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SUMMARY

Ovarian Cancer is the most lethal gynecological disease among women due to difficult to diagnose at early stage and lack of molecular biomarkers. To overcome this limitations and obstacles, research is required to gain a better understanding of ovarian carcinogenesis mechanisms both genetics and epigenetics in avian species, especially laying hens which are the best animal model for research on human ovarian carcinogenesis. The aims of this study with laying hens were to determine: 1) the expression of DNA methyltransferases (DNMTs), cyclins, cyclin dependent kinases and their inhibitors, and pleiotrophin (PTN) gene in normal and cancerous ovaries; 2) whether these genes are regulated by post-transcriptional actions of specific microRNAs; and 3) methylation patterns of several specific genes.

DNMTs are key regulators of DNA methylation and have crucial roles in carcinogenesis, embryogenesis and epigenetic modification. Results of the present study demonstrated increased expression of DNMT genes in cancerous ovaries of laying hens and post-transcriptional regulation of those genes by specific microRNAs, as well as control of hypermethylation of the promoters of tumor suppressor genes.

The cell cycle system is controlled in a timely manner by three groups of cyclins, cyclin dependent kinases and their inhibitors. Abnormal alterations of cell cycle regulatory mechanisms are a common feature of many diseases including numerous tumor types such as ovarian cancer. Results of the present study demonstrated increased expression of cell cycle-related genes in cancerous ovaries of laying hens and indicate that
expression of these genes is post-transcriptionally regulated by specific microRNAs.

PTN is a developmentally-regulated growth factor which is widely distributed in various tissues and also detected in many kinds of carcinomas. Our results indicated that PTN is most abundant in the GE of adenocarcinoma of cancerous, but not normal ovaries of hens. Bisulfite sequencing revealed that 30- and 40% of -1311 and -1339 CpG sites are demethylated in ovarian cancer cells, respectively. Further, several microRNAs, specifically miR-499 and miR-1709 were discovered to influence PTN expression via its 3'-UTR which suggests that post-transcriptional regulation influences PTN expression in chickens.

Collectively, this present study provides new insights into epigenetic regulation and functional roles in ovarian carcinogenesis in laying hens that are highly relevant to the development of therapies for treatment of ovarian cancers in humans. As well as, it absolutely provides better approach to identify epithelial derived human ovarian cancer.

**Keywords:** ovarian cancer, chicken, DNA methyltransferase, cell cycle, pleiotrophin

**Student number:** 2011-22979
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3’UTR</td>
<td>3’ Untranslated Region</td>
</tr>
<tr>
<td>5-meC</td>
<td>5-Methyl Cytosine</td>
</tr>
<tr>
<td>ACTB</td>
<td>Beta-actin</td>
</tr>
<tr>
<td>AkT</td>
<td>Serine/Threonine protein kinase Akt</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>Breast cancer1/2</td>
</tr>
<tr>
<td>CCNA2</td>
<td>Cyclin A2</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
</tr>
<tr>
<td>CCND2</td>
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<tr>
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<td>CCNE2</td>
<td>Cyclin E2</td>
</tr>
<tr>
<td>CDK1</td>
<td>Cyclin-dependent kinase 1</td>
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</tr>
<tr>
<td>CDK5</td>
<td>Cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>Cyclin-dependent kinase inhibitor 1B (p27, Kip1)</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstilbestrol</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
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</table>
DNA  Deoxyribonucleic acid
DNMT1  DNA (cytosine-5-)-methyltransferase 1
DNMT3A  DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B  DNA (cytosine-5-)-methyltransferase 3 beta
DNMT3L  DNA (cytosine-5-)-methyltransferase 3-like
EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor
EGFR  Epidermal growth factor receptor
EMT  Epithelial–mesenchymal transition
ER  Estrogen receptor
ERBB2  Erythroblastic leukemia viral oncogene homolog2
FACS  Flow cytometry
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
GE  Glandular epithelium
GFP  Green fluorescent protein
HDAC  Histone deacetylases
IgG  Immunoglobulin G
LE  Luminal epithelium
miR  MicroRNA
mRNA  Messenger RNA
MSP  Methylation specific PCR
PCNA  Proliferating Cell Nuclear Antigen
PI3K  Phosphoinositide 3 kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PTN</td>
<td>Pleiotrophin</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RB1</td>
<td>Retinoblastoma 1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SMAD4</td>
<td>SMAD family member 4</td>
</tr>
<tr>
<td>SSC</td>
<td>Standard saline citrate</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein p53</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>TSG</td>
<td>Tumor suppressor gene</td>
</tr>
<tr>
<td>USF</td>
<td>Upstream stimulatory factor</td>
</tr>
<tr>
<td>WL</td>
<td>White Leghorn</td>
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CHAPTER 1

GENERAL INTRODUCTION
Ovarian cancer is the deadliest gynecological disease and the fifth leading cause of cancer-related death in women. Difficult to control with chemotherapeutic and surgical strategies, ovarian cancer poses significant challenges in early stage diagnosis. It is typically detected at later metastasized stages in other organs through blood vesicles or lymph nodes, and is directly linked to a high mortality rate. Seventy percent of ovarian cancer patients are diagnosed at advanced stages while only 30% of these will survive longer than five years following diagnosis. However, ovarian cancer detection at early stages increases survival rates to over 70%. In order to allay its fatal effects, biomarkers for early stage detection and appropriate animal models for research on human ovarian cancer are required. In addition, further genetic and epigenetic insights are needed for a better understanding of ovarian carcinogenesis mechanisms.

We focused on the domestic chicken to develop an appropriate animal model for human ovarian cancer research. Previously, popular animal models have included primates, rodents and other avian species. As an experimental animal model, the primate shares similar physiological characteristics and anatomical features with humans, but biological and ethical limitations involving non-spontaneous ovarian cancer development demands a higher cost. Laboratory rodents have the advantages of easy handling, high efficiency of genetic changes, and low cost. However, they are impractical in ovarian cancer research due to a wide variety of histological types, low incidence, and long periods required for the appearance of tumors. The laying hen is a more appropriate corresponding human experimental model for ovarian cancer studies. Approximately 83% of laying hens display incidence of genital tumors, which is of exceptional value compared with other species. Moreover, several well-developed molecular biomarkers for human ovarian cancer diagnoses are
also expressed in cancer ovarian chicken tissues.

In order to better understand ovarian carcinogenesis, we focused on multiple genetic and epigenetic mechanisms. Various genetic alterations are involved in ovarian carcinogenesis. For example, during menstruation and ovulation, the disruption and repair of the epithelial surface of ovaries leads to genomic damages, DNA mutations, and insertion and deletion of the surface ovarian epithelium, increasing the risk of epithelial ovarian cancer. In genetic alterations, abnormal cell cycles and their alteration of regulatory genes such as cyclins, cyclin-dependent kinases and cyclin-dependent kinases inhibitors are common features of various cancer types. We analyzed methylation patterns in normal and cancer ovaries to discover epigenetic mechanisms of ovarian cancer. DNA methylation has critical roles in embryogenesis, organogenesis and carcinogenesis including ovarian cancer and is regulated by several types of DNA methyltransferases (DNMTs). DNMT1 mainly regulates methylation patterns while DNMT3A and DNMT3B mediate de novo methylation patterns. Recently, research has been conducted on the relationship between epigenetics and microRNAs to determine whether they directly or indirectly regulate epigenetic mechanisms such as methylation and post-transcriptional gene regulation in ovarian cancer. Thus we identified several small non-coding RNAs that targeted DNMT genes, which were down-regulated in epithelial ovarian cancer.

In this study, to discover multiple ovarian cancer mechanisms, we conducted several progressing series of experiments. We review general and histological characteristics of ovarian cancer, multiple mechanisms in genetic and epigenetic ovarian cancer development, experimental animal models, and cell cycle regulatory genes in Chapter 2. In Chapter 3, we
examine epigenetic functions and multiple mechanisms of DNMTs in chicken ovarian carcinoma, and in Chapter 4, we distinguish and demonstrate expression patterns and post-transcriptional regulation of cell cycle regulatory genes in avian epithelial ovarian carcinomas. Moreover, we identify the avian pleiotrophin gene, and determine tissue-specific expression patterns as a result of estrogen in the oviduct and epithelia of ovarian carcinomas in Chapter 5.
CHAPTER 2

LITERATURE REVIEW
1. Ovarian Cancer Characteristics and Classification

1.1. General Characteristics

Ovarian cancer is the fifth deadliest cancer for females in the United States. Approximately 1 in 70 women have a lifetime risk of ovarian cancer, and 1 in 100 women die from it. The most probable cause of death upon patient diagnosis is advanced stages of cancer development, which has metastasized to other organs. Approximately 70% of ovarian cancer patients are diagnosed at the advanced stages, of which only 30% are expected to survive past five years. More than 50% of ovarian cancers appear after the age of 40, which rises sharply in occurrence during perimenopausal and postmenopausal periods in women; the peak incidence of epithelial ovarian cancer occurs at age 60. However, mortality rates of ovarian cancer sharply drop after 65 (Jemal et al., 2008; Parazzini et al., 1991).

Approximately 3% of ovarian cancers, such as choriocarcinomas, originate from germ cells, with 7% originating from sertoli or granulosa cell tumors which come from sex chord stromal cells, whereas 90% of ovarian cancers are germinal epithelia-derived (EOC) or originate in the fallopian tube epithelium (FTE). EOCs are divided into histological subgroups with endometrioid, serous, clear cell, mucinous, and undifferentiated carcinomas (Auersperg, 2011; Auersperg et al., 2001).

The high rate of EOC incidence likely results from continuous ovulation and menstrual cycles that lead to genomic damage and
mutations in genes in the ovarian surface epithelium. DNA damage caused by reactive oxygen species are unavoidable effects of physiological metabolism resulting from hormones and ovulatory products. To protect from such damage, animals maintain several mechanisms including the production of antioxidant enzymes or molecular regulatory systems. However, these are less than complete protection (Cooke et al., 2003; Marnett, 2000; Murdoch et al., 2005). In addition, numerous genetic and epigenetic alterations are involved in ovarian carcinogenesis such as gene mutation, activation or inactivation of miRNAs, and alteration of DNA methylation or histone acetylation.

1.2. Classification of Epithelial Ovarian Cancer

Four histological subtypes classify epithelial ovarian cancer, of which serous compose approximately 60%, endometrioid: 10%–20%, clear cell: less than 10%, mucinous: less than 5%, and undifferentiated carcinomas: less than 1% (Bocker, 2002).

Endometrioid ovarian cancer is the second most common gynecological cancer. It is influenced by endocrines such as estrogen and progesterone and shows distinct differentiation with nuclear pleiomorphism as well as characteristics including less myometrium invasion and low potential for lymph node metastasis. In the histological features of the endometrioid type, complex glandular, microglandular foci and solid growth patterns exist (Barua et al., 2009; Sherman, 2000).

Serous carcinoma is the most common and lethal ovarian cancer. These cancers are characterized by high levels of abnormal alteration of
DNA copy number and low levels of gene point mutations. It exhibits papillary structures in histological features, nuclear atypia, and abnormal slit glandular locations (Barua et al., 2009).

2. Genetic and Epigenetic Mechanisms of carcinogenesis

2.1. Genetic Mechanisms

Various genetic alterations are involved in ovarian carcinogenesis. During a menstrual cycle and continuous ovulations, disruption and repair of the epithelial surface of ovaries may lead to genomic damage and DNA mutations in the surface of the ovarian epithelium, thus increasing the risk of epithelial ovarian cancer (Auersperg et al., 2001; Vanderhyden et al., 2003). These may contribute to abnormal gene expression and ovarian epithelial carcinogenesis. Gene expression alterations are major features of ovarian carcinogenesis and their identification is useful for clinical trials of ovarian cancer. In addition, oncogene dysregulation, gene amplification, promoter insertion, point mutation, chromosomal translocation, and loss of function in tumor suppressor genes play central roles in cancer development.

In general terms, alterations of tumor suppressor genes and oncogenes lead to carcinogenesis. BRCA1 and BRCA2, and p53 mutations are common features of high-grade serous ovarian cancers. K-Ras overexpression (Enomoto et al., 1991), alteration of Her-2/neu
(Lancaster et al., 2006), as well as mutation of c-myc, AKT, PTEN and CTNNB1 have also been discovered as critical regulators of ovarian carcinogenesis (Xing and Orsulic, 2005). Activation and abnormal overexpression via gene amplification (RAB25, FGF1, PI3R1 and AURKA), and genomic mutations (CDKN2A, K-Ras, SMAD4 and KIT) have also been discovered to play a role. Genetic alterations involved in tumorigenesis include deletion, mutation, and loss of heterozygosity (PTNE, BRCA1, BRCA2, TP53 and ARH1) (Bast et al., 2009).

The epithelial mesenchymal transition (EMT) also correlates with ovarian carcinogenesis (Liliac et al., 2012). It is a necessary process for morphological organogenesis and embryogenesis (Thiery and Sleeman, 2006). During cancer metastasis and invasion of ovarian cancer, EMT is detected in most cases and involves cellular reprogramming, loss of epithelial characteristics and production of the extracellular matrix (Kalluri and Weinberg, 2009). Thus, E-cadherin is significantly increased in chicken ovarian cancer and this is associated with human ovarian cancer (Ansenberger et al., 2009).

2.2. Epigenetic Mechanisms

Recently the field of epigenetics has become highly relevant for clinical trials in cancer research. As a result, histone deacetylase (HDAC) and DNA methylation inhibitors have undergone rapid development in the anti-cancer drug industry. In contrast to genetic regulation, epigenetic modification alters gene expression without any changes to DNA sequences. Epigenetic mechanisms are involved in DNA methylation,
histone acetylation and microRNA dysregulation. Some research groups have demonstrated that epigenetics is closely associated with the development and progression of ovarian cancer, and gradual stimulation is involved in advanced stages and tumorigenesis grades (Balch et al., 2009).

DNA methylation is added to the carbon-5 cytosine ring of CpG islands by DNA methyltransferase (DNMT). Tumor suppressor genes are detected with hyper-methylation patterns and oncogenes display hypomethylation patterns in cancer development. It has become evident that hypermethylation can lead to gene transcriptional silencing while hypomethylation may accelerate gene transcriptional levels in tumorigenesis including ovarian cancer. DNMTs are key regulators of DNA methylation and have crucial roles in carcinogenesis, embryogenesis, and epigenetic modification. DNMTs have three established forms: DNMT1, DNMT3A and DNMT3B. DNMT1 mainly plays an enzymatic role in the maintenance of DNA methylation, and DNMT3A and DNMT3B function in de novo methylