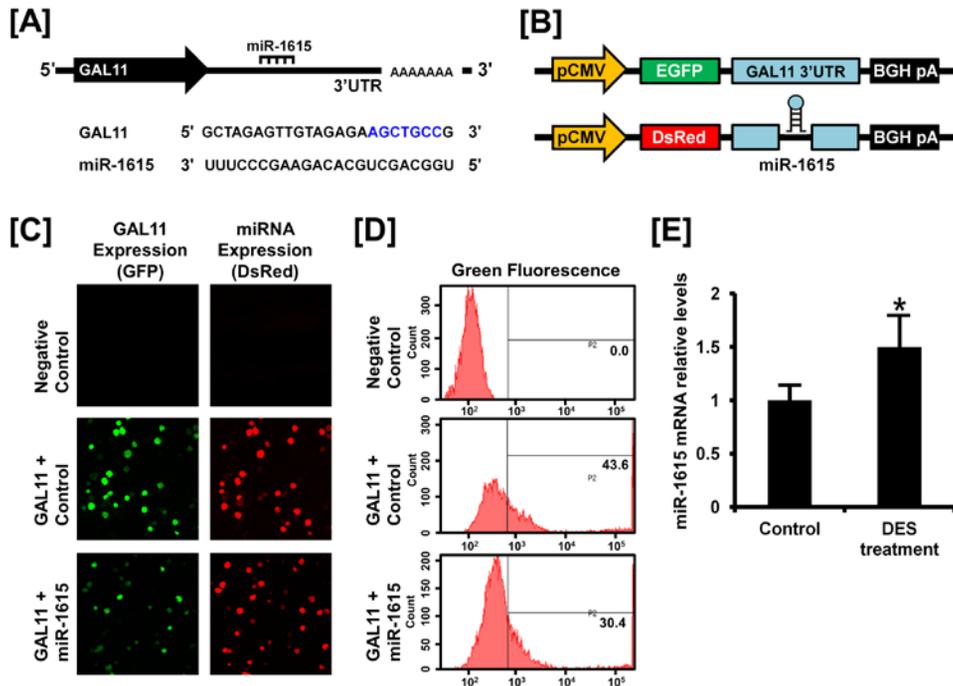
[E]



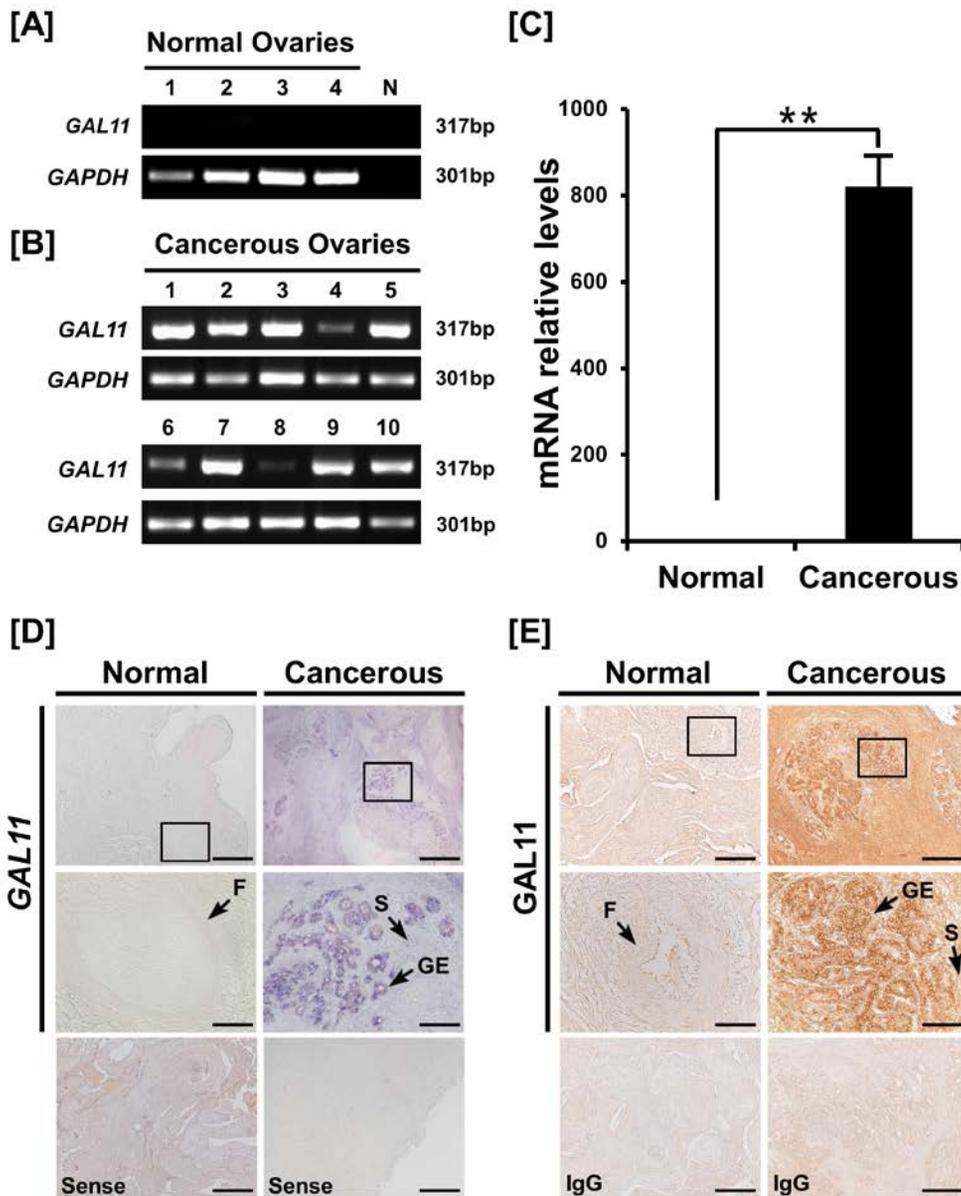
**Figure 4-1. Expression of GAL11 in various organs of chickens.** *GAL11* mRNAs were detected in different organs of male [A] and female [B] chickens by RT-PCR. [C] Results of RT-PCR analysis using cDNA templates from each segment of the chicken oviduct (infundibulum, magnum, isthmus, and shell gland) with chicken specific *GAL11* and *GAPDH* primers. [D] *In situ* hybridization analyses of *GAL11* in the chicken oviduct. Cross-sections of the four major segments of the chicken oviduct were hybridized with antisense or sense *GAL11* cRNA probes. [E] Immunoreactive *GAL11* protein was detected in the chicken oviduct. For the IgG control, preimmune rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium. Scale bar represents 200  $\mu\text{m}$  (the first horizontal panels, sense and IgG) or 50  $\mu\text{m}$  (the second horizontal panels, sense and IgG).



**Figure 4-2. Effect of DES on tissue specific expression of GAL11.** Both RT-PCR [A and B] and q-PCR [C and D] analyses were performed using cDNA templates from oviducts of DES-treated and control chicks. These experiments were conducted in triplicate and normalized to control *GAPDH* expression. [E] *In situ* hybridization analyses revealed cell-specific expression of *GAL11* mRNA in oviducts of DES-treated and control chicks. Cross-sections of the four major segments of oviducts (infundibulum, magnum, isthmus, and shell gland) from chicks treated with DES or vehicle were hybridized with antisense or sense *GAL11* cRNA probes. [F] Immunoreactive GAL11 protein was detected in oviducts of DES-treated and control chicks. For the IgG control, preimmune rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium. Scale bar represents 200  $\mu\text{m}$  (the first horizontal panels, sense and IgG) or 50  $\mu\text{m}$  (the second horizontal panels, sense and IgG).

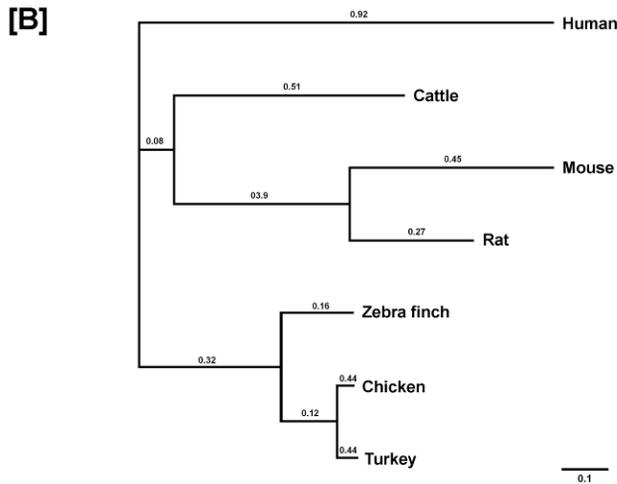


**Figure 4-3.** *In vitro* target assay to determine effects of *miR-1615* on *GAL11* transcript. [A] Diagram of *miR-1615* binding sites in the 3'-UTR of the *GAL11* gene. [B] Expression vector maps for eGFP with *GAL11* 3'-UTR and Ds-Red for each miRNA. [C and D] After co-transfection of pcDNA-eGFP-3'-UTR for the *GAL11* transcript and pcDNA-DsRed-miRNA for *miR-1615*, the fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D]. [E] Quantitative analysis of *miR-1615* expression between non-treated (Control) and DES-treated oviducts of chick was performed. These experiments were conducted in triplicate and normalized to control U6 snRNA expression. The asterisks denote statistically significant differences (\* $P < 0.05$ ).



**Figure 4-4. Expression and quantitation of GAL11 in normal and cancerous ovaries from laying hens.** [A] RT-PCR analyses were performed using cDNA templates from normal and cancerous ovaries of laying hens using *GAL11* and *GAPDH*-specific primers for chickens. Lanes 1-4 show results of analysis of four normal ovaries with distilled water as a negative control (N). [B] Lanes 1-10 are from

analyses of 10 different cancerous ovaries. Expression of *GAL11* mRNA was abundant in all carcinomas, but not in normal ovaries. Legend for panel B: Lane 1, clear cell carcinoma (Stage IV); Lane 2, endometrioid/serous/mucinous carcinoma (Stage III); Lane 3, endometrioid carcinoma (Stage I); Lane 4, serous carcinoma (Stage I); Lane 5, mucinous/endometrioid carcinoma (Stage IV); Lane 6, endometrioid carcinoma (Stage IV); Lane 7, endometrioid carcinoma (Stage III); Lane 8, clear cell carcinoma (Stage IV); Lane 9, serous/mucinous carcinoma (Stage IV); and Lane 10, serous/mucinous/endometrioid carcinoma (Stage III). [C] The q-PCR analysis was performed using cDNA templates from normal and cancerous ovaries of laying hens (mean±SEM;  $P < 0.01$ ). [D] *In situ* hybridization analyses of *GAL11* mRNA in normal and cancerous ovaries of hens. Cross-sections of normal and cancerous ovaries of hens hybridized with antisense or sense chicken *GAL11* cRNA probes demonstrated abundant *GAL11* mRNA predominantly in GE of cancerous ovaries, but not in LE, stroma or blood vessels. [E] Immunoreactive GAL11 protein in normal and endometrioid cancerous ovaries of hens. For the IgG control, preimmune rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: F, follicle; GE, glandular epithelium; S, stroma. Scale bar represents 200  $\mu\text{m}$  (the first horizontal panels, sense and IgG) or 50  $\mu\text{m}$  (the second horizontal panels, sense and IgG).



**Figure 4-5. Multiple sequence alignment and tissue-specific expression of GAL11 in chickens.** [A] The amino acid sequences of GAL11 (beta-defensin 11) from each of seven species (chicken, human, mouse, rat, cattle, zebra finch and turkey) are presented based on alignments determined using Geneious Alignment with BLOSUM (Blocks Substitution Matrix) and gap penalties. Amino acid sequences in the shaded boxes represent those that are identical for chicken, avian and mammalian *beta-defensin 11*, and dashes indicate gaps in the sequences. [B] This phylogenetic tree of *beta-defensin 11* was generated from alignments of primary sequences of chicken, human, mouse, rat, cattle, zebra finch and turkey beta-defensin 11 proteins using bootstrap analysis with 1000 replicates.

**Table 4-1. Pairwise comparison of GAL11 between chicken and other species.**

<b>Species</b>	<b>Symbol</b>	<b>Identity (%)</b>	<b>GenBank No.</b>
Chicken	GAL11	-	NP_001001779.1
vs. Human	DEFB11	38	NP_001032817.1
vs. Mouse	Defb11	50	NP_631967.1
vs. Rat	Defb11	32	NP_001032594.1
vs. Cattle	DEFB11	44	XP_003587988.1
vs. Zebra Finch	beta-defensin 11	71	XP_002186664.1
vs. Turkey	gallinacin-11-like	93.3	XP_003204671.1

## **CHAPTER 5**

# **Differential Expression of Secreted Phosphoprotein 1 in Response to Estradiol- 17 $\beta$ and in Ovarian Tumors in Chickens**

## 1. Abstract

Secreted phosphoprotein 1 (SPP1), a highly phosphorylated protein containing a polyaspartic acid sequence and a conserved RGD motif, plays important roles in physiological processes such as inflammatory responses, calcification, organ development, immune cell function and carcinogenesis. Results of the present study indicate expression of SPP1 mRNA in various organs such as oviduct, small intestine and kidney from chickens, particularly in the glandular epithelium (GE) of the shell gland and, to a lesser extent, in luminal epithelium (LE) of the infundibulum and magnum, and GE of the isthmus of the oviduct. We determined that DES (diethylstilbestrol, a synthetic nonsteroidal estrogen) decreases *SPP1* expression in the oviduct and that SPP1 mRNA and protein are significantly more abundant in GE of ovarian endometrioid carcinoma, but not the other cancerous and normal ovaries of hens. Further, *microRNA-140* was discovered to influence *SPP1* expression via its 3'-UTR which suggests that post-transcriptional regulation influences *SPP1* expression in chickens. Collectively, results of this study indicate that SPP1 is novel in that its expression is down-regulated by estrogen in epithelial cells of the chicken oviduct and that it is up-regulated in chicken ovarian endometrioid tumor that could be used for monitoring effects of therapies for this disease in laying hens.

## 2. Introduction

Secreted phosphoprotein 1 (*SPP1*), also known as osteopontin, is a highly phosphorylated small integrin-binding ligand (SIBLING), N-linked glycoprotein originally isolated from bones of rats (Butler, 1989). The *SPP1* gene includes seven exons and it is located on chromosome 4q13 in humans and chromosome 5 in mice, and it has a polyaspartic acid sequence and a highly conserved RGD motif (Sodek et al., 2000). *SPP1* plays essential roles in various biological events, such as cell–cell interactions, immune responses, carcinogenesis, wound healing, bone metabolism and calcification (Sodek et al., 2000). In chickens, *SPP1* participates in formation and calcification of the egg shell in the shell gland of the reproductive tract of laying hens (Pines et al., 1995). Further, immunoreactive *SPP1* protein is detected mainly in the core of the non-mineralized shell membrane fibers, in the base of the mammillae and in the outermost part of the palisade of the egg shell (Fernandez et al., 2003). Although the number of amino acid residues in mammalian and avian *SPP1* proteins are similar, *SPP1* exists as 44-75 kDa forms due to post-translational modifications (Sodek et al., 2000) and proteolytic cleavage at its thrombin cleavage site. This indicates that *SPP1* is synthesized and secreted by epithelia of the shell gland and accumulated in the egg shell membrane as it becomes calcified during egg formation and oviposition.

The reproductive tract of the female chicken is a unilateral organ with one functional ovary and one oviduct. The chicken oviduct is well-known as an excellent research model for studies of organ development, morphogenesis, and hormonal

responsiveness (Dougherty and Sanders, 2005). It has four segments: infundibulum, magnum, isthmus and shell gland. Each segment has an important role in formation of the egg by secretion of egg proteins and formation of soft shell and calcified shell (Chousalkar and Roberts, 2008). During development of the chicken oviduct, estrogen is essential as it stimulates proliferation and cytodifferentiation of epithelial to tubular gland cells and expression of oviduct-specific genes (Palmiter and Wrenn, 1971b; Socher and Omalley, 1973). Therefore, this study was conducted to: (1) determine tissue- and cell-specific expression of the *SPP1* gene in various organs of the chicken; (2) examine effects of estrogen on expression of *SPP1* mRNA and protein during oviduct development in chicks; (3) determine whether *SPP1* is regulated by posttranscriptional action of specific microRNA and (4) compare differential expression of *SPP1* in normal and cancerous ovaries from hens. Results of this study provide novel insights into the chicken *SPP1* gene with respect to its tissue- and cell-specific expression and regulation of its expression by estrogen and microRNA during development of the chicken oviduct and in chicken ovarian carcinogenesis.

### 3. Materials and Methods

#### *Experimental animals and animal care*

The experimental use of chickens for this study was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). All White Leghorn (WL) chickens were exposed to a light regimen of 15 h light and 9 h dark with *ad libitum* access to feed and water.

#### *Tissue samples*

**Study one.** Following euthanasia of mature WL hens, tissue samples were collected from brain, heart, liver, kidney, small intestine, gizzard, ovary, oviduct and testis of 1–2 year-old males (n = 3) and females (n = 3).

**Study two.** Female chicks were identified by PCR analysis using W chromosome-specific primer sets (Lee et al., 2009). Treatment with DES and recovery of the oviduct (n = 5) were conducted as reported previously (Sanders and Mcknight, 1988; Song et al., 2011).

**Study three.** A total 136 chickens (88 chickens aged over 36 months and 48 chickens aged over 24 months), which had completely stopped laying eggs were euthanized for biopsy and cancerous (n = 10) ovaries were collected. As a control, normal (n = 5) ovaries were also collected from egg-laying hens. We examined the tumor stage in 10 chickens with cancerous ovaries using characteristic features of chicken ovarian cancer (Barua et al., 2009; Lim et al., 2012f).

### ***RNA isolation***

Total cellular RNA was isolated from frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations.

### ***Semi-quantitative and quantitative RT-PCR analysis***

The level of expression of *SPP1* mRNA in various organs from chickens, including the oviduct, was assessed using semi-quantitative RT-PCR as described previously (Song et al., 2007).

### ***In situ hybridization analysis***

Location of *SPP1* mRNA in sections (5  $\mu$ m) of chicken oviduct was determined by radioactive in situ hybridization analysis as described previously (Song et al., 2007).

### ***Immunohistochemistry***

Immunocytochemical localization of SPP1 protein in the chicken oviduct was performed as described previously (Song et al., 2007).

### ***MicroRNA target validation assay***

The 3'-UTR of *SPP1* was cloned and confirmed by sequencing. The 3'-UTR was subcloned between the eGFP gene and the bovine growth hormone poly-A tail in pcDNA3-eGFP (Clontech, Mountain View, CA) to generate the eGFP-miRNA target 3'-UTR (pcDNAeGFP-3'-UTR) fusion constructs as described previously (Lee et al., 2011). For the dual fluorescence reporter assay, the fusion constructs containing the DsRed gene and *miR-140* were designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miRNA). At 48 h post-transfection, dual fluorescence was detected by fluorescence microscopy and calculated by FACSCalibur flow cytometry (BD Biosciences). For flow cytometry, the cells were fixed in 4% paraformaldehyde and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

### ***Statistical analyses***

Data presented for quantitative PCR analysis are expressed as mean±SEM unless otherwise stated. Differences in the variances between normal and cancerous ovaries were analyzed using the *F* test, and differences between means were subjected to the Student's *t* test. Differences with a probability value of  $P < 0.05$  were considered statistically significant.

## 4. Results

### *Analysis of SPP1 expression in adult chicken organs (study one)*

To determine tissue-specific expression of *SPP1* mRNA in various organs of male and female chickens, RT-PCR analysis was performed. The analysis revealed that *SPP1* mRNA was abundant in kidneys from male and female chickens and also gizzard and oviduct of females (Fig. 5-1A and 5-1B). *SPP1* mRNA was also detected in small intestines from both sexes, and, to a lesser extent, testis of males and livers of both sexes. In oviduct of hens, *SPP1* is involved in eggshell formation and calcification (Pines et al., 1995). Therefore, subsequent aspects of this study focused on expression and potential roles of *SPP1* in the oviduct and ovary of hens.

### *Localization of SPP1 mRNA and protein expression in adult chicken oviduct (study one)*

We first determined cell-specific expression of *SPP1* mRNA in each segment of chicken oviduct; infundibulum (site of fertilization), magnum (production of components of egg-white), isthmus (formation of the shell membrane), and shell gland (formation of the egg shell) using in situ hybridization analysis (Fig. 5-1C). *SPP1* mRNA was most abundant in the glandular epithelium (GE) of the shell gland, and also at a lower abundance in luminal epithelium (LE) of the infundibulum and magnum, and GE of the isthmus. Little or no mRNA was detected in stromal cells, blood vessels or immune cells of the oviduct. Next, immunohistochemistry was used

to determine the localization of immunoreactive SPP1 protein in the chicken oviduct (Fig. 5-1D). Interestingly, SPP1 protein is present in the LE of all segments of the oviduct, but less abundant in GE of the isthmus and shell gland. The mouse IgG used as a negative control did not detect SPP1.

### ***Effects of DES on SPP1 expression in the chick oviduct (study two)***

Next we examined the effects of DES on *SPP1* expression in the chicken oviduct. As illustrated in Figure 5-2A and 5-2B, RT-PCR and realtime PCR analyses revealed that DES decreased expression of *SPP1* mRNA in the oviduct as compared to control chicks ( $P < 0.001$ ). Consistent with these results, *in situ* hybridization analyses revealed abundant expression of *SPP1* mRNA in control chick oviducts (Fig. 5-2C). However, *SPP1* mRNA was also localized in GE of the shell gland of DES-treated chick oviducts and present at a lower abundance in LE and GE of the infundibulum, magnum, and isthmus from oviducts of DES-treated chicks. In contrast, immunoreactive SPP1 protein was detected in LE of oviducts of control chicks and the infundibulum of the oviducts of chicks treated with DES (Fig. 5-2D). In addition, SPP1 protein was abundant in GE of the magnum, isthmus and shell gland of DES-treated oviducts and, to a lesser extent, in LE of each segment of the oviduct.

### ***Post-transcriptional regulation of microRNA affecting SPP1***

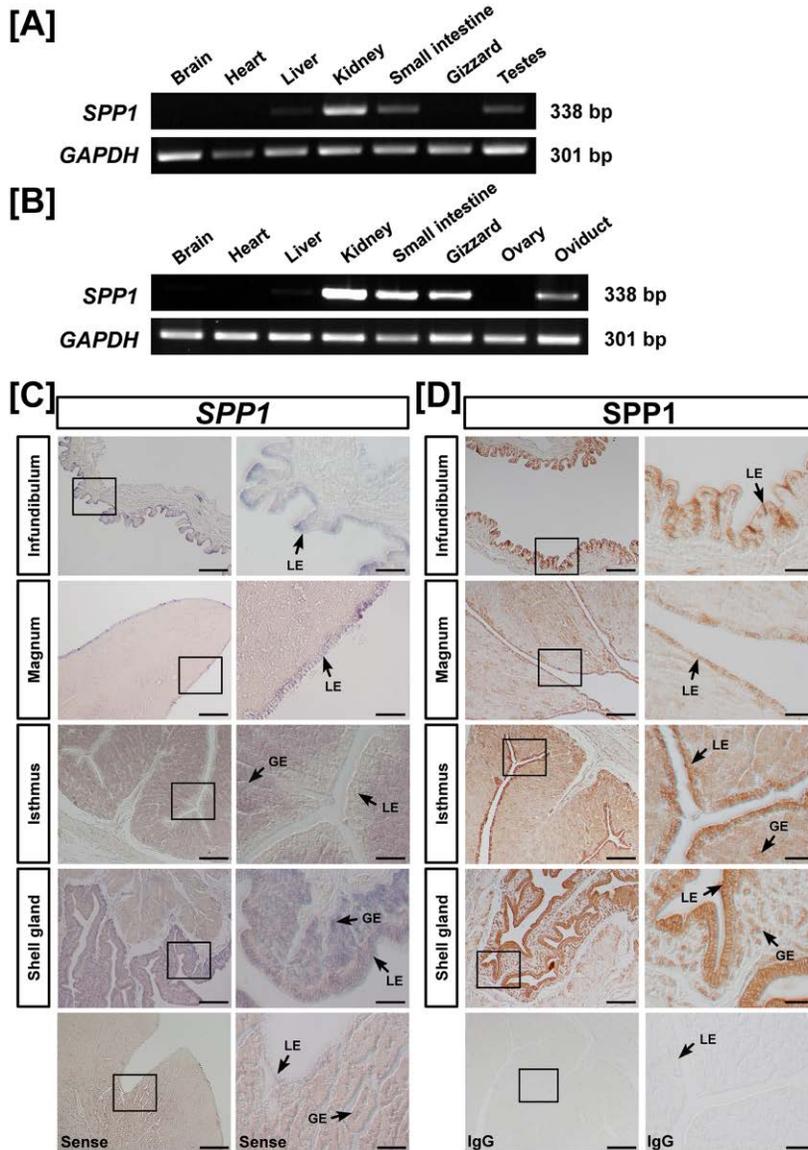
To investigate the possibility that *SPP1* expression is regulated at the post-transcriptional level by miRNAs, we performed a miRNA target validation assay.

Analysis of potential miRNA binding sites within the 3'-UTR for *SPP1* using a miRNA target prediction database (miRDB; <http://mirdb.org/miRDB>) revealed only one putative binding site for *miR-140* (Fig. 5-3A). Therefore, we determined if this *miR-140* influenced *SPP1* expression via its 3'-UTR. A fragment of the *SPP1* 3'-UTR harboring binding site for the *miR-140* was cloned downstream of the green fluorescent protein (GFP) reading frame, thereby creating a fluorescent reporter for function of the 3'-UTR region (Fig. 5-3B). After co-transfection of eGFP-SPP1-3'-UTR and DsRed-miRNA, the intensity of GFP expression and percentage of GFP-expressing cells were analyzed by fluorescence microscopy and FACS. As shown in Figure 5-3C and 5-3D, in the presence of *miR-140*, the intensity and percentage of GFP-expressing cells (32.4% in control vs. 10.1% in *miR-140*) decreased ( $P < 0.01$ ). This result indicates that *miR-140* directly bind to the *SPP1* transcript and post-transcriptionally regulate *SPP1* gene expression.

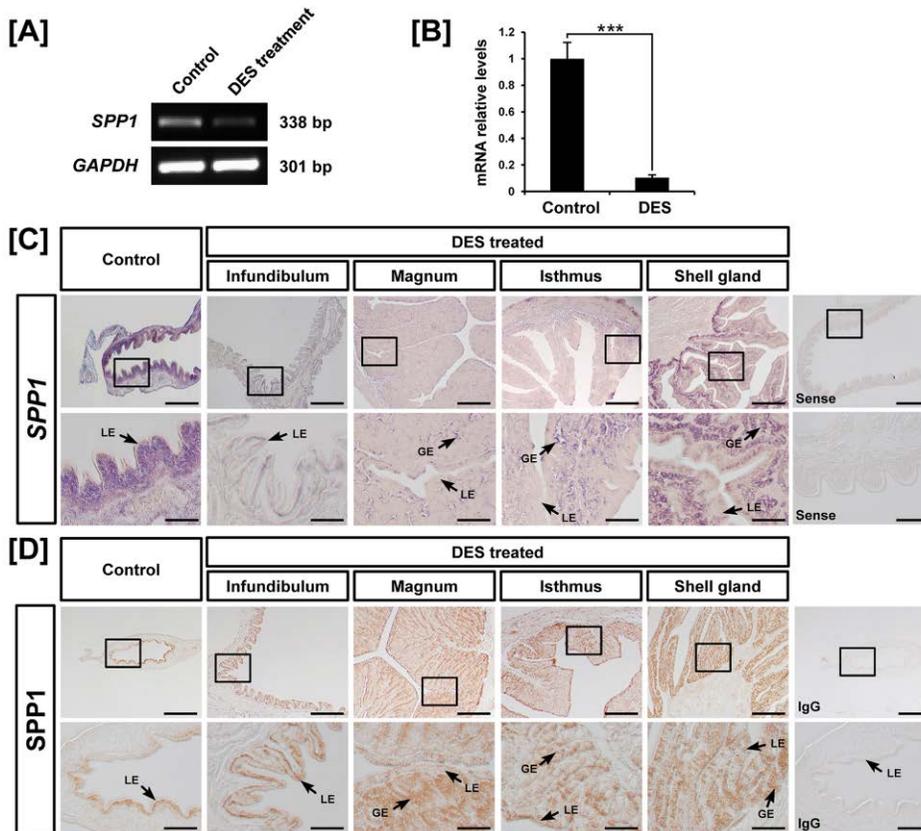
***Differential expression and localization of SPP1 mRNA and protein in normal and cancerous ovaries of hens (study three)***

To examine if *SPP1* is up- and down-regulated in ovarian cancer cells of chickens, because the chicken is the only animal that spontaneously develops ovarian cancer of the surface epithelium of the ovaries at a high rate as occurs in women (Stammer et al., 2008), we performed RT-PCR and quantitative PCR analyses. Results of RT-PCR analyses revealed that expression of *SPP1* mRNA was predominantly found in five ovarian endometrioid carcinomas, but there was little or no expression in serous, mucinous or clear cell carcinomas and normal ovaries (Fig. 5-4A and 5-4B).

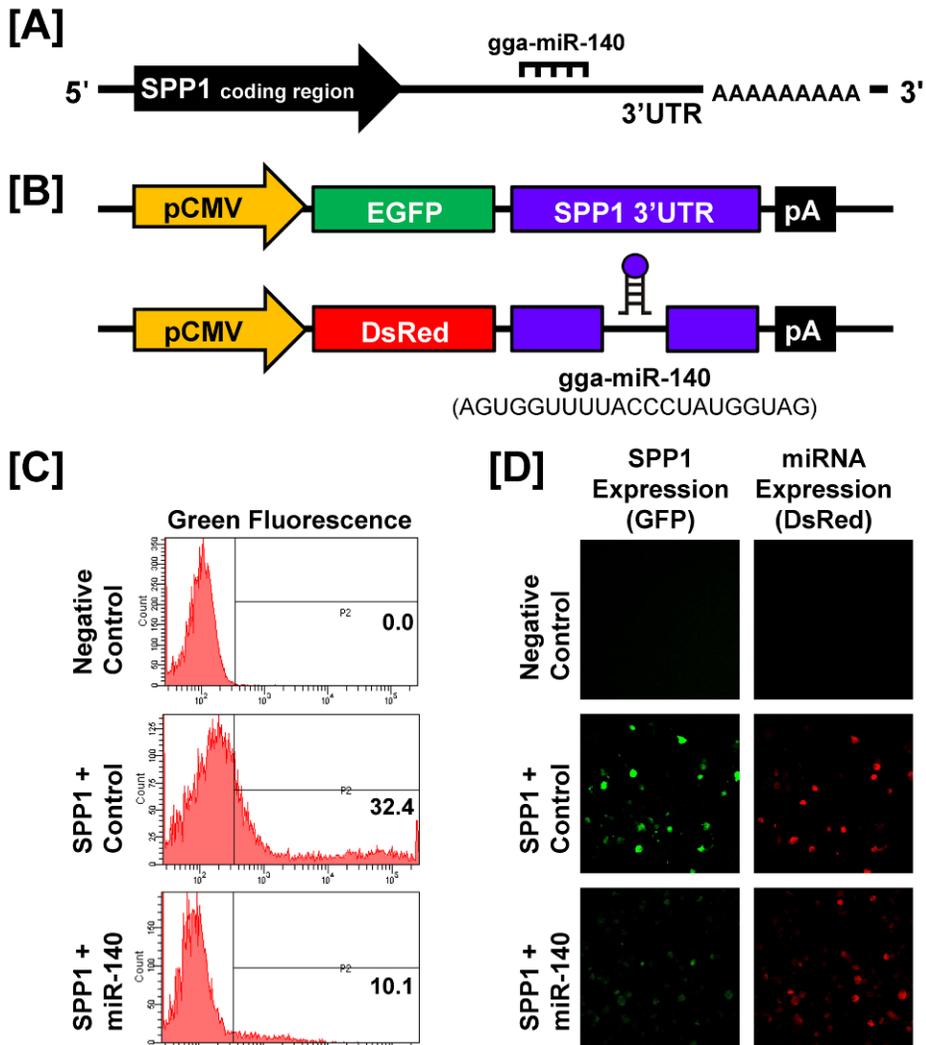
Further, quantitative PCR showed that expression of *SPP1* mRNA was greater ( $P < 0.05$ ) in endometrioid cancerous ovaries from hens (Fig. 5-4C). To determine cell-specific expression of *SPP1* mRNA and protein, we performed *in situ* hybridization analysis and immunohistochemistry. There was abundant *SPP1* mRNA localized predominantly in GE of cancerous ovaries, but not in LE, stroma or blood vessels (Fig. 5-4D). Consistent with this result, immunoreactive SPP1 protein was abundant in GE of cancerous ovaries, but not in any other cell types in the same tissues (Fig. 5-4E).



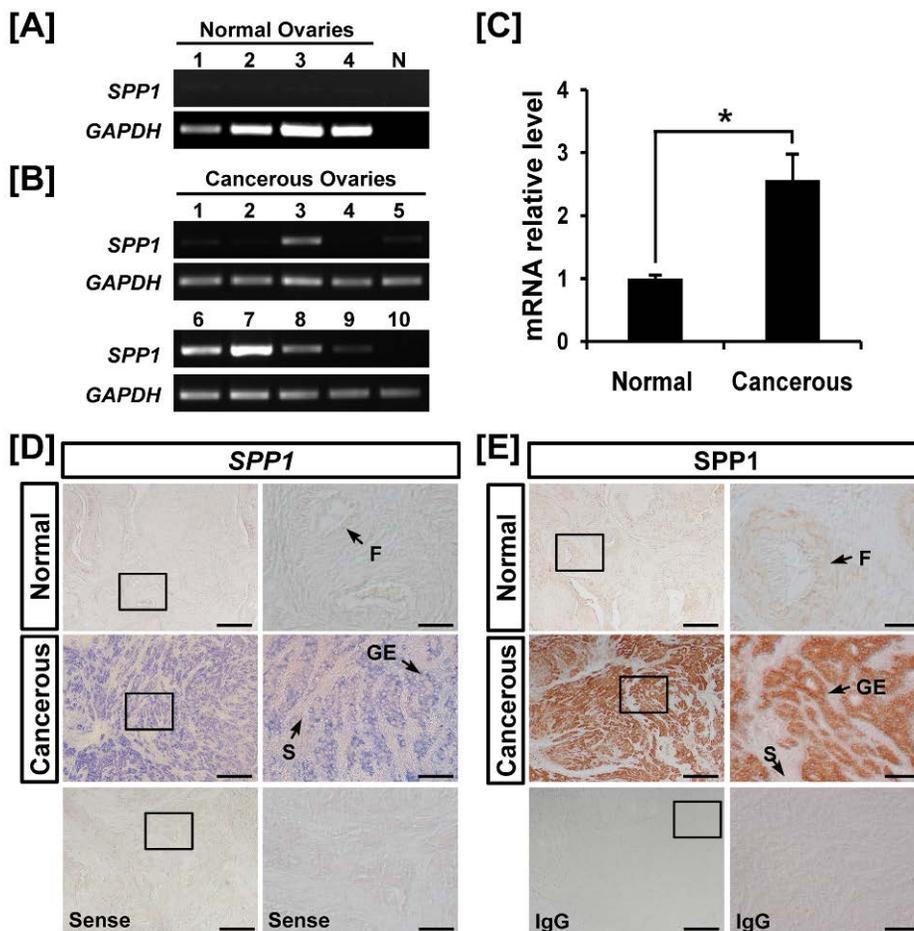
**Figure 5-1. Expression of SPP1 in various organs of male and female of chickens.** Results of RT-PCR analysis using cDNA templates from different organs of male [A] and female [B] chickens with chicken *SPP1* and chicken *GAPDH*-specific primers. [C] *In situ* hybridization analyses of *SPP1* mRNAs in the chicken oviduct. [D] Immunoreactive SPP1 protein in the chicken oviduct. Legend: LE, luminal epithelium; GE, glandular epithelium; *Scale bar* represents 200  $\mu\text{m}$  (the first columnar panels) and 50  $\mu\text{m}$  (the second columnar panels).



**Figure 5-2. Effect of DES on tissue- and cell-specific expression of *SPP1* in the chicken oviduct.** Both RT-PCR [A] and quantitative-PCR [B] analyses were performed using cDNA templates from DES-treated and control chicken oviducts (mean±SEM;  $P < 0.001$ ). [C] *In situ* hybridization analyses of *SPP1* mRNA in oviducts of DES-treated and control chicks. Cross-sections of the four segments of chicken oviduct (infundibulum, magnum, isthmus, and shell gland) treated with DES or vehicle were hybridized with antisense or sense chicken *SPP1* cRNA probes. (D) Immunoreactive *SPP1* protein in oviducts of DES-treated and control chicks. Legend: LE, luminal epithelium; GE, glandular epithelium. *Scale bar* represents 200  $\mu\text{m}$  (the first horizontal panels) and 50  $\mu\text{m}$  (the second horizontal panels).



**Figure 5-3. *In vitro* target assay of microRNAs on the *SPP1* transcript.** [A] Diagram of *miR-140* binding site in *SPP1* 3'-UTR. [B] Expression vector maps for eGFP with *SPP1* 3'-UTR and Ds-Red with *miR-140*. [C and D] After co-transfection of pcDNA-eGFP-3'UTR for the *SPP1* transcript and pcDNA-DsRed-miRNA for the *miR-140*, the fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D].



**Figure 5-4. Expression and quantitation of *SPP1* in normal and cancerous ovaries from hens.** [A] and [B] RT-PCR analyses were performed using cDNA templates from normal and cancerous ovaries of laying hens using chicken *SPP1* and *GAPDH*-specific primers. [A] Lanes 1 to 4 show the results of analyses of four normal ovaries with N as a negative control. [B] Lanes 1–10 are from analyses of 10 different cancerous ovaries from laying hens. Legend for panel B: 1, clear cell carcinoma (Stage IV); 2, serous carcinoma (Stage III); 3, endometrioid carcinoma (Stage I); 4, serous carcinoma (Stage I); 5, mucinous carcinoma (Stage IV); 6, endometrioid carcinoma (Stage IV); 7, endometrioid carcinoma (Stage III); 8,

endometrioid/clear cell carcinoma (Stage IV); 9, serous/mucinous carcinoma (Stage IV); 10, serous carcinoma (Stage III). [C] The q-PCR analysis for *SPP1* mRNA was performed using cDNA templates from normal and cancerous ovaries of laying hens (mean±SEM;  $P < 0.001$ ). [D] *In situ* hybridization analyses of *SPP1* mRNA in normal and cancerous ovaries of hens. Cross-sections of normal and cancerous ovaries of hens hybridized with antisense or sense chicken *SPP1* cRNA probes. [E] Immunoreactive *SPP1* protein in normal and cancerous ovaries of hens. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: GE, glandular epithelium. *Scale bar* represents 200  $\mu\text{m}$  (the first horizontal panels, sense and IgG) or 50  $\mu\text{m}$  (the second horizontal panels, sense and IgG).

## 5. Discussion

Results of the present study demonstrate tissue- and cell-specific expression of *SPP1* in normal chickens. Our results also revealed that *SPP1* gene expression is post-transcriptionally regulated by *miR-140* critical to development of the chick oviduct in response to estrogen. Furthermore, these results are the first to identify high levels of expression of *SPP1* gene in ovarian endometrioid carcinoma as compared to normal ovaries of hens. In chickens, SPP1 isolated from chicken bone exists as two phosphorylated proteins of approximately 66 kDa and 60 kDa (Gotoh et al., 1990) and has seven of nine consecutive residues of aspartic acid, an RGD (Arg-Gly-Asp) integrin recognition motif and four recognition sequences for phosphorylation with the greatest similarity to mammalian SPP1 (Rafidi et al., 1994). Expression of *SPP1* in a variety of tissues from different organisms suggests that it has important roles in multiple functions such as inflammation responses, calcification, organ development, and carcinogenesis (Sodek et al., 2000). In this study, chicken *SPP1* gene expression was detected in various organs from male and female chickens. Although the expression of *SPP1* gene in the oviduct and eggshell of chickens has been reported (Fernandez et al., 2003; Lavelin et al., 1998; Pines et al., 1995), results of the present study indicate that *SPP1* was not only detected only in the shell gland, but also expressed at lower abundance in LE of the infundibulum and magnum, and GE of the isthmus. Moreover, immunoreactive SPP1 protein is readily detectable in LE of all segments of the hen oviduct.

Since the discovery of *SPP1*, a number of comparative studies have offered

insights into the potential hormonal and molecular mechanisms underlying *SPP1*-mediated cell adhesion, organ remodeling, and cell-extracellular matrix interactions within the reproductive tract such as uterus and embryo/placenta. For examples, in the ovine uterus, *SPP1* mRNA is expressed by endometrial GE during pregnancy (Johnson et al., 1999b). These results suggest that ovarian steroid hormone such as progesterone and/or interferon tau, maternal recognition signal from developing trophoctoderm in ruminant species, induce expression and secretion of *SPP1* by uterine glands during the peri-implantation period and that *SPP1* induces cell-cell adhesion between LE of the uterus and trophoctoderm of the embryo to facilitate superficial implantation in ruminants (Johnson et al., 1999a; Johnson et al., 1999b). *SPP1* also has a critical role in the reproductive tract of chickens. Pines *et al.* reported that *SPP1* is synthesized and secreted by LE cells of the shell gland and accumulates in the egg shell for calcification (Pines et al., 1995). However, little is known about the effect of steroid hormones on *SPP1* gene expression in the chicken oviduct. Therefore, we investigated effects of estrogen on expression of *SPP1* mRNA and protein during oviductal development in chicks. In this study, a synthetic nonsteroidal estrogen, diethylstilbestrol (DES) was used. McKnight in 1978 and Kohler in 1969 reported that the developmental pattern of the neonatal chick oviduct in response to DES implants was similar to that in response to natural estrogen based on confirmation of differentiation of tubular glands and ciliated cell, and the expression of egg white protein in the magnum (Kohler et al., 1969; Palmiter et al., 1978). Indeed, the developmental pattern of chick oviduct in response to DES treatment in our study is very similar to the previous reports (Kohler et al., 1968; Oka and Schimke, 1969a, b, c). As illustrated in Figure 5-2, DES decreased expression of *SPP1* mRNA and protein

in the oviduct as compared to control chicks. These results are consistent with our previous differential gene profiling data on the chicken oviduct treated with the synthetic estrogen agonist DES (Song et al., 2011). Therefore, these results indicate that estrogen down-regulates *SPP1* gene expression during development of the chicken oviduct; however, the underlying mechanisms for DES-induced down-regulated *SPP1* expression are not known.

In a wide variety of fundamental processes and biological events in vertebrates, such as cellular survival, growth, development and differentiation, microRNAs (miRs) play pivotal roles in post-transcriptional regulation and pathways (Bartel, 2004). As shown in Figure 5-3, co-transfection of eGFP-*SPP1*-3'-UTR and DsRed-miRNA decreased the percentage of GFP-positive cells and GFP fluorescence density in *miR-140* transfected cells, when compared to controls. These results indicate that *miR-140* bind directly to the 3'-UTR of the *SPP1* transcript and post-transcriptionally regulate *SPP1* gene transcription. Thus, we propose that the *miR-140* is closely related to the regulatory pathways of oviduct development and differentiation in chickens; however, this requires further investigation.

The chicken is a unique animal model for research on human ovarian cancer, because it spontaneously develops epithelial cell-derived ovarian cancer as occurs in women (Barua et al., 2009). In general, natural menopause in women occurs between 40 and 55 years of age when estrogen and progesterone production decreases progressively with advancing age of the ovaries. Likewise, epithelial-derived ovarian cancer in hens develops spontaneously at a high rate after they experience a severe

depression in egg production when more than 2 years of age. Therefore, the laying hen is a unique model for research on human ovarian cancer aimed at development of biomarkers and anti-cancer drugs for prevention, early diagnosis, and therapies to treat the disease. In the present study, *SPP1* mRNA and protein are most abundant in GE of ovarian endometrioid carcinoma, but not normal ovaries of hens. These results suggest that *SPP1* increases only in chicken ovarian endometrioid cancer cells that could be used for monitoring effects of therapies for the disease.

Collectively, our current data demonstrate that the *SPP1* gene is differentially expressed in the reproductive tract of the female chicken with respect to tissue- and cell-specific expression and hormonal and post-transcriptional regulation, and also increases dramatically in hens with progressive endometrioid carcinoma of the ovary. Therefore, results of the present study provide new insights into *SPP1* with respect to regulation and functional roles in oviduct development and egg formation cascades, and potential application for therapies of ovarian endometrioid cancer in chickens.

## **CHAPTER 6**

# **Differential Expression of Alpha 2 Macroglobulin in Response to Diethylstilbestrol and in Ovarian Carcinomas in Chickens**

## 1. Abstract

Alpha 2 macroglobulin (A2M; also known as ovostatin), a homotetrameric protein with four disulfide-linked subunits, has the unique feature of inactivating/inhibiting most known proteases including serine-, threonine-, cysteine-, aspartic- and metalloproteases. In chickens, A2M has been identified and characterized biochemically, but little is known of its functional role(s) in the oviduct, hormonal regulation of expression or its expression in ovarian carcinomas in chickens. Therefore, we investigated estrogen regulation of A2M gene expression during development of the chicken oviduct, and its expression in normal and cancerous ovaries from chickens. To determine tissue-specific expression of A2M in chickens, we collected various organs from male and female chickens and performed RT-PCR analyses. We found that A2M is most abundant in the chicken oviduct, specifically luminal (LE) and glandular epithelia (GE), but it was not detected in any other tissues of either sex by RT-PCR and *in situ* hybridization analyses. We then determined that DES (diethylstilbestrol, a synthetic nonsteroidal estrogen) increased A2M mRNA only in LE and GE of the oviduct of chicks RT-PCR, qPCR and *in situ* hybridization analyses. Further, expression of A2M was most abundant in GE of endometrioid adenocarcinoma of cancerous, but not normal ovaries of hens. Collectively, results of the present study indicate that A2M is novel estrogen-stimulated gene expressed in LE and GE of the chicken oviduct and may be used for monitoring effects of therapies for ovarian cancer in laying hens.

## 2. Introduction

The alpha 2 macroglobulins (A2M) are proteins in blood that act as protease inhibitors in mammals (Armstrong, 2006; Sottrup-Jensen, 1989; Tayade et al., 2005; Vanleuven et al., 1988). In humans, the *A2M* gene is a single-copy gene located on chromosome 12p12-13 that encodes for a functional homotetramer protein with disulfide-linked 180-kDa subunits (Matthijs et al., 1992; Sottrup-Jensen et al., 1984). Even though A2M is produced predominantly by the liver, it may also be expressed in the reproductive tract, heart, and brain, and may have important roles in many physiological processes and medical illnesses including Alzheimer's disease (Blacker et al., 1998; Matthijs et al., 1992; Umans et al., 1995). Of particular note, A2M increases in blood serum of women with inflammatory and neoplastic lesions of the ovary (Zbroja-Sontag, 1983). It also stimulates production of activin and inhibin in pre-ovulatory follicles (McElhinney et al., 2002; Vaughan and Vale, 1993) and controls coordinate changes in uterine vasculature during pregnancy (Tayade et al., 2005). In addition, Umans *et al.* reported that A2M deficient mice were viable and produced normal size litters with normal sex ratios over three generations (Umans et al., 1995). Moreover, A2M regulates the function of cortical granule proteases and other trypsin-like proteases activated in sea urchin eggs during fertilization (Yamada and Aketa, 1988). In chickens, A2M is also known as an ovostatin or ovomacroglobulin. It is found in the oviduct and egg white, but not in other tissues or serum (Nagase et al., 1983) and it has a strong anti-collagenase activity (Nagase et al., 1985; Nagase and Harris, 1983). However, little is known about regulation of its expression by steroid hormones in the oviduct or its expression in normal and

cancerous ovaries.

As the primary female sex hormone, estrogen regulates reproductive behavior. It is responsible for proliferation and differentiation of several cell types associated with osteoporosis, diabetes, cardiovascular disease, and reproductive carcinomas (Dougherty and Sanders, 2005; Herynk and Fuqua, 2004; Hewitt et al., 2005; Louet et al., 2004; Pearce and Jordan, 2004; Wise et al., 2005). The chicken oviduct is well-known as an excellent research model for studies of organ development and hormonal responsiveness (Dougherty and Sanders, 2005). During development of the chicken oviduct, estrogen stimulates proliferation and cytodifferentiation of epithelial cells to tubular gland cells and expression of oviduct-specific genes (Palmiter and Wrenn, 1971b; Socher and Omalley, 1973). However, progesterone interferes with normal estrogen-mediated cytodifferentiation of tubular gland cells (Oka and Schimke, 1969a, b, c; Palmiter and Wrenn, 1971b). Estrogen also affects calcium metabolism for eggshell formation and ovipositioning or egg laying (Bar, 2009; Hincke et al., 2010). In addition, estrogen administration to sexually immature chicks stimulates growth of the oviduct by inducing cellular hyperplasia and hypertrophy (Dougherty and Sanders, 2005; Song et al., 2011). The mammalian oviduct undergoes diverse biological changes in response to sex steroids during the estrous cycle and early pregnancy as these actions are pivotal to establishing an optimal microenvironment for events ranging from gamete transport to early embryonic development (Buhi et al., 1997). To investigate the biological actions and signaling pathways of estrogen, the chicken is one of the best animal models (Dougherty and Sanders, 2005). Indeed, steroid hormones are involved in many

physiological and developmental processes accompanying modification of tissue-specific and conditional control of gene expression and homeostasis (Dougherty and Sanders, 2005; Okada et al., 2005). Although general effects of estrogen and progesterone on the reproductive tract of vertebrates are well documented, details of their interactions that affect cell signaling pathways in avian species are unclear.

The objectives of this study were to: 1) determine tissue- and cell-specific expression of the *A2M* gene in chickens; 2) determine whether estrogen regulates expression of *A2M* during oviductal development in chicks; and 3) compare expression of *A2M* in normal and cancerous ovaries from laying hens. Results of this study indicate that *A2M* is a novel estrogen-stimulated gene during development of the chicken oviduct and that it may be an initial candidate gene for further study of the development of epithelial ovarian cancer in hens.

### 3. Materials and Methods

#### *Experimental animals and animal care and use*

The experimental use of chickens for this study was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). White Leghorn (WL) chickens were subjected to standard management practices at the University Animal Farm, Seoul National University, Korea with respect to management of hens for reproduction, incubation of eggs and rearing of chicks, as well as standard operating protocols in our laboratory. All chickens had *ad libitum* access to feed and water.

#### *Tissue samples*

**Study one.** Following euthanasia of mature WL hens, tissue samples were collected from brain, heart, liver, kidney, muscle, small intestine, gizzard, ovary, oviduct and testis of 1- to 2-year-old males (n = 3) and females (n = 3). Subsets of these samples were frozen or fixed in 4% paraformaldehyde for further analyses. Frozen tissue samples were cut into 5- to 7-mm pieces, frozen in liquid nitrogen vapor, and stored at -80°C. The other samples were cut into 10 mm pieces and fixed in fresh 4% paraformaldehyde in PBS (pH 7.4). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5 µm.

**Study two.** Female chicks were identified by PCR analysis using W

chromosome-specific primer sets (Lee et al., 2009). Treatment with DES and recovery of the oviduct were conducted as reported previously (Sanders and Mcknight, 1988; Seo et al., 2009). Briefly, a 15 mg DES pellet was implanted subcutaneously in the abdominal region of 1-week-old female chicks for release of hormone for 20 days (Kohler et al., 1969; McKnight, 1978; Sanders and Mcknight, 1988). Five chicks in each group were euthanized using 60%-70% carbon dioxide. Subsets of these samples were frozen or fixed in 4% paraformaldehyde for further analyses. Frozen tissue samples were cut into 5- to 7-mm pieces and frozen in liquid nitrogen. The other samples were cut into 10- to 15-mm pieces and fixed in fresh 4% paraformaldehyde in PBS (pH 7.4). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5  $\mu$ m.

**Study three.** In this study, a total of 136 chickens (88 chickens over 36 months of age and 48 chickens over 24 months of age), which had completely stopped laying eggs were euthanized for biopsy and cancerous (n = 10) ovaries were collected. As a control, normal (n = 5) ovaries were also collected from egg-laying hens. We examined tumor stage in 10 hens with cancerous ovaries based on characteristic features of chicken ovarian cancers (Barua et al., 2009). In three hens, ovarian tumor cells were classified as Stage III as they had metastasized to the gastrointestinal tract and superficial surface of the liver with profuse ascites in the abdominal cavity. In five hens, the tumors had metastasized to distant organs such as liver parenchyma, lung, gastrointestinal tract and oviduct with profuse ascites, so these were classified at Stage IV tumors. The other two hens did not have tumors in any other organs; therefore, their ovarian tumors were classified as Stage I. Subsets of these samples were frozen

or fixed in 4% paraformaldehyde for further analyses. Frozen tissue samples were cut into 5- to 7-mm pieces and frozen in liquid nitrogen. The other samples were cut into 10 mm pieces and fixed in 4% paraformaldehyde in PBS (pH 7.4). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5  $\mu\text{m}$  and stained with hematoxylin and eosin. Epithelial ovarian cancers in chickens were classified based on their cellular subtypes and patterns of cellular differentiation with reference to ovarian malignant tumor types in humans (Barua et al., 2009).

### ***RNA isolation***

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

### ***Sequence analysis***

For pair-wise comparisons, the amino acid sequences of A2M genes from each species were aligned using Geneious Pro Version 5.04 with default penalties for gap and the protein weight matrix of BLOSUM (Blocks Substitution Matrix).

### ***Semiquantitative RT-PCR analysis***

The level of expression of *A2M* mRNA in various organs from chickens, including the oviduct, was assessed using semi-quantitative RT-PCR as described previously (Song et al., 2007). The cDNA was synthesized from total cellular RNA (2 µg) using random hexamer (Invitrogen, Carlsbad, CA) and oligo (dT) primers and AccuPower<sup>®</sup> RT PreMix (Bioneer, Daejeon, Korea). The cDNA was diluted (1:10) in sterile water before use in PCR. For *A2M*, the sense primer (5'-CTG GCT CAC TGC CTT TGT GT-3') and antisense primer (5'-CCG TCA ACT TCC TTT GCT GA-3') amplified a 405-bp product. For *GAPDH*, the sense primer (5'-TGC CAA CCC CCA ATG TCT CTG TTG-3') and antisense primer (5'-TCC TTG GAT GCC ATG TGG ACC AT-3') amplified a 301-bp product. The primers, PCR amplification and verification of their sequences were conducted as described previously (Song et al., 2007). PCR amplification was conducted using approximately 60 ng cDNA as follows: (1) 95°C for 3 min; (2) 95°C for 20 sec, 61°C for 40 sec, and 72°C for 1 min for 35 cycles (*A2M*), 30 cycles (*GAPDH*); and (3) 72°C for 5 min. Then, equal amounts of reaction product were analyzed using a 1% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a Gel Doc<sup>™</sup> XR+ system with Image Lab<sup>™</sup> software (Bio-Rad).

### ***Quantitative RT-PCR analysis***

Total RNA was extracted from each oviduct of control and DES-treated

chicks using TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized using AccuPower<sup>®</sup> RT PreMix (Bioneer, Daejeon, Korea). Gene expression levels were measured using SYBR<sup>®</sup> Green (Sigma, St. Louis, MO, USA) and a StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The *GAPDH* gene was simultaneously analyzed as a control and used for normalization of data. Expression of each target gene and *GAPDH* was analyzed in triplicate. Using the standard curve method, we determined levels of expression of the examined genes using the standard curves and C<sub>T</sub> values, and normalized them based on *GAPDH* expression levels. The PCR conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 20 sec, 60°C for 40 sec, and 72°C for 1 min using a melting curve program (increasing the temperature from 55°C to 95°C at 0.5°C per 10 sec) and continuous fluorescence measurement. The ROX dye (Invitrogen) was used as a negative control for measurements of fluorescence. Sequence-specific products were identified by generating a melting curve in which the C<sub>T</sub> value represented the cycle number at which a fluorescent signal was significantly greater than background, and relative gene expression was quantified using the 2<sup>-ΔΔCT</sup> method (Livak and Schmittgen, 2001). For the control, the relative quantification of gene expression was normalized to the C<sub>T</sub> of the control oviduct.

### ***In situ hybridization analysis***

For hybridization probes, PCR products were generated from cDNA with the primers used for RT-PCR analysis. The products were gel-extracted and cloned into

pGEM-T vector (Promega). After verification of the sequences, plasmids containing 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') then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). Tissues were collected and fixed in 4% paraformaldehyde. The tissues were embedded in paraffin and sectioned at 5  $\mu$ m on APES-treated (silanized) slides. The sections were then deparaffinized in xylene and rehydrated to diethylpyrocarbonate (DEPC)-treated water through a graded series of alcohol. The sections were treated with 1% Triton X-100 in PBS for 20 min and washed two times in DEPC-treated PBS. After washing in DEPC-treated PBS, the sections were digested with 5  $\mu$ g/ml Proteinase K (Sigma) in TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) at 37°C. After post-fixation in 4% paraformaldehyde, sections were incubated twice for 5 min each in DEPC-treated PBS and incubated in TEA buffer (0.1 M triethanolamine) containing 0.25% (v/v) acetic anhydride. The sections were incubated in a prehybridization mixture containing 50% formamide and 4 $\times$  standard saline citrate (SSC) for at least 10 min at room temperature. After prehybridization, the sections were incubated with a hybridization mixture containing 40% formamide, 4 $\times$  SSC, 10% dextran sulfate sodium salt, 10 mM DTT, 1 mg/ml yeast tRNA, 1 mg/ml salmon sperm DNA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2 mg/ml RNase-free bovine serum albumin and denatured DIG-labeled cRNA probe for overnight at 42°C in a humidified chamber. After hybridization, sections were washed for 15 min in 2 $\times$  SSC at 37°C, 15 min in 1 $\times$  SSC at 37°C, 30 min in NTE buffer (10 mM Tris, 500 mM NaCl and 1 mM EDTA) at 37°C and 30 min in 0.1 $\times$  SSC at 37°C. After blocking with 2% normal sheep serum

(Santa Cruz Biotechnology, INC.), the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche). 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).

### *Statistical analyses*

Differences in the variance between control and DES-treated oviducts were analyzed by analysis of variance, and differences between means were subjected to Student's *t* test. The probability value of  $P < 0.05$  was considered statistically significant. Excel (Microsoft, Redmond, WA, USA) was used for statistical analyses.

## 4. Results

### *Multiple sequence alignment, pairwise comparisons, and phylogenetic analysis*

The chicken *A2M* gene was found in the genomic region spanning 35,791 bp on chromosome 1. The gene consists of 35 exons and the mRNA has 4,715 bp encoding a protein with 1,454 amino acid residues. The primary sequence of chicken A2M was compared to those of some mammalian species. In pairwise comparisons of chicken A2M with eight other vertebrate species, chicken A2M protein has moderate homology to mammalian A2M proteins (42.4-45.4%, Table 6-1). In particular, chicken A2M protein contains the highly conserved macroglobulin 1 (MG1) and MG2 domains, A2M family N-terminal region, and A2M receptor binding domain found in mammalian A2M proteins (Fig. 6-7). In the phylogenetic tree generated from primary sequences of available vertebrate A2M proteins, chicken A2M was placed between mammalian and amphibian species consistent with the general pattern of molecular evolution in vertebrates (Fig. 6-8).

### *A2M mRNA expression in chickens (study one)*

To determine tissue-specific expression of *A2M* mRNA in chickens, we collected various organs from male and female chickens and performed RT-PCR analysis. As shown in Figure 6-1, *A2M* mRNA is abundantly expressed in the oviduct and, to a lesser extent, in the brain of female chickens. However, specific expression was not detected in any other organs analyzed for either sex. Therefore, further studies

focused on *A2M* in the chicken oviduct.

### ***Localization of A2M mRNA expression in the chicken oviduct***

The oviduct of egg-laying hens includes the infundibulum (site of fertilization), magnum (production of components of egg-white), isthmus (formation of the soft shell membrane), and shell gland (formation of the egg shell). Using RT-PCR analysis, it was determined that *A2M* mRNA is expressed abundantly in the infundibulum, magnum, and isthmus of the chicken oviduct (Fig. 6-2A). At a lower abundance, *A2M* mRNA is also expressed in the shell gland. In addition, *in situ* hybridization analysis revealed that *A2M* mRNA is most abundant in glandular epithelium (GE) of the magnum but expressed to a lesser extent in GE of the isthmus and luminal epithelium (LE) and GE of the shell gland (Fig. 6-2B). Little or no mRNA was detected in stromal cells, blood vessels, immune cells or myometrium (smooth muscle) of the oviduct.

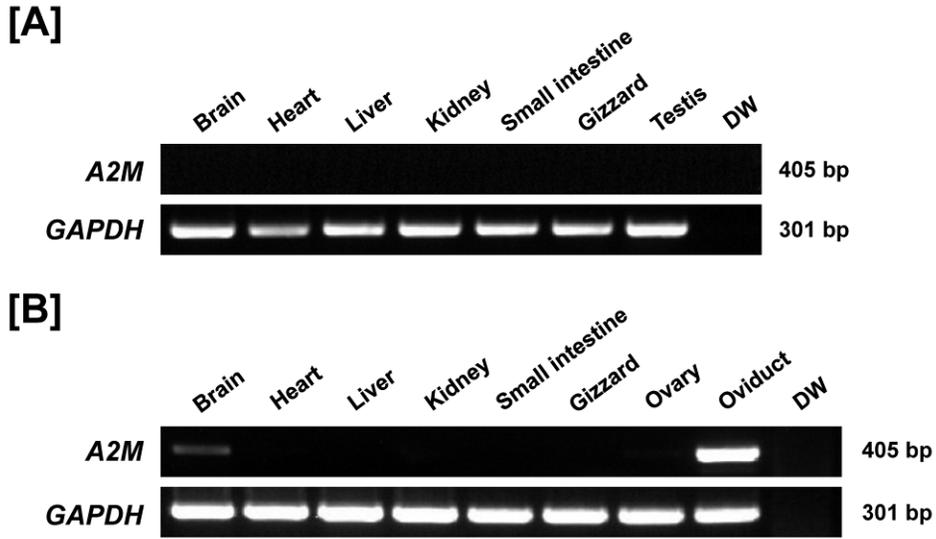
### ***Effects of DES on A2M mRNA expression in the chicken oviduct (study two)***

Oviduct-specific and cell-type specific expression of *A2M* in the oviductal segments of hens suggested regulation by estrogen during development of the oviduct in chicks. We reported that DES induces development and differentiation of the chicken oviduct (Seo et al., 2009) and discovered candidate genes regulating oviduct development in chickens (Song et al., 2011). Therefore, we examined *A2M* gene expression in the oviduct of 37-day-old chicks that received a subcutaneous implant of

15 mg DES in the abdominal region for 20 days (Seo et al., 2009). We performed RT-PCR analyses using cDNAs from control- (n = 5) and DES-treated oviducts (n = 5), and then each segment of oviducts from DES-treated chicks (Fig. 6-3A and 6-3B). In addition, as illustrated in Figure 6-3C, quantitative PCR analysis revealed that DES stimulated a 22-fold increase ( $P < 0.001$ ) in *A2M* mRNA in oviducts as compared to oviducts from control chicks. DES treatment also stimulated a 123-fold increase ( $P < 0.001$ ) in *A2M* mRNA in the magnum (Fig. 6-3D). Further, *in situ* hybridization analyses revealed abundant expression of *A2M* mRNA only in GE of the isthmus and a lower abundance in GE of the magnum of chick oviducts treated with DES (Fig. 6-4).

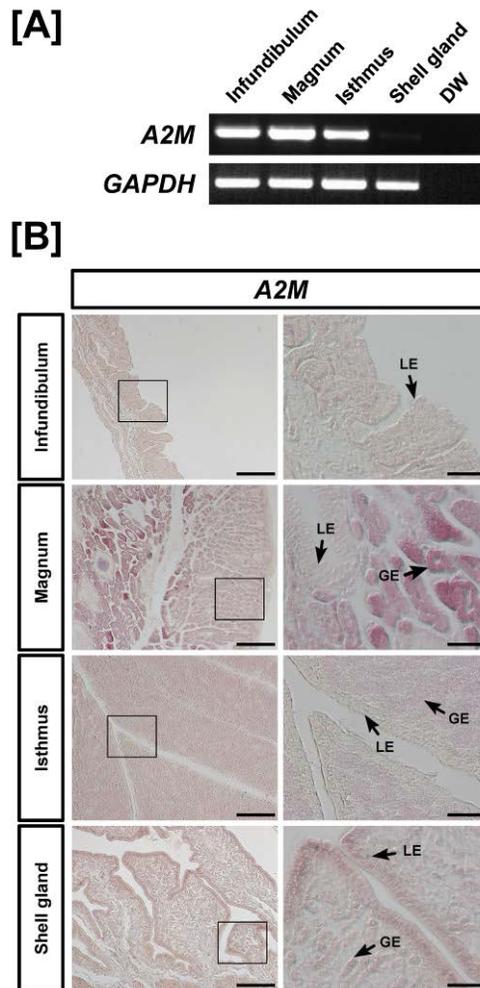
***Differential expression of A2M mRNA in normal and cancerous ovaries of hens (study three)***

It was next determined if *A2M* is a biomarker of ovarian cancer in laying hens which is the only animal model that spontaneously develops epithelia-derived ovarian cancer at a high rate as occurs in women (Fredrickson, 1987). As illustrated in Figure 6-5B, RT-PCR analyses indicated that *A2M* mRNA expression was abundant in endometrioid carcinomas (lane 2, -3, -6, and -7), which is characterized by glandular patterns resembling those of the endometrium, but there was little or no expression of *A2M* in serous, mucinous or clear cell carcinomas and normal ovaries (Fig. 6-9). Further, *in situ* hybridization analysis revealed abundant *A2M* mRNA localized predominantly to GE of cancerous ovaries, but not LE, stroma or blood vessels (Fig. 6-6).

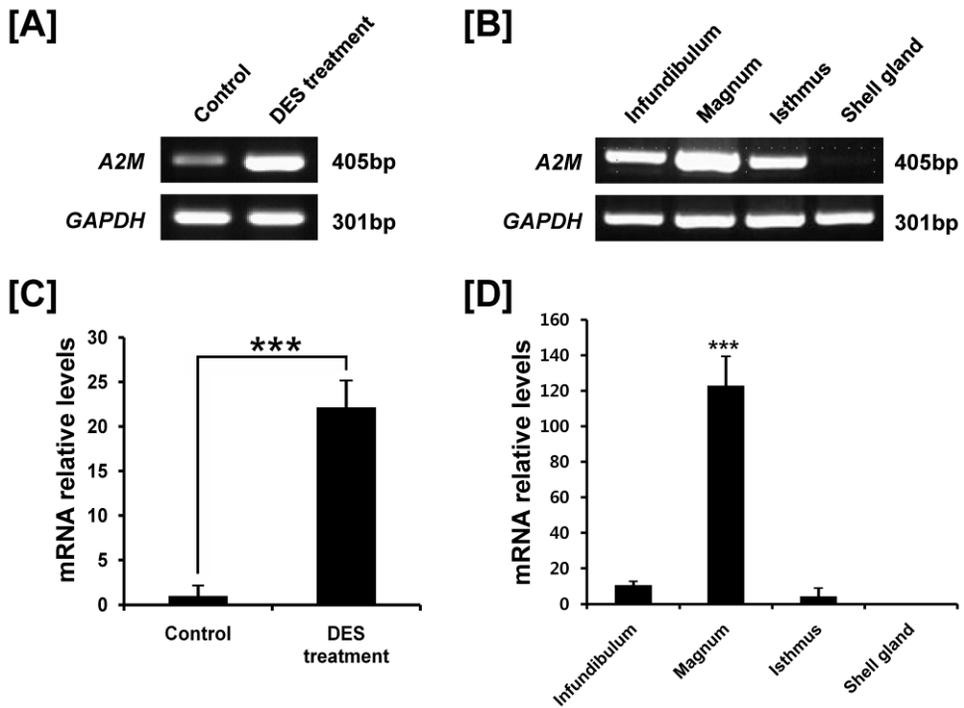


**Figure 6-1. Expression of *A2M* in various organs of male and female chickens.**

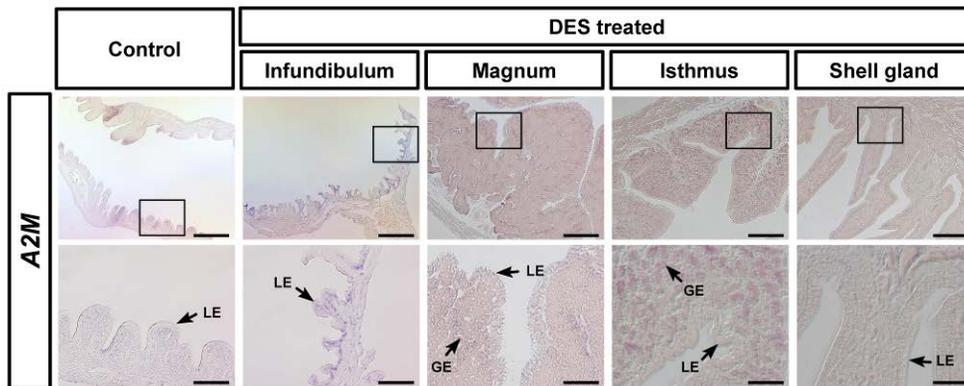
Results of RT-PCR analysis using cDNA from different organs of male [A] and female [B] chickens indicate that *A2M* mRNA is expressed abundantly in the oviduct and, to a lesser extent, in the brain of female chickens. However, specific expression was not detected in any other organs analyzed for either sex.



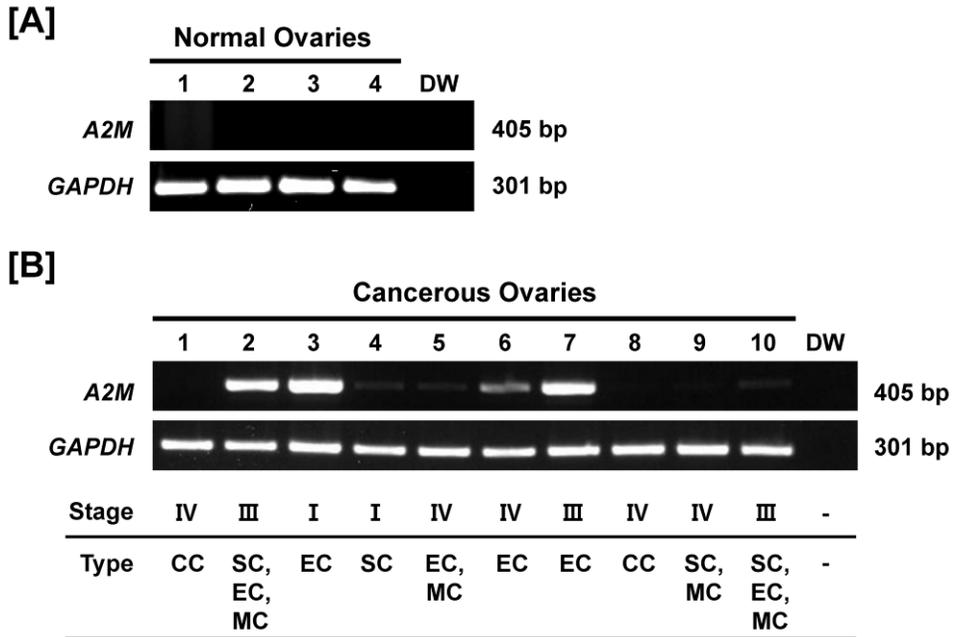
**Figure 6-2. RT-PCR and *in situ* hybridization analyses of *A2M* mRNAs in the chicken oviduct.** Results of RT-PCR analysis using cDNA from different segments from female chicken [A] indicate that *A2M* mRNA is expressed abundantly in the infundibulum, magnum, and isthmus of the oviduct. [B] Cross-sections of the infundibulum, magnum, isthmus and shell gland of the chicken oviduct hybridized with antisense or sense chicken *A2M* cRNA probes. Legend: LE, luminal epithelium; GE, glandular epithelium; Scale bar represents 200  $\mu\text{m}$  (the first columnar panels) and 50  $\mu\text{m}$  (the second columnar panels).



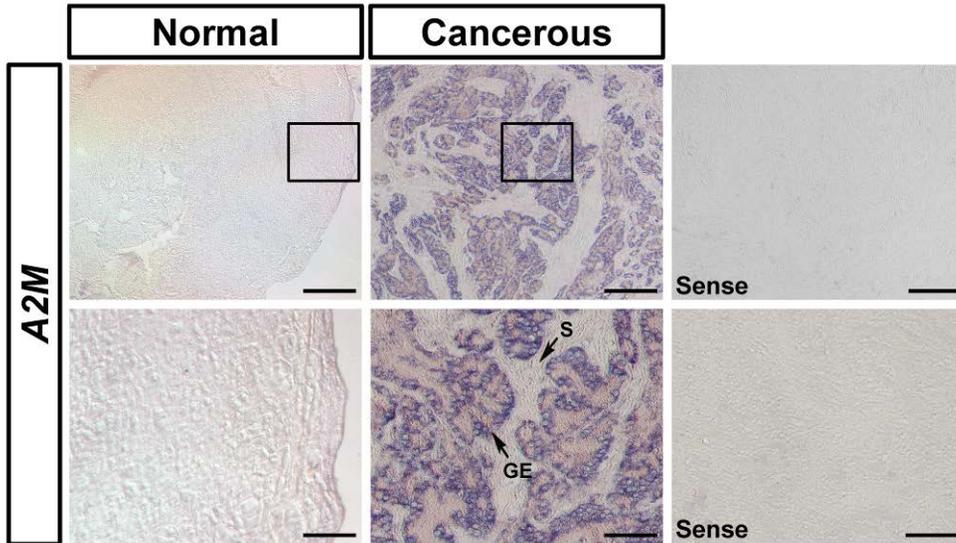
**Figure 6-3. Effect of DES on tissue specific expression of chicken A2M based on RT-PCR and qPCR analyses.** Both RT-PCR [A and B] and q-PCR [C and D] analyses were performed using cDNA templates from DES-treated and control chicken oviducts (mean±SEM;  $P < 0.001$ ) to determine that DES induced about a 22-fold increase in oviductal A2M mRNA as compared to control chicks.



**Figure 6-4. *In situ* hybridization analyses of *A2M* mRNA in oviducts of DES-treated and control chicks.** *In situ* hybridization with cross sections of the infundibulum, magnum, isthmus, and shell gland of the chicken oviduct with antisense or sense chicken *A2M* cRNA probes revealed abundant expression of *A2M* mRNA only in glandular epithelium (GE) of isthmus and lower expression in GE of the magnum of chick oviducts treated with DES. Legend: LE, luminal epithelium; GE, glandular epithelium; Scale bar represents 200  $\mu\text{m}$  (the first horizontal panels) and 50  $\mu\text{m}$  (the second horizontal panels).

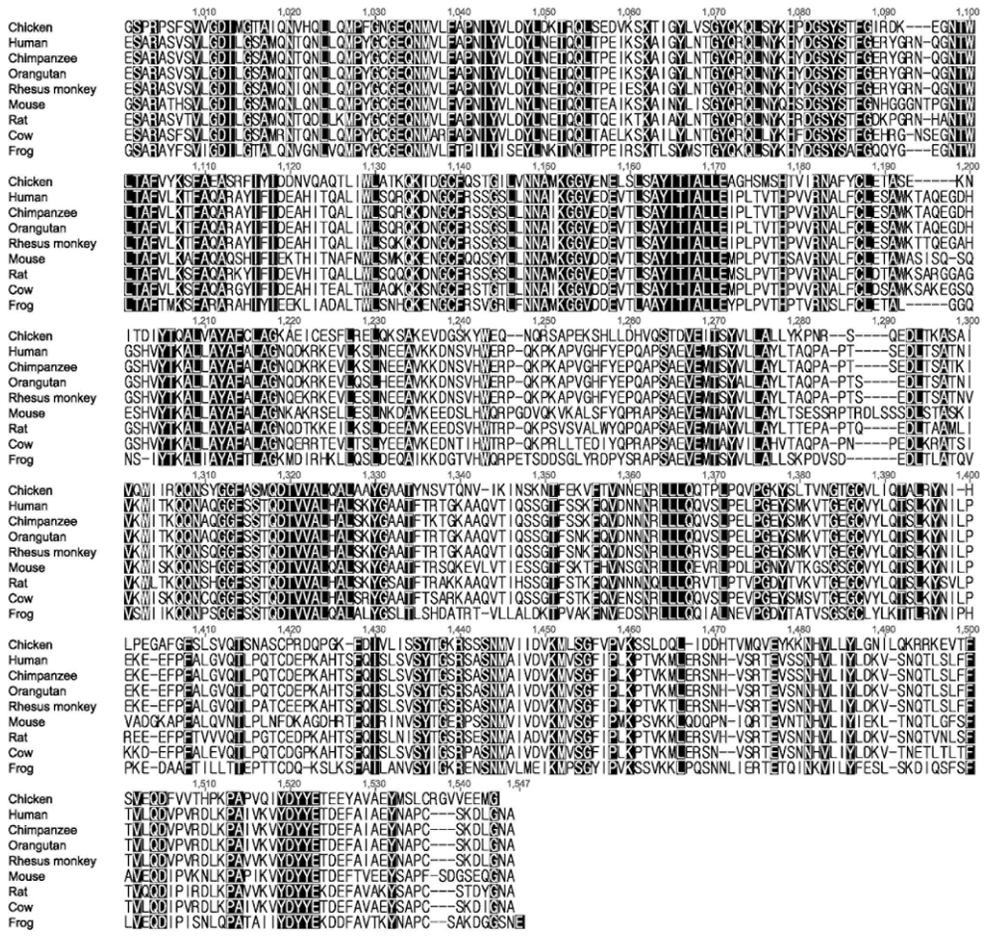


**Figure 6-5. Expression and quantitation of *A2M* mRNA in normal and cancerous ovaries from hens.** [A] RT-PCR analyses were performed using chicken *A2M* and *GAPDH*-specific primers. Lanes 1 to 4 show results of analysis of four normal ovaries with distilled water as a negative control. [B] Lanes 1-10 are from analyses of 10 different cancerous ovaries. Expression of *A2M* mRNA was predominantly in endometrioid carcinoma, with little or no expression in serous, mucinous or clear cell carcinomas and normal ovaries (mean±SEM;  $P < 0.05$ ). Legend for panel B: Lane 1, clear cell carcinoma (Stage IV); Lane 2, endometrioid/serous/mucinous carcinoma (Stage III); Lane 3, endometrioid carcinoma (Stage I); Lane 4, serous carcinoma (Stage I); Lane 5, mucinous/endometrioid carcinoma (Stage IV); Lane 6, endometrioid carcinoma (Stage IV); Lane 7, endometrioid carcinoma (Stage III); Lane 8, clear cell carcinoma (Stage IV); Lane 9, serous/mucinous carcinoma (Stage IV); and Lane 10, serous/mucinous/endometrioid carcinoma (Stage III).

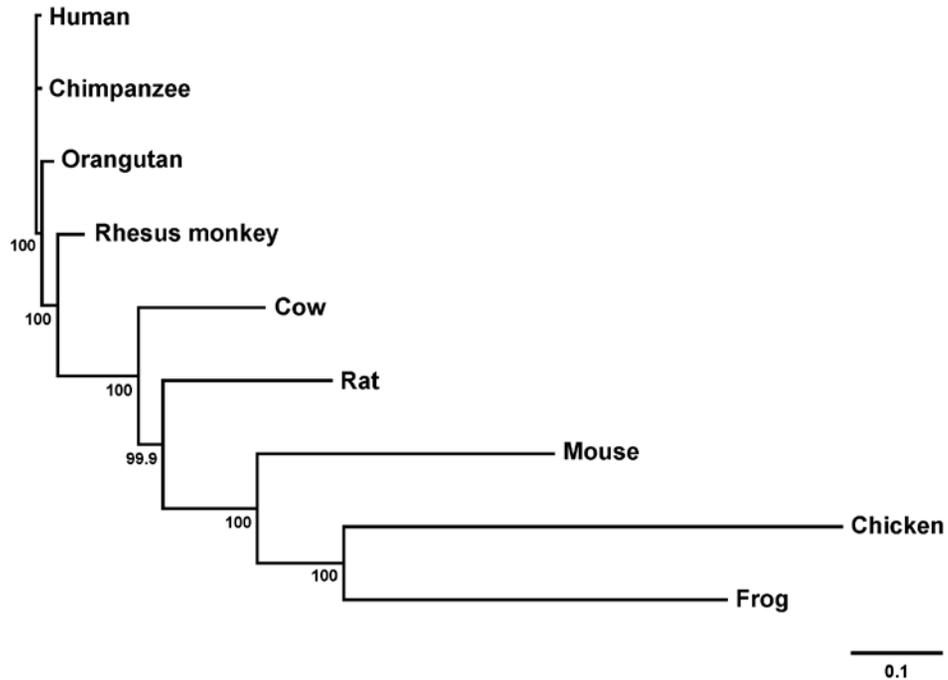


**Figure 6-6.** *In situ* hybridization analyses of *A2M* mRNA in normal and cancerous ovaries of hens. Cross-sections of normal and cancerous ovaries of hens hybridized with antisense or sense chicken *A2M* cRNA probes demonstrated abundant *A2M* mRNA predominantly in GE of cancerous ovaries, but not in LE, stroma or blood vessels. Legend: F, follicle; GE, glandular epithelium; S, stroma; Scale bar represents 200  $\mu\text{m}$  (the first horizontal panels and sense) or 50  $\mu\text{m}$  (the second horizontal panels and sense).

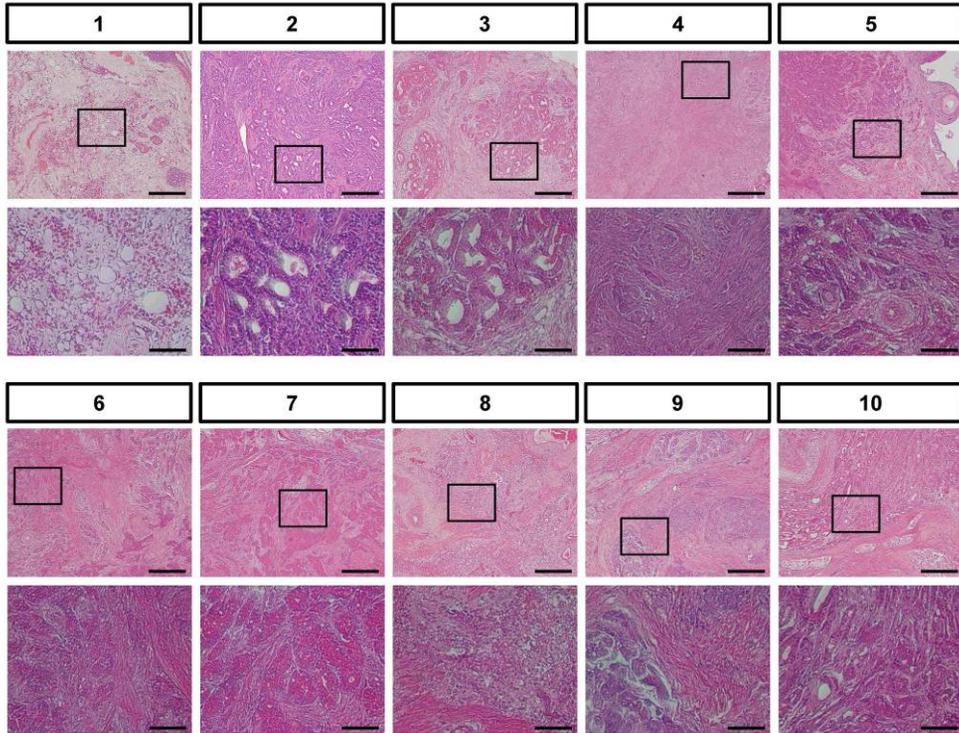




**Figure 6-7. Multiple sequence alignment of chicken, mammalian and amphibian A2M proteins.** Chicken A2M protein has moderate homology to mammalian A2M proteins (42.4-45.4%) and contains the highly conserved MG1 and MG2 domains, the A2M family N-terminal region, and the A2M receptor binding domain found in mammalian A2M. Shaded sequences indicate identical amino acid sequences among all species examined. Dashes represent gaps among the sequences. The conserved functional domains in A2M proteins were identified using the Pfam-A family matrix and NCBI conserved domain database.



**Figure 6-8.** The phylogenetic tree generated from alignments of primary sequences of chicken, mammalian and amphibian A2M proteins. Results of phylogenetic tree analysis indicated that chicken A2M was between mammalian and amphibian species consistent with the general pattern of molecular evolution in vertebrates. Bootstrap values from 1,000 replicates are shown at the appropriate branches.



**Figure 6-9. Histological types of ovarian cancers in chickens used in this study.**

Briefly, clear cell carcinoma showed vacuolated cells consisting of nuclear atypia. Serous carcinoma was a solid mass of cells with nuclear atypia. Endometrioid carcinoma had many glands and mucinous carcinoma was differentiated around the stromal region. Each image shows two different regions within each type of carcinoma. Legend: Lane 1, clear cell carcinoma; Lane 2, serous/endometrioid/mucinous carcinoma; Lane 3, endometrioid carcinoma; Lane 4, serous carcinoma; Lane 5, endometrioid/mucinous carcinoma; Lane 6, endometrioid carcinoma; Lane 7, endometrioid

**Table 6-1. Pairwise comparisons of A2M among chicken and several mammalian species**

<b>Species</b>	<b>Symbol</b>	<b>Identity (%)</b>	<b>GenBank No.</b>
<i>Gallus gallus</i> (Chicken)	A2M	-	NP_990557.1
vs. <i>Homo sapiens</i> (Human)	A2M	45.2	NP_000005.2
vs. <i>Pan troglodytes</i> (Chimpanzee)	A2M	45.4	XP_001139819.1
vs. <i>Pongo abelii</i> (Orangutan)	A2M	45.2	NP_001126929.2
vs. <i>Macaca mulatta</i> (Rhesus monkey)	A2M	45.1	XP_001114328.1
vs. <i>Mus musculus</i> (Mouse)	a2m	43.2	NP_783327.2
vs. <i>Rattus norvegicus</i> (Rat)	A2M	43.5	NP_036620.2
vs. <i>Bos Taurus</i> (Cow)	A2M	42.5	NP_001103265.1
vs. <i>Xenopus laevis</i> (Frog)	A2M	42.4	NP_001165531.1

## 5. Discussion

Results of the present study are the first to demonstrate tissue- and cell-specific expression of *A2M* mRNA in oviducts from normal chickens and to identify high levels of expression of *A2M* gene in cancerous ovaries of laying hens. Expression of *A2M* mRNA is unique to the chicken oviduct. In humans, *A2M* is synthesized primarily in the liver (Petersen et al., 1988; Sain-van der Velden et al., 1998) and as a major plasma protein, it makes up roughly 8-10% of the total protein in human blood (Anderson and Anderson, 2002). However, in chickens, *A2M* (also known as ovomacroglobulin or ovostatin) is synthesized in the oviduct and found in egg whites (Mann, 2007; Nagase and Harris, 1983; Saxena and Tayyab, 1997). Indeed, the magnum segment of the chicken oviduct synthesizes and secretes components of egg-white such as ovalbumin, conalbumin, lysozyme, and ovomucoid (Kohler et al., 1968) and the isthmus is the site of formation of the soft shell membrane. As illustrated in Figure 6-2, *A2M* mRNA was most abundant in GE of the magnum and isthmus, but also expressed to a lesser extent in both LE and GE of the shell gland. Given the high level of expression of *A2M* in these two segments of the oviduct, *A2M* may interact with other proteases to maintain a balance of proteases and protease inhibitors involved in egg formation after ovulation.

It is well known that estrogen is required for normal development of reproductive organs in female mammals and birds (Kohler et al., 1969). In general, the biological actions of estrogen are mediated by its cognate receptors, estrogen receptors alpha and beta which activate and recruit a variety of transcription factors that bind to

estrogen response elements in the 5' upstream region of target genes (Dougherty and Sanders, 2005; Hewitt et al., 2005). Indeed, several steroid hormones, including estrogen, are involved in many physiological and developmental events requiring modification of cell-type and tissue-specific gene expression (Dougherty and Sanders, 2005; Okada et al., 2005). Consistent with these results, we reported that exogenous DES affects growth, development and differentiation of the chicken oviduct (Seo et al., 2009) and discovered candidate genes and pathways regulating oviduct development in chickens (Song et al., 2011). In this study, DES induced *A2M* mRNA in GE of the infundibulum, magnum, and isthmus of the chick oviduct. These results indicate that *A2M* is likely regulated by estrogen in the oviduct and that it is likely involved in growth, differentiation and function of the chicken oviduct.

Ovarian cancer is the most lethal gynecological malignancy and the leading cause of cancer-related deaths among woman (Cvetkovic, 2003; Goodman et al., 2003; Wong and Auersperg, 2003), but it is rarely diagnosed at an early stage (Goodman et al., 2003). Therefore, over 75% of woman diagnosed are at an advanced stage of the disease because it is generally asymptomatic (Bast et al., 2002) and there is no specific biomarker(s) for early detection (Barua et al., 2009; Pepe et al., 2001). Although genetically manipulated rodent models have been used to elucidate some aspects of the etiologies and pathogenesis of ovarian cancer, the non-spontaneous feature of their ovarian cancer limits its clinical application (Barua et al., 2009; Stakleff and Von Gruenigen, 2003; Vanderhyden et al., 2003). Meanwhile, the laying hen is a unique animal model for research on human ovarian cancer, because laying hens spontaneously develop epithelial cell-derived ovarian cancer as occurs in women

(Barua et al., 2009; Fredrickson, 1987; Giles et al., 2004). We found that lysosomal cysteine cathepsin B (CTSB) (Ahn et al., 2010) and serpin peptidase inhibitor, clade B, member 11 (SERPINB11) (Lim et al., 2012f) are highly expressed in cancerous ovaries of hens. In the present study, as for SERPINB11, *A2M* is predominantly localized in endometrioid carcinoma, but there was little or no expression in serous, mucinous or clear cell carcinomas and normal ovaries (Figure 6-5A and 6-5B). In addition, *A2M* mRNA is abundant in GE of cancerous ovaries, but not in LE, stroma or blood vessels. These results strongly support the idea that proteases and their inhibitors play fundamental roles in a wide variety of biological events including cancer progression and metastasis due to their capacity to degrade extracellular matrix proteins (Lopez-Otin and Matrisian, 2007). Although human *A2M* is a well-known major cytotoxic factor in serum which inhibits the DNA synthesis in mouse ovarian tumor cells (Koo, 1983a, b), Hawkrigde and coworkers (Hawkrigde et al., 2010) reported that *A2M* increased in plasma over a 6 month period in laying hens with late-stage epithelial ovarian cancer. This result provides protein level evidence for up-regulation of a predicted form of *A2M* in plasma of laying hens that developed Stage IV ovarian cancer as compared to concentrations in plasma of healthy control laying hens. Collectively, our current results indicate that *A2M* is likely involved in gland morphogenesis and angiogenesis in chicken carcinogenesis. Alternatively, it is possible that *A2M* interacts reciprocally with other proteases in cancerous ovaries of chickens as it exhibits some inhibitory activity against a variety of proteinases (including serine-, cysteine-, aspartic- and metalloproteinases) (Deboer et al., 1993).

Results of the present study indicate that *A2M* is a novel estrogen-regulated

gene during development of the chicken oviduct and that it is likely a critical regulator of growth and developmental aspects of epithelial cells of the ovaries of laying hens as they transition from normal to a cancerous state. These results also provide a roadmap for our future research to investigate the precise role(s) of A2M in underlying mechanisms responsible for estrogen-mediated development of the chicken oviduct and as a biomarker to assess the effectiveness of therapies for endometrioid-type ovarian cancer in laying hens.

## **CHAPTER 7**

# **Tissue Specific Expression and Estrogen Regulation of SERPINB3 in the Chicken Oviduct**

## 1. Abstract

Serine protease inhibitors (SERPINs) comprise the largest superfamily of protease inhibitors and appear to be ubiquitously expressed in a variety of species. Of these, squamous cell carcinoma antigen 1 (SCCA1), also known as a SERPINB3, was first identified in squamous cell carcinoma tissue from the cervix of women. However, there is little known about the expression and hormonal regulation of SERPINB3 in chickens. Therefore, the avian *SERPINB3* gene was compared with those of other species with respect to structure, phylogenetic evolution and tissue- and cell-specific expression in hens. Chicken SERPINB3 has moderate homology to mammalian SERPINB3 proteins (36–47%). Of particular note, *SERPINB3* mRNA was most abundant in the chicken oviduct and cell-specific expression was in glandular (GE) and luminal (LE) epithelial cells of the oviduct of laying hens. Treatment of young chicks with DES (diethylstilbestrol, a synthetic nonsteroidal estrogen) induced *SERPINB3* mRNA and protein in GE and LE, but not in other cell types of the oviduct. Western blot analyses determined that immunoreactive SERPINB3 protein was also increased by DES in LE and GE of the oviduct of chicks. Collectively, these results indicate that SERPINB3 is an estrogen-induced gene expressed only in LE and GE of the chicken oviduct and implicate SERPINB3 in regulation of oviduct development and egg formation.

## 2. Introduction

Serine protease inhibitors (SERPINs) are a large family of proteins involved in a variety of physiological processes in all organisms (Gettins, 2002b; Masumoto et al., 2003; Vidalino et al., 2009). Their conserved tertiary structure consists of three conserved beta-sheets and eight or nine alpha-helices, and an exposed region termed the reactive center loop (Masumoto et al., 2003; Silverman et al., 2001). Since the first identification of serpins by Hunt and Dayhoff (Hunt and Dayhoff, 1980), a number of serpins have been identified and verified using detailed phylogenetic analyses. Human serpins are divided into nine clades (A–I) (Cuccioloni et al., 2004; Vidalino et al., 2009) with proteins that play crucial roles in the immune system and in apoptosis (Mangan et al., 2008). Of these, clade B serpins (SERPINB), previously known as the ov-serpin family (Remold-O'Donnell, 1993), include 13 *SERPINB* genes, based on in silico analyses of human genomic DNA (Silverman et al., 2004). They are found predominantly in intracellular regions and mainly inhibit targeted proteases (Bird et al., 2001). Recently, Benarafa and Remold-O'Donnell (Benarafa and Remold-O'Donnell, 2005) reported 10 *SERPINB* genes in the chicken genome that are located within approximately 150 kb of a single locus and contain the gene structure of ov-serpins with either seven or eight exons and the signature protein sequence of serpins.

The oviduct of oviparous animals, such as chickens, is an excellent model for studying mechanisms of steroid hormone actions underlying oviduct development and oviposition (egg laying) (Dougherty and Sanders, 2005). These mechanisms orchestrate the proliferation and cyto-differentiation of oviductal epithelial cells into

tubular gland cells and each structural component of the avian oviduct is involved in production of egg white and eggshell, as well as oviposition (Chousalkar and Roberts, 2008; Fertuck and Newstead, 1970; Kohler et al., 1969). It is well known that estrogen is a primary sex hormone in females that stimulates the development and maintenance of function of the chicken oviduct (Dougherty and Sanders, 2005; Seo et al., 2009). Especially, estrogen triggers formation of tubular glands, as well as differentiation of the oviductal epithelium into goblet and ciliated cells (Palmiter and Wrenn, 1971b). In addition, estrogen induces synthesis of egg white proteins, including ovalbumin, conalbumin, ovomucoid, and lysozyme, in the magnum of the oviduct of immature chicks (Palmiter, 1972).

Based on differential gene profiling of the chicken oviduct to identify the avian homolog of human SCCA1 (Squamous Cell Carcinoma Antigen 1), *SERPINB3* was identified as a highly expressed transcript in chicks treated with the synthetic estrogen agonist, diethylstilbestrol (Song et al., 2011). *SERPINB3* was first identified and cloned from squamous cell carcinoma tissue of the human uterus (Kato and Torigoe, 1977; Suminami et al., 1991). D'Alessandro *et al.* reported that *SERPINB3* plays a crucial role in the cascade of events required for the formation of egg yolk and egg white (D'Alessandro et al., 2010). However, little is known about the hormonal regulation of expression of *SERPINB3* in chickens, although there are many reports that the protein participates in formation of egg yolk and egg white proteins in liver and in the magnum of the oviduct, respectively. Therefore, the objectives of this study were to: (1) compare the primary sequences of chicken *SERPINB3* with those of selected mammalian species; (2) determine tissue and cell-specific expression of the

*SERPINB3* gene in various organs from male and female chickens; and (3) determine whether estrogen regulates expression of *SERPINB3* mRNA and protein during oviduct growth and differentiation during development of the chick. Results of this study provide novel information on the *SERPINB3* gene with respect to its sequence, tissue- and cell- specific expression and regulation of its expression by estrogen during development of the chicken oviduct.

### 3. Materials and Methods

#### *Experimental animals and animal care*

The experimental use of chickens for this study was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). White Leghorn (WL) chickens were subjected to standard management practices at the University Animal Farm, Seoul National University, Korea with respect to management of hens for reproduction, incubation of eggs and rearing of chicks, as well as standard operating protocols in our laboratory. All chickens had *ad libitum* access to feed and water.

#### *Tissue samples*

Following euthanasia of the WL chickens, tissue samples were collected from brain, heart, liver, kidney, muscle, small intestine, gizzard, ovary, oviduct and testis of 1- to 2- year-old males (n = 3) and females (n = 3). Subsets of these samples were frozen or fixed in 4% paraformaldehyde for further analyses. Frozen tissue samples were cut into 5- to 7-mm pieces, frozen in liquid nitrogen vapor, and stored at -80°C. The other samples were cut into 10mm pieces and fixed in fresh 4% paraformaldehyde in PBS (pH 7.4). After 24 h, fixed tissues were dehydrated through a series of graded alcohols and xylene, and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5 µm.

### ***Diethylstilbestrol (DES) treatment and oviduct retrieval***

Female chicks were identified by PCR analysis using W chromosome-specific primer sets (Lee et al., 2009). Treatment with DES and recovery of the oviduct were conducted as reported previously (Sanders and Mcknight, 1988; Seo et al., 2009). Briefly, a 15 mg DES pellet was implanted subcutaneously in the abdominal region of 1-week-old female chicks for release of hormone for 10 days. The DES pellet was removed from one-half of the chicks after 10 days, and a 30 mg dose of DES was implanted for release of hormone for 10 additional days (Seo et al., 2009). Five chicks in each group were euthanized using 60–70% carbon dioxide. Subsets of these samples were frozen or fixed in 4% paraformaldehyde for further analyses. Frozen tissue samples were cut into 5- to 7-mm pieces and frozen in liquid nitrogen. The other samples were cut into 10- to 15-mm pieces and fixed in fresh 4% paraformaldehyde in PBS (pH 7.4). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5  $\mu$ m.

### ***RNA isolation***

Total cellular RNA was isolated from frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively. The quantity and purity of RNA were measured by using Nanodrop 2000 (Thermo Scientific, DE, USA). We confirmed

RNA purity with 260/280 nm ratio greater than 1.8.

### *Sequence analysis*

For pair-wise comparisons and multiple sequence alignment, the amino acid sequences of *SERPINB3* genes from each species were aligned using Geneious Pro Version 5.04 with default penalties for gap and the protein weight matrix of BLOSUM (Blocks Substitution Matrix). A phylogenetic tree was constructed using the neighbor-joining method [40] of the Geneious Pro Version 5.04. To determine the confidence level for each internal node on the phylogenetic tree, 1000 nonparametric bootstrap replications were used (Felsenstein, 1985).

### *Semiquantitative RT-PCR analysis*

The level of expression of *SERPINB3* mRNA in various organs from chickens, including the oviduct, was assessed using semiquantitative RT-PCR as described previously (Song et al., 2007). The cDNA was synthesized from total cellular RNA (2 µg) using random hexamer (Invitrogen, Carlsbad, CA) and oligo (dT) primers and AccuPower<sup>®</sup> RT PreMix (Bioneer, Daejeon, Korea). The cDNA was diluted (1:10) in sterile water before use in PCR. For *SERPINB3*, the sense primer (5'-GAG CAT GTT GGT GCT GTT GC-3') and antisense primer (5'-TTG AAC CTG TCG CCT CAG TG-3') amplified a 317-bp product. For *GAPDH* (housekeeping gene; glyceraldehyde 3-phosphate dehydrogenase), the sense primer (5'-TGC CAA CCC CCA ATG TCT CTG TTG-3') and antisense primer (5'-TCC

TTG GAT GCC ATG TGG ACC AT-3') amplified a 301-bp product. The primers, PCR amplification and verification of their sequences were conducted as described previously (Song et al., 2007). The PCR amplification of *SERPINB3* and *GAPDH* was conducted using approximately 60 ng cDNA as follows: (1) 95 °C for 3 min; (2) 95 °C for 20 s, 60 °C for 40 s and 72 °C for 1 min for 33 cycles; and (3) 72 °C for 10 min. Then, equal amounts of reaction product were analyzed using a 1% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a Gel Doc™ XR + system with Image Lab™ software (Bio-Rad).

### ***Quantitative RT-PCR analysis***

Total RNA was extracted from each oviduct of control and DES-treated chicks using TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized using AccuPower® RT PreMix (Bioneer, Daejeon, Korea). Gene expression levels were measured using SYBR® Green (Sigma, St. Louis, MO, USA) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The *GAPDH* gene was simultaneously analyzed as a control and used for normalization. Expression of each target gene and *GAPDH* was analyzed in triplicate. Using the standard curve method, we determined levels of expression of the examined genes using the standard curves and C<sub>T</sub> values, and normalized them based on *GAPDH* expression levels. 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 0.5 °C per 10 s) and continuous fluorescence measurement. The ROX dye (Invitrogen) was used as a negative control for measurements of fluorescence. Sequence-specific products were identified by generating a melting curve in which the  $C_T$  value represented the cycle number at which a fluorescent signal was significantly greater than background, and relative gene expression was quantified using the  $2^{-\Delta\Delta C_T}$  method (Livak and Schmittgen, 2001). For the control, the relative quantification of gene expression was normalized to the  $C_T$  of the control oviduct.

### ***In situ hybridization analysis***

For hybridization probes, PCR products were generated and were gel-extracted and then cloned into pGEM-T vector (Promega) as described previously (Ahn et al., 2010). After verification of the sequences, plasmids containing the correct gene sequences were amplified with T7- and SP6-specific primers then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). After hybridization and blocking, the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche). The signal was visualized by exposure to a solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4mM nitroblue tetrazolium, and 2 mM levamisole (Sigma).

### ***Immunohistochemistry***

Immunocytochemical localization of SERPINB3 protein in the chicken oviduct was performed as described previously (Song et al., 2006) using an anti-human SERPINB3 monoclonal antibody (0.5 mg/ml; catalog number: ab55733; Abcam plc, Cambridge, UK) at a final dilution of 1:500 (1 µg/ml). Antigen retrieval was performed using the boiling citrate method as described previously. Accordingly, mouse IgG (1 mg/ml) was diluted and used with the same concentration (1 µg/ml) as the SERPINB3 antibody as the negative control.

### ***Western blot analyses***

Whole oviduct extracts of proteins and immunoblot assays were performed as described previously (Song et al., 2009). Oviducts from DES-treated and control chickens were rinsed with cold PBS and lysed by homogenization for 5 min in ice cold lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 0.2 mM phenylmethylsulfonylfluoride, 50 mM NaF, 30 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 µg/ml leupeptin, and 1 µg/ml pepstatin). Cell lysates were clarified by centrifugation (16,000g, 15 min, 4°C). The protein content was determined using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Proteins were denatured, separated using 10% SDS-PAGE and transferred to nitrocellulose. Blots were developed using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce, Rockford, IL) and quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a ChemiDoc EQ system and Quantity One software (Bio-Rad, Hercules, CA). Immunoreactive SERPINB3 protein was detected using an anti-

human SERPINB3 monoclonal antibody (catalog number: ab55733; Abcam plc, Cambridge, UK) at a final dilution of 1:1000. As a loading control, western blotting with mouse anti-beta actin IgG (catalog number: sc-47778) was performed.

### *Statistical analyses*

Differences in the variance between control and DES-treated oviducts were analyzed using the *F* test, and differences in the means were subjected to Student's *t* test. The probability value of  $P < 0.05$  was considered statistically significant. Excel (Microsoft, Redmond, WA, USA) was used for statistical analyses.

## 4. Results

### *Sequence comparison, pair-wise alignment and phylogenetic tree analysis of SERPINB3*

The full length amino acid sequence of avian SERPINB3 protein and those from mammalian species were aligned and compared. All SERPINB3 proteins had a reactive center loop site between amino acids 343 and 372 (Fig. 7-1). The homology of SERPINB3 proteins among mammalian species compared with chicken SERPINB3 protein was analyzed by pair-wise sequence alignment and found to be 36 to 47 percent (Table 7-1). The values in Table 7-1 represent ratios of identified regions between chicken SERPINB3 protein and homologous regions of amino acids for other species. The phylogenetic tree analysis was performed by using the neighbor-joining method and primary amino acid sequences of SERPINB3 for the chicken and for six other vertebrate species. As shown in Figure 7-2, the phylogenetic tree indicates that chicken SERPINB3 was placed among mammalian species consistent with the general pattern of molecular evolution in vertebrates.

### *SERPINB3 mRNA expression in various organs from chickens*

Expression of *SERPINB3* mRNA in brain, heart, liver, kidney, small intestine, gizzard, ovary, oviduct and testis of 1- to 2- year-old males (n = 3) and females (n = 3) was determined. Results of RT-PCR analyses indicated high levels of expression of *SERPINB3* mRNA in oviduct, ovary and gizzard from female chickens and to a lesser

extent, in brain and liver from females and testes from male chickens. However, specific expression was not detected in any other non-reproductive organs analyzed for either sex. Thus, our next study focused on the chicken oviduct (Fig. 7-3).

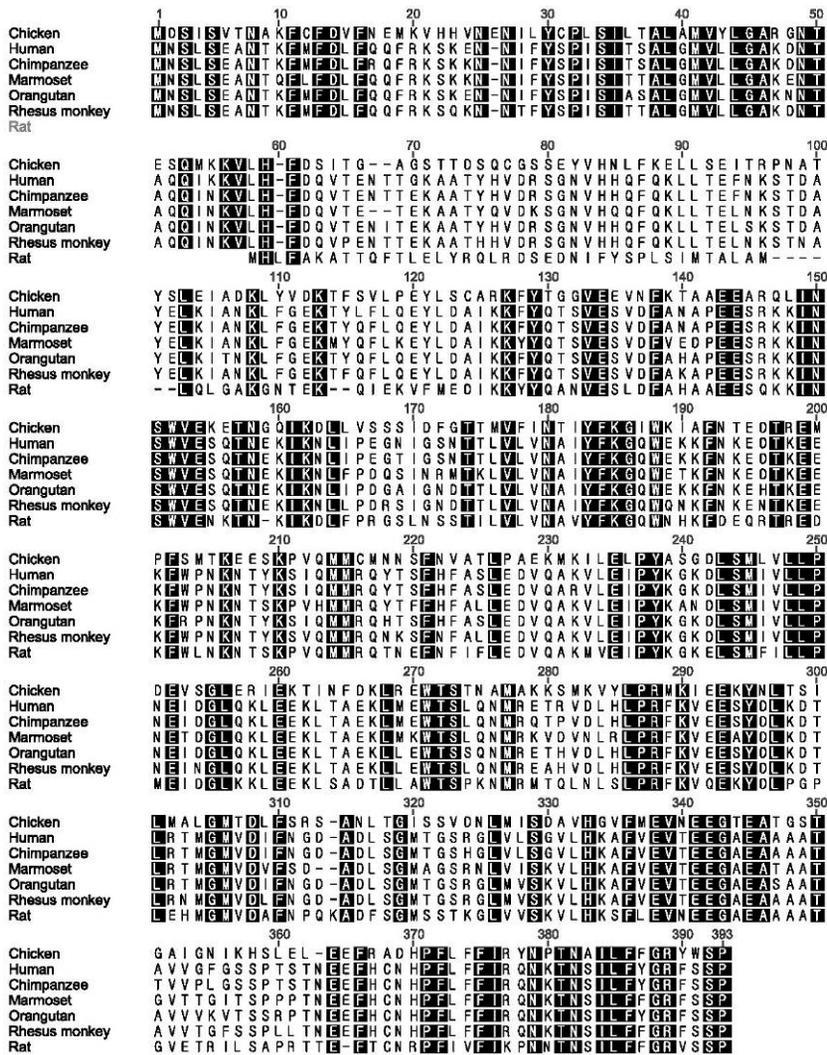
#### ***Localization of SERPINB3 mRNA and protein expression in the chicken oviduct***

The oviduct of a laying hen includes the infundibulum (reception of ovulated egg for fertilization), magnum (synthesis and deposition of egg-white proteins), isthmus (synthesis of two soft shell membranes), and shell gland or uterus (formation and calcification of egg shell). Cell-specific expression of *SERPINB3* mRNA and protein in oviducts from laying hens was determined using *in situ* hybridization analysis and immunohistochemistry. *SERPINB3* mRNA was most abundant in the glandular epithelia (GE) of magnum, isthmus and shell gland (Fig. 7-4), as well as luminal epithelia (LE) of the infundibulum and shell gland and the same pattern of expression was found for *SERPINB3* protein. Immunohistochemistry determined that immunoreactive *SERPINB3* protein was most abundant in LE of magnum and isthmus (Fig. 7-5). In infundibulum and shell gland, consistent with results of *in situ* hybridization analysis, *SERPINB3* protein was predominantly localized in LE and LE/GE, respectively. In the negative control, mouse IgG was used and no signal was detected.

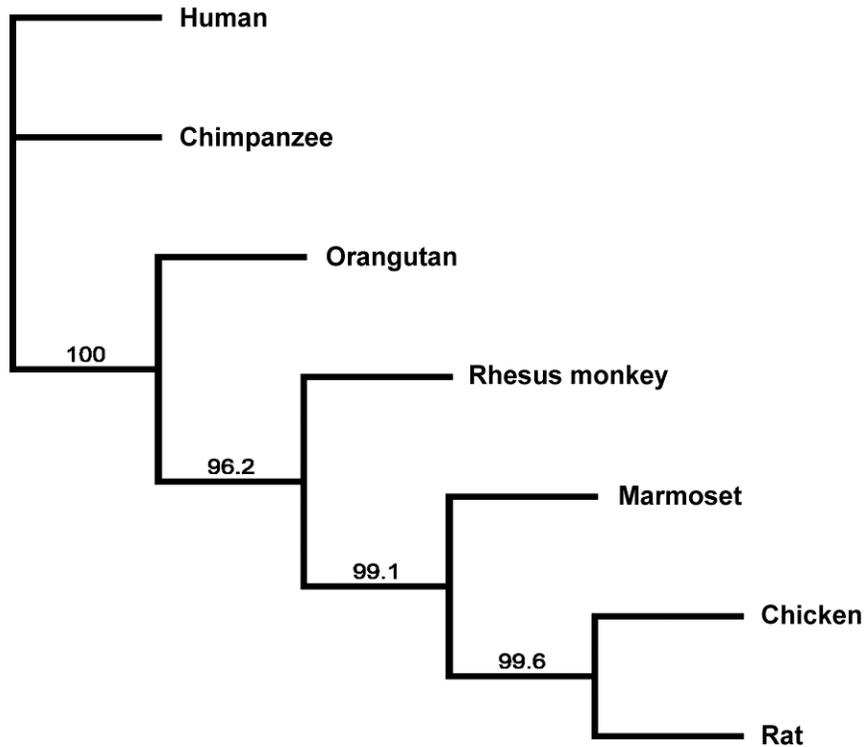
#### ***Effects of diethylstilbestrol on SERPINB3 mRNA expression in the chicken oviduct***

Tissue-specific expression in various organs from chickens and differential

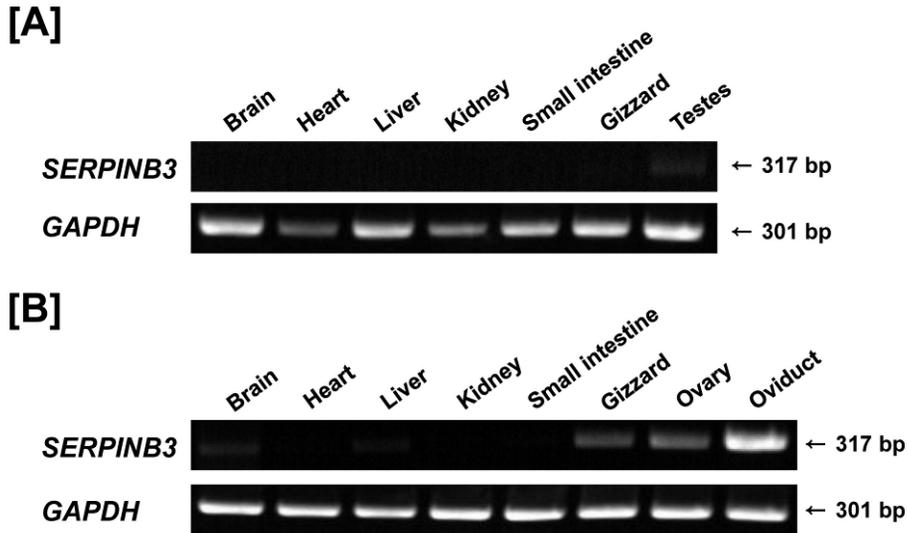
expression and localization of *SERPINB3* gene in the distinct regions and cells of the oviduct suggested regulation by estrogen during development of the oviduct in chicks. We reported that DES affects growth and differentiation of the chicken oviduct (Seo et al., 2009) and identified candidate genes and pathways regulating oviduct development in chickens (Song et al., 2011). Therefore, we investigated the effects of DES on *SERPINB3* expression in the chicken oviduct. Quantitative PCR analysis revealed that DES induced about a 175-fold increase ( $P < 0.001$ ) in total oviductal *SERPINB3* mRNA, as compared to control chicks (Fig. 6A and 6B). In addition, DES treatment resulted in 2217-fold and 171-fold increases ( $P < 0.001$ ) in *SERPINB3* mRNA in the magnum and isthmus of the oviduct, respectively (Fig. 7-6C and 7-6D). *In situ* hybridization analyses revealed that *SERPINB3* mRNA was expressed abundantly only in GE of the magnum and the isthmus of chick oviducts treated with DES (Fig. 7-7). In addition, *SERPINB3* mRNA was expressed at a lower level in the LE of the infundibulum. Consistent with results from *in situ* hybridization analyses, immunoreactive *SERPINB3* protein was most abundant in GE and LE of the magnum and isthmus (Fig. 7-8). Furthermore, as illustrated in Figure 7-9, Western blot analysis detected a greater ( $P < 0.01$ ) abundance of *SERPINB3* protein in oviducts of DES-treated as compared to control chicks.



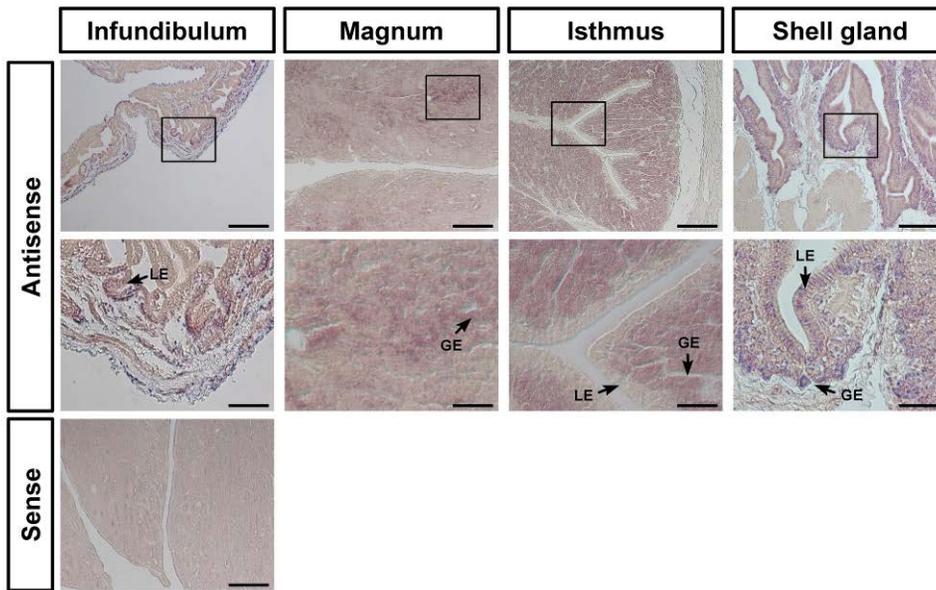
**Figure 7-1. Multiple sequence alignment of SERPINB3 amino acids for comparison among chicken and mammalian species.** The amino acid sequences of SERPINB3 from each of seven species (chicken, human, chimpanzee, marmoset, orangutan, rhesus monkey and rat) are presented based on alignments determined using Geneious Alignment (Drummond et al., 2010) with BLOSUM (Blocks Substitution Matrix) and gap penalties. Amino acid sequences in the shaded boxes represent those that are identical among sequences for chicken and mammalian SERPINB3. Dashes indicate gaps in the sequences.



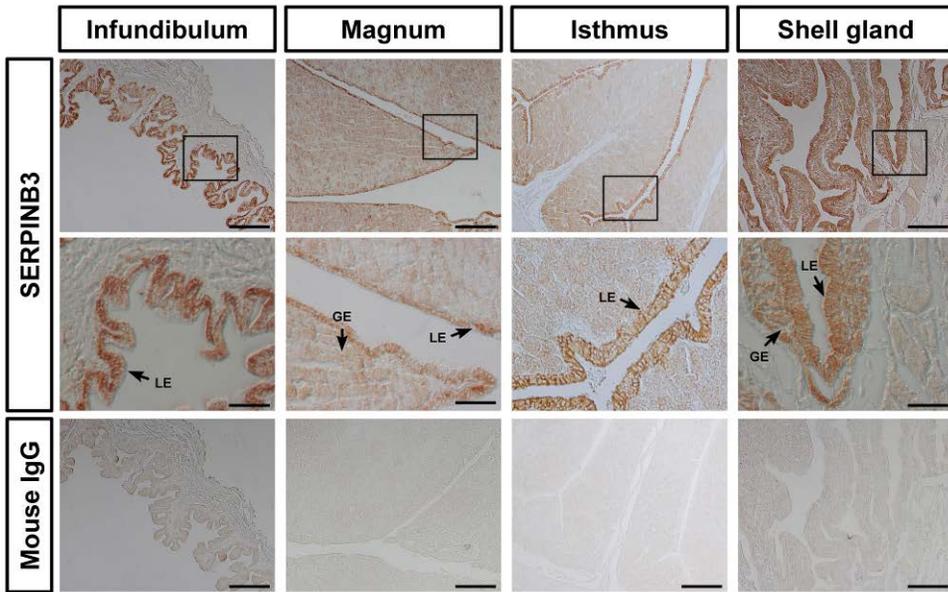
**Figure 7-2. The phylogenetic tree of SERPINB3.** The phylogenetic tree was generated from alignments of primary sequences of chicken, human, chimpanzee, marmoset, orangutan, rhesus monkey and rat SERPINB3 proteins using a bootstrap analysis with 1000 replicates. The results indicate that chicken SERPINB3 is placed among mammalian species consistent with the general pattern of molecular evolution in vertebrates.



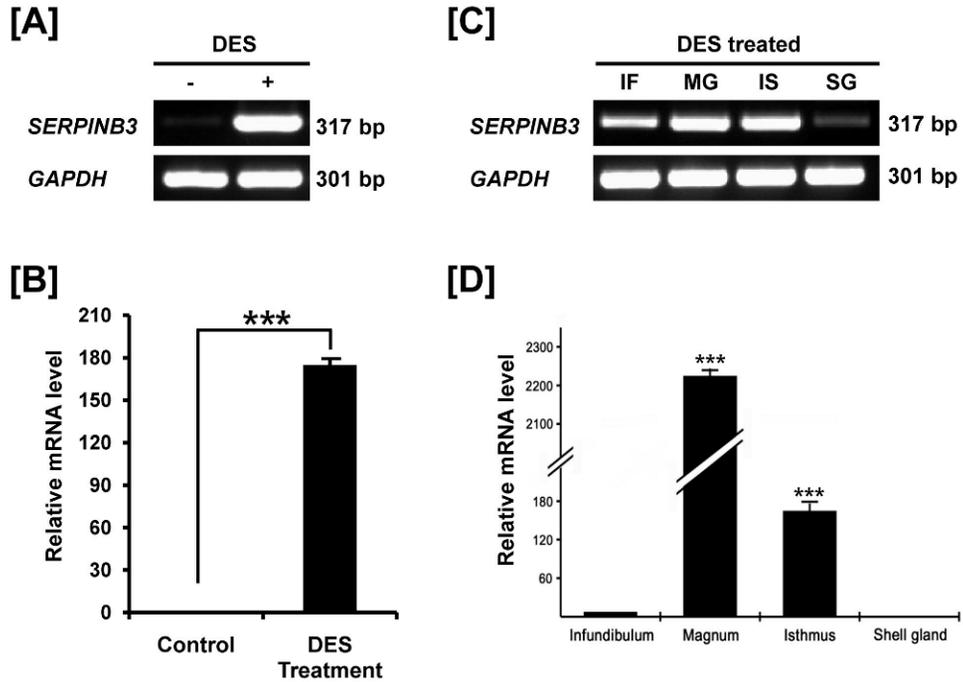
**Figure 7-3. Expression of *SERPINB3* in various organs of both male and female chickens.** Results of RT-PCR analysis using cDNA templates from different organs of both male [A] and female [B] chickens with chicken *SERPINB3* and chicken *GAPDH*-specific primers. The results indicate abundant expression of *SERPINB3* in the oviduct, ovary and gizzard from female chickens and to a lesser extent, in brain and liver from females and testes from male chickens, but no expression in any other tissues from either sex.



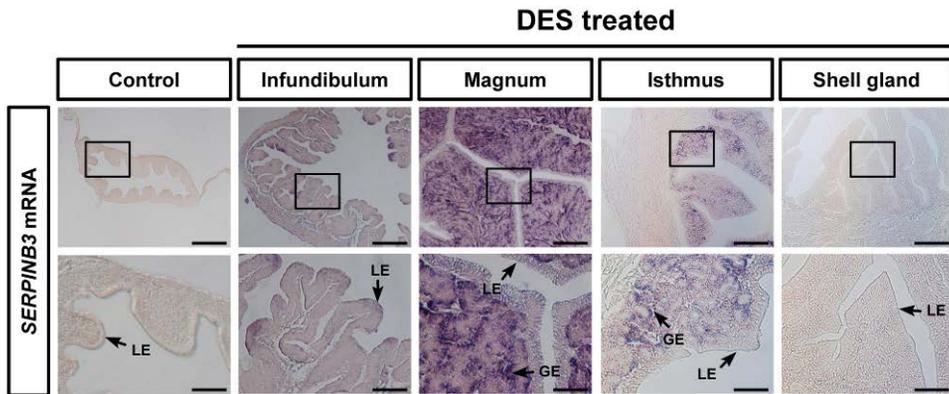
**Figure 7-4.** *In situ* hybridization analyses of *SERPINB3* mRNAs in the chicken oviduct. Cross-sections of the four components of the chicken oviduct (infundibulum, magnum, isthmus and shell gland) were hybridized with antisense or sense chicken *SERPINB3* cRNA probes. The results indicate cell-specific expression of *SERPINB3* mRNA in luminal (LE) and/or glandular (GE) epithelia of the chicken oviduct. See Materials and Methods for a complete description. Legend: LE, luminal epithelium; GE, glandular epithelium; *Scale bar* represents 200  $\mu\text{m}$  (the first horizontal panels and sense) and 50  $\mu\text{m}$  (the second horizontal panels).



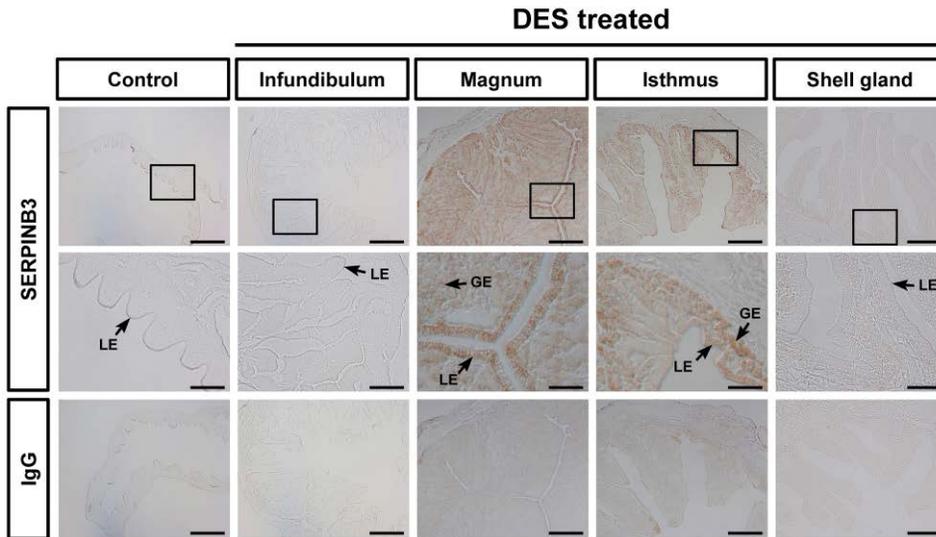
**Figure 7-5. Immunoreactive SERPINB3 protein in the chicken oviduct.** For the IgG control, normal mouse IgG was substituted for the primary antibody. Sections were not counterstained. The results indicate cell-specific localization of SERBINB3 protein in luminal (LE) and/or glandular (GE) epithelia of the chicken oviduct. Legend: LE, luminal epithelium; GE, glandular epithelium; *Scale bar* represents 200  $\mu\text{m}$  (the first horizontal panels and IgGs) and 50  $\mu\text{m}$  (the second horizontal panels).



**Figure 7-6. Effect of DES on tissue specificity of chicken *SERPINB3* by RT-PCR and quantitative PCR analyses.** Both RT-PCR [A and C] and q-PCR [B and D] analyses were performed using cDNA templates from DES-treated and control chicken oviducts (mean±SEM;  $P < 0.001$ ). The results clearly indicate that DES induces expression of *SERPINB3* mRNA in the chicken oviduct.



**Figure 7-7. *In situ* hybridization analyses of *SERPIN3* mRNA in oviducts of DES-treated and control chicks.** Cross-sections of the four segments of chicken oviduct (infundibulum, magnum, isthmus, and shell gland) treated with DES or vehicle were hybridized with antisense or sense chicken *SERPIN3* cRNA probes. Legend: LE, luminal epithelium; GE, glandular epithelium; *Scale bar* represents 200  $\mu\text{m}$  (the first horizontal panels and sense) and 50  $\mu\text{m}$  (the second horizontal panels).



**Figure 7-8. Immunoreactive SERPINB3 protein in oviducts of DES-treated and control oviducts.** For the IgG control, normal mouse IgG was substituted for the primary antibody. Sections were not counterstained. See Materials and Methods for complete description. Legend: LE, luminal epithelium; GE, glandular epithelium; *Scale bar* represents 200  $\mu\text{m}$  (the first horizontal panels and IgGs) and 50  $\mu\text{m}$  (the second horizontal panels).



**Figure 7-9. Detection of SERPINB3 in the oviducts of DES treated and control oviducts.** Proteins were separated by 10% SDS-PAGE under reducing conditions. Immunoreactive SERPINB3 protein, detected using the anti-human SERPINB3 monoclonal antibody, is strongly expressed in oviducts of chicks treated with DES. As a loading control, western blotting with mouse anti-beta actin (ACTB) IgG was performed.

**Table 7-1. Pairwise comparison of SERPINB3 between chicken and the mammalian species.**

<b>Species</b>	<b>Symbol</b>	<b>Identity (%)</b>
Chicken ( <i>Gallus gallus</i> )	SERPINB3	-
vs. Human ( <i>Homo sapiens</i> )	SERPINB3	45.7
vs. Chimpanzee ( <i>Pantroglodytes</i> )	SERPINB3	46.1
vs. Rhesus monkey ( <i>Macaca mulatta</i> )	SERPINB3	47
vs. Orangutan ( <i>Pongo abelii</i> )	SERPINB3	45
vs. Marmoset ( <i>Callithrix jacchus</i> )	SERPINB3	47
vs. Rat ( <i>Rattus norvegicus</i> )	Serpinb3	36

## 5. Discussion

Results of the present study indicate that SERPINB3 expression is regulated by estrogen in a tissue- and cell-specific manner in the chicken oviduct. In addition, comparison of chicken and mammalian *SERPINB3* genes with respect to structure, phylogenetic evolution, and sex- and tissue-specific expression provides strong support for our hypothesis that SERPINB3 is conserved among species and required for growth, differentiation, and development of the immature chick oviduct, as well as functional aspects of the mature oviduct of hens in response to estrogens produced by the ovary during the reproductive cycle of chickens.

In the present study, as illustrated Figure 7-1, we determined that chicken SERPINB3 has moderate homology to mammalian SERPINB3 proteins including the highly conserved reactive center loop site. SERPINS, a large group of glycoproteins with similar structures identified by Hunt and Dayhoff in 1980 (Hunt and Dayhoff, 1980) are mainly serine and cysteine peptidase inhibitors with a single common core domain (three  $\beta$ -sheets and 8 to 9  $\alpha$ -helices) found in Eukarya, Eubacteria, Archaea and Poxviridae (Askew et al., 2007; Gettins, 2002b; Law et al., 2006). Most serpins are commonly present in plasma and play important roles in pivotal physiological processes such as angiogenesis, immune responses, blood coagulation and fibronolysis (Askew et al., 2007). Based on phylogenetic analyses, the human genome encodes 17 SERPIN groups or “clades”, termed SERPINA through SERPINP, encoding 29 inhibitory and 7 non-inhibitory SERPINS (Law et al., 2006). The clade was originally proposed on the basis of similarities in amino acid sequences, lack of

the signal sequence, common structural features, and similar gene organization (Vidalino et al., 2009). In addition, the phylogenetic tree constructed using the neighbor-joining method showed that chicken and rodent SERPINB3s are clustered together and form a larger cluster with a sister group of primate SERPINB3s (Fig. 7-2). These results indicate that chicken SERPINB3 diverged from and is placed among mammalian species with the general pattern of molecular evolution in vertebrates.

As illustrated Figure 7-3, -4, and -5, results of the current study showed that *SERPINB3* was most abundant in the chicken oviduct and cell-specific expression was in glandular and luminal epithelial cells of the oviduct of laying hens. These results are of special interest as they support results of previous studies of SERPINB5 and SERPINB11 (Askew et al., 2007; Luo et al., 2007). That is, we found that chicken SERPINB3 protein was most abundant in the cytoplasmic compartment of epithelial cells in the oviduct using immunofluorescent microscopy (unpublished data). Most human SERPINs are secreted and circulate in the blood, but clade B serpins exist predominantly in the cytoplasmic or nucleo-cytoplasmic areas of cells as intracellular proteins that lack signal peptides and mainly inhibit target proteases (Bird et al., 2001; Silverman et al., 2004; Uemura et al., 2000). Unlike most inhibitory serpins, several serpins such as SERPINB5 and SERPINB11 are intracellular non-inhibitory SERPINBs (Askew et al., 2007; Luo et al., 2007). In humans, SERPINB3 was first identified and purified from squamous cell carcinoma tissue of the cervix (Kato and Torigoe, 1977; Suminami et al., 1991). SERPINB3 inhibits papain-like cysteine proteases such as papain, cathepsin K (CTSK), CTSL and CTSS (Gettins, 2002b; Schick et al., 1998). Of these clade B serpins, a subgroup of ovalbumin-related serpins

(*ov-serpins*), including orthologs of SERPINB1 (*MNEI*), SERPINB2 (*PAI-2*), SERPINB5 (*maspin*), and SERPINB6 (*PI-6*), are highly conserved between human and chicken genomes. However, there is no ortholog of SERPINB3 between humans and chickens (Benarafa and Remold-O'Donnell, 2005). Moreover, chicken ovalbumin, the related gene Y, and the related gene X (Heilig et al., 1980) are known as *SERPINB14*, *SERPINB14b*, and *SERPINB14c*, respectively; on authority of the SERPIN nomenclature guidelines and comparative genome sequence analysis (Benarafa and Remold-O'Donnell, 2005; Silverman et al., 2001). On the other hand, the human oviduct is usually involved in many important events in female reproductive biology by providing an optimal environment for sperm capacitation, fertilization and early cleavage stage development of embryos (Suarez, 2008). Unlike the structure of mammalian oviduct, the avian oviduct is a highly differentiated unilateral organ with five functionally unique regions; infundibulum, magnum, isthmus, shell gland or uterus and vagina (oviposition) (Chousalkar and Roberts, 2008). The sequential activities of these distinct regions of the oviduct are responsible for normal egg formation including deposition of egg white proteins surrounding the yolk, formation of the protective soft shell membranes and then the hard shell membrane of the egg. Therefore, the present results indicate that the expression of SERPINB3 is likely critical to the reproductive biology of egg-lay hens and egg formation prior to oviposition (egg laying).

Our results demonstrate that treatment of young chicks with a synthetic nonsteroidal estrogen, diethylstilbestrol (DES) induces *SERPINB3* mRNA and protein in glandular and luminal epithelia (Fig. 7-6, -7, -8, and -9). McKnight (1978) and

Kohler (1969) reported that the developmental pattern of the neonatal chick oviduct in response to DES implants was similar to that in response to natural estrogen based on differentiation of tubular glands and ciliated cells in the chicken oviduct, and the expression of egg white protein by cells in the magnum (Kohler et al., 1969; McKnight, 1978; Palmiter et al., 1978). Indeed, the developmental pattern of the chick oviduct in response to DES treatment in our study is very similar to that reported previously (Kohler et al., 1968; Oka and Schimke, 1969a, b, c). In addition, it was our assumption that endogenous estrogen in neonatal chicks was insufficient to induce development of the oviduct. Generally, endogenous estrogen is secreted mainly by ovarian follicles in chickens in response to endogenous FSH or LH (Robinson and Etches, 1986). However, pre-ovulatory follicles were not present on ovaries of either control or DES-treated neonatal chicks in the present study. Therefore, DES is an agonist for estradiol that has an equivalent effect on development of the oviduct in neonatal chicks and our results and conclusions are comparable to those in previous reports in which the investigators used estradiol.

The avian oviduct is an excellent model system for studying the hormonal regulation of expression of specific genes in comparison to their regulation of expression in mammals. Estrogen, the primary sex steroid hormone affecting the avian reproductive tract, along with progesterone, induces cytodifferentiation of epithelia in tubular glands, stimulation of secretion of oviduct-specific egg-white proteins (ovalbumin, conalbumin, lysozyme, and ovomucoid) and development of the immature chick oviduct (Dougherty and Sanders, 2005). In addition, estrogen is involved in various biological processes including regulation of development and

differentiation of germ cells and their niches (Berruti, 2006; Edson et al., 2009) and homeostasis of calcification of bone (Edwards, 2005). The physiological and biochemical actions of estrogen are mediated by its cognate receptors, estrogen receptors alpha (ESR1) and -beta (ESR2) (Hall and McDonnell, 1999) to effect physiological changes via different regulatory cascades that transactivate diverse transcription factors and induce expression of various target genes. Until recently, the majority of studies investigating estrogen-mediated development of the chicken oviduct focused on the phenomena and developmental status of folliculogenesis and egg production. The results demonstrated that estrogen controls growth and development of preovulatory follicles and production of egg yolk precursors in the liver of hens (Evans et al., 1988; Yoshimura et al., 1995) and calcium and phosphorous metabolism required for formation of the egg shell (Bar, 2009; Hincke et al., 2010). In our previous studies, we identified the avian homolog of human *SERPINB3* as being highly expressed in chicks treated with DES (Song et al., 2011). In the present study, we demonstrated that DES induced *SERPINB3* gene expression in the oviduct, especially in the shell gland, as compared to control chicks (Fig. 7-6), and *SERPINB3* mRNA and protein were expressed abundantly in glandular and luminal epithelial cells of the magnum, isthmus, and shell gland of chick oviducts treated with DES (Fig. 7-7 and 7-8). These results indicate that DES binds strongly to ESR1 as an agonist that mimics effects of 17 $\beta$ -estradiol (Dougherty and Sanders, 2005; Niemela and Elo, 1986; Oka and Schimke, 1969b) and orchestrates the expression of many genes including *SERPINB3* that determine development and differentiation of the chicken oviduct.

Based on results of the present study, we conclude that there is differential tissue- and cell-specific expression of *SERPINB3* mRNA and protein in various regions of the chicken oviduct, but predominantly and abundantly in oviductal epithelial cells. Further, we suggest that *SERPINB3* has dynamic and functional effects on development, differentiation and secretion of egg white proteins during egg formation. Consistent with our results from microarray analysis that *SERPINB3* expression changes significantly during oviduct development in chickens, current results indicate that estrogen induces expression of *SERPINB3* mRNA and protein in a tissue- and cell-specific manner that is coordinate with development, differentiation and function of the various anatomical components of the oviduct in chickens that are critical to the reproductive biology of laying hens.

## **CHAPTER 8**

# **Avian SERPINB11 Gene: Characteristics, Tissue-Specific Expression, and Regulation of Expression by Estrogen**

## 1. Abstract

Serpins, a group of proteins with similar structural and functional properties, were first identified based on their unique mechanism of action: their inhibition of proteases. While most serpins have inhibitory roles, certain serpins are not involved in canonical proteolytic cascades but perform diverse functions including storage of ovalbumin in egg white, transport of hormones (thyroxine- and cortisol-binding globulin), and suppression of tumors. Of these, serpin peptidase inhibitor, clade B, member 11 (SERPINB11) is not an inhibitor of known proteases in humans and mice, and its function is unknown. In the present study, the SERPINB11 gene was cloned, and its expression profile was analyzed in various tissues from chickens. The chicken SERPINB11 gene has an open reading frame of 1346 nucleotides that encode a protein of 388 amino acids that has moderate homology (38.8%–42.3%) to mammalian SERPINB11 proteins. Importantly, *SERPINB11* mRNA is most abundant in the chicken oviduct, specifically luminal and glandular epithelia, but it was not detected in any other chicken tissues of either sex. We then determined effects of diethylstilbestrol (DES; a synthetic nonsteroidal estrogen) on SERPINB11 expression in the chicken oviduct. Treatment of young chicks with DES induced *SERPINB11* mRNA and protein only in luminal and glandular epithelial cells of the oviduct. Collectively, these results indicate that the novel estrogen-induced *SERPINB11* gene is expressed only in epithelial cells of the chicken oviduct and implicate SERPINB11 in regulation of oviduct development and differentiated functions.

## 2. Introduction

As the primary female sex hormone, estrogen controls a variety of biological events such as cell proliferation and differentiation, protection against apoptosis, and diabetes (Hewitt et al., 2005; Louet et al., 2004). The action of estrogen is mediated by its cognate nuclear receptors, estrogen receptors alpha or beta, and it then binds to specific estrogen response elements in DNA to activate transcription of target genes (Hall and McDonnell, 1999). The mammalian oviduct undergoes diverse biological changes in response to sex steroids during the estrous cycle and early pregnancy, as these actions are pivotal in establishing an optimal microenvironment from gamete transport to early embryonic development (Buhi et al., 1997). The chicken is known to be one of the best animal models for investigating the biological actions and signaling pathways of estrogen (Dougherty and Sanders, 2005). In the chicken oviduct, estrogen induces both cell proliferation and differentiation and has anti-apoptotic effects (Monroe et al., 2002; Monroe et al., 2000). In particular, estrogen stimulates formation of tubular glands and differentiation of epithelial cells into goblet and ciliated cells in the chicken oviduct (Palmiter and Wrenn, 1971b). In addition, estrogen affects calcium metabolism for eggshell formation (Bar, 2009; Hincke et al., 2010).

SERPINs are serine and cysteine peptidase inhibitors with a single common core domain consisting of three  $\beta$ -sheets and 8 to 9  $\alpha$ -helices that have been identified in a variety of species based on the inhibitors' unique mechanism of action (Gettins, 2002b; Law et al., 2006). SERPINs are commonly present in plasma, where they support biological processes such as angiogenesis, immune responses, and fibrinolysis.

More than 1500 types of SERPINs have been identified, including 36 human SERPINs, as well as those in plants, fungi, bacteria, and viruses (Askew et al., 2007; Gotoh et al., 1990; Irving et al., 2000). Thus, SERPINs comprise the largest and most diverse superfamily of protease inhibitors (Rawlings et al., 2004). The human genome encodes 17 SERPIN clades, termed SERPINA to SERPINP, encoding 29 inhibitory and 7 non-inhibitory SERPINs (Law et al., 2006). Interestingly, SERPINs differ from other common inhibitors in that they inhibit their target peptidase or protease by serving as their substrates. Most human SERPINs are secreted and circulate in the blood, but clade B serpins lack signal peptides (Silverman et al., 2004) and thus exist in cytoplasm or nucleocytoplasmic area as intracellular proteins (Bird et al., 2001; Uemura et al., 2000). Even though recent reports indicate that the clade B SERPIN family is highly conserved in gene arrangement, the functions of many of them are unknown (Askew et al., 2007; Kaiserman et al., 2002).

Our laboratory used differential gene profiling data for the chicken oviduct to identify the avian homolog of human *SERPINB11* transcript as being highly expressed in chicks treated with the synthetic estrogen agonist diethylstilbestrol (DES) (Song et al., 2011). Little is known about the expression and function of *SERPINB11* in most species, except for humans and mice (Askew et al., 2007); therefore, the objectives of this study were (1) to compare the primary *SERPINB11* sequences of chicken with those of selected mammalian species; (2) to determine tissue- and cell-specific expression of the *SERPINB11* gene in various organs of the chicken; and (3) to determine whether estrogen regulates expression of *SERPINB11* mRNA and protein during oviductal development in chicks. Results of this study provided novel insights

into the *SERPINB11* gene with respect to its sequence, tissue-specific expression, and regulation of its expression during development of the chicken oviduct.

### 3. Materials and Methods

#### *Experimental Animals and Animal Care*

The experimental use of chickens for this study was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). White Leghorn (WL) chickens were subjected to standard management practices at the University Animal Farm, Seoul National University, Korea. The management, reproduction, and embryo manipulation procedures adhered to standard operating protocols of our laboratory. All chickens were exposed to a 15L:9D regimen with *ad libitum* access to feed and water.

#### *Tissue Samples*

Following euthanasia of the WL chickens, tissue samples were collected from brain, heart, liver, kidney, muscle, small intestine, gizzard, ovary, oviduct, and testis of 1- to 2-yr-old males (n = 3) and females (n = 3). Subsets of these samples were frozen or fixed in 4% paraformaldehyde for further analyses. Frozen tissue samples were cut into 5- to 7-mm pieces, frozen in liquid nitrogen vapor, and stored at -80°C. The other samples were cut into 10-mm pieces and fixed in fresh 4% paraformaldehyde in PBS (pH 7.4). After 24 h, fixed tissues were changed to a solution of 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5 µm.

### ***DES Treatment and Oviduct Retrieval***

Female chicks were identified by PCR analysis using W chromosome-specific primer sets (Lee et al., 2009). Treatment with DES and recovery of the oviduct were conducted as reported previously (Sanders and Mcknight, 1988; Seo et al., 2009). Briefly, a 15-mg DES pellet was implanted subcutaneously in the abdominal region of 1-wk-old female chicks for 10 days. The DES pellet was removed from all chicks for 10 days, and then a 30-mg dose was administered for 10 additional days (Seo et al., 2009). Five chicks in each group were euthanized using 60%–70% carbon dioxide. Subsets of these samples were frozen or fixed in 4% paraformaldehyde for further analyses. Frozen tissue samples were cut into 5- to 7-mm pieces and frozen in liquid nitrogen. The other samples were cut into 10- to 15-mm pieces and fixed in fresh 4% paraformaldehyde in PBS (pH 7.4). After 24 h, fixed tissues were changed to a solution of 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffinembedded tissues were sectioned at 5  $\mu$ m.

### ***RNA Isolation***

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

### *Sequence Analysis*

For pair-wise comparisons and multiple sequence alignment, the amino acid sequences of *SERPINB11* genes from each species were aligned using Geneious Pro version 5.04 software (Biomatters Ltd), with default penalties for gap, and the protein weight matrix of Blocks Substitution Matrix (BLOSUM; Biomatters Ltd). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) of Geneious Pro version 5.04 software. To determine the confidence level for each internal node on the phylogenetic tree, 1000 nonparametric bootstrap replications were used (Felsenstein, 1985).

### *Semiquantitative RT-PCR Analysis*

The expression levels of *SERPINB11* mRNA in various organs from chickens, including the oviduct, were assessed using semiquantitative RT-PCR as described previously (Song et al., 2007). The cDNA was synthesized from total cellular RNA (2 µg) by using random hexamer (Invitrogen, Carlsbad, CA) and oligo(dT) primers and AccuPower RT PreMix (Bioneer, Daejeon, Korea). cDNA was diluted (1:10) in sterile water before use in PCR. For *SERPINB11* expression, the sense primer (5'-CGG AGA CCT GAG CAT GTT GG-3') and antisense primer (5'-TAT CAC CCC TGT GGA GCC TG-3') amplified a 337-bp product. For the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) housekeeping gene, the sense primer (5'-TGC CAA CCC CCA ATG TCT CTG TTG-3') and antisense primer (5'-

TCC TTG GAT GCC ATG TGG ACC AT-3') amplified a 301-bp product. Primers, PCR amplification, and verification of their sequences were conducted as described previously (Song et al., 2007). PCR amplification was conducted using approximately 60 ng of cDNA consisting of (1) 95°C for 3 min, (2) 95°C for 20 sec, then 60°C for 40 sec (for *SERPINB11* and *GAPDH*) and 72°C for 1 min for 33 cycles, and (3) 72°C for 10 min. Then, equal amounts of reaction product were analyzed using a 1% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light, using a Gel Doc XR+ system with Image Lab software (Bio-Rad).

### ***Quantitative RT-PCR Analysis***

Total RNA was extracted from each oviduct of control and DES-treated chicks by using TRIzol (Invitrogen) and purified using an RNeasy mini-kit (Qiagen). Complementary DNA was synthesized using AccuPower RT PreMix (Bioneer, Daejeon, Korea). Gene expression levels were measured using SYBR Green (Sigma, St. Louis, MO) and a StepOnePlus real-time PCR System (Applied Biosystems, Foster City, CA). The *GAPDH* gene was simultaneously analyzed as a control and used for normalization. Expression levels of each target gene and the *GAPDH* gene were analyzed in triplicate. Using the standard curve method, we determined levels of expression of the examined genes by using the standard curves and threshold cycle ( $C_T$ ) values and normalized them based on *GAPDH* expression levels. PCR conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 30 sec, 60°C for 30

sec, and 72°C for 30 sec, using melting curve software (increasing the temperature from 55°C to 95°C at a rate of 0.5°C per 10 sec) and continuous fluorescence measurement. 6-Carboxy-X-rhodamine dye (Invitrogen) was used as a negative control for measurements of fluorescence. Sequence-specific products were identified by generating a melting curve in which the  $C_T$  value represented the cycle number at which a fluorescent signal was significantly greater than that of the background, and relative gene expression was quantified using the  $2^{-\Delta\Delta C_T}$  method (Livak and Schmittgen, 2001). For the control, the relative quantification of gene expression was normalized to the  $C_T$  value of the control oviduct.

### ***In situ hybridization analysis***

For hybridization probes, PCR products were generated and were gel-extracted and then cloned into pGEM-T vector (Promega) as described previously (Ahn et al., 2010). After verification of the sequences, plasmids containing the correct gene sequences were amplified with T7- and SP6-specific primers then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). After hybridization and blocking, the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche). The signal was visualized by exposure to a solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4mM nitroblue tetrazolium, and 2 mM levamisole (Sigma).

### ***Immunohistochemistry***

Immunocytochemical localization of SERPINB11 protein in the chicken oviduct was performed as described previously (Song et al., 2006), using goat anti-human SERPINB11 polyclonal antibody (catalog no. sc-85140; Santa Cruz Biotechnology) at a final dilution 1:100 (2 µg/ml). Antigen retrieval was performed using the boiling citrate method as described previously (Song et al., 2006). Negative controls included replacement of the primary antibody with purified non-immune goat immunoglobulin G (IgG) at the same final concentration.

### ***Western Blot Analyses***

Whole oviduct extracts and immunoblot assays were prepared and performed as described previously (Song et al., 2009). Oviducts from chickens treated with DES and from control chickens were rinsed with cold PBS and lysed by homogenization for 5 min in ice-cold lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 0.2 mM phenylmethylsulfonylfluoride, 50 mM NaF, 30 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 µg/ml leupeptin, and 1 µg/ml pepstatin). Cell lysates were clarified by centrifugation (16,000×g, 15 min, 4°C). Protein content was determined by using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Proteins were denatured, separated using 10% SDS-PAGE, and transferred to nitrocellulose membranes. Blots were developed using enhanced chemiluminescence detection (SuperSignal West Pico; Pierce, Rockford, IL) and quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light, using a

ChemiDoc EQ system and Quantity One software (Bio-Rad, Hercules, CA). Immunoreactive SERPINB11 protein was detected by using goat anti-human SERPINB11 polyclonal antibody (catalog no. sc-85140; Santa Cruz Biotechnology) at a final dilution 1:1000. As a loading control, Western blotting with mouse anti-beta actin IgG (catalog no. sc-47778; Santa Cruz Biotechnology) was performed.

### ***Statistical Analyses***

All statistical analyses were performed using Student *t*-test SAS software (SAS Institute, Cary, NC). Differences were considered significant at a *P* value of < 0.05.

## 4. Results

### *Multiple Sequence Alignment, Pairwise Comparisons, and Phylogenetic Analysis*

The chicken *SERPINB11* gene was found in the genomic region spanning 7071-bp on chromosome 2. The gene consists of eight exons, and the mRNA has 1346-bp encoding a protein with 388 amino acid residues. The primary chicken *SERPINB11* sequence was compared to those of some other mammalian species. Chicken SERPINB11 protein contained a reactive center loop required for inhibitor function in members of the serpin gene family (Lawrence et al., 1994) and as found in mammalian SERPINB11 (see Supplemental Figure 8-1). In pairwise comparisons of chicken SERPINB11 proteins with those of 10 other vertebrates, chicken SERPINB11 protein was found to have moderate (38.8%-42.3% [Table 8-1]) homology to mammalian SERPINB11 proteins. The phylogenetic tree constructed with the neighbor-joining method is shown in Figure 8-1. Human and chimpanzee SERPINB11 genes clustered together and formed a larger cluster with those of orangutan and an even larger cluster with those of sister groups for baboon and rhesus monkey. However, the chicken SERPINB11 protein is in a separate branch but closer to those of rodents than to primates. These results indicate that chicken SERPINB11 diverged from mammalian SERPINB11 at very early stage in its evolution.

### *SERPINB11 mRNA Expression in Chickens*

Analysis of tissue-specific expression patterns of *SERPINB11* mRNA in

various organs of male and female chickens by using RT-PCR analysis (Fig. 8-2) revealed that *SERPINB11* mRNA was expressed only in the oviduct of female chickens but not in ovary or testes and not in brain, heart, liver, kidney gizzard, or small intestine in either sex. Therefore, this study focused on the chicken oviduct.

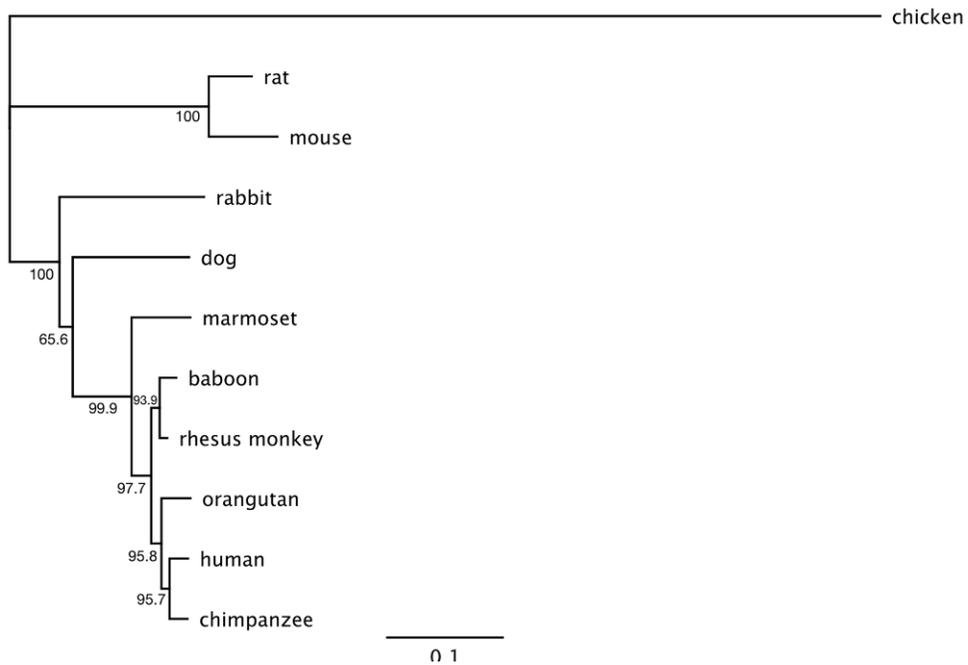
### ***Localization of SERPINB11 mRNA and Protein Expression in the Chicken Oviduct***

The oviduct of egg-laying hens includes the infundibulum (site of fertilization), magnum (production of components of egg white), isthmus (formation of the shell membrane), and shell gland (formation of the egg shell). To determine cell-specific expression of *SERPINB11* mRNA and protein in chicken oviduct, we performed *in situ* hybridization analysis and immunohistochemistry. *SERPINB11* mRNA was most abundant in glandular epithelium (GE) of the magnum and isthmus, and it was also expressed in luminal epithelium (LE) of the infundibulum and LE/GE of the shell gland (Fig. 8-3). Little or no mRNA was detected in stromal cells, blood vessels, immune cells, or myometrium of the oviduct. SERPINB11 protein was most abundant in LE of the infundibulum and GE of the shell gland and magnum (Fig. 8-4). However, of greatest interest was finding SERPINB11 protein present in LE of the isthmus. Goat IgG used as a negative control did not detect any signal.

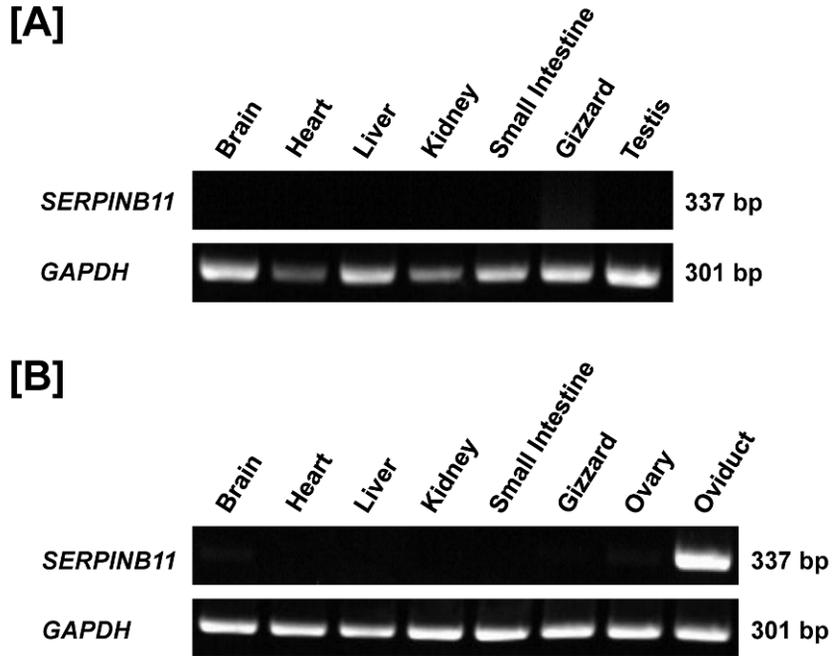
### ***Effects of DES on SERPINB11 mRNA Expression in the Chicken Oviduct***

Oviduct-specific and cell-type-specific SERPINB11 expression in the oviductal segments of hens suggested regulation by estrogen during development of

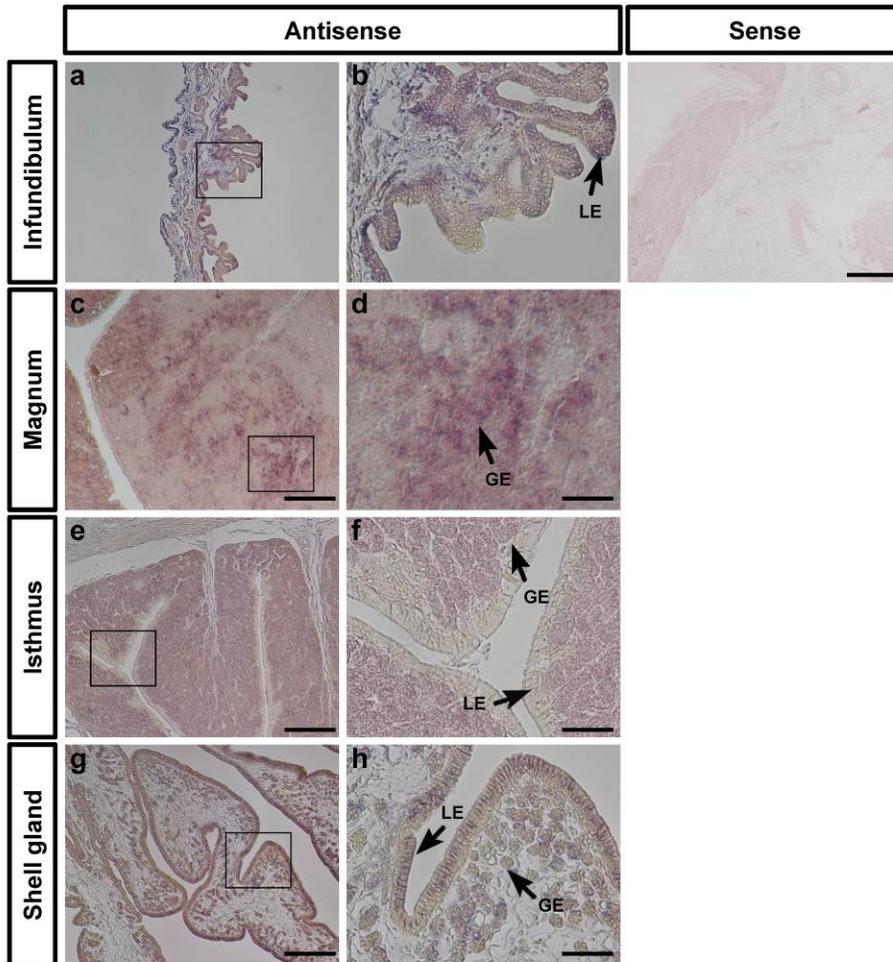
the oviduct in chicks. We previously reported that exogenous DES affects growth, development, and differentiation of the chicken oviduct (Seo et al., 2009) and discovered candidate genes and pathways regulating oviduct development in chickens (Song et al., 2011). Therefore, we examined the effects of DES on SERPINB11 expression in the chicken oviduct. In order to determine if DES regulated expression of SERPINB11 in the oviduct, we exposed 1-wk-old female chicks to two periods of stimulation by using subcutaneous implants of 15-mg and 30-mg DES in the abdominal region for 10 days each (Seo et al., 2009). As shown in Figure 8-5, real-time PCR analysis revealed that DES induced an approximately 522-fold increase ( $P < 0.001$ ) in oviductal *SERPINB11* mRNA compared to that in control chicks (Fig. 8-5C). In addition, DES stimulated approximately 17-, 18.5-, and 110-fold increases ( $P < 0.01$ ) in *SERPINB11* mRNA in the magnum, isthmus, and shell gland, respectively (Fig. 8-5D). *In situ* hybridization analyses revealed that *SERPINB11* mRNA was expressed abundantly only in GE of the magnum, isthmus, and shell gland of chick oviducts treated with DES (Fig. 8-6). Consistent with results from *in situ* hybridization analyses, immunoreactive SERPINB11 protein was detected predominantly in LE/GE of the magnum, GE of the isthmus and shell gland, and also at a lower level of abundance in LE of the isthmus and shell gland (Fig. 8-7). Furthermore, as illustrated in Figure 8-8, Western blot analysis detected abundant SERPINB11 protein in oviducts of DES-treated chicks ( $P < 0.001$ ).



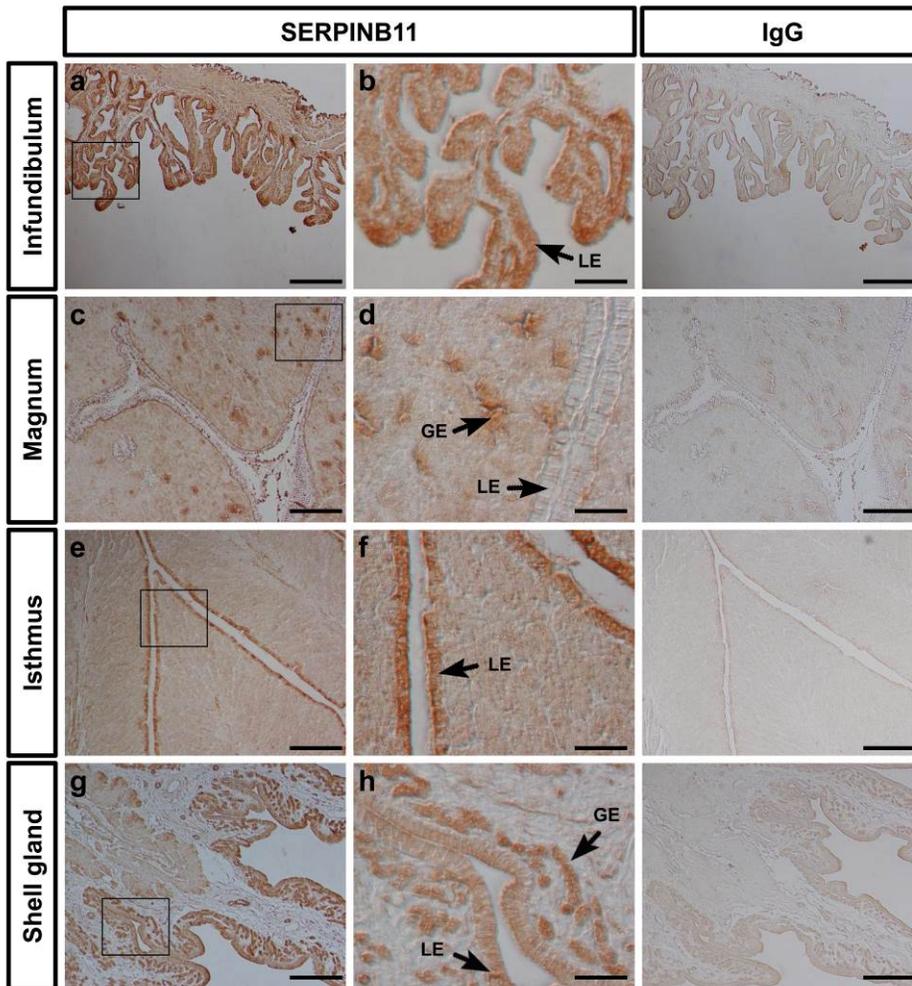
**Figure 8-1. The phylogenetic tree was generated from alignments of primary sequences of the chicken and mammalian SERPINB11 protein.** Amino acid sequences were obtained from each GenBank file (Table 1). The phylogenetic tree was constructed by the neighbor-joining method using Geneious software. Numbers next to the branches indicate bootstrap values from 1000 replicates. Bar shows a genetic distance.



**Figure 8-2.** Levels of *SERPINB11* expression are shown in various organs of both male and female chickens. Results of RT-PCR analysis using cDNA templates from different organs of both male [A] and female [B] chickens with chicken *SERPINB11* and chicken *GAPDH*-specific primers are shown.

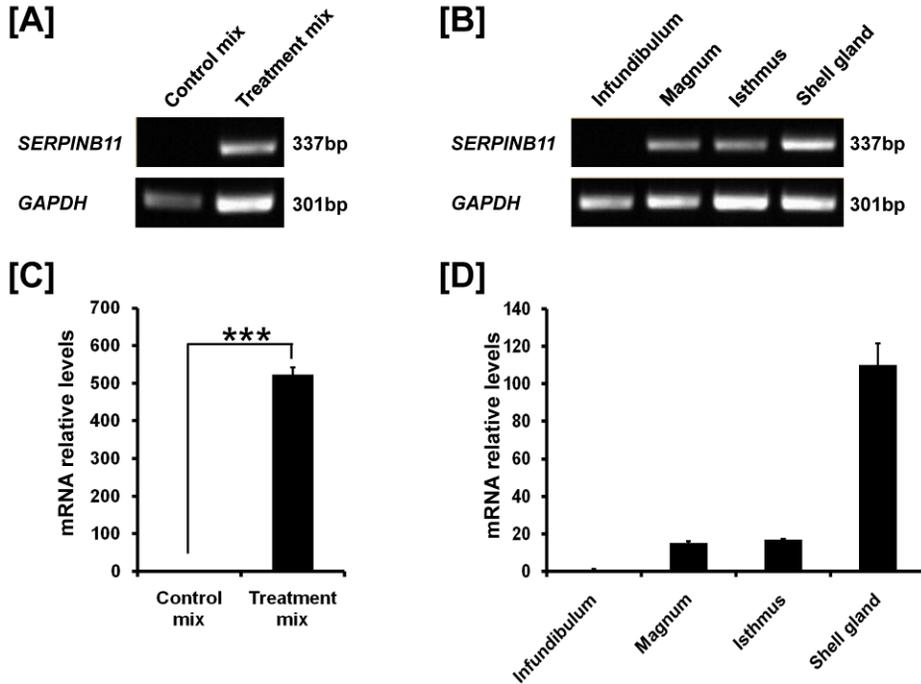


**Figure 8-3. *In situ* hybridization analyses of *SERPINB11* mRNAs in the chicken oviduct are shown.** Cross-sections of the four components of the chicken oviduct (infundibulum, magnum, isthmus, and shell gland) were hybridized with antisense or sense chicken *SERPINB11* cRNA probes (see Materials and Methods for details). LE, luminal epithelium; GE, glandular epithelium. *Scale bars* represent 200  $\mu\text{m}$  (a, c, e, and g and sense) and 50  $\mu\text{m}$  (b, d, f, and h).

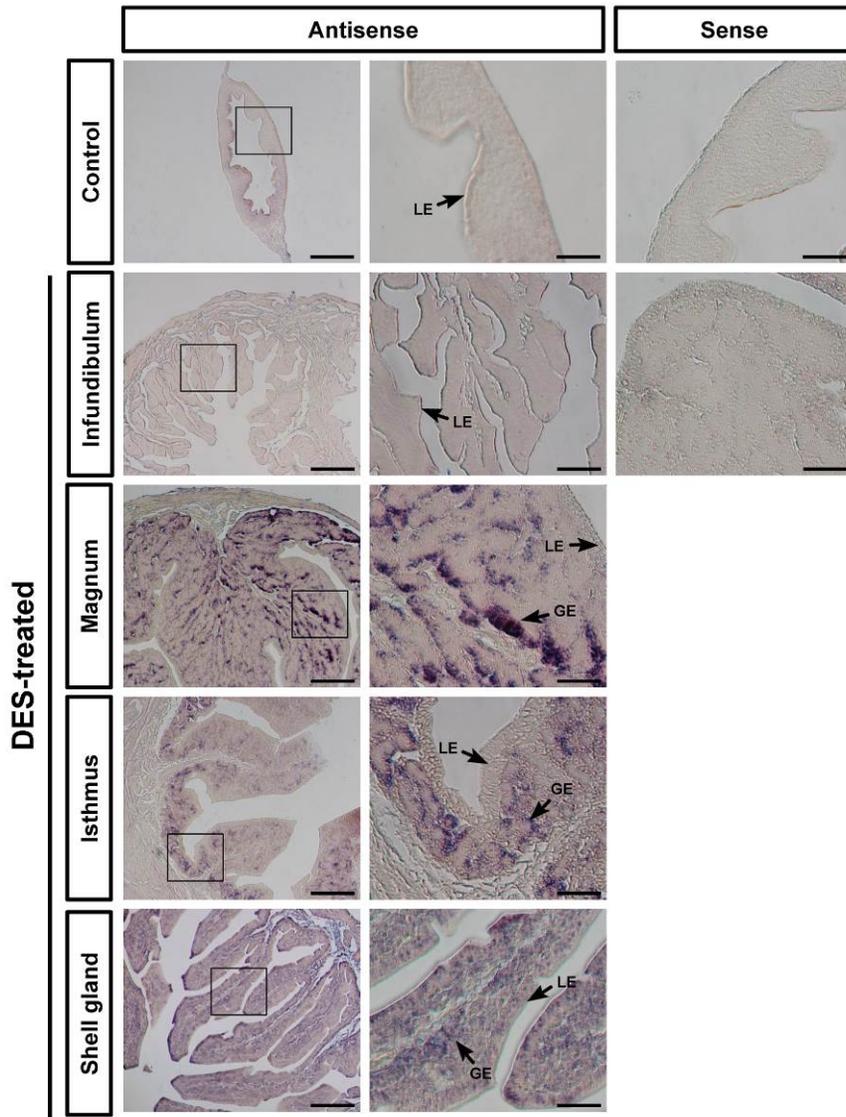


**Figure 8-4. Immunoreactive SERPINB11 protein is shown in the chicken oviduct.**

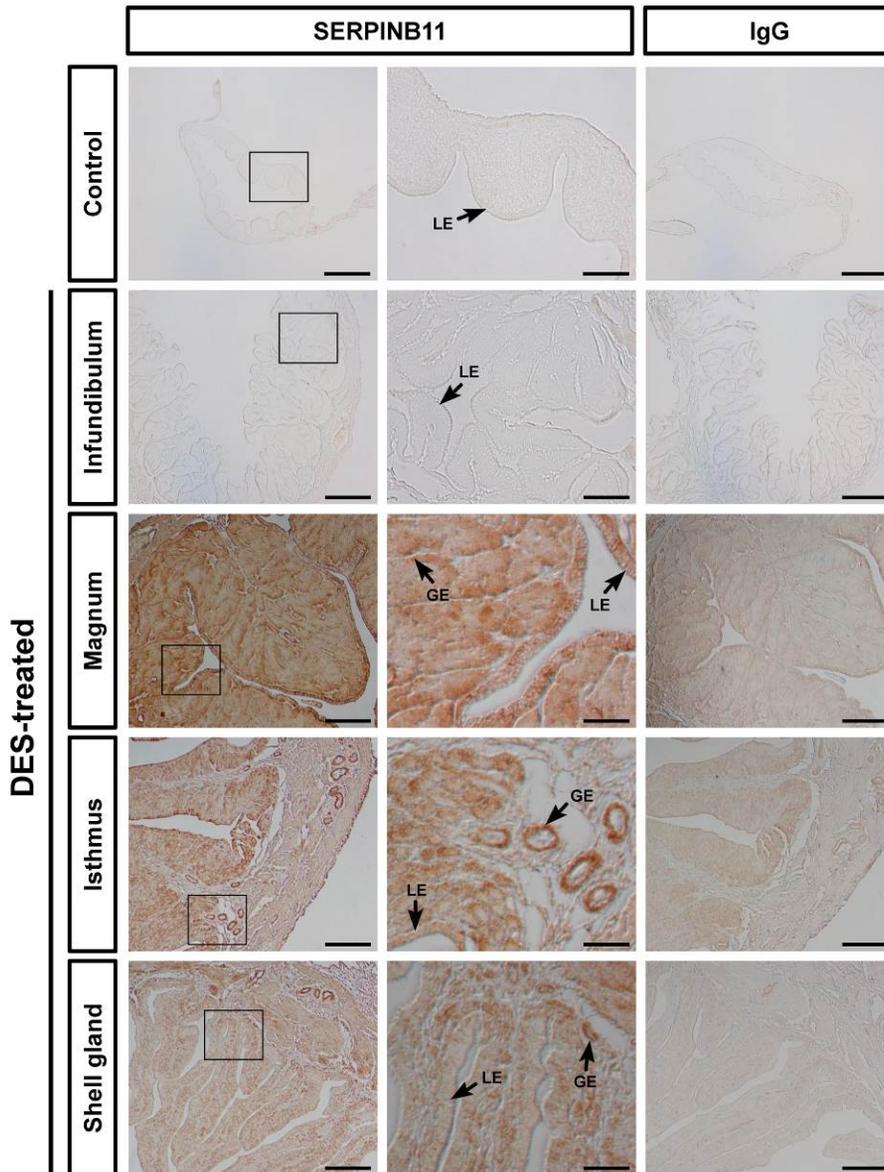
For the IgG control, normal goat IgG was substituted for primary antibody. Sections were not counterstained (see Materials and Methods for details). LE, luminal epithelium; GE, glandular epithelium. *Scale bars* represent 200  $\mu\text{m}$  (a, c, e, and g and IgG) and 50  $\mu\text{m}$  (b, d, f, and h).



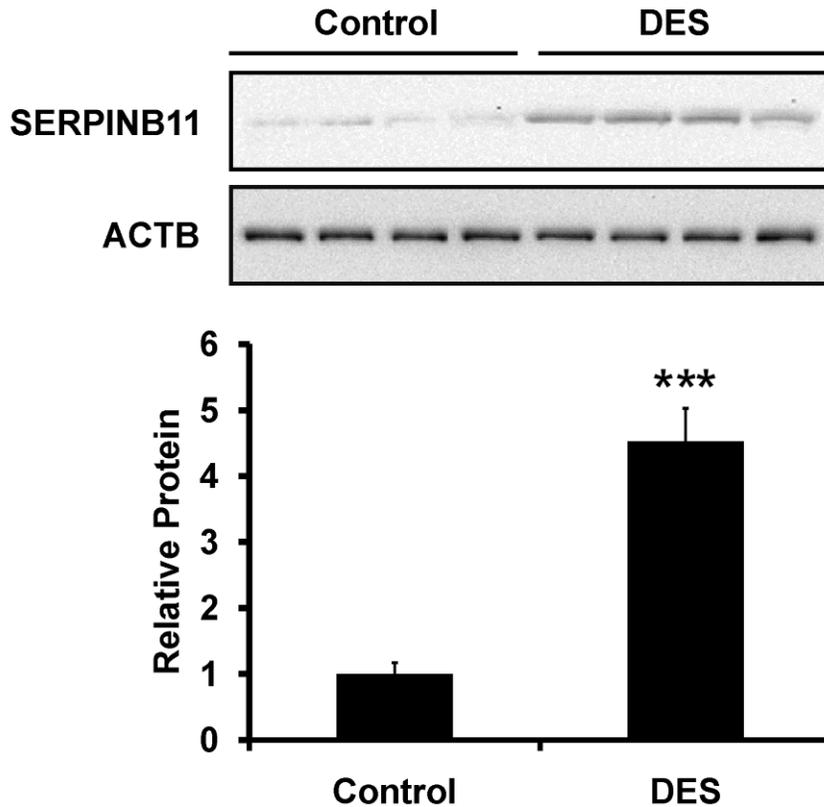
**Figure 8-5.** Effect of DES is shown on tissue specificity of chicken *SERPINB11* expression by RT-PCR and quantitative PCR analyses. Both RT-PCR [A and B] and quantitative PCR [C and D] analyses were performed using cDNA templates from DES-treated and control chicken oviducts (values are means $\pm$ SEM;  $P < 0.001$ ). See Materials and Methods for details.



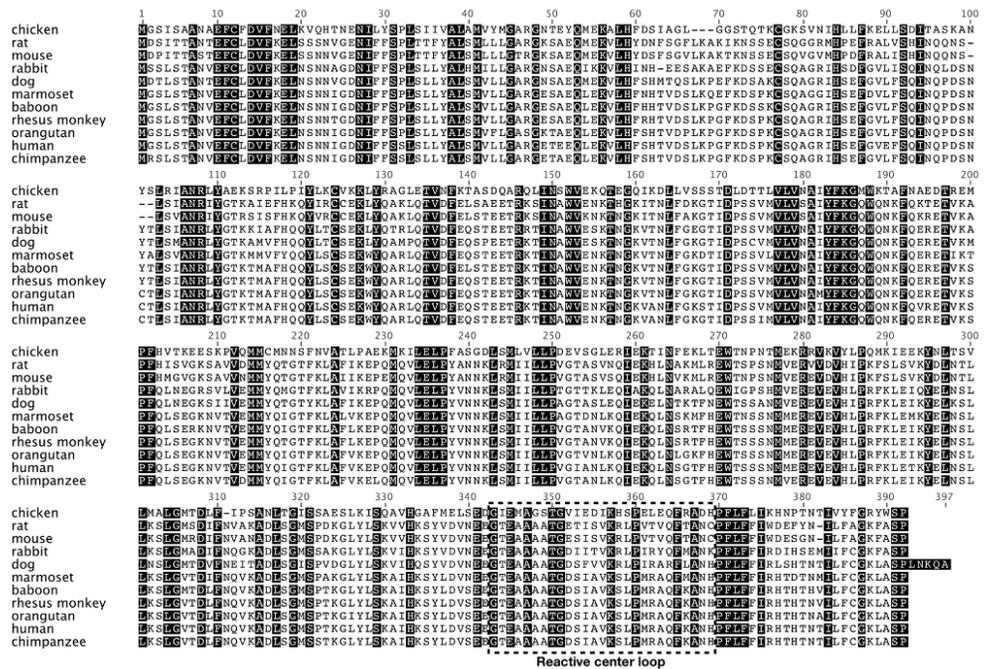
**Figure 8-6. *In situ* hybridization analyses of *SERPINB11* mRNA are shown in oviducts of DES-treated and control chicks.** Cross-sections of the four segments of chicken oviduct (infundibulum, magnum, isthmus, and shell gland) treated with DES or vehicle were hybridized with antisense or sense chicken *SERPINB11* cRNA probes (see Materials and Methods for details. LE, luminal epithelium; GE, glandular epithelium. *Scale bars* represent 200  $\mu\text{m}$  (columns 1 and 3) and 50  $\mu\text{m}$  (column 2).



**Figure 8-7. Immunoreactive SERPINB11 protein is shown in oviducts of DES-treated and control oviducts.** For the IgG control, normal goat IgG was substituted for the primary antibody. Sections were not counterstained (see Materials and Methods for details). LE, luminal epithelium; GE, glandular epithelium. *Scale bars* represent 200  $\mu\text{m}$  (columns 1 and 2) and 50  $\mu\text{m}$  (column 3).



**Figure 8-8. Detection of SERPINB11 in the oviducts of DES-treated and control oviducts is shown.** Proteins were separated by 10% SDS-PAGE under reducing conditions. Immunoreactive SERPINB11 protein was detected using goat anti-human SERPINB11 polyclonal antibody. As a loading control, Western blotting with mouse anti-beta actin (ACTB) IgG was performed. Blots were imaged to calculate the normalized values presented in the graph (bottom) by measurements of levels of SERPINB11 protein relative to those of ACTB protein. DES treatment produced more than 4.5-fold increase in the level of SERPINB11 protein ( $P < 0.001$ ) in reproductive tract of chicks. Asterisks denote an effect of treatment. Error bars show standard deviation.



**Figure 8-9. Multiple sequence alignment of chicken and mammalian SERPINB11 proteins.** The amino acid sequences of SERPINB11 proteins from chicken (*Gallus gallus*), rat (*Rattus norvegicus*), baboon (*Papio Anubis*), rabbit (*Oryctolagus cuniculus*), rhesus monkey (*Macaca mulatta*), mouse (*Mus musculus*), human (*Homo sapiens*), marmoset (*Callithrix jacchus*), orangutan (*Pongo abelii*), chimpanzee (*Pan troglodytes*), and dog (*Canis familiaris*) were aligned using Geneious Pro Version 5.04 with default penalties for gap and the protein weight matrix of BLOSUM (Blocks Substitution Matrix). Shaded amino acid sequences are identical among all species examined. Dashes represent gaps among the sequences. The conserved functional domains in SERPINB11 proteins were identified using the Pfam-A family matrix and NCBI conserved domain database.

**Table 8-1. Pairwise comparisons of SERPINB11 among chicken and several mammalian species.**

<b>Species</b>	<b>Symbol</b>	<b>Identity (%)</b>	<b>GenBank No.</b>
Chicken	SERPINBP1	-	XP_418984.2
vs. Human	SERPINB11	42.3	NP_536723.2
vs. Chimpanzee	SERPINB11	42.1	XP_523958.2
vs. Orangutan	SERPINB11	41.6	XP_002828332.1
vs. Rhesus monkey	SERPINB8P1	42.3	NP_001180852.1
vs. Baboon	SERPINB8P1	42.1	NP_001162386.1
vs. Marmoset	SERPINB8P1	41.8	XP_002757358.1
vs. Dog	SERPINB11	42.1	XP_541073.2
vs. Rabbit	SERPINB8P1	40.2	NP_001164756.1
vs. Rat	SERPINB11	39.1	NP_001100637.1
vs. Mouse	SERPINB11	38.8	NP_080143.1

## 5. Discussion

Results of the present study are the first to compare chicken and mammalian *SERPINB11* genes with respect to structure and phylogenetic evolution, as well as tissue-specific expression of *SERPINB11* mRNA and protein in chickens and regulation of their expression by estrogen in a tissue- and cell-specific manner in the chicken oviduct. These results support our hypothesis that SERPINB11 is required for growth, development, and functional aspects of the mature oviduct of hens in response to estrogen-mediated changes in oviduct function during the reproductive cycle in chickens.

In the present study, we found expression of the *SERPINB11* gene in chickens to be regulated by estrogen during development of the oviduct. The avian oviduct is a highly differentiated linear organ with compartments that undergo structural, cellular, and biochemical changes in response to sex hormones during egg formation and oviposition (Chousalkar and Roberts, 2008). The oviduct of mature egg-laying hens includes five functionally specific regions: infundibulum (site of fertilization), magnum (production of components of egg-white), isthmus (formation of the soft shell membrane), shell gland or uterus (formation of calcified egg shell), and vagina (oviposition). The oviductal epithelial cells differentiate into various cell types including luminal epithelium, tubular glands lined with epithelial cells, goblet cells, and ciliated cells in the magnum (Palmiter and Wrenn, 1971b). Of these, epithelial cells of the tubular glands synthesize and secrete large amounts of critical egg white protein such as ovalbumin, conalbumin, lysozyme, and ovomucoid (Kohler

et al., 1968) and perhaps transport nutrients such as glucose and amino acids to be incorporated into the egg white. The chicken is a well-established animal model for embryology, reproductive biology, and transgenesis, but little is known about chicken oviduct with respect to the highly hormonal regulation of its specific anatomical components due to the lack of fundamental research related to production of components of egg white by tubular gland cells of the magnum.

Estrogen is required for normal reproductive organ development in female mammals and birds (Kohler et al., 1969). In addition, estrogen is essential for protective effects against apoptosis of neuronal, endothelial, and testicular cells (Alvarez et al., 1997; Henderson, 1997; Pentikainen et al., 2000; Wada et al., 1996). Overproduction of estrogens and early exposure of the female to estrogens may cause uterine abnormalities, such as failure of the magnum to calcify the soft shell and a general impairment in the ability of hens to produce eggs (Gildersleeve et al., 1985; Rissman et al., 1984). These results indicate that estrogen is essential to uterine homeostasis and for inhibition of apoptosis of cells of the avian reproductive tract (Dougherty and Sanders, 2005). An overall reduction in secretion of estradiol and progesterone results in regression of the ovary in chickens (Etches et al., 1984). Generally, the apoptotic process begins with activation of initiator caspases (CASPs) such as CASP1, CASP2, CASP8, and CASP10, which mediate the activation of effector CASPs including CASP3, CASP6, and CASP7, which then induce apoptosis (Hengartner, 2000). Insufficient estrogen levels result in up-regulation of CASP1 and CASP2 in the regressing oviduct of chickens (Monroe et al., 2002); however, the mechanism(s) responsible is not known (Anish et al., 2008).

Since Hunt and Dayhoff (Wong and Auersperg, 2003) discovered that certain molecules share significant amino acid sequence similarity to the major proteins in chicken egg white such as ovalbumin, over 1000 SERPINs have been identified. The major SERPINs regulate chemical processes by inhibiting/blocking the function of enzymes, specifically, serine proteases. Most SERPINs are plasma proteins that play pivotal roles in physiological processes such as blood coagulation, fibrinolysis, and inflammation (Law et al., 2006). Upon binding to their target proteins, an irreversible change in the structure of the target protein–serpin complex prevents the target protein from completing any further reactions, and it is removed from the bloodstream (Gettins, 2002b). While most SERPINs control proteolytic cascades, certain SERPINs do not have an inhibitory function on proteases (Askew et al., 2007; Auersperg et al., 2001; Silverman et al., 2004); instead they perform diverse functions such as storage (ovalbumin, in egg white) and hormone binding (cortisol-binding globulin) (Auersperg et al., 2001; Gettins, 2002b).

Homeostatic regulation of serine proteinases is achieved mainly through interactions with inhibitors belonging to the large metazoan, plant, and virus serpin superfamily (Potempa et al., 1994). Some clade B SERPINs are thought to be secreted because of their regulatory effects on cell-cell and cell-matrix interactions, and others can be detected in both intracellular and extracellular areas of tissues (Belin, 1993; Zou et al., 1994). In addition, anti-proteinase systems of most tissues have several combinations of SERPIN B clade proteins (Silverman et al., 2004). For example, most human clade B SERPINs inhibits serine and/or papain-like cysteine proteinases to

protect cells from exogenous and endogenous protease-mediated damage (Silverman et al., 2004). Clustering of 13 *SERPINB* genes occurs in two regions of chromosome 6p25 and 18q21 in the human genome (Shioji et al., 2005; Silverman et al., 2004). The human *SERPINB11* gene, located on chromosome 18q21, is highly conserved with its mouse ortholog, but it has no known inhibitory activity (Askew et al., 2007).

In this study, tissue-specific expression of *SERPINB11* mRNA and protein was found in most parts of the chicken oviduct. Except for the infundibulum, *SERPINB11* is specifically expressed in GE and LE of the oviduct treated with DES. Based on these results, we suggest that *SERPINB11* has functional roles in oviduct development, differentiation, and function critical to the reproductive biology of the laying hen. In a previous study, we examined transcript changes in chick oviducts after exposure to DES (Song et al., 2011) that binds strongly to estrogen receptor alpha to act as an agonist with similar effects similar to that of 17 $\beta$ -estradiol (Dougherty and Sanders, 2005; Niemela and Elo, 1986; Oka and Schimke, 1969b). Through microarray analysis, we found that the *SERPINB11* expressed changed significantly during oviduct development in chickens. Result of the present study indicate that DES induces significant increases in expression of *SERPINB11* mRNA and protein in a tissue- and cell-specific manner that is coordinate with development, differentiation, and function of the various anatomical components of the oviduct in chickens.

## **CHAPTER 9**

# **Novel Genes and Hormonal Regulation for Gonadal Development during Embryogenesis in Chickens**

## 1. Abstract

Asymmetrical gonadal morphogenesis is well known in female chickens in contrast to males where both gonads develop symmetrically. Differential development of gonads provides a model for understanding the responsible cellular and molecular mechanisms. However, only a few genes have been reported to determine differential morphology between female and male gonads in chicken and their mechanism of action are unclear. Therefore, we examined changes in transcripts in the asymmetric female gonads between E6 and E9 using microarray analysis. A total of 568 and 760 transcripts were up-regulated in the left and right gonads at E9 as compared with E6. Based on results from gene ontology (GO) analysis, we focused on five genes (*PITX2*, *SNCA*, *TOMIL1*, *TTR* and *ZEB1*) that are related to cellular proliferation and embryonic development. To define the validity of the gene expression pattern discovered by microarray analysis, quantitative RT-PCR and *in situ* hybridization analyses were performed. Specifically, *TOMIL1*, *TTR* and *ZEB1* expression increased in the left female gonad between E6 and E9 and their expression was similarly localized to that for *CYP19A1* and gonadotropin receptors. On the other hand, *PITX2*, *SNCA*, *TOMIL1* and *TTR* increased significantly in both male gonads between E6 and E9. In addition, recombinant FSH and LH stimulated proliferation of gonadal cells and influenced expression of selected genes in chickens. This suggests that hormonal regulation is involved in growth and development in the embryonic gonad of chickens. Collectively, the results show how differential expression of genes affects morphogenesis of the left and right gonads in chicken embryos. These results provide novel insights into candidate genes regulating gonad development and differentiation

in chickens.

## 2. Introduction

In vertebrates, left-right (L-R) asymmetric morphogenesis is important for development of various organs including the heart, liver, spleen and gut. The L-R asymmetric development is one of the orthogonal axes including anterior-posterior and dorsal-ventral axes for the formation of body. It is conserved in vertebrates by genes encoding for various transcription factors and growth factors (Levin, 2005; Raya and Izpisua Belmonte, 2006). For example, Nodal is a member of the transforming growth factor-beta superfamily expressed in the left lateral plate mesoderm (LPM) (Hamada et al., 2002b). At the node, an early asymmetric signal is generated and conveyed to the LPM through the function of Nodal signaling molecules in mammals. Lefty1 and Lefty2, as representative Nodal signaling molecules, are induced by Nodal signaling and their expression is regulated by Smad-5 in mice (Chang et al., 2000; Takaoka et al., 2006). In addition, the homeobox gene PITX2 is downstream of Nodal and cooperates with Nodal to develop the L-R axis (Liu et al., 2001a; Patel et al., 1999a). Asymmetric expression of PITX2 is involved in the process of organ morphogenesis such as heart, gut and lung (Campione et al., 1999b).

In mammals, reproductive organs such as ovary and testis are positioned symmetrically in both sexes. Unlike mammals, bilateral development of the paired gonads is dependent on sex in chickens. Before sex determination, the gonads of chick embryos have no L-R asymmetrical morphology in either sex. The onset of sex specific differentiation becomes apparent histologically at embryonic day 6.5 (E6.5).

Most female birds (ZW) have an asymmetric functional left ovary whereas the right gonad degenerates during embryonic development. In other words, only the left outer epithelial cell layer (cortex) proliferates to form an ovary whereas the right cortex fails to develop into an ovary. Therefore most female germ cells assemble around the cortex of the left ovary from day 10. On the other hand, testicular development in male (ZZ) chicks arises symmetrically and medulla cords thicken due to differentiation of sertoli cells within the cords (Merchantlarios et al., 1984; Smith and Sinclair, 2004a). During gonadal morphogenesis in chicken, the left-right symmetry during gonadal development in embryogenesis of chicks is fascinating. While only the left gonad develops in female chicks, the early stage epithelium of the undeveloped right gonad is thinner than for the left gonad. Genes expressed asymmetrically during development of the gonads in chicks include bone morphogenic protein 7 (BMP7) (Hoshino et al., 2005), paired-like homeodomain transcription factor 2 (PITX2) (Levin, 2005), forkhead transcription factor L2 (FOXL2) (Wang et al., 2007), sry-box 9 (SOX9) (Smith and Sinclair, 2004a), sonic hedgehog (SHH) (Levin et al., 1995), and estrogen receptor alpha (ESR1) (Gonzalez-Moran, 2005). For example, ESR1 is expressed in both sexes until E7. After that ESR1 is only expressed in the cortex of the left gonad of female chicken embryos and it is not expressed in either gonad in male chick embryos (Andrews et al., 1997; Nakabayashi et al., 1998a). And misexpression of PITX2 in the left lateral plate mesoderm prevents development of the right gonad in female chick embryos and that gonad then degenerates (Rodriguez-Leon et al., 2008).

There are a few genes which have been identified as regulators for

asymmetric development of chicken embryonic gonads. Nevertheless, understanding how gonadal development occurs asymmetrically is unclear. Since the chicken genomic sequence is known, high-throughput analyses using chicken DNA microarrays provide valuable insight into changes in gene expression related to various biological processes. Therefore, the objectives of present study were to: 1) examine asymmetric changes in transcripts in the female gonad between E6 and E9 using microarray analyses; 2) investigate cell- and tissue-specific expression of five selected genes considered to be involved in the development of gonads of female and male chick embryos during embryogenesis; 3) compare the patterns of expression of cytochrome p450, family 19, subfamily a, polypeptide 1 (CYP19A1), follicle stimulating hormone receptor (FSHR) and the luteinizing hormone receptor (LHCGR) with expression of five genes associated with gonadal development; and 4) determine whether FSH and LH regulate proliferation of embryonic gonadal cells and expression of the five target genes. Collectively, results of this study identified asymmetrically expressed genes that likely affect gonadal morphogenesis between left and right gonads during embryogenesis and how expression of those genes is regulated by gonadotropins during gonadal morphogenesis.

### 3. Materials and Methods

#### *Experimental Animals and Animal Care*

The experimental use of chickens for this study was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). White Leghorn (WL) chickens were subjected to standard management practices at the University Animal Farm, Seoul National University, Korea. All chickens were exposed to a light regimen of 15 h light and 9 h dark with *ad libitum* access to feed and water, and subjected to standard poultry husbandry guidelines.

#### *Sex Determination*

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

### ***Tissue Samples***

The left and right gonads were collected separately from the mesonephric kidney of chicken embryos at E6 and E9 in a 1.5 ml tube containing diethylpyrocarbonate treated PBS (DEPC-PBS). Then we centrifuged the sample at  $1,080 \times g$  for 5 min to allow collection of each gonad from the bottom of tubes. After removal of the DEPC-PBS, the gonads were stored at  $-80^{\circ}\text{C}$  until RNA was extracted. Also we collected whole embryos and fixed them in freshly prepared 4% paraformaldehyde in PBS (pH 7.4). After 24 h, embryos fixed in 4% paraformaldehyde were changed to 70% ethanol for 24 h and then dehydrated in a graded series of increasing concentrations of ethanol. Then, embryos were incubated in xylene for 3 h and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at  $5 \mu\text{m}$ .

### ***RNA Isolation***

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

### ***Microarray Analysis***

Microarray analysis was performed using Affymetrix GeneChip® Chicken Genome Arrays (Affymetrix, Santa Clara, CA, USA). Data were generated by SeoulLin Bioscience Corporation (Seoul, Korea) and dChip software was used for the analysis (Li and Wong, 2001). For biological variation, total RNA was extracted from 135 samples from E6 and 105 samples from E9 using TRIzol (Invitrogen, Carlsbad, CA, USA) and purified using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). We selected differentially expressed genes at each time point by two-sample comparisons with a lower threshold of greater than a two-fold change with a 90% confidence interval (corresponding to  $P < 0.05$ ) and a mean difference between collection times for each tissue of greater than 100. We used the student's *t*-test to control for false positives. Differentially regulated genes identified in the microarray analyses were analyzed using the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Mountain View, CA, USA). Canonical pathway analyses identified the pathways from the IPA library of canonical pathways that were most highly represented in the data set.

### ***Quantitative RT-PCR Analysis***

Total RNA was extracted from each left and right gonads of embryonic day 6 and 9 of both sexes using TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized using a Superscript® III First-Strand Synthesis System (Invitrogen). Gene expression levels were measured using SYBR® Green (Sigma, St. Louis, MO, USA) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The glyceraldehydes 3-phosphate dehydrogenase (*GAPDH*) gene was analyzed simultaneously as a control

and used for normalization of data. *GAPDH* expression is assumed to be most stable among other housekeeping genes and it is used commonly for normalizing for variations in loading. Each target gene and *GAPDH* were analyzed in triplicate. Using the standard curve method, we determined expression of the examined genes using the standard curves and  $C_T$  values, and normalized them using *GAPDH* expression. The PCR conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 20 sec, 60°C for 40 sec, and 72°C for 1 min using a melting curve program (increasing the temperature from 55°C to 95°C at 0.5°C per 10 sec) and continuous fluorescence measurement. ROX dye (Invitrogen) was used as a negative control for the fluorescence measurements. Sequence-specific products were identified by generating a melting curve in which the  $C_T$  value represented the cycle number at which a fluorescent signal was statistically greater than background, and relative gene expression was quantified using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001). For the control, the relative quantification of gene expression was normalized to the  $C_T$  value for the control oviduct. Information on the primer sets is provided in Table 9-1.

### ***In situ hybridization analysis***

For hybridization probes, PCR products were generated and were gel-extracted and then cloned into pGEM-T vector (Promega) as described previously (Ahn et al., 2010). After verification of the sequences, plasmids containing the correct gene sequences were amplified with T7- and SP6-specific primers then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN). After hybridization and blocking, the sections

were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche). The signal was visualized by exposure to a solution containing 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.4mM nitroblue tetrazolium, and 2 mM levamisole (Sigma).

### ***Proliferation Assay***

Proliferation assays were conducted using a Cell Proliferation ELISA, BrdU kit (Roche) according to the manufacturer's recommendations. Briefly, gonadal cells were seeded in a 96-well microplate (tissue culture grade, flat bottom). Cells were then treated with either recombinant FSH (catalog number: F8174; Sigma, St. Louis, MO, USA), LH (catalog number: L5269; Sigma, St. Louis, MO, USA) or FSH plus LH in a final volume of 100  $\mu$ l/well (n = 3 well per treatment). The concentration of each hormone was determined by performing a preliminary dose-response experiment. After labeling of cells with BrdU, cells were fixed, incubated with anti-BrdU-POD working solution for 90 min. The anti-BrdU-POD binds to the BrdU incorporated in newly synthesized cellular DNA and the immune complexes are then detected following addition of substrate. BrdU incorporation was quantified by measuring the absorbance at the respective wavelength using an ELISA reader (Bio-Rad).

### ***Scanning Electron Microscopy***

Collected samples (embryonic day 6 males and females, embryonic day 9 males and females) were fixed primarily at 4°C for 2 to 4 h in modified Karnovsky's

fixative (2% glutaraldehyde and 2% formaldehyde in 0.05M sodium cacodylate buffer, pH 7.2), washed three times with cacodylate buffer, fixed secondarily for 2 h with 1% osmium tetroxide in cacodylate buffer, and stained overnight with 0.5% uranyl acetate at 4°C. To observe specimens for scanning electron microscopy (SEM), samples were dried twice with 100% isoamyl acetate for 15 min in a critical point dryer, mounted on metal stubs, coated with gold, and observed under field emission FE-SEM (SUPRA 55VP; Carl Zeiss) at the National Instrumentation Center for Environmental Management (NICEM) at Seoul National University.

### *Statistical Analyses*

All quantitative data were subjected to analysis of variance (ANOVA) according to the general linear model (PROC-GLM) of the SAS program (SAS Institute, Cary, NC). All tests of significance were performed using the appropriate error terms according to the expectation of the mean square for error. Data are presented as mean  $\pm$  SEM unless otherwise stated. Differences in the variance between E6 and E9 for each gonad were analyzed using the *F* test, and differences in the means were subjected to Student's *t* test. Differences were considered significant at  $P < 0.05$ .

## 4. Results

### *Morphology of the chicken embryonic gonads from both sexes of E6 and E9*

To compare the morphology of chicken embryonic gonads between embryonic day 6 and embryonic day 9, we photographed their morphological structure using a stereoscopic microscope. As shown in Figure 9-1A, there were no differences in shape of left and right gonads in male and female embryos at E6 indicating that the gonads developed symmetrically in both sexes until E6. On the other hand, at E9 the female embryonic gonad developed asymmetrically such that the left gonad was thicker and bigger than the right gonad on E9. In contrast, the left and right gonads of male embryos were morphologically equal at E9.

### *Embryonic gonadal gene expression is altered at E6 and E9 in female chicken*

Hierarchical clustering analysis of genes showed that most changes in gene expression occurred when comparing female gonads at E6 and E9 (Fig. 9-1B). Microarray analysis determined noteworthy differences in numerous transcripts between left and right gonads at E6 and E9 during development of chick embryos (Fig. 9-1C). There was a two-fold or greater difference ( $P < 0.05$ ) in 819 transcripts in the left gonad between E6 and E9, with 568 up-regulated and 251 down-regulated transcripts. There were also changes in 1,074 transcripts between E6 and E9 for the right gonad, with 760 up-regulated transcripts and 314 down-regulated transcripts. Then we analyzed genes co-regulated in each combination for differential expression

due to embryonic day for both gonads and for the left gonad, destined to develop into a functional ovary, at the two embryonic days using Venn diagrams (Fig. 9-1D and 9-2E). Seventy-seven transcripts changed in both the left and the right gonad between E6 and E9, and 479 transcripts changed differentially between the left and the right gonad between E6 and at E9.

***Functional categories of genes were altered significantly between left and right gonads during embryogenesis***

Between the left and right gonad at embryonic days 6 and 9, the developmentally regulated gonadal genes were categorized into specific functional groups using gene ontology analysis (GO analysis) that included physiological system development, molecular and cellular function, and disease/disorder categories (Fig. 9-2). In the physiological system development and function categories, transcripts were related to cellular proliferation and growth, cellular development, organ development, nervous system development and function and embryonic development (Fig. 9-2A and 9-2B). In the category of molecular and cellular functions, transcripts that changed were involved in cell death, skeletal and muscular disorders, cellular movement, cell-to-cell signaling and interaction and cell cycle (Fig. 9-2C and 9-2D). In addition, abundant transcripts sorted into the diseases and disorders category including genetic disorder, neurological disease, cancer, reproductive system disease and gastrointestinal disease (Fig. 9-2E and 9-2F).

***Verification of Selected Genes***

Through microarray analysis we identify significant differences in various transcripts and gene ontology between the left and right gonads at E6 and E9 in both sexes of chick embryos. To verify differential mRNA expression of selected genes including *PITX2*, synuclein alpha (*SNCA*), target of myb1 (chicken)-like 1 (*TOM1L1*), transthyretin (*TTR*) and zinc finger E-box binding homeobox 1 (*ZEB1*) that are related to cellular proliferation and embryonic development, quantitative RT-PCR and *in situ* hybridization analyses are performed for each gonad between E6 and E9 (Fig. 9-3). Among the five genes, *TOM1L1*, *TTR* and *ZEB1* mRNAs increased 7.8- ( $P < 0.05$ ), 11.6- ( $P < 0.01$ ) and 2.3-fold ( $P < 0.01$ ) in left gonad at E9 as compared with E6 in female chicken embryos (Fig. 9-3A-9-3E). Also, *PITX2* and *TOM1L1* increased 1.14- ( $P < 0.05$ ) and 1.6-fold ( $P < 0.01$ ) and *TTR* increased 5.8-fold ( $P < 0.01$ ). On the other hand, *SNCA* and *ZEB1* mRNA expression decreased 0.3-fold ( $P < 0.05$  and  $P < 0.01$ , respectively) in the right gonad at E9 as compared to E6.

As illustrated Figure 9-3, cell-specific expression of the five mRNAs in female gonads at E6 and E9 was demonstrated by *in situ* hybridization analysis (Fig. 9-3F-9-3J). Their expression patterns were consistent with change in mRNA expression detected by quantitative RT-PCR. *TOM1L1*, *TTR* and *ZEB1* mRNAs increased to a greater degree in the left gonad at E9 as compared with E6 and all were localized mainly to germ cells (GCs) in cortex region of left gonad.

In male embryos, expression of *PITX2*, *TOM1L1* and *TTR* mRNAs increased 18.1- ( $P < 0.001$ ), 8.0- ( $P < 0.001$ ) and 9.8-fold ( $P < 0.01$ ) at E9 in the left gonad as

compared with the left gonad at E6. Similarly, those three genes also increased 10.2- ( $P < 0.01$ ), 7.2- ( $P < 0.01$ ) and 10.3-fold ( $P < 0.05$ ) in the right gonad at E9 as compared to E6 (Fig. 9-4A-9-4E). However, expression of only *ZEB1* mRNA decreased 0.8- ( $P < 0.05$ ) and 0.4-fold ( $P < 0.05$ ) in left and right gonads at E9 as compared with E6 of embryonic development. There was no significant difference in expression *SNCA* between E6 and E9. *In situ* hybridization analysis demonstrated expression of *PITX2*, *SNCA*, *TOMIL1* and *ZEB1* mRNAs in germ cells of both gonads at E6 (Fig. 9-4F-9-4J). And, at E9, *PITX2*, *SNCA*, *TOMIL1* and *TTR* mRNAs were expressed strongly in the seminiferous cords (Sc) which develop into seminiferous tubules in mature males. In contrast to the increase in *ZEB1* expression in the left gonad of female embryos at E9, lower expression was detected in seminiferous cords in male gonads at E9.

#### ***The expression of steroidogenic enzymes and pituitary glycoprotein hormone receptors***

As an endocrine organ, the gonad has a role in synthesis of sex steroid hormones and it is a target of gonadotropins. To determine whether gonadal function involves secretion of primary hormones during gonadal morphogenesis, we compared expression patterns of aromatase (*CYP19A1*) and receptors for FSH (*FSHR*) and LH (*LHCGR*) using quantitative RT-PCR and *in situ* hybridization analyses (Fig. 9-5). First there was differential mRNA expression of each of the genes in female (Fig. 9-5A) and male (Fig. 9-5B) embryonic gonads. *CYP19A1* and *LHCGR* were increased 55.7- ( $P < 0.01$ ) and 6.4-fold ( $P < 0.001$ ) whereas *FSHR* was decreased 0.5-fold in the

left gonad at E9 as compared with E6 in female chicken embryos. In male embryos, expression of *CYP19A1* and *LHCGR* mRNA increased 18.8- ( $P < 0.001$ ) and 1.4-fold ( $P < 0.05$ ) at E9 in the left as compared with the right gonad at E6. These genes were similarly increased 16.6- ( $P < 0.001$ ) and 1.8-fold ( $P < 0.01$ ) in the right gonad of female embryos on E9. However, *FSHR* mRNA levels decreased 0.8- ( $P < 0.05$ ) and 0.6-fold at E9 both left and right gonads. Cell specific localization of *CYP19A1*, *FSHR* and *LHCGR* was demonstrated in female (Fig. 9-5C) and male (Fig. 9-5D) gonads during chicken embryogenesis. The *CYP19A1* mRNA was expressed in the left and right gonad of both sexes at E6, but at E9 its expression was highly localized to the cortex region of the left gonad in female chicken embryos and to the seminiferous cords in male gonads at E9. *FSHR* mRNA was localized to germ cells of the left gonads of embryos on E6 and E9. Expression of *FSHR* mRNA was more abundant in the left gonadal cortex than in the right gonadal cortex of female embryos at E9 and in males at E9 there was little *FSH* mRNA was detected in seminiferous cords of male gonads. *LHCGR* mRNA was expressed weakly as compared to other mRNAs and at E9 expression of *LHCGR* was more abundant in the gonadal cortex of the left gonad in female embryos while *LHCGR* mRNA was quite low in both gonads of male embryos at E9.

#### ***Effect of FSH and LH on gonadal cell proliferation in chicken embryos***

Cell proliferation assays were performed to determine biological effects of FSH and LH on development of embryonic gonads in chickens. Treatment of chicken embryonic gonadal cells with FSH increased cell numbers by approximately 130% at

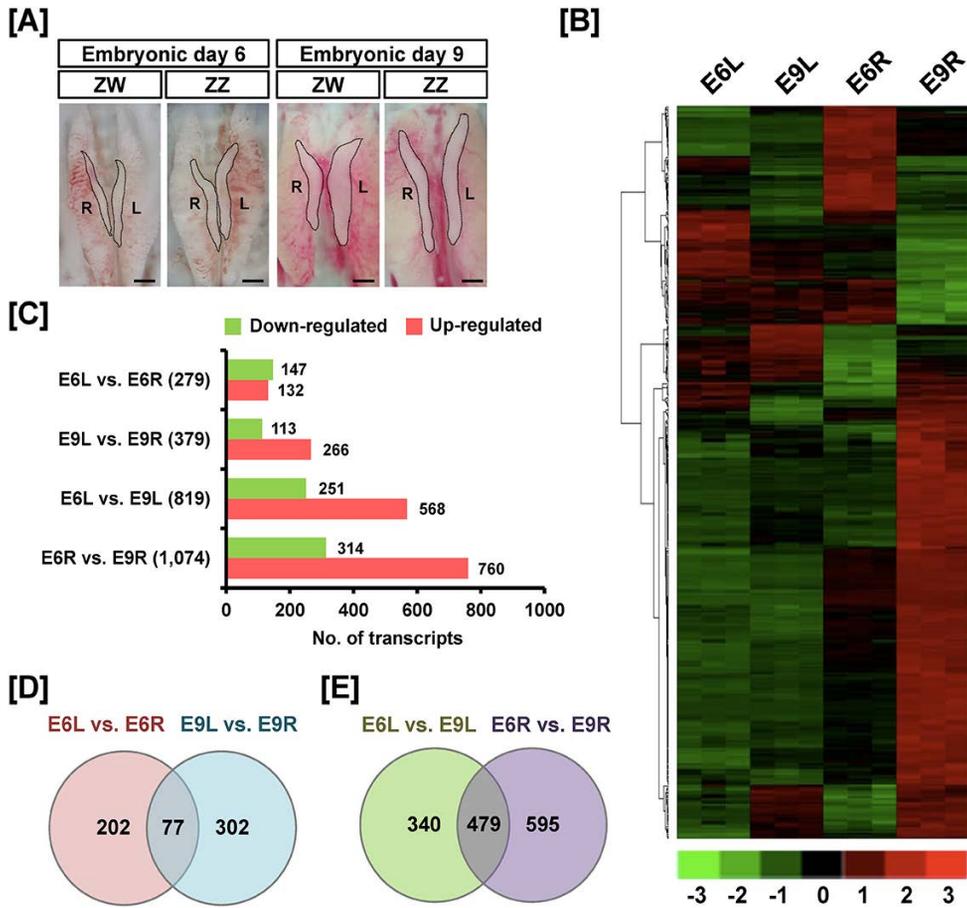
6 h of treatment (Fig. 9-6A). Similarly, LH increased gonadal cell numbers at 6 h and by approximately 160% at 24 h after treatment ( $P < 0.01$ ) (Fig. 9-6B). In addition, the combined FSH and LH treatment also stimulate proliferation of gonadal cells ( $P < 0.05$ ) (Fig. 9-6C). These results indicate that FSH and LH stimulate proliferation of chicken embryonic gonadal cells as the gonads grow and develop.

### ***FSH and LH activate target genes in embryonic gonadal cells in chickens***

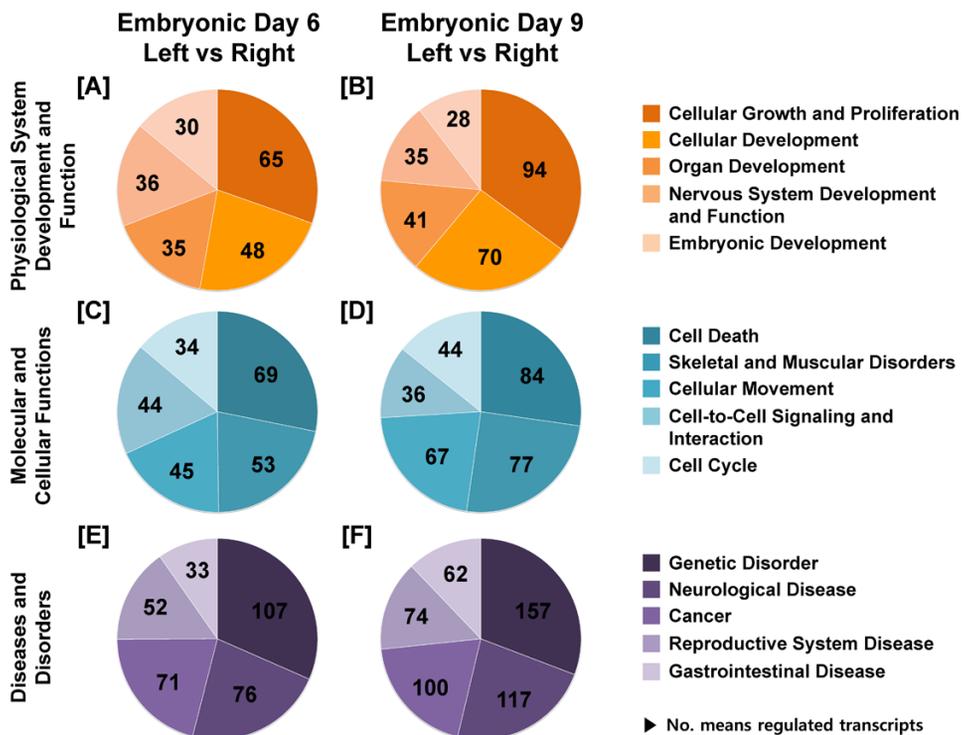
We next investigated effects of FSH and LH expression of the five selected genes associated with chicken embryonic gonadal cells using quantitative RT-PCR (Fig. 9-7). Expression of *TOMIL1* and *TTR* mRNAs were slightly decreased in response to FSH, while *PITX2* and *SNCA* mRNA levels increased at 6 h after treatment then expression decreased. There was no effect of FSH on *ZEB1* expression. LH inhibited expression of four of the five selected genes (Fig. 9-7) by 33% to 90%; however, expression of *TOMIL1* mRNA increased in response to LH by 190% ( $P < 0.001$ ) at 24 h after treatment of gonadal cells. The combination of FSH and LH on chicken embryonic gonadal cells was to increase expression of *ZEB1* mRNA at 6 h after which time expression decreased. Similarly, expression of *PITX2* decreased 67% at 6 h after treatment ( $P < 0.001$ ) with FSH and LH, and as was expression of *SNCA* (83%) and *TTR* (60%). The expression of *TOMIL1* was significantly (660%;  $P < 0.01$ ) increased at 24 h after treatment with FSH and LH. These results indicate that candidate genes regulating development of gonads in chick embryos are influenced positively and negatively by LH and the combination of FSH and LH.

*Ultra-structure of embryonic gonads and gene expression during gonadal morphogenesis.*

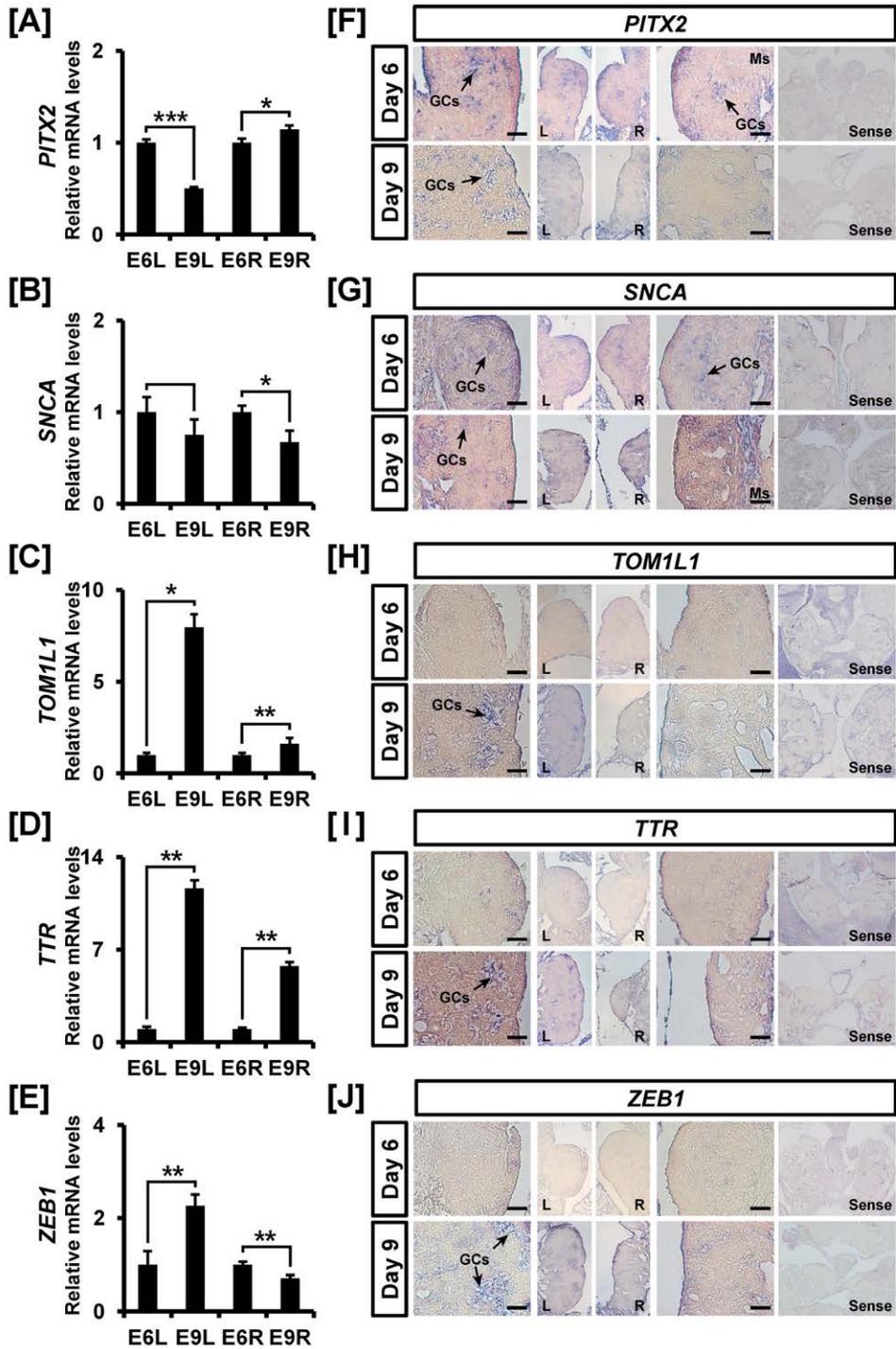
Detailed morphological differences were identified based on ultra-structural properties of the embryonic gonad at both E6 and E9 using field emission-scanning electron microscopy (FE-SEM). At E6, surface structure of left and right gonads were spherical and covered densely with pili (Fig. 9-8). When compared to surface of gonads from E6 embryos, the morphology of the surface epithelium on the right gonad of female embryos was completely transformed into a flattened form and pili were rarely detected. In examining the pattern of expression of selected genes in both left and right gonads at E6 and E9, it was determined that expression of most genes increased in the developing female and male embryonic gonads of chickens. The *PITX2*, *TOM1L1*, *TTR* and *ZEB1* genes were expressed asymmetrically between the left and right on female gonad at E9. On the other hand, *SNCA* expression was not different between the two gonads at E9 in female chicken embryos. In males, expression of most genes increased at E9 in both embryonic gonads.



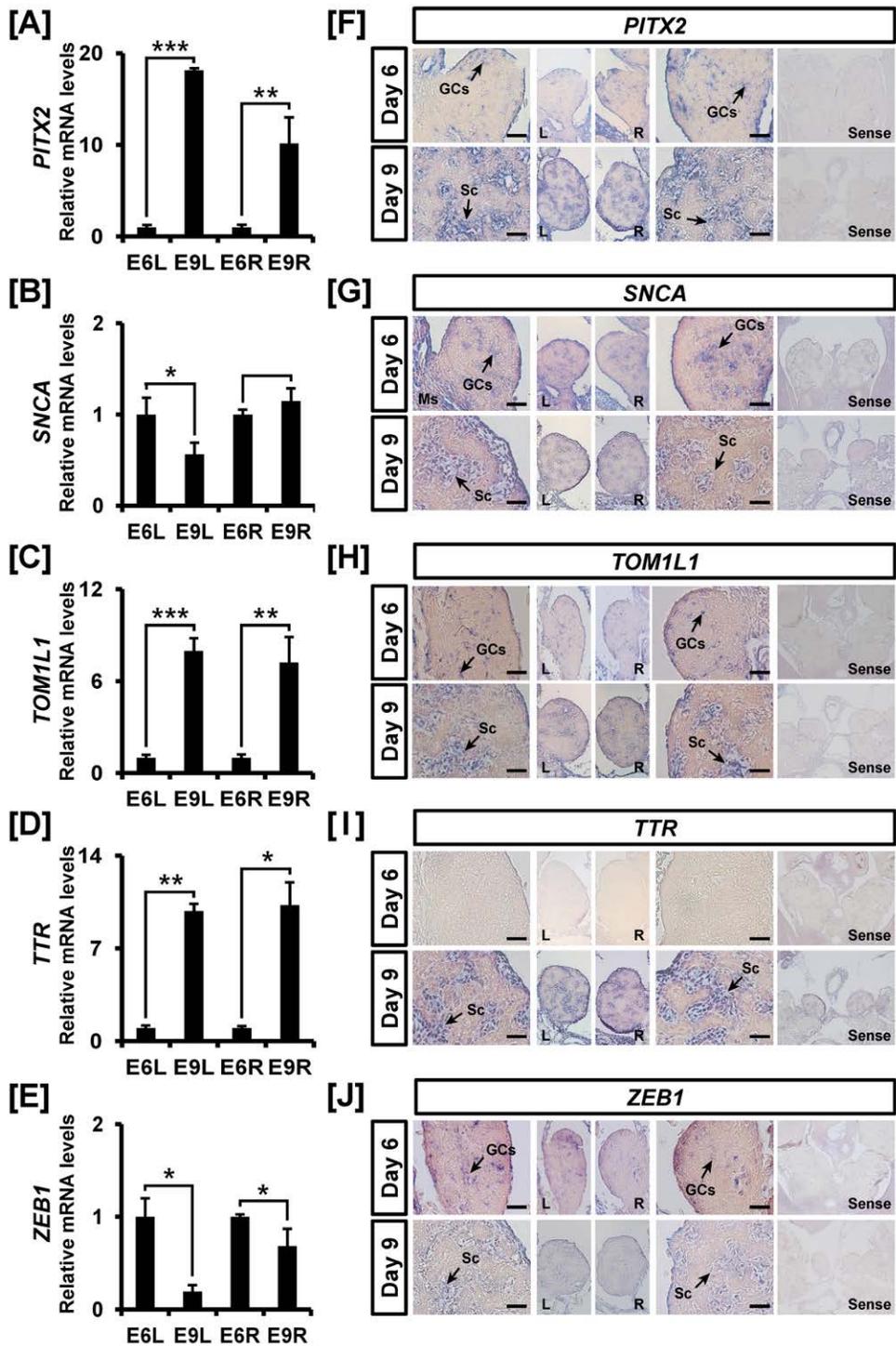
**Figure 9-1. Asymmetrical morphology of chicken embryonic gonads and identification of genes for which expression changed significantly in the left and right gonads during gonadal morphogenesis in chicken embryos.** Morphology of left and right gonads at E6 and E9 was determined using a stereoscopic microscope [A]. Hierarchical clustering analysis of genes indicated changes in expression of these genes in the left and right gonads between E6 and E9 [B]. The number of genes for which expression increased or decreased significantly in the left and the right gonads of the chicken embryos between E6 and E9 while embryogenesis was determined. [C]. The Venn diagrams show the relationship between genes that changed in the left and right gonad at the same embryonic day [D] and in the left and right gonads between E6 and E9 [E].



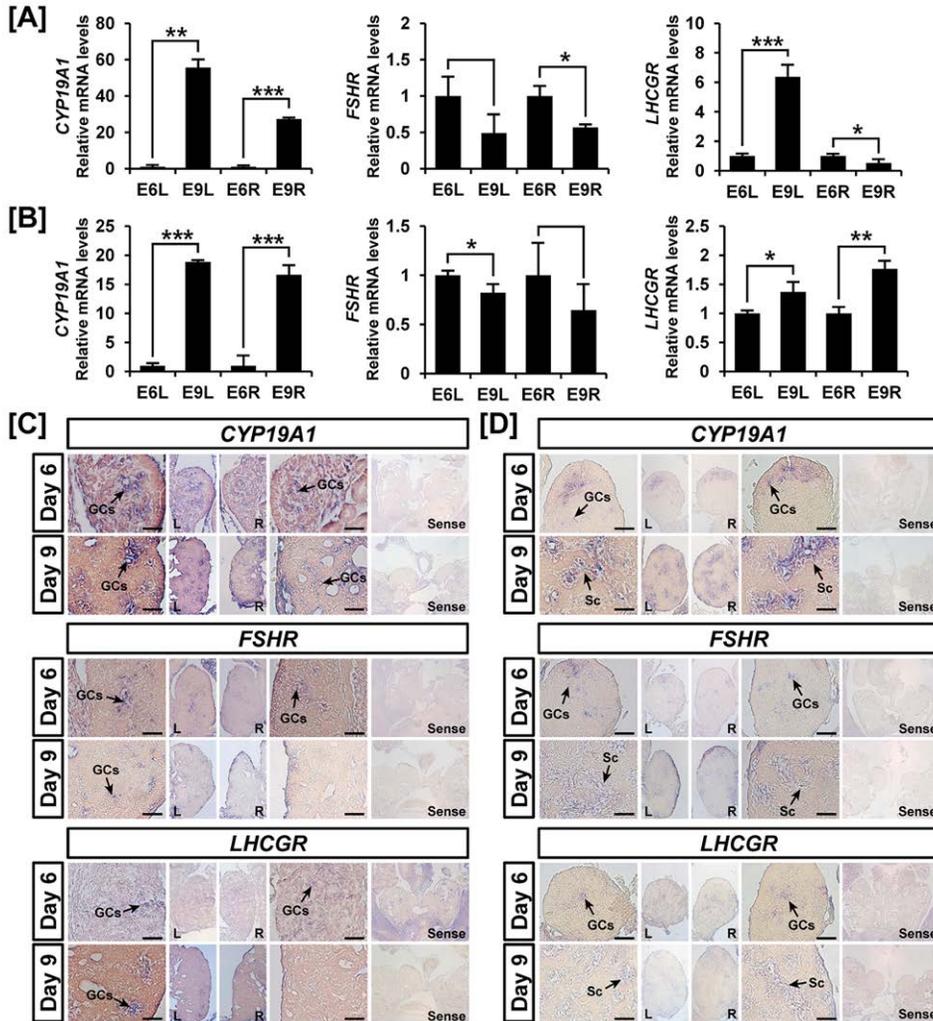
**Figure 9-2. Functional categorization of the genes that changed during gonadal morphogenesis.** Expression of some genes changed between left and right gonads at either E6 [A, C and E] or E9 [B, D and F]. Genes that changed significantly were annotated and assigned to various functional categories using gene ontology.



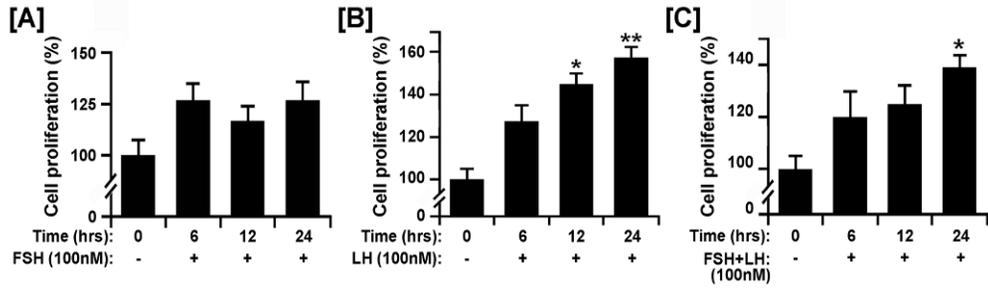
**Figure 9-3. Comparison of relative expression and localization of *PITX2*, *SNCA*, *TOM1L1*, *TTR* and *ZEB1* mRNAs in female embryonic gonads during embryogenesis.** A comparison of mRNA expression between E6 and E9 in female embryonic gonads was performed by quantitative RT-PCR using cDNA templates from left and right gonads at embryonic days 6 and 9 (values are mean  $P < 0.05$ ) [A]. Cross sections of left and right gonads at E6 and E9 in female embryos were hybridized with antisense or sense cRNA probes. The asterisks denote statistically significant differences ( $***P < 0.001$ ,  $** P < 0.01$  and  $* P < 0.05$ ). GCs, germ cells; Ms, mesonephros. *Scale bar* represents 20  $\mu\text{m}$ .



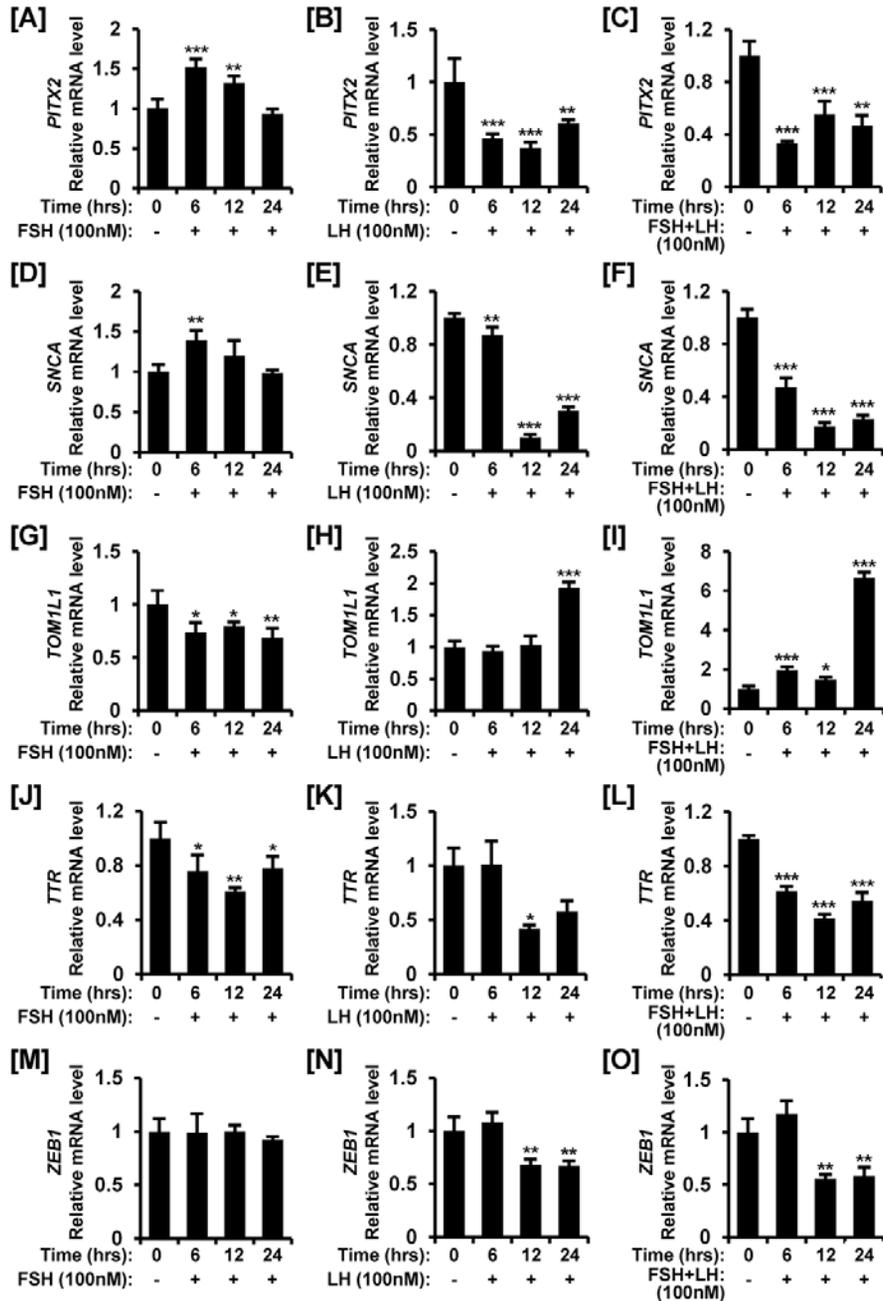
**Figure 9-4. Comparison of relative expression and localization of *PITX2*, *SNCA*, *TOM1L1*, *TTR* and *ZEB1* mRNAs in male embryonic gonads during embryogenesis.** Relative changes in mRNA expression for each gene were determined using quantitative RT-PCR [A]. Expression of most genes increased in gonads at E9 as compared with E6. Cell-specific localization of selected genes was determined using *in situ* hybridization analysis [B]. The changes in expression were assessed using quantitative RT-PCR and genes of interest were localized primarily to the seminiferous cords in male embryonic gonads. The asterisks denote statistically significant differences ( $***P < 0.001$ ,  $** P < 0.01$  and  $* P < 0.05$ ). GCs, germ cells; Sc, seminiferous cord; Ms, mesonephros. *Scale bar* represents 20  $\mu\text{m}$ .



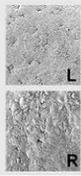
**Figure 9-5. Analysis of relative levels of expression and cell-specific expression of mRNAs for *CYP19A1*, *FSHR* and *LHCGR* in female and male gonads during gonadal development.** Comparisons of mRNA expression levels for each gene were determined by quantitative RT-PCR in female [A] and male [B] chicken embryos. Cross-sections of the embryonic gonads in female [C] and male [D] chicken were hybridized with antisense or sense cRNA probes (See Materials and Methods for details). GCs, germ cells; Sc, seminiferous cord. *Scale bar* represents 20  $\mu\text{m}$ .



**Figure 9-6. Time-dependent effects of FSH, LH and FSH plus LH on proliferation of gonadal cells from embryonic day 6.** Monolayers of 60% confluent embryonic gonadal cells were treated with 100nM FSH [A], LH [B] or FSH with LH [C]. Cell numbers were determined at 0, 6, 12 and 24 h after treatment and data were expressed as a percent change relative to non-treated control cells (100%).



**Figure 9-7.** Analysis of time-dependent expression of *PITX2*, *SNCA*, *TOM1L1*, *TTR* and *ZEB1* in response to FSH, LH and FSH plus LH in gonadal cells from embryonic day 6 at 0, 6, 12 and 24 h after treatment. The asterisks denote statistically significant differences (\*\*\* $P < 0.001$ , \*\* $P < 0.01$  and \* $P < 0.05$ ).

Sex	Embryonic day 6				Embryonic day 9			
	Female (ZW)		Male (ZZ)		Female (ZW)		Male (ZZ)	
								
Gene	Left	Right	Left	Right	Left	Right	Left	Right
PITX2	++	+	+	++	+	-	+++	+++
SNCA	+	+	+++	+++	+	+	+++	+++
TOM1L1	-	-	+	+	++	+	++	++
TTR	-	-	-	-	++	+	+++	+++
ZEB1	+	+	-	-	++	+	+	+

**Figure 9-8. Summary of gene expression patterns and ultrastructure of embryonic gonads during gonadal morphogenesis in chicken embryos.** The morphological differences were identified based on ultra-structural properties of the embryonic gonad following scanning electron microscope imaging of tissues from E6 and E9. The intensity of expression of selected genes was analyzed by *in situ* hybridization. The plus (+) means existence of expression in the gonad tissue and minus (-) means little or no expression in the chicken embryonic gonad.

**Table 9-1. Primer sequence for quantitative RT-PCR**

<b>Gene</b>		<b>Sequence (5' to 3')</b>	<b>Accession No.</b>	<b>Size</b>
<b>CYP19A1</b>	F	CCTCTGCTGGAGATGGTTTT	NM_001001761.1	67 bp
	R	CTGATCCACTTTAGTCACTCTGA		
<b>FSHR</b>	F	CCGGGACATTCCCACCA	NM_205079.1	70 bp
	R	GATGAGAGTCATTCCGAAGGGA		
<b>LHCGR</b>	F	GCTCAACGTCATAGCCTTCC	NM_204936.1	80 bp
	R	CTACCAGCTCAGGGTTCTGC		
<b>PITX2</b>	F	CGCAGAGCCAAATGGAGGAA	NM_205010.1	100 bp
	R	CATCGTAGGGCTGCATCAGG		
<b>SNCA</b>	F	TGGCAGAAGCAGCAGGAAAG	NM_204673.1	136 bp
	R	ACCACAGCTCCCCAACATT		
<b>TOM1L1</b>	F	ATCCCAGATACAACCTGCCC	XM_415646.2	98 bp
	R	TTCGGTCACATCCACCATTC		
<b>TTR</b>	F	CCAAATGCCCTCTCATGGTGA	NM_205335.2	119 bp
	R	CCCAGTAGCAAAGTCCTGCC		
<b>ZEB1</b>	F	CCAGCAAGACCACAGATACGA	NM_205131.1	174 bp
	R	CGGGCTACCACCACTAAAAA		

**Table 9-2. Primers sequence used for generating cRNA probes**

<b>Gene</b>		<b>Sequence (5' to 3')</b>	<b>Accession No.</b>	<b>Size</b>
<b>CYP19A1</b>	F	ATGCACAAGCTTGAATTCTTCCC	NM_001001761.1	185 bp
	R	TCTGTACTCTGCACCGTCTCAG		
<b>FSHR</b>	F	CGGCTAAATAAAAACGGGAT	NM_205079.1	303 bp
	R	ACAGCAATGGCTAGGATAGGT		
<b>LHCGR</b>	F	CTTGCGTATGACAACCATACC	NM_204936.1	245 bp
	R	AGCTAGGCAGGGACTCCAGTG		
<b>PITX2</b>	F	GTGCTCCTCCTCACCTTCTT	NM_205010.1	414 bp
	R	TTCCTCCATTTGGCTCTGCG		
<b>SNCA</b>	F	TGGCAGAAGCAGCAGGAAAG	NM_204673.1	439 bp
	R	GGGCACATTGGA ACTCAGCA		
<b>TOM1L1</b>	F	AGTGGGGGCAATTCATGCAC	XM_415646.2	475 bp
	R	GTGGGCAGAGGCAACAAAGA		
<b>TTR</b>	F	CATGGTGAAAGTGCTGGATGC	NM_205335.2	303 bp
	R	GGACTGAGGAGAGCAGCGAT		
<b>ZEB1</b>	F	AAAGAGAGGCAGAGGAGCGT	NM_205131.1	476 bp
	R	GCAGGCTACCGTGTTTCCAT		

## 5. Discussion

Results of the present study indicate that changes in expression of PITX2, SNCA, TOM1L1, TTR and ZEB1 are associated with and may be important for development and differentiation of embryonic gonads in chickens and provide insight into differential gene expression in female embryos that experience asymmetric development of their gonads. Our results also provide evidence that FSH, LH and the combination of FSH and LH stimulate gonadal cell proliferation and influence expression of the selected genes. These results support our hypothesis that cellular and molecular mechanisms regulated by expression of selected genes are important in regulating asymmetrical gonadal morphogenesis during development of the chick embryo.

In chicken embryos, gonadal sex differentiation differs morphologically between sexes. In males, the gonads develop symmetrically and medulla cords thicken beginning on E6.5. But, gonads in female chicken embryos develop asymmetrically with only the left gonad giving rise to a functional ovary (Fig. 9-1A). As shown in Figures 9-1B to 9-1E, microarray analysis detected a two-fold or greater difference in 819 transcripts in the left gonad between E6 and E9 (568 genes up-regulated and 251 genes down-regulated transcripts), and in 1,074 transcripts between E6 and E9 for the right gonad (760 genes up-regulated transcripts and 314 genes down-regulated). Only 77 transcripts changed in both the left and right female gonads between E6 and E9, while 479 transcripts changed between the left and the right gonads between E6 and E9. The most highly expressed genes were found to be associated with cellular growth,

cell proliferation and development. Results of the microarray analysis focused attention to five genes considered as key to sex determination in chicken. These genes, *PITX2*, *SNCA*, *TOM1L1*, *TTR* and *ZEB1*, were validated using quantitative RT-PCR and *in situ* hybridization analyses.

The *PITX2* gene encodes for a bicoid-type homeobox protein that regulates asymmetries between left and right gonadal development in both sexes of chicken (Guioli and Lovell-Badge, 2007). It is needed to develop the gonadal primordium epithelium that is a key regulator of cell adhesion, affinity, and cell recognition events (Rodriguez-Leon et al., 2008). *PITX2* is downstream of Nodal genes expressed in the left lateral plate mesoderm; therefore, both of genes are markers for development of the left-side (Concha et al., 2000). In the present study, *PITX2* mRNA decreased about 0.5-fold ( $P < 0.001$ ) in the left gonad, but increased about 1.1-fold ( $P < 0.05$ ) in right gonad at E9 as compared to E6 in female gonads. On the other hand, *PITX2* mRNA expression was higher in left than right gonad in E9 female chicken embryos (Fig. 9-3A and 9-3B) which supports evidence that it is related to development of the left LPM. During establishment of the left-right axis of animals, *PITX2* is involved in asymmetrically gonadal development only on the left side.

The *SNCA* gene encodes for a family of small intracellular proteins and it is highly conserved and abundantly expressed in the nervous system of vertebrates (Clayton and George, 1998). In addition, *SNCA* has been found in Parkinson's disease and several neurodegenerative diseases. *SNCA* protein is found primarily in brain tissue and it has been mapped to 4q21.3-q22 in human (Chen et al., 1995). This gene

is expressed in different tissues during development in humans. In human fetal tissues, SNCA is strongly expressed in brain, liver and heart and weakly expressed in testis, adrenal gland, lung and kidney. In adults, the strongest expression is only in the brain (Baltic et al., 2004). The expression pattern of SCNA is similar during embryogenesis between chickens and mammals. In each segment of brain in chicken embryos, SNCA is abundantly expressed whereas it is not expressed in other tissues (Tiunova et al., 2000). However, there are no reports of SCNA in the reproductive tract of chicken. In the present study, we have first identified *SNCA* mRNA in the chicken gonad and found it to be expressed equally in the left and right female and male gonads, but specifically in germ cells of embryonic gonads. Therefore, it may not participate in determining asymmetric expression of the gonads in either female or male gonads in chicken embryos.

Another gene of interest is *TOM1L1* (also known as Srcasm). TOM1L1 is a member of the TOM protein family comprised of TOM1, TOM1L1 and TOM1L2. These genes contain a VHS (Vps27-Hrs-STAM) domain that has a role in membrane trafficking and GAT (GGA and TOM1) domain [this sentence is not clear as GAT is not defined nor is GGA (Kato et al., 2004; Wang et al., 2010)]. The role of TOM1L1 is related to interactions with Src family kinases (SFK) which then regulate several cellular functions including differentiation, migration, proliferation and cell survival (Seykora et al., 2002; Thomas and Brugge, 1997). In addition, SFK are involved in growth factor receptor signaling for proteins such as EGF and PDGF that induce DNA synthesis and receptor mediated endocytosis (Bromann et al., 2004). Fyn, one member of the SFK family, can phosphorylate TOM1L1 and its defects are evident in mice

with a mutated Fyn gene. TOM1L1 enhances Fyn kinase activity and it also modulates EGF and SFK signaling (Seykora et al., 2002). We have shown here that *TOM1L1* abundance increases in the left gonad of females and both gonads of males at E9 as compared with E6 in chicken embryos. These results suggest that TOM1L1 has an important role in development of the gonads in chicken embryos.

Transthyretin (TTR) is a thyroid hormone-binding protein as are other plasma proteins such as albumin and thyroxine-binding globulin (Larsson et al., 1985). TTR is expressed mainly in the liver and the choroid plexus among various species including chickens and amino acid sequences of TTR are highly conserved among chicken, human, mouse and rat (73-74%) (Duan et al., 1991b). TTR participates in the transport of thyroid hormones and retinol (vitamin A) that regulates development of testicular function (Blake et al., 1978; Livera et al., 2002). An overdose of vitamin A leads to testicular lesions whereas a deficiency of vitamin A cause cessation of spermatogenesis and a decrease in germ cells (Marinos et al., 1995; Morales and Griswold, 1987; Ricci et al., 1999). In the metabolism of vitamin A, TTR helps to stimulate germ cells by complexing with retinol binding protein (RBP) which also transports vitamin A in the plasma. In a previous study, both RBP and TTR were detected in the interstitial spaces between the seminiferous tubules in rat testes (Kato et al., 1985). In our study, *TTR* mRNA was highly expressed in both gonads of males at E9 and it is likely required for normal development of the testis in chickens.

ZEB1 is a transcription factor involved in a two-member family of zinc-finger homeodomain. It is encoded for by *tcf8* which regulates development, cell

proliferation and reproduction (Sekido et al., 1996). ZEB1 has pivotal roles in developmental differentiation such as hematopoiesis, chondrogenesis, osteogenesis, myogenesis, migration and growth by regulating expression of its downstream targets (Vandewalle et al., 2009). In chickens, ZEB1 is expressed in mesodermal tissues, notochord, neuroectoderm, neural crest, somites, lateral plate and nephrotome (Funahashi et al., 1993a). Also ZEB1 belongs to elements of the ovalbumin genes and it is necessary for induction of ovalbumin related genes which are regulated by steroid hormones. Human ZEB1 is induced by progesterone whereas chicken ZEB1 is induced by estrogen that is stimulatory to proliferation and differentiation of tubular glands in the chicken oviduct (Chamberlain and Sanders, 1999; Richer et al., 2002b). In present study, *ZEB1* mRNA increased about 2.3-fold ( $P < 0.01$ ) in the left gonad at E9 in females. However, mRNA expression level was lower in the right gonad of females and in gonads of male chicken embryos at E9 as compared with E6. These results indicate its expression increased only in the left gonad of female chicken embryos in which the oviduct and ovary are differentiated from left urogenital ridge and gonad, respectively. Therefore, we speculated that ZEB1 has an important role in development of left gonad in female chickens. In support of those results, we identified expression of genes for aromatase (CYP19A1) and FSHR and LHCGR in the gonads of chickens.

In birds, the estrogen, FSHR and LHCGR play pivotal roles in sexual differentiation of females. Inhibition of estrogen synthesis occurs due to low expression of CYP19A1 and failure of conversion of testosterone to E2 (Abinawanto et al., 1996; Bruggeman et al., 2002; Elbrecht and Smith, 1992). At the onset of

morphological differentiation (E6.5), CYP19A1 exists only in female gonads and the absence of CYP19A1 leads to phenotypic sex reversal in female chickens (Nakabayashi et al., 1998b; Nishikimi et al., 2000; Smith et al., 1997). In the present study, CYP19A1 was expressed in the left and right gonads of both sexes at E6. After their morphological differentiation at E6.5 the expression of CYP19A1 was localized in the cortex region of the female gonads and the seminiferous cords of male gonads at E9. In female and male embryonic gonads in chicken, FSH and LH regulate the secretion of sex steroids (Grzegorzewska et al., 2009; Pierce and Parsons, 1981) via interactions with their respective receptors, FSHR and LHCGR composed of seven transmembrane spanning domains (Akazome et al., 2002; Zhang et al., 1997). As expected, *FSHR* was expressed in both gonads of males and females chickens at E6 and at E9 in the present study. But, expression of *FSHR* was greater in the left gonad of female chickens at E9 where it has an essential role in gonadal morphogenesis by inducing cell proliferation and synthesis of sex steroid in the left ovary (Anderson and Anderson, 2002; Puebla-Osorio et al., 2002; Sanchez-Bringas et al., 2006). The *LHCGR* mRNA was found on the both gonads in each sex at E6 and E9. The expression of *LHCGR* mRNA was localized to germ cells of gonads of both sexes at E6, and it is weakly expressed in medullar cords of both left and right gonads in males. On the other hand, *LHCGR* is more highly expressed in left gonad of female chickens, particularly in the cortex region of left gonad. In this study, CYP19A1, FSHR, and LHCGR had similar patterns of expression as TOM1L1, TTR and ZEB1 that localized primarily to the left gonad in female gonads at E9. These results support our hypothesis that these six genes participate in development of the functional left gonad in female chickens.

Treatment of day 6 gonadal cells with recombinant FSH, LH and the combination of FSH and LH stimulated gonadal cell proliferation. FSH and LH are known regulators of folliculogenesis and spermatogenesis (Gulyas et al., 1977; Thackray et al., 2010) with FSH inducing growth, development, and differentiation of granulosa cells in ovarian follicles of the ovary and sertoli cells in the seminiferous tubules of the testis (Mendez-Herrera et al., 1998; Pedernera et al., 1999). LH is crucial for ovulation of the ovarian follicle and for stimulating secretion of testosterone by leydig cells in the male testis (Casarini et al., 2011). In addition, FSH and LH stimulate germ cell proliferation in both sexes and modulate germ cell mitosis and meiosis during morphogenesis of chicken embryonic gonads (He et al., 2013; Mendez et al., 2003). In the present study, we found that FSH and LH influenced expression of mRNAs for the target genes expressed by gonadal (Fig. 9-7). The expression of *PITX2* and *SNCA* mRNA was slightly increased by FSH, while mRNA levels for *TOMIL1* and *TTR* were reduced by FSH, and there was no effect of FSH on *ZEB1* expression. Interestingly, the combined effects of FSH and LH on gonadal cells were to decrease expression of *PITX2*, *SNCA* and *TTR* and exert a modest effect on expression of *ZEB1* mRNA. On the other hand, *TOMIL1* expression was significantly affected by LH and the combination of FSH and LH. These results suggest that gonadotropins modulate proliferation of germ cells in the embryonic gonad and regulate expression of selected genes involved in gonad development and differentiation during embryogenesis in chicken.

In summary, results from present microarray study detected several novel

candidate genes that may regulate development of embryonic gonads during embryogenesis in chickens. Expression of those genes was regulated by gonadotropins to effect gonadal morphogenesis in chicken embryos. The identified genes will be the subject of further studies to mechanisms which they influence for gonadal development in both sexes during embryogenesis in chickens.

## **CHAPTER 10**

# **Identification of Novel Regulatory Genes in Development of the Chicken Reproductive Tract**

## 1. Abstract

The chicken reproductive system is unique in maintaining its functions including production of eggs or sperm, fertilization of the egg by sperm maintained in sperm nests, production of hormones regulating its growth, development and function, and reproduction. Development of the reproductive organs is a highly regulated process that results in differentiation and proliferation of germ cells in response to predominant regulatory factors such as hormones and transcription factors. However, only a few genes are known to determine morphogenesis of the chicken reproductive tract and their mechanisms are unknown. Therefore, in the present study, we investigated the expression patterns of five genes including PITX2, SNCA, TOM1L1, TTR and ZEB1 that are associated with gonadal morphogenesis in both sexes of chickens between embryonic days 6 and 9, embryonic days 14 and 18, and in immature (12-week-old) and mature (50-week-old) chickens, as well as the reproductive tract including ovary, oviduct and testes of the respective sexes by qRT-PCR, *in situ* hybridization and immunofluorescence analyses. The expression of SNCA, TOM1L1 and ZEB1 genes was higher in immature and mature female reproductive tracts than expression of PITX and TTR. In addition, different temporal and spatial patterns of expression of the five genes were observed during maturation of testis in chickens. Specifically, SNCA, TOM1L1 and TTR were highly expressed in testes of 12-week-old chickens. Moreover, several chicken specific microRNAs (miRs) were demonstrated to affect expression of target gene mRNAs by directly binding to the 3'-UTR of their target genes through actions at the post-transcriptional level as follows: *miR-140*, *miR-200a*, *miR-1796* and *miR-1812* for PITX2; *miR-153*

and *miR-1643* for SNCA; *miR-1680\** for TTR; and *miR-200b* and *miR-1786* for ZEB1. These results suggest that five-selected genes play an important role in development of the male and female reproductive tract in chickens and expression of most candidate genes is regulated at the post-transcriptional level through specific microRNAs.

## 2. Introduction

The chicken is an invaluable animal model for research on embryology and reproductive developmental biology. In avian models, sexual differentiation of the reproductive system is initiated in the embryonic gonads from embryonic day 6.5 (E6.5, HH30) to be either ovaries or testes and this occurs asymmetrically in females and symmetrically in males. It depends on which component of the embryonic gonad, cortex or medullary, is colonized by germ cells that migrate there through bloodstream (Carlson and Stahl, 1985; Fujimoto et al., 1976; Smith and Sinclair, 2004b). In the female (ZW), germ cells asymmetrically populate the left and right gonads. The thickened outer cortex only develops in the left gonad while medulla cords form lacunae. However, the right gonad regresses and fails to develop the cortex. Female germ cells enter meiosis that commences between E14 and E18 in the left gonad (Callebau.M, 1967; Hughes, 1963; Ukeshima and Fujimoto, 1991). Then, by beginning folliculogenesis, granulosa and theca cells surrounding the oocyte are formed from cells in the cortex of the left functional gonad. There are several candidate genes, forkhead box L2 (FOXL2), wingless-related MMTV integration site 4 (WNT4), proprotein convertase subtilisin/kexin type 6 (PCSK6) and bone morphogenetic protein 6 (BMP6) known to regulate follicular development (Diaz et al., 2011; Ocon-Grove et al., 2012; Smith and Sinclair, 2004b).

The chicken oviduct has a major role in that it is a reproductive tract that produces eggs. The oviduct derives from the Mullerian duct that develops only on the left side in female chicks whereas the right Mullerian duct degenerates in female

chicks and both of the Mullerian ducts degenerate in male chicks. The immature oviduct develops rapidly after 16 weeks of age and egg laying begins when chickens are 16- to 20-weeks-old (Ha et al., 2008; Whittow, 2000). The oviduct of laying hens consists of four specific segments which are the infundibulum (fertilization), magnum (production of egg-white proteins), isthmus (formation of the soft shell membrane) and shell gland (formation of the outer egg shell) (Palmiter and Wrenn, 1971a). Development of the oviduct is stimulated in response to estrogen and previous studies have shown the regulatory genes, serpin peptidase inhibitor, clade B (ovalbumin), member 3 (SERPINB3) (Lim et al., 2012b), SERPINB11 (Lim et al., 2011d), adenosylhomocysteinase-like 1 (AHCYL1) (Jeong et al., 2012a) alpha 2 macroglobulin (A2M) (Lim et al., 2011b) and pleiotrophin (PTN) (Lee et al., 2012b) are highly expressed during development of the immature oviduct in chickens.

In contrast to female reproductive organs, gonadal morphogenesis leading to a mature testis is symmetrical between left and right gonads and originates in thickened medullary cords via proliferation of sertoli cells in the cords that are anlage of the seminiferous tubules of male embryos (ZZ). Testes of 6-week-old chicken have seminiferous tubules that include a simple layer of spermatogonia, sertoli cells, basal lamina and myoid cells. In testes of 50-week-old chickens all stage of spermatogenesis from spermatogonia to spermatozoa are found along with sertoli cells surrounded by basal lamina and myoid cells (Gonzalez-Moran et al., 2008; Smith and Sinclair, 2004b). In development of testis, testes-determining genes such as doublesex and mab-3 related transcription factor 1 (DMRT1) (Z-linked gene) and sex determining region Y-box 9 (SOX9) (sertoli cell differentiation factor) participate in

testicular morphogenesis (Chue and Smith, 2011). However, cell- and tissue-specific regulation for spermatogenesis remains unknown.

In a previous study, we identified several novel genes including paired-like homeodomain transcription factor 2 (PITX2), synuclein alpha (SNCA), target of myb1 (chicken)-like 1 (TOM1L1), transthyretin (TTR) and zinc finger E-box binding homeobox 1 (ZEB1) that may regulate gonadal morphogenesis in the both sexes of chicken embryos. However, these genes have not been investigated with respect to their influence on development of chicken reproductive tract. Therefore, in order to determine the role of five genes as important regulators of morphogenesis of reproductive organs, we determined differential patterns of mRNA expression and verified cell- and tissue-specific localization of mRNAs and proteins encoded by the five genes of interesting during development of female and male reproductive tracts in chickens. Moreover, we investigated post-transcriptional regulation of expression of four of the genes (PITX2, SNCA, TTR and ZEB1) using a miRNA target validation assay. Results of present study provide novel insights into PITX2, SNCA, TOM1L1, TTR and ZEB1 genes with respect to their tissue-specific expression during differentiation of germ cells into mature reproductive organs and post-transcriptional regulation of their expression by specific miRNAs in chickens.

### 3. Material and Methods

#### *Experimental Animals and Animal Care*

The experimental use of chickens for this study was approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5). White Leghorn (WL) chickens were subjected to standard management practices at the University Animal Farm, Seoul National University, Korea. All chickens were exposed to a light regimen of 15 h light and 9 h dark with *ad libitum* access to feed and water, and subjected to standard poultry husbandry guidelines.

#### *Tissue Samples*

The left and right gonads were collected separately from the mesonephric kidney of chicken embryos at E14 and E18 in a 1.5 ml tube containing diethylpyrocarbonate treated PBS (DEPC-PBS). Then we centrifuged the sample at 1,080 x g for 5 min to allow collection of each gonad from the bottom of the tubes. After removal of the DEPC-PBS, the gonads were stored at -80°C until RNA was extracted. Also we collected whole embryos and fixed them in freshly prepared 4% paraformaldehyde in PBS (pH 7.4). Tissue samples were collected from ovary, oviduct and testis of 12- and 50-week-old females (n=4) and males (n=4). The collected samples were either stored at -80°C until RNA was extracted or fixed immediately upon collection in freshly prepared 4% paraformaldehyde in PBS (pH 7.4). After 24 h, the samples fixed in 4% paraformaldehyde were changed to 70%

ethanol for 24 h and then dehydrated in a graded series of increasing concentrations of ethanol. Embryos were then incubated in xylene for 3h and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5 µm.

### ***RNA Isolation***

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

### ***Quantitative RT-PCR Analysis***

Total RNA was extracted from gonads on embryonic day 14 and 18 from both sexes and ovaries, oviducts and testes from 12- and 50-week-old females and males using TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized using a Superscript<sup>®</sup> III First-Strand Synthesis System (Invitrogen). Gene expression levels were measured using SYBR<sup>®</sup> Green (Sigma, St. Louis, MO, USA) and a StepOnePlus<sup>™</sup> Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The *glyceraldehydes 3-phosphate dehydrogenase* (*GAPDH*) gene was analyzed simultaneously as a control and used for normalization of data. *GAPDH* expression is most stable among other housekeeping genes and it is used commonly for normalizing for variations in loading. Each target gene and

*GAPDH* were analyzed in triplicate. Using the standard curve method, we determined expression of the examined genes using the standard curves and Ct values, and normalized them using *GAPDH* expression. The PCR conditions were 95°C for 3 min, followed by 40 cycles at 95°C for 20 sec, 60°C for 40 sec, and 72°C for 1 min using a melting curve program (increasing the temperature from 55°C to 95°C at 0.5°C per 10 sec) and continuous fluorescence measurement. ROX dye (Invitrogen) was used as a negative control for the fluorescence measurements. Sequence-specific products were identified by generating a melting curve in which the Ct value represented the cycle number at which a fluorescent signal was statistically greater than background, and relative gene expression was quantified using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001). For the control, the relative quantification of gene expression was normalized to the Ct value for the control oviduct. Information on the primer sets was provided previously (Table 9-1).

### ***In Situ Hybridization Analysis***

For hybridization probes, PCR products were generated from cDNA with the primers used for RT-PCR analysis. The products were extracted from the gel and cloned into TOPO TA cloning vector (Invitrogen). After verification of the sequences, plasmids containing gene sequences were linearized and transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN, USA) with T7 or SP6 polymerase (Roche Applied Science). Information on the probes has been published (Table 9-2). Tissues were collected and fixed in freshly prepared 4% paraformaldehyde, embedded in paraffin and sectioned at 5  $\mu$ m on APES-treated

(silanized) slides. The sections were then deparaffinized in xylene and rehydrated to diethylpyrocarbonate (DEPC)-treated water through a graded series of alcohol. The sections were treated with 1% Triton X-100 in PBS for 20 min and washed two times in DEPC-treated PBS. After washing in DEPC-treated PBS, the sections were digested with 5 µg/ml Proteinase K (Roche Applied Science) in TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) at 37°C. After post-fixation in 4% paraformaldehyde, sections were incubated twice for 5 min each in DEPC-treated PBS and incubated in TEA buffer (0.1M triethanolamine) containing 0.25% (v/v) acetic anhydride. The sections were incubated in a prehybridization mixture containing 50% formamide and 4X standard saline citrate (SSC) for at least 10 min at room temperature. After prehybridization, the sections were incubated overnight at 42°C in a humidified chamber in a hybridization mixture containing 40% formamide, 4X SSC, 10% dextran sulfate sodium salt, 10mM DTT, 1 mg/ml yeast tRNA, 1mg/ml salmon sperm DNA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2mg/ml RNase-free bovine serum albumin and denatured DIG-labeled cRNA probe. After hybridization, sections were washed for 15 min in 2X SSC at 37°C, 15min in 1X SSC at 37°C, 30 min in NTE buffer (10mM Tris, 500mM NaCl and 1mM EDTA) at 37°C and 30 min in 0.1X SSC at 37°C. After blocking with 2% normal sheep serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche Applied Science). The signal was visualized following 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 Chemical Co., St. Louis, MO, USA).

### ***Immunofluorescence Analysis***

The localization of five proteins in the reproductive tract of both sexes during their development was evaluated by immunofluorescence (IF) using anti-human PITX2 polyclonal antibody (sc-8748; Santa Cruz Biotechnology), anti-human SNCA polyclonal antibody (ab21975; Abcam), anti-human TOM1L1 polyclonal antibody (ab126972; Abcam), anti-human TTR polyclonal antibody (ab9015; Abcam) and anti-human ZEB1 polyclonal antibody (ab81972; Abcam). Antigen retrieval was performed using boiling 10mM sodium citrate buffer pH 6.0 for 10 min after which the slides were cooled on the bench top for 20 min. After antigen retrieval the slides were washed three times in 1X PBS for 5 min. Slides were incubated in blocking buffer (10 % normal serum from the same species as the secondary antibody in 1X PBS) for 1 h. After the blocking solution was aspirated, slides were incubated overnight at 4 °C with primary antibody. The slides were then rinsed three times in 1X PBS for 5 min each. Slides were then incubated with Alexa Fluor 488 rabbit anti-goat IgG secondary antibody (A11078, Invitrogen) for PITX2 and ZEB1, goat anti-rabbit IgG secondary antibody (A11034, Invitrogen) for TOM1L1 and donkey anti-sheep IgG secondary antibody (A11015, Invitrogen) for SNCA and TTR at a 1:200 dilution for 1 h at room temperature in the dark. Slides were then washed and overlaid with Prolong Gold Antifade with DAPI. For primary antibody, images were captured using a Zeiss confocal microscope LSM710 (Carl Zeiss) fitted with a digital microscope camera AxioCam using Zen 2009 software.

### ***MicroRNA Target Validation Assay***

The 3'-UTR of PITX2, SNCA, TTR and ZEB1 were cloned and confirmed by sequencing. Each 3'-UTR was subcloned between the eGFP gene and the bovine growth hormone (bGH) poly-A tail in pcDNA3eGFP (Clontech, Mountain View, CA, USA) to generate the eGFP-miRNA target 3'-UTR (pcDNA-eGFP-3'UTR) fusion constructs. For the dual fluorescence reporter assay, the fusion constructs containing the DsRed gene and target miRNAs were designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miRNA). The pcDNA-eGFP-3'UTR and pcDNA-DsRed-miRNA (4 $\mu$ g) were co-transfected into 293FT cells using the calcium phosphate method. When the DsRed-miRNA is expressed and binds to the target site of the 3'-UTR downstream of the GFP transcript, green fluorescence intensity decreases due to degradation of the GFP transcript. At 48 h post-transfection, dual fluorescence was detected by fluorescence microscopy and calculated by FACSCalibur flow cytometry (BD Biosciences). For flow cytometry, the cells were fixed in freshly prepared 4% paraformaldehyde and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

### ***Statistical Analyses***

All quantitative data were subjected to analysis of variance (ANOVA) according to the general linear model (PROC-GLM) of the SAS program (SAS Institute, Cary, NC, USA). All tests of significance were performed using the appropriate error terms according to the expectation of the mean square for error. Data are presented as mean  $\pm$  SEM unless otherwise stated. Differences with a probability value of  $P < 0.05$  were considered statistically significant.

## 4. Results

### *Comparative expression of PITX2 during development of reproductive organs in both sexes of chicken*

To demonstrate the differential patterns of expression of mRNAs and proteins encoded by target genes (PITX2, SNCA, TOM1L1, TTR and ZEB1) during development of female and male reproductive tract in chickens, we conducted quantitative RT-PCR, *in situ* hybridization and immunofluorescence analyses. First, we investigated the expression pattern of PITX2 gene during morphogenesis of reproductive organ. As illustrated in Figure 10-1A, *PITX2* mRNA expression decreased as development of the ovary and oviduct advanced in the chickens. Quantitative *PITX2* mRNA levels indicated 0.98-fold in embryonic gonads at 18 (E18), 0.3- ( $P < 0.001$ ) and 0.2-fold ( $P < 0.001$ ) in ovary and oviduct of 12-week-old (12 wks) and 0.2- ( $P < 0.001$ ) and 0.3- ( $P < 0.001$ ) fold in the ovary and oviduct of 50-week-old (50 wks) respectively compared with *PITX2* mRNA expression at E14 embryonic gonads. In addition, *PITX2* mRNA was abundant in the cortex of the left gonad on E14, while little expression was detected in other tissues. Similarly, PITX2 protein was expressed in the cortex of the left female gonad at E14 and E18; however, there was low abundance of PITX2 protein in ovarian follicles of 12-week-old chickens and PITX2 protein was not detected in other tissues (Fig. 10-1B and 1C). In male chickens, *PITX2* mRNA gradually decreased from E14 gonads to differentiated testes at 12 wk, but then increased dramatically in testes of 50-week-old male chickens with mature testes. Expression of *PITX2* mRNA revealed 0.2-, 0.01- ( $P <$

0.001) and 1.2- ( $P < 0.001$ ) fold in the gonads at E18, and testis at 12- wk and 50 wk chickens, respectively compared with the expression of *PITX2* in embryonic gonads at E14 (Fig. 10-1D). *PITX2* was localized mainly in the seminiferous cord of E14 and E18 gonads. In immature testis (12-week-old testis), there was low abundance of *PITX2* protein in seminiferous tubules, but *PITX2* protein was very abundant in seminiferous tubules of mature testes from 50-week-old chickens (Fig. 10-1E and 1F).

***Comparative expression of SNCA during development of reproductive organs in both sexes of chicken***

As illustrated in Figure 2A, the result from quantitative RT-PCR analysis indicated that expression of *SNCA* mRNA decreased 0.2-fold in left gonads at E18, 0.8-fold ( $P < 0.05$ ) in oviduct of 12 week and 0.6- and 0.24-fold ( $P < 0.05$ ) in the adult ovary and oviduct of 50 week respectively compared with *SNCA* expression in the gonads at E14. On the other hand, *SNCA* mRNA expression increased 11.2-fold ( $P < 0.001$ ) in the ovary of 12-week-old chickens. *In situ* hybridization and immunofluorescence analyses detected *SNCA* mRNA and protein localized mainly in the cortex region of embryonic gonads and both were highly expressed in ovarian follicles of the immature chicken oviduct (Fig. 10-2B and 2C). Moreover, expression of *SNCA* decreased 0.3- and 0.1-fold in the gonads at E18 and testis of 50-week-old respectively compared with *SNCA* expression in the gonads at E14 (Fig. 10-2D). However, *SNCA* abundantly increased 26.3-fold ( $P < 0.001$ ) in the testis of 12-week-old chickens compared with expression of *SNCA* in E14 gonads. In accordance with mRNA expression, *SNCA* protein was localized to the seminiferous cord of male

gonads at E14 and abundance decreased to E18. Interestingly, SNCA protein was abundant in the seminiferous tubules of 12-week-old testis and weakly expressed in sertoli cells of 50-week-old testes (Fig. 10-2E and 2F).

***Comparative expression of TOMIL1 during development of reproductive organs in both sexes of chicken***

Tissue specific expression of *TOMIL1* mRNA in female embryonic gonad at E18, immature ovary and oviduct of 12-week-old and adult ovary and oviduct of 50-week-old chickens was found to increase 0.7-, 0.9-, 9.4- ( $P < 0.001$ ), 4.9- ( $P < 0.01$ ) and 0.3-fold, respectively compared with *TOMIL1* expression in the gonads at E14 by quantitative RT-PCR (Fig. 10-3A). In addition, *TOMIL1* expression was strong in the oviduct of 12-week-old chickens and moderately expressed in the follicles of 12- and 50-week-old female chickens (Fig. 10-3B and 3C). In the reproductive tract of male chickens, *TOMIL1* expression increased 1.1- ( $P < 0.001$ ), 5.2- ( $P < 0.001$ ) and 0.3- ( $P < 0.05$ ) fold in male embryonic gonads at E18, and in testis of 12- and 50-week-old male chickens compared with *TOMIL1* expression in the gonads at E14 (Fig. 10-3D). *TOMIL1* mRNA and protein was most abundant in the seminiferous cord of embryonic gonads at E14 and E18, seminiferous tubules of immature testis (12 wks) and spermatogonia of testes from 50-week-old male chickens (Fig. 10-3E and 3F).

***Comparative expression of TTR during development of reproductive organs in both sexes of chicken***

Chicken TTR gene expression was demonstrated in the reproductive tract during development of ovaries, oviduct and testes. In females, *TTR* was rarely expressed during development of the ovary and oviduct. The expression levels indicated 0.01- ( $P < 0.001$ ), 0.08- ( $P < 0.01$ ), 0.02-, 0.3- ( $P < 0.001$ ) and 0.02- fold changes in expression of *TTR* mRNA in the embryonic gonads at E18, 12-week-old ovary and oviduct and 50-week-old ovary and oviduct compared with *TTR* expression in the embryonic gonads at E14 (Fig. 10-4A). Cell-specific expression based on results from *in situ* hybridization and immunofluorescence analyses, revealed that *TTR* is mainly expressed the cortex of embryonic gonads, whereas its expression rarely detected in other tissues of female reproductive tract (Fig. 10-4B and 4C). Next, *TTR* mRNA was evaluated during testis development in chickens. The results showed 0.08-, 5.1- ( $P < 0.001$ ) and 1.3- ( $P < 0.01$ ) fold changes in expression in the embryonic gonads at E18 and testis of 12- and 50-week-old chickens, respectively compared with the expression at E14 (Fig. 10-4D). In accordance with quantitative mRNA expression, *TTR* was localized predominantly to the seminiferous cords of embryonic gonads (E14), seminiferous tubules of immature testis (12 wks) and Sertoli cells of adult testes (50 wks) as shown in Figures 10-4E and 4F.

***Comparative expression of ZEB1 during development of reproductive organs in female and male chickens***

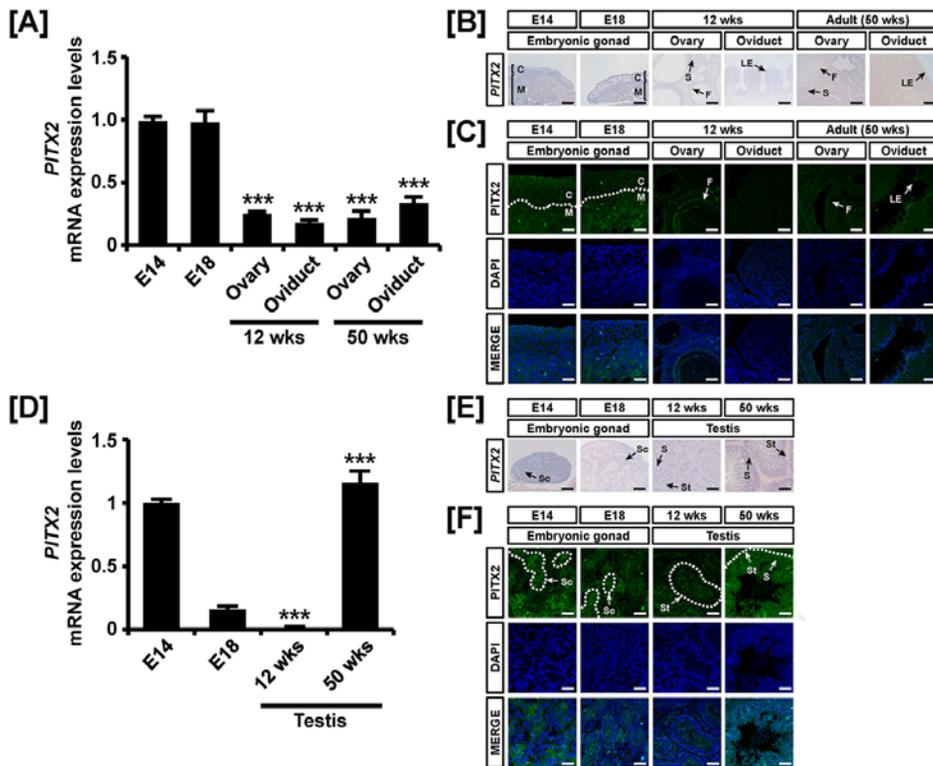
Tissue-specific expression of *ZEB1* in the female and male reproductive tracts of hens during development was demonstrated using quantitative RT-PCR, *in situ* hybridization and immunofluorescence analyses. As illustrated in Figure 10-5A,

*ZEB1* mRNA expression increased in the female chicken reproductive tract of 12- and 50-week-old chickens as compared to embryonic gonads. Its expression increased 1- ( $P < 0.05$ ), 11.9- ( $P < 0.01$ ), 13- ( $P < 0.001$ ), 2.2- ( $P < 0.05$ ) and 7.6- ( $P < 0.001$ ) fold in gonads at E18, 12-week-old ovaries and oviducts and 50-week-old ovaries and oviducts respectively compared with *ZEB1* expression in the gonads at E14. *ZEB1* mRNA and protein were highly expressed in luminal epithelium of the oviduct at 12- and 50-weeks of age and in ovarian follicles of ovaries from 12-week-old female chickens (Fig. 10-5B and 5C). In addition, *ZEB1* was weakly expressed in the cortex and medullary region of gonads at E14 and E18. In male chickens, *ZEB1* expression increased gradually from E18 gonads to adult testes. Expression of *ZEB1* mRNA revealed 0.2- ( $P < 0.001$ ), 0.6- ( $P < 0.01$ ) and 1.3- ( $P < 0.01$ ) fold in the gonads at E14 and E18 and testis of 12- and 50-week-old respectively compared with *ZEB1* expression in the gonads at E14 (Fig. 10-5D). *ZEB1* mRNA and protein were localized predominantly to seminiferous cord of E14 gonads and sertoli cells of testes from 50-week-old male chickens (Fig. 10-5E and 5F). These results suggest that *ZEB1* participates in development of both the oviduct and testis in chickens.

### ***Post-transcriptional regulation of genital ridge development regulatory genes by chicken microRNAs***

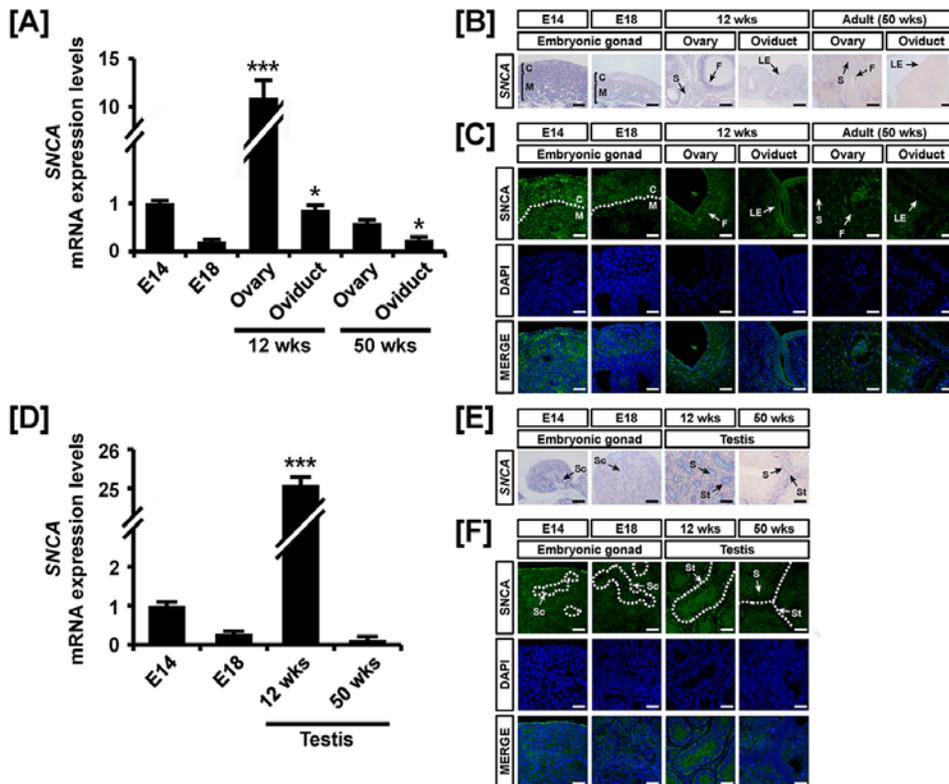
We next investigated the possibility that expression of *PITX2*, *SNCA*, *TTR* and *ZEB1* is regulated at the post-transcriptional level microRNAs (miRNAs) using a miRNA target validation assay. In order to find target miRNAs and their binding sites within the 3'-UTR of *PITX2*, *SNCA*, *TTR* and *ZEB1* genes, the miRNA target

prediction database (miRDB: <http://mirdb.org/miRDB/>) was used. It revealed several putative binding sites for miRNAs including *miR-140*, *miR-200a*, *miR-1796* and *miR-1812* for PITX2, *miR-153* and *miR-1643* for SNCA, *miR-1680\** for TTR and *miR-200b* and *miR-1786* for ZEB1 (Fig. 10-6 to -9). However, no specific target miRNA was detected for TOM1L1. Thus, we determined if these specific miRNAs influence expression of PITX2, SNCA, TTR and ZEB1 via their 3'-UTR. A fragment of each 3'-UTR with binding sites for the miRNAs was cloned downstream of the green fluorescent protein (GFP) reading frame, thereby creating a fluorescent reporter for function of the 3'-UTR region (Fig. 10-6B, 10-7B, 10-8B and 10-9B). After co-transfection of eGFP-3'-UTR and DsRed-miRNA, analyses for intensity of GFP expression and percentage of GFP-expressing cells were conducted using FACS and fluorescence microscopy. In the presence of *miR-140*, *miR-200a*, *miR-1796* and *miR-1812*, the intensity and percentage of GFP-PITX2-expressing cells decreased 26%, 34%, 35% and 40%, respectively by each miRNA as compared with the control (Fig. 10-6). Also, *miR-153* and *miR-1643* decreased the intensity and percentage of GFP-SNCA-expressing cells 58% and 61% (Fig. 10-7). In addition, *miR-1680\** decreased the intensity and percentage of cells expressing TTR by 58% (Fig. 10-8). Furthermore, *miR-200b* and *miR-1786* decreased the intensity and percentage of GFP-ZEB1-expressing-cells by 63% and 66%, respectively (Fig. 10-9). These results indicate that specific miRNAs associated with target transcripts may be involved in development of reproductive organs in chickens and regulate their expression at the post-transcriptional level during morphogenesis of the ovary, oviduct and testis.



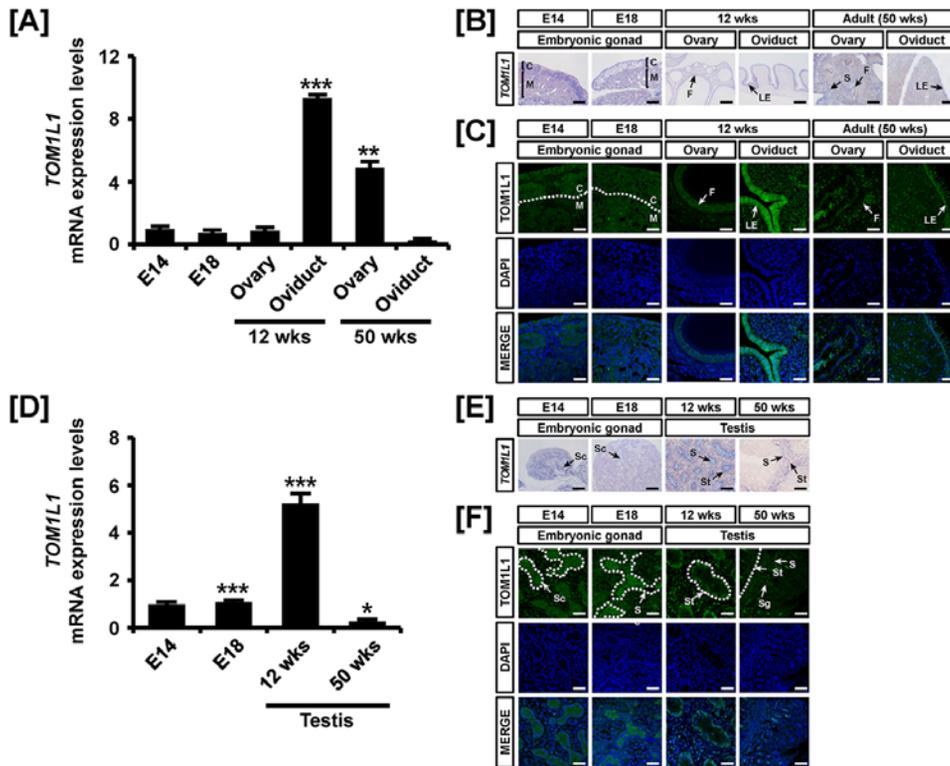
**Figure 10-1. Quantitative analysis of mRNA expression and cell-specific localization of mRNA and protein for PITX2 in female and male reproductive tracts during their development.** [A] Quantitative RT-PCR was conducted using cDNA templates from female gonads at embryonic days 14 and 18, 12-week-old ovary and oviduct and 50-week-old ovary and oviduct. [B and C] Cell-specific expression of *PITX2* mRNA and protein in the development of female reproductive tract was demonstrated by *in situ* hybridization [B] and immunofluorescence analyses [C]. Cell nuclei were stained with DAPI (blue). [D-F] Quantitation and localization of *PITX2* expression were analyzed in the male reproductive tract of chickens during their development by qRT-PCR [D], *in situ* hybridization [E] and immunofluorescence analyses [F]. The asterisks denote statistically significant

differences (\*\*\*)  $p < 0.001$  and \*\*  $p < 0.01$ ). Legend: C, cortex; F, follicle; LE, luminal epithelium; M, medullar; S, Sertoli cell; Sc, seminiferous cord; St, seminiferous tubule. Scare bar represents 100  $\mu\text{m}$  for [B and E] and 50  $\mu\text{m}$  for [C and F].



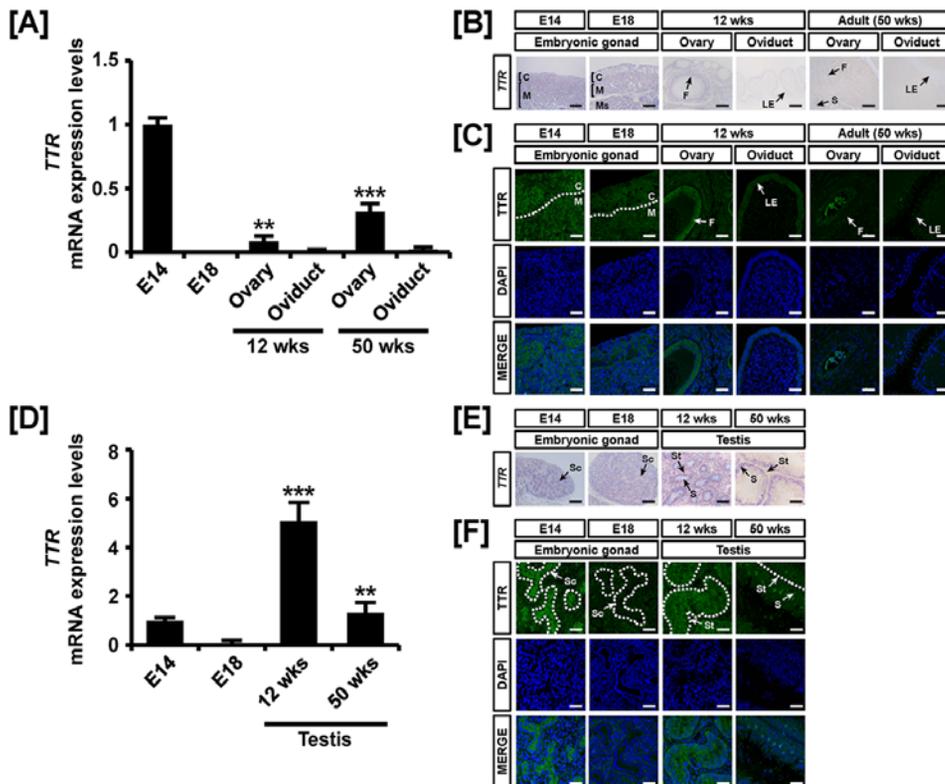
**Figure 10-2. Quantitative analysis of mRNA expression and cell-specific localization of mRNA and protein for SNCA in female and male reproductive tracts during their development.** [A] Quantitative RT-PCR was conducted using cDNA templates from female gonads at embryonic days 14 and 18, 12-week-old ovary and oviduct and 50-week-old ovary and oviduct. [B and C] Cell-specific expression of *SNCA* mRNA and protein in development of the female reproductive tract was demonstrated by *in situ* hybridization [B] and immunofluorescence analyses [C]. Cell nuclei were stained with DAPI (blue). [D-F] Quantitation and localization of SNCA expression were analyzed in the male reproductive tract of chickens during their development by qRT-PCR [D], *in situ* hybridization [E] and immunofluorescence analyses [F]. The asterisks denote statistically significant

differences (\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$ ). Legend: C, cortex; F, follicle; LE, luminal epithelium; M, medullar; S, Sertoli cell; Sc, seminiferous cord; St, seminiferous tubule. Scare bar represents 100  $\mu\text{m}$  for [B and E] and 50  $\mu\text{m}$  for [C and F]. See *Materials and Methods* for a complete description of the methods.



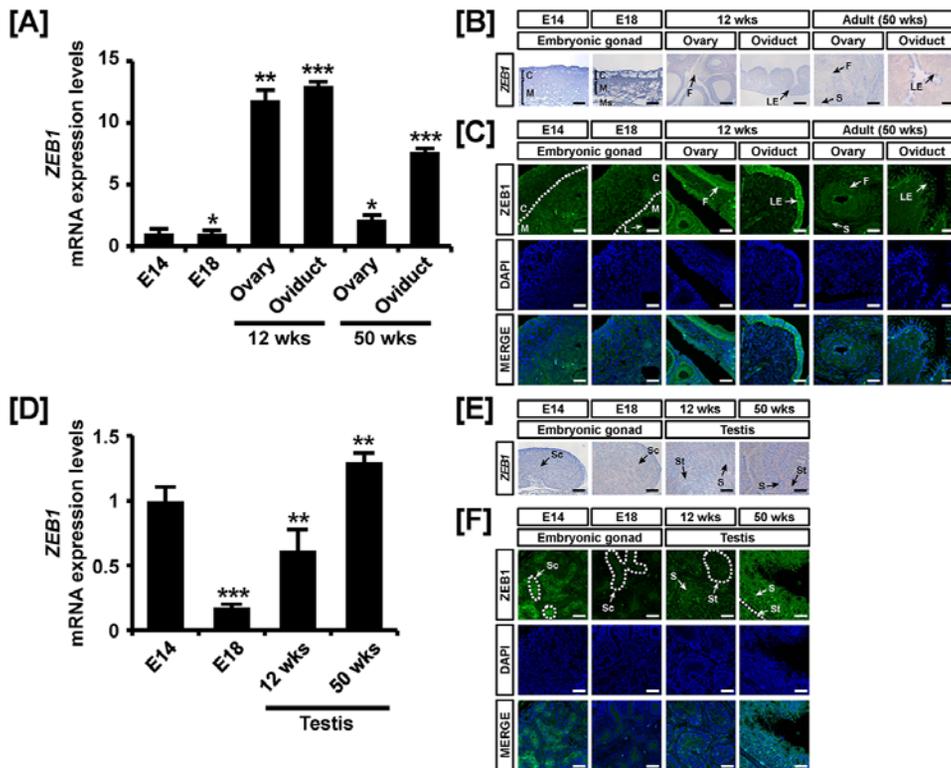
**Figure 10-3. Quantitative analysis of mRNA expression and cell-specific localization of mRNA and protein for TOM1L1 in female and male reproductive tracts during their development.** [A] Quantitative RT-PCR was conducted using cDNA templates from female gonads at embryonic days 14 and 18, 12-week-old ovary and oviduct and 50-week-old ovary and oviduct. [B and C] Cell-specific expression of *TOM1L1* mRNA and protein in development of the female reproductive tract was demonstrated by *in situ* hybridization [B] and immunofluorescence analyses [C]. Cell nuclei were stained with DAPI (blue). [D-F] Quantitation and localization of TOM1L1 expression were analyzed in the male reproductive tract of chickens during their development by qRT-PCR [D], *in situ* hybridization [E] and immunofluorescence analyses [F]. The asterisks denote statistically significant

differences (\*\*\*)  $p < 0.001$ , \*\*  $p < 0.01$  and \*  $p < 0.05$ ). Legend: C, cortex; F, follicle; LE, luminal epithelium; M, medullar; S, Sertoli cell; Sc, seminiferous cord; Sg, spermatogonia; St, seminiferous tubule. Scare bar represents 100  $\mu\text{m}$  for [B and E] and 50  $\mu\text{m}$  for [C and F]. See *Materials and Methods* for a complete description of the methods.



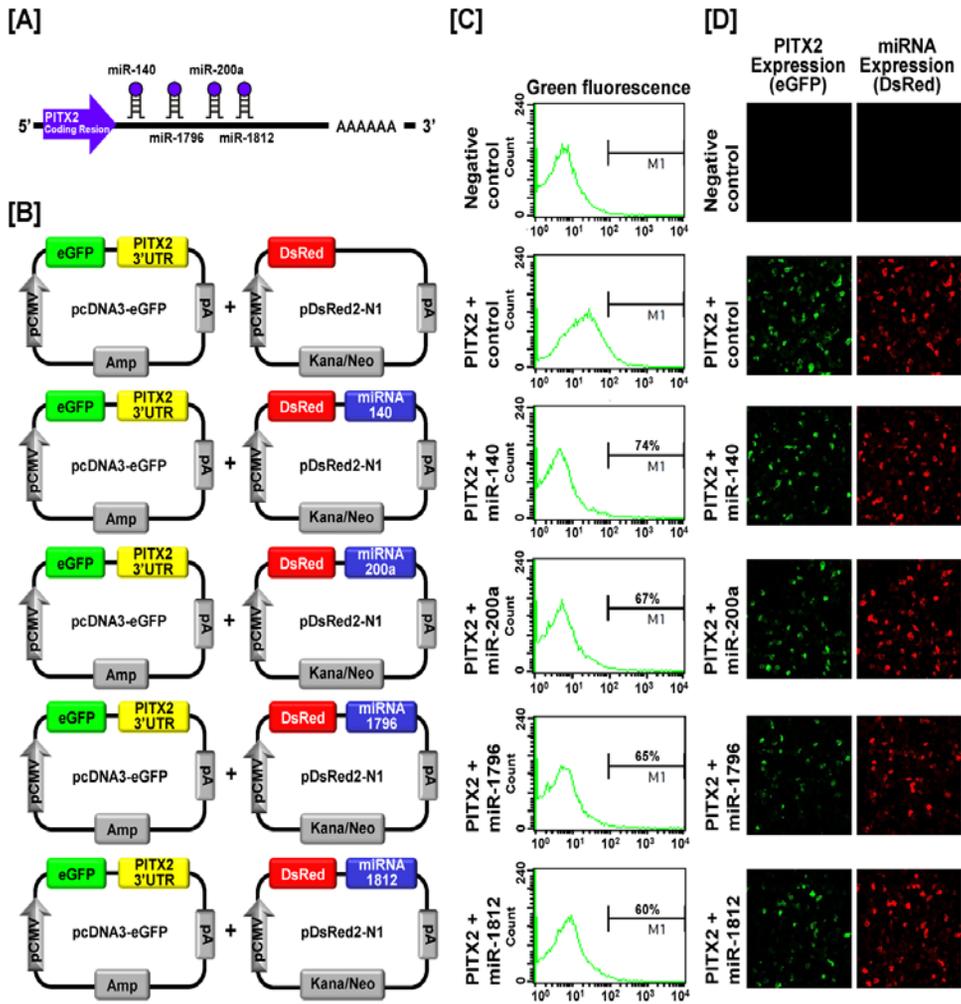
**Figure 10-4. Quantitative analysis of mRNA expression and cell-specific localization of mRNA and protein for TTR in female and male reproductive tracts during their development.** [A] Quantitative RT-PCR was conducted using cDNA templates from female gonads at embryonic days 14 and 18, 12-week-old ovary and oviduct and 50-week-old ovary and oviduct. [B and C] Cell-specific expression of *TTR* mRNA and protein in the development of female reproductive tract was demonstrated by *in situ* hybridization [B] and immunofluorescence analyses [C]. Cell nuclei were stained with DAPI (blue). [D-F] Quantitation and localization of *TTR* expression were analyzed in the male reproductive tract of chickens during their development by qRT-PCR [D], *in situ* hybridization [E] and immunofluorescence analyses [F]. The asterisks denote statistically significant differences (\*\*\*)  $p < 0.001$

and \*\*  $p < 0.01$ ). Legend: C, cortex; F, follicle; LE, luminal epithelium; M, medullar; S, Sertoli cell; Sc, seminiferous cord; St, seminiferous tubule. Scare bar represents 100  $\mu\text{m}$  for [B and E] and 50  $\mu\text{m}$  for [C and F]. See *Materials and Methods* for a complete description of the methods.

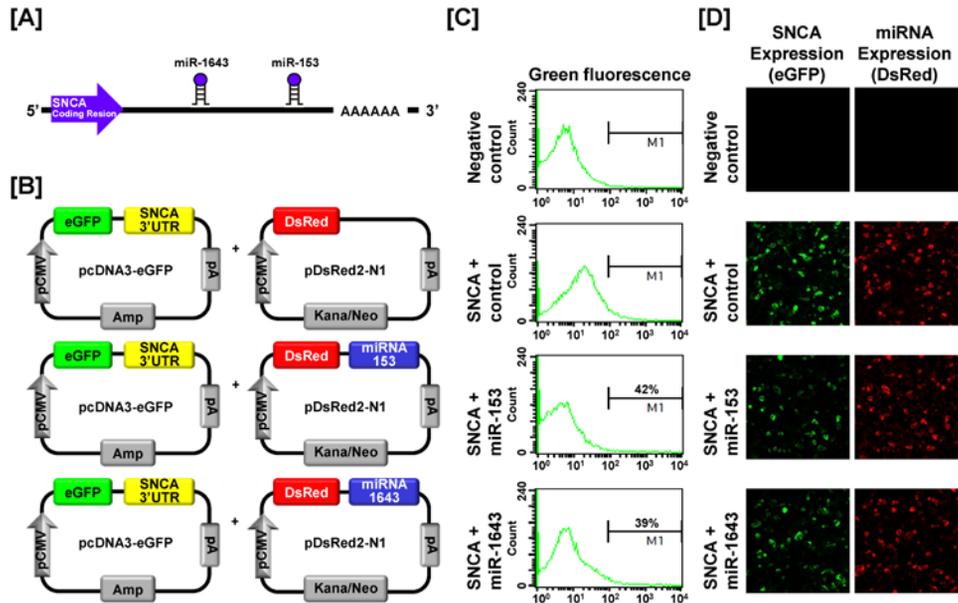


**Figure 10-5. Quantitative analysis of mRNA expression and cell-specific localization of mRNA and protein for ZEB1 in female and male reproductive tracts during their development.** [A] Quantitative RT-PCR was conducted using cDNA templates from female gonads at embryonic days 14 and 18, 12-week-old ovary and oviduct and 50-week-old ovary and oviduct. [B and C] Cell-specific expression of ZEB1 mRNA and protein in development of female reproductive tract was demonstrated by *in situ* hybridization [B] and immunofluorescence analyses [C]. Cell nuclei were stained with DAPI (blue). [D-F] Quantitation and localization of ZEB1 expression were analyzed in the male reproductive tract of chickens during their development by qRT-PCR [D], *in situ* hybridization [E] and immunofluorescence analyses [F]. The asterisks denote statistically significant

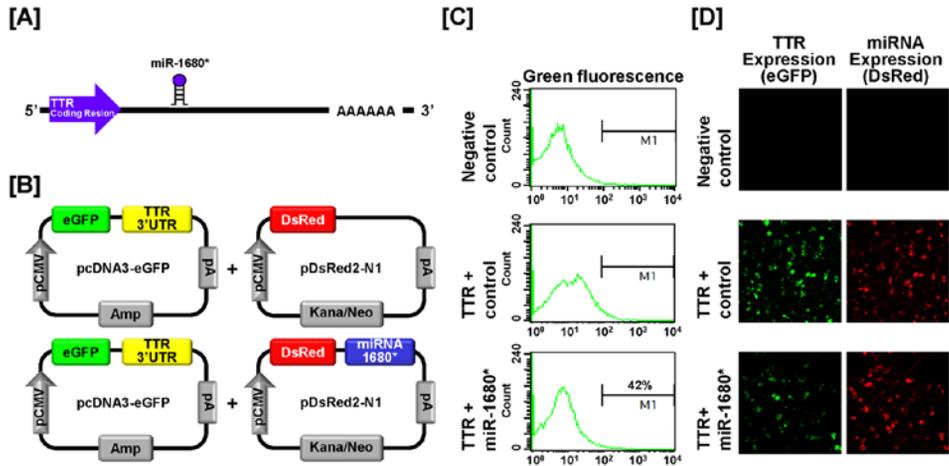
differences (\*\* $p < 0.01$ , \*\*\*  $p < 0.001$  and \*  $p < 0.05$ ). Legend: C, cortex; F, follicle; LE, luminal epithelium; M, medullar; S, Sertoli cell; Sc, seminiferous cord; St, seminiferous tubule. Scare bar represents 100  $\mu\text{m}$  for [B and E] and 50  $\mu\text{m}$  for [C and F]. See *Materials and Methods* for a complete description of the methods.



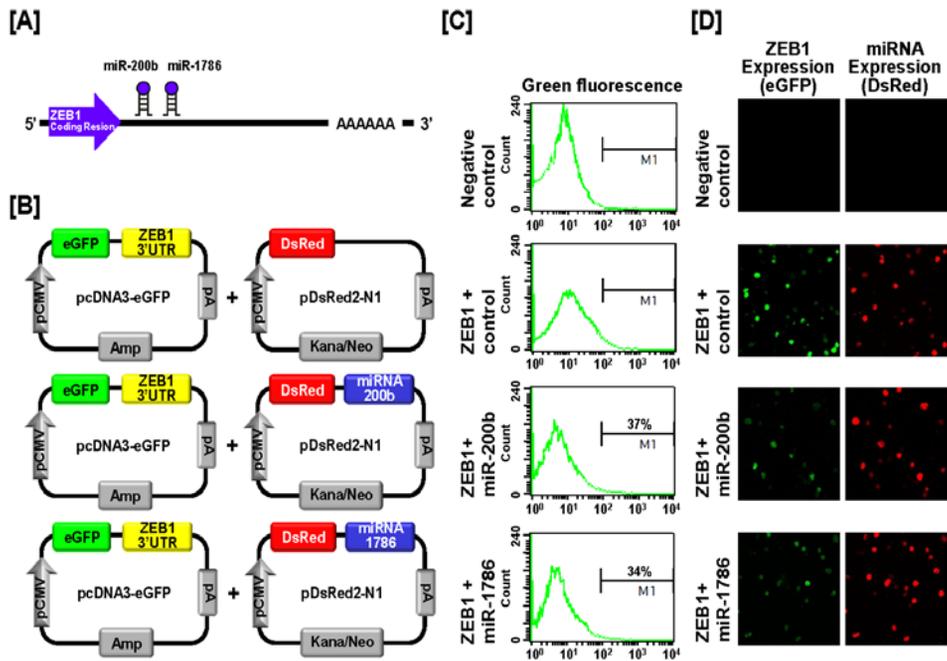
**Figure 10-6. *In vitro* target assay for *miR-140*, *miR-200a*, *miR-1796* and *miR-1812* on the *PITX2* transcript.** [A] Diagram showing *miR-140*, *miR-200a*, *miR-1796* and *miR-1812* binding sites in *PITX2* 3'-UTR. [B] Schematic expression of vector maps for eGFP with *PITX2* 3'-UTR and DsRed with each miRNA. [C and D] The fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D] after co-transfection of pcDNA-eGFP-3'-UTR for the *PITX2* transcript and pcDNA-DsRed-miRNA for the *miR-140*, *miR-200a*, *miR-1796* and *miR-1812*.



**Figure 10-7. *In vitro* target assay for *miR-153* and *miR-1643* on the SNCA transcript.** [A] Diagram showing *miR-153* and *miR-1643* binding sites in SNCA 3'-UTR. [B] Schematic expression of vector maps for eGFP with SNCA 3'-UTR and DsRed with each miRNA. [C and D] The fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D] after co-transfection of pcDNA-eGFP-3'-UTR for the SNCA transcript and pcDNA-DsRed-miRNA for the *miR-153* and *miR-1643*.



**Figure 10-8.** *In vitro* target assay for *miR-1680\** on the TTR transcript. [A] Diagram showing *miR-1680\** binding sites in TTR 3'-UTR. [B] Schematic expression of vector maps for eGFP with TTR 3'-UTR and DsRed with each miRNA. [C and D] The fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D] after co-transfection of pcDNA-eGFP-3'-UTR for the TTR transcript and pcDNA-DsRed-miRNA for *miR-1680\**.



**Figure 10-9.** *In vitro* target assay for *miR-200b* and *miR-1786* on the *ZEB1* transcript. [A] Diagram showing *miR-200b* and *miR-1786* binding sites in *ZEB1* 3'-UTR. [B] Schematic expression of vector maps for eGFP with *ZEB1* 3'-UTR and DsRed with each miRNA. [C and D] The fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D] after co-transfection of pcDNA-eGFP-3'-UTR for the *ZEB1* transcript and pcDNA-DsRed-miRNA for the *miR-200b* and *miR-1786*.

## 5. Discussion

Results of the current study revealed differential temporal and spatial expression patterns for key genes, PITX2, SNCA, TOM1L1, TTR and ZEB1, that are important with respect to development and differentiation of chicken reproductive tract in both sexes. In addition, the results indicate that among five selected genes, expression of PITX2, SNCA, TTR and ZEB1 is post-transcriptionally regulated via specific miRNAs binding directly the 3'-UTR of these target genes. These results support our hypothesis that molecular patterning of the reproductive system is affected by prominent transcripts crucial for development of female and male reproductive organs in chickens.

The chicken is a firmly entrenched animal model for research in embryology and reproductive developmental biology, but little is known about regulatory genes that control development of the reproductive tract in female and male chickens. In our previous study (Lim and Song, 2013, Chapter 9), we reported novel genes and hormonal regulation of gonad morphogenesis in chicken embryos. To demonstrate the differential patterns of expression of mRNAs and proteins of PITX2, SNCA, TOM1L1, TTR and ZEB1 during development and differentiation of germ cells and primordial tissues to mature reproductive organs, we performed quantitative RT-PCR, *in situ* hybridization and immunofluorescence analyses.

PITX2 is a bicoid-type homeobox gene that plays a crucial role for asymmetric development of several organs including heart, gut, spleen and lung in

vertebrates (Campione et al., 1999a). It is positioned downstream of a member in transforming growth factor-beta superfamily called Nodal that regulates formation of the left lateral plate mesoderm and interacts with the Nodal protein during morphogenesis of left-right axis (Hamada et al., 2002a; Liu et al., 2001b; Patel et al., 1999b). Thus, PITX2 participates in mechanisms for left-right asymmetric cell proliferation. Importantly, in the female chicken embryo, PITX2 regulates asymmetric gonadal morphogenesis due to its expression only in the left gonad where it induces cyclin D1 expression (Ishimaru et al., 2008). Moreover, expression of PITX2 is also found in the granulosa and theca cells of postnatal ovaries and in germ cells in seminiferous tubules of postnatal testes, as well as embryonic gonads in mice (Nandi et al., 2011). In this study, temporal and spatial expression of PITX2 was identified during development of reproductive organs in an age- and sex-dependent manner. PITX2 expression was relatively low in the female reproductive tract whereas its expression was mainly in the seminiferous cords of embryonic gonads and seminiferous tubules of testes. With these results, we proposed that PITX2 has regulatory functions in development of embryonic and postnatal reproductive organs in chickens.

SNCA belongs to a family of small and highly conserved proteins in vertebrates including alpha-, beta- and gamma-synuclein. The SNCA gene has 7 exons (5 protein-coding) and is expressed mainly in the brain, particularly in the hippocampus, caudate nucleus, amygdala, substantia nigra and thalamus in adult humans (Lavedan, 1998). The SCNA gene has been specifically related to several neurodegenerative diseases such as Parkinson's disease via three point mutations

(A53T, A30P and E46K) (Li et al., 2001; Zarranz et al., 2004) and Alzheimer's disease through accumulation of the gene in humans (Masliah et al., 1996). In addition, testosterone increases *SNCA* mRNA expression in the brain of chipping sparrows to affect their song system (Hartman et al., 2001). In the chicken, expression of *SNCA* in the majority of neurons in brain and spinal cord during embryogenesis has been reported (Tiunova et al., 2000). However, there are no published results on *SNCA* and development of reproductive organs of any animal or human model. We reported previously that *SNCA* was expressed in both male and female embryonic gonads in chickens. Therefore, we investigated expression of *SNCA* during development of the reproductive organs from embryonic gonads to adult male and female reproductive tracts. *SNCA* was predominantly detected in ovary and testis of 12-week-old chickens which suggests that *SNCA* might have an important role in morphogenesis of chicken ovary and testis.

*TOM1L1* is also known as Src-activating and signaling molecule (*SRCASM*). This gene is an activator and substrate for Src family tyrosine kinases (SFKs) that include nine members that have significant roles in mitogenesis and morphological alterations via induction of growth factors (Franco et al., 2006). *TOM1L1* is tyrosine-phosphorylated in response to EGFR ligand as a SFK substrate downstream of EGFR. Increased expression of *TOM1L1* activates endogenous SFKs preferably for phosphorylating Fyn and Src. Therefore, *TOM1L1* links with EGFR and SFK-dependent signaling in differentiation of keratinocytes (Elmarghani et al., 2009; Li et al., 2005). In addition, *TOM1L1* has a role as a regulatory adaptor bridging activated EGFR in endocytosis by EGF stimulus (Liu et al., 2009). In the

present study, we determined that TOM1L1 was strongly expressed in luminal epithelium of the immature oviduct and follicles of adult ovaries in female chickens and that it was abundant in testes of 12-week-old male chickens. These results show that TOM1L1 likely has a role in regulating development of the immature oviduct, ovarian folliculogenesis and seminiferous tubules in chickens.

TTR (also called prealbumin) is one of the transporters of thyroid hormones and cooperates with retinol-binding protein (RBP) and vitamin A (retinol). TTR directly binds the thyroid hormones ( $T_3$  and  $T_4$ ) in the central channel constituted by tetrameric assembly of the monomers (Blake et al., 1978; Duan et al., 1991a; Ferguson et al., 1975), and it indirectly provides vitamin A as a RBP (Kanai et al., 1968). TTR has a well-established role in regulating spermatogenesis through effects on retinol metabolism in the adult testis of rats. Circulating retinol binds to a complex of RBP and TTR which is positioned in the peritubular cells involving an intracellular CRBP which has a high affinity for binding retinol (Blaner et al., 1987). The peritubular cells secrete retinol as a complex form to the Sertoli cells that oxidize retinol into retinoic acid which stimulates differentiation of germ cells (Livera et al., 2002). Therefore, TTR assists development of germ cells within the developing seminiferous tubules. In females, it is not known if TTR regulate oogenesis or embryogenesis. However, thyroid hormones that TTR transports from serum into the oocyte play a crucial role in embryogenesis in various species, especially as it is accumulated in the yolk during oogenesis in chickens (Vieira et al., 1995). In our study, TTR was highly expressed during testes development, predominantly in the seminiferous tubules of immature testes. Otherwise, in the female reproductive tract,

expression of TTR was weak in immature and mature ovaries. These results indicate that the TTR gene might have an important role in development and maturation of the postnatal testis in chickens.

ZEB1 (also known as EF1, TCF8, AREB6, Nil-2-a) is a transcription factor binding to DNA via two zinc finger clusters, one at the N-terminus and one at the C-terminus, and it can modulate transcription of target genes by binding directly to 5'-CACCT sequences in their promoter regions (Funahashi et al., 1993b). ZEB1 plays an important role in development, cell proliferation, differentiation, migration and reproduction (Sekido et al., 1996; Vandewalle et al., 2009). In addition, ZEB1 protein induces cell migration during development and cancer progression by repressing expression of E-cadherin in epithelial cells (Comijn et al., 2001; Eger et al., 2005; Guaita et al., 2002; Spoelstra et al., 2006). ZEB1 is regulated by steroid hormones, estrogen (Chamberlain and Sanders, 1999), progesterone (Richer et al., 2002a) and androgen (Anose and Sanders, 2011). In chickens, estrogen induces proliferation and differentiation of tubular gland cells associated with production of egg white protein and stimulates ZEB1 expression leading to activation of transcription of downstream targets in the chick oviduct (Chamberlain and Sanders, 1999; Schutz et al., 1978). In this study, ZEB1 was highly expressed in both the immature and mature female reproductive tract as compared to the embryonic gonads suggesting a key role in development of the oviduct of adult female chickens. Moreover, in male chickens, ZEB1 expression gradually increased with progressive development of the testes from an E18 gonad to an adult testis. Thus, ZEB1 may play a crucial role in egg production through effects on development of the oviduct, as well as all stages of development of

the testis in male chickens.

Based on results from validation of gene expression during development of chicken reproductive organs, we next investigated whether target genes undergo post-transcriptional regulation by specific microRNAs. MicroRNAs (miRNAs) are small non-coding single stranded RNAs of 18-23 nucleotides that play a role as post-transcriptional regulators and transformers of cell fate through modulation of target-mRNA translation in various cells and tissues. In other words, miRNAs have crucial regulatory effects in a variety of biological events including growth, development, differentiation and control of cell cycle by modulating gene expression (Bartel, 2009b; Garzon et al., 2006b; Gregory et al., 2005a). For example, expression of miRNAs during gonadal development in chickens and mammals has been reported (Huang et al., 2010; Torley et al., 2011; Tripurani et al., 2010). In addition, previous research suggests that several miRNAs regulate mechanisms required for development and differentiation of the oviduct and ovarian cancer in female chickens (Jeong et al., 2012a; Lee et al., 2012b; Lim et al., 2012c; Lim et al., 2012e). Moreover, *miR-34c* down-regulates genes related in germ cell differentiation and its expression was detected mainly in the later stages of meiosis in spermatogenesis in chickens (Bouhallier et al., 2010). Based on previous reports, miRNAs might play a role during morphogenesis of the ovary, oviduct and testis in chickens. However, few miRNAs have been investigated with respect to their regulation of target genes and mechanisms whereby that act remain unknown. In this study, we performed an *in vitro* target assay of miRNAs to determine if *PITX2*, *SNCA*, *TTR* and *ZEB1* transcripts are regulated at the post-transcriptional level by target miRNAs. As illustrated in Figures 10-6 to -9,

specific target miRNAs of chickens attenuate intensity of GFP-*PITX2*, -*SNCA*, -*TTR* and -*ZEB1* expressing cells. These results indicate that at least one to four miRNAs directly bind to the developmental-regulatory genes of reproductive organs and post-transcriptionally regulate their expression during development of the male and female reproductive tracts of chickens.

In conclusion, our results provide evidence for temporal and spatial expression of five genes that influence development of reproductive organs of chickens from the embryonic stage to the immature and mature stages of development. Expression of *PITX2*, *SNCA*, *TTR* and *ZEB1* were modulated via post-transcriptional regulation by specific target miRNAs which warrant further study. These results suggest roles for five important genes that likely regulate development of reproductive organs in chickens.

# **CHAPTER 11**

## **Conclusion**

Ovarian carcinogenesis leads to dynamic alterations in morphology, physiology and function of the female reproductive tract. Results of the present study demonstrate tissue- and cell-specific expression of prognostic factors (SERPINB3, SERPINB11, GAL11, SPP1 and A2M) which are associated with and may be essential for development of EOC in women and laying hens. Our results also indicate that these genes are regulated by mechanisms affecting both the genome and epigenome including post-transcriptional regulation via miRNAs and methylation or demethylation of CpG sites of target genes. In addition, most suggested genes for detection of ovarian cancer are also related in the development of the chicken oviduct in response to estrogen which can act via its receptors to induce malignant transformations in cells of the ovaries. These results support our hypothesis that selected genes are critical regulators for development and differentiation of epithelial cells of the ovaries as they transition from the normal to the cancerous state during oncogenesis in women and laying hens.

To demonstrate function of our selected genes, laying hen as the best animal model was selected to elucidate the mechanisms responsible for both EOC and growth and development of the oviduct in response to estrogen. Specifically, laying hens are the most relevant model to identify biomarkers for EOC because they experience continuous daily ovulation that has the possibility of inducing genetic mutations and damage to the ovarian surface epithelium that are responsible for the spontaneous nature of developing EOC as occurs in women experiencing menstrual cycles and ovulations regularly (Barua et al., 2009; Murdoch et al., 2005). As compared to the laying hen model, rodents are inappropriate to verify clinical relevance of markers of

EOC because they do not spontaneously develop ovarian cancer, but must be engineered genetically to demonstrate etiologies and pathogenesis of ovarian cancer (Barua et al., 2009; Stakleff and Von Gruenigen, 2003; Vanderhyden et al., 2003).

In this study, expression patterns of *SERPINB3*, *SERPINB11*, *GAL11*, *SPP1* and *A2M* were identified between normal and cancerous ovaries of laying hens. The expression of mRNAs for those genes increased significantly in cancerous as compared with normal ovaries. Further, those genes are localized predominantly to the glandular epithelium of cancerous ovaries of laying hens whereas they were not expressed in normal ovaries. According to results of our studies, the five selected genes may be involved in gland morphogenesis in laying hens.

Some genes associated with EOC are regulated by epigenetic effects. First, *miR-101*, *miR-1668* and *miR-1681* influenced *SERPINB3* expression, *miR-1615* modulated *GAL11* expression and *miR-140* regulated *SPP1* expression via their 3'-UTR regions which suggests that post-transcriptional regulation of gene expression influences their expression in laying hens. In addition, bisulfite sequencing revealed differences in methylation status of -110 CpG sites in the promoter region of *SERPINB11* in epithelial-derived ovarian cancer cells as compared to normal cells. These results indicate that *SERPINB3* and *SERPINB11* expression is influenced by epigenetic regulatory mechanisms.

Expression patterns of *SERPINB3* and *SERPINB11* protein were determined in ovarian cancer cells from laying hens and women. Immunoreactive

SERPINB3 protein was localized specifically to the nucleus whereas SERPINB11 protein was predominantly localized to the cytoplasmic compartment in cancer cells of laying hens and ovarian cancer cells from women (OVCAR-3, SKOV-3 and PA1). These results indicate that SERPINB3 protein may activate transcription factors or inhibit apoptosis leading to development of EOC in laying hens and women and that SERPINB11 detected in the cytoplasmic compartment may be involved in carcinogenesis although the mechanisms whereby expression of SERPINB11 proteins is regulated by epigenetic programming is unknown.

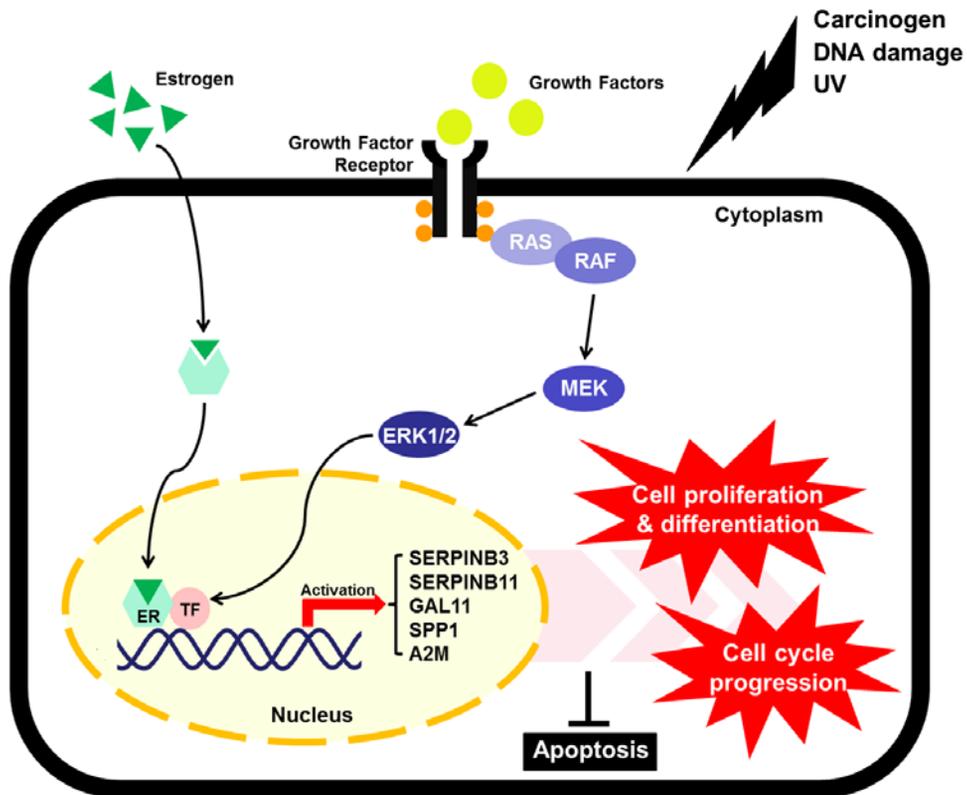
Estrogen is the primary steroid hormone affecting the avian female reproductive tract. Generally, it regulates various biological events such as protection against apoptosis, osteoporosis, diabetes and Alzheimer's disease (Hewitt et al., 2005; Louet et al., 2004; Wise et al., 2005). Estrogen also regulates carcinogenesis of various gynecologic cancers and it is associated with markers for prognosis of breast cancer (Mason et al., 1983), endometrial cancer (Smith et al., 1975) and ovarian cancer (Ho, 2003) through actions dependent on two nuclear receptors, ESR1 and ESR2 (Muramatsu and Inoue, 2000). Estrogens influence the incidence of ovarian cancer as it is a factor stimulating oncogenesis in the ovary (Salehi et al., 2008). For example, taking estrogen as hormone replacement therapy within a group of menopausal women increases the risk of ovarian cancer (Lacey et al., 2002). However, taking oral contraceptives for more than five years reduces the incidence of ovarian cancer during the premenopausal period (Schildkraut et al., 2002; Spillman et al., 2010). In addition, high levels of estrogen increase angiogenesis, endothelial cell migration and proliferation of ovarian surface epithelium by regulating various growth

factors (Cullinan-Bove and Koos, 1993; Hsueh et al., 1981; Hyder et al., 2000; Liu et al., 1994). Moreover, expression of estrogen receptors indicates moderate in normal and benign epithelial cancers whereas their expression increases significantly in malignant ovarian carcinomas (Damiao et al., 2007).

Consistent with the relationship between ovarian cancer and estrogen, expression of selected genes in the chick oviduct that respond to estrogen was identified because the chicken oviduct is a well-established model for investigating the biological actions and signaling pathways induced by estrogens. Results of current studies show that SERPINB3, SERPINB11, GAL11, and A2M expression increased significantly in the chick oviduct in response to estrogen that also stimulated development of the chick oviduct. However, SPP1 expression was slightly decreased in response to estrogen. These results support those of previous studies indicating that estrogen induces development and differentiation of ovarian cancer and that SERPINB3, SERPINB11, GAL11, and A2M might be involved in ovarian carcinogenesis induced by estrogen which increase and decrease daily with the ovulatory cycle of laying hens.

Collectively, results of the present study revealed regulation of expression and function of five selected genes during progression of development of EOC and that their expression depends on transactivation of estrogen via estrogen receptors as shown in Figure 10-1 (Simpkins et al., 2013). However, further studies are required to elucidate the clinical application of discoveries of these target genes in the diagnosis and treatment of EOC. Therefore, our future research will: 1) examine biological

targets of selected genes in human and chicken EOC cells through knockdown using siRNAs; 2) determine functional mechanism whereby the genes of interest affect carcinogenesis through various signaling pathways for target genes in human EOC cells; 3) clinically evaluate, through clinical prospective studies of women, the utility of these genes in diagnosing development of EOC and in assessing effectiveness of therapeutics for inhibiting or preventing progression of EOC to increase rates of survival and prevention of recurrence of EOC; and 4) generate knockout models of target genes to understand function of the five genes associated with EOC.



**Figure 11-1. Schematic illustrating mechanism for expression and function of regulatory genes for development of the oviduct and for development of epithelial-derived ovarian cancer.** Carcinogens, DNA damage, estrogen and ultra-violet light (UV) likely activate estrogen- and MAPK cascade signaling pathway that regulate cell proliferation and differentiation, cell cycle progression and apoptosis in EOC through stimulation of expression of SERPINB3, SERPINB11, GAL11, SPP1 and A2M genes. Legend: RAS, synaptic Ras-GTPase-activating protein; RAF, mitogen-activated protein kinase (MAPK) kinase kinase; MEK, MAPK kinase; ERK1/2, extracellular signal-regulated kinase; ER, estrogen receptor; TF, transcription factor.

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## 초 록

난소암은 난소에서 발생하는 악성 종양으로 주기적인 배란으로 인한 난소 상피의 파열 및 이에 따른 유전자 변이로부터 발생한다. 난소암은 조기진단 시 완치율이 90%정도로 매우 높다. 하지만 발병 이후, 특이적인 초기 증상이 거의 없으며 조기 진단 가능한 종양 표지인자 (바이오마커)가 미비하여 대다수의 난소암 환자들은 복수와 다른 생체 내 기관으로 암세포의 전이를 보이는 말기에 진단되며, 이는 5년 생존율을 30% 이하로 감소시킨다. 난소암의 90%이상은 난소의 표면 상피로부터 자연적으로 발생한다. 이는 난소의 표면 상피로부터 발생하는 닭의 난소암과 유래가 일치하며 상피성 난소암의 조직학적 특징, 암 전이 경향 등의 공통된 유사성은 산란 닭이 난소암 발달의 진단용 마커 또는 치료제 개발 등의 난소암 연구에 있어 적절한 동물 모델임을 나타낸다.

그러므로 본 연구에서는 상피성 난소암 조기 진단 바이오마커를 개발하기 위해 먼저, 산란 닭의 정상 및 암화 된 난소를 사용하여 후보 유전자의 발현 유무를 확인하였고, 이 후, 산란 닭의 정상 및 난소암 세포주와 인간의 난소암 세포주 내에서 후보 유전자의 발현을 비교분석 하였다. 또한 후생유전학적 분석 방법 중 하나로 마이크로알엔에이를 이용하여 난소암 관련 유전자의 전사 후 조절 기전을 규명하며 상피성 난소암의 분자유전학적 기전을 이해하는데 많은 정보를 제공하였다.

먼저, 본 연구에서는 인간 난소암과 같은 유래의 상피성 난소암 연구에 적절한 생물모델인 닭을 이용하여 상피성 난소암에서 SERPINB3 유전자의 기능적 역할을 연구하였다. SERPINB3는 정상난소에서 발현이 전무하였지만 암화된 난소암 조직에서는 과발현 되는것으로 확인되었다. 또한 SERPINB3의

발현은 *miR-101*, *miR-1668*, *miR-1681*에 의해 프로모터 지역 내 전사 후 조절을 통해 중재됨을 확인할 수 있었다. 게다가 인간 및 닭의 난소암 세포주 내 SERPINB3의 발현패턴을 비교한 결과 다른 두 종의 난소암 세포주 핵에서 SERPINB3 단백질이 특이적으로 발현됨을 알 수 있었다. 이와 더불어 109명의 상피성 난소암 환자의 조직샘플을 사용하여 본 유전자의 발현을 확인한 결과, 강한 SERPINB3의 발현은 난소암의 플래티늄 내성과 생존율을 위한 예지 인자로 역할을 할 것이라 결론 지을 수 있었다. 따라서 SERPINB3는 난소암 발병에 중요한 역할을 수행할 것이고 플래티늄 내성 예지 및 상피성 난소암 환자의 생존을 위한 진단에 중요한 바이오마커로 작용할 것이다.

두번째 연구에서는 SERPINB11이 바이오마커로서 작용할 수 있는 가능성에 대해 살펴보았다. 이를 위해 닭의 정상 난소와 난소선암조직에서 SERPINB11 유전자의 발현패턴과 후생유전학적 조절 메커니즘을 확인하였다. SERPINB11은 닭의 난소 선암조직의 상피선 부분에서 특이적으로 강한 발현이 있는 것으로 확인되었다. 또한 bisulfite sequencing을 통해 정상세포와는 다르게 난소암 세포에서 -110 CpG 부위의 30% 정도가 메틸화 된 것을 확인할 수 있었다. 그리고 SERPINB11이 인간의 난소암 세포주에서 (OVCAR-3, SKOV-3, PA-1) 닭의 난소암 세포주와 동일하게 세포질에서 과발현 하는 것을 확인함으로써 SERPINB11이 인간 난소암의 진단 또는 치료효과 모니터링 마커로서 활용할 수 있을 것이라 사료된다.

세번째로, 선천성면역 시스템에 중요한 역할을 할 뿐만 아니라 상피세포와 섬유아세포의 증식을 활성화시키는 조류의 beta-defensins (AvBDs)의 한 일원으로 GAL11 유전자와 난소암의 연관성을 확인하였다.

GAL11 유전자의 발현은 암탉의 일반 난소가 아닌 자궁내막형의 난소암 선상피세포에서 주로 확인되었다. 또한 GAL11의 표적 마이크로알엔에이, *miR-1615*를 통해 프로모터 부위를 조절하여 GAL11 유전자가 전사 후 조절 기작에 의해 영향을 받는다는 사실을 규명하였다.

네번째로, 염증반응, 석회화, 기관발달, 면역세포기능 및 발암작용 등과 같은 여러 생리적 과정들에 있어 중요한 기능을 담당하는 SPP1 유전자에 대해 조사하였다. SPP1은 산란하는 닭의 정상난소에서와는 달리 상피성 난소암 조직에서 그 mRNA 발현양이 두드러지게 증가하였다. 특히 난소암 조직 내의 선상피세포에서 특이적으로 SPP1의 mRNA와 단백질이 강하게 발현하는 것을 밝혔다. 더불어, 본 유전자 또한 선행실험들과 같이 *miR-140*에 의한 전사 후 조절 기전을 통해 발현이 조절되는 것으로 확인되었다.

마지막으로, A2M 유전자는 serine-, threonine-, cysteine-, aspartic- 그리고 metalloproteases 등과 같은 대부분의 단백질 분해 효소들을 활성화 혹은 억제시키는 독특한 특징을 지닌다. Metalloprotease와 같은 단백질 분해 효소들은 세포외 기질과 기저막의 분해 과정에 관련되어, 종양의 혈관 형성 및 암세포의 침범과 전이 촉진 등 암 발생과 밀접한 관련이 있다. 본 연구 결과에서는 A2M 유전자의 발현이 정상 닭 난소에 비해 암화된 난소의 선상피세포 조직에서 두드러지게 나타남을 확인하였다.

결론적으로, 본 연구를 통해 발굴된 유전자 SERPINB3, SERPINB11, GAL11, SPP1, A2M은 상피성 난소암 발달에 있어 중요한 역할을 할 것이며, 상피성 난소암의 조기 진단 및 암의 진행과 예후를 판단할 수 있는 바이오마커 및

신약개발을 위한 선행 기초연구로서 생명공학, 약학 및 의과학 등의 다양한 분야에 많은 공헌을 할 수 있을 것이라 기대된다.

**주요어:** 상피성 난소암, SERPINB3, SERPINB11, GAL11, SPP1, A2M

**학 번:** 2010-22859

## 감사의 글

분자내분비학 및 세포전달실험실 내에서 학위과정 동안 생활하며 기본적인 실험 테크닉부터 논문작성 완성까지 수많은 과정에 있어 다양한 지식을 습득하며 더욱 성장하는 기회를 지닐 수 있었습니다. 지난 시간 동안 부족한 제가 하나의 프로젝트를 시작하여 마무리 지을 수 있는 연구자가 되도록 도움을 주신 분들에게 깊은 감사의 말씀을 올리고자 합니다.

먼저 제가 현 위치에 도달하는데 있어 많은 가르침을 건네주시며 아낌없는 지원과 학자로서의 면모를 스스로 보여주시고 제게 또 다른 꿈을 일깨워 주신 지도 교수님, 송원화 교수님께 깊은 감사의 말씀 드립니다. 또한 공동 지도교수님으로서 학위논문 작성에 있어 많은 조언을 주신 이형주 교수님께도 깊은 감사의 말씀 드립니다. 그리고 프로젝트 진행 및 논문 작성에 있어 많은 가르침을 주신 Texas A&M University의 Bazer 교수님께도 감사 드립니다. 그리고 바쁘신 와중에 저의 논문 심사를 위해 귀중한 시간을 할애하여 조언을 주신 이창규 교수님, 가학현 교수님, 최영석 교수님께도 감사의 말씀 드립니다.

다음으로 학위과정 동안 실험실에서 회로애락을 함께한 분자내분비학 및 세포전달실험실 멤버들에게 감사의 마음을 전합니다. 환한 미소와 함께 실험 진행에 많은 도움을 주신 김진영 교수님, 감사합니다. 때로는 호랑이 선배로서, 때로는 친구처럼 실험실을 꾸러가는 동반자로서의 역할을 함께한 은서언니, 묵묵히 자신의 일과 함께 조력자로서의 임무를 다해준 우영이, 다혈질의 소유자이지만 마음만은 여린 진영언니, 실험실의 분위기 메이커인 동시에 시어머니 역할을 도맡아 준 철홍오빠, 막내로서 어리광을 벗어나 어엿한

실장으로서의 모습을 보여주고 있는 승민이, 실험실의 미래를 밝혀줄 청이에게  
고마움을 전합니다.

마지막으로 언제나 든든한 지원군으로서 항상 저를 믿어주시고  
응원해주신 부모님께 진심으로 감사 드리며 든든한 버팀목으로 자리매김하고 있는  
남동생 재형이에게 고마움을 전합니다.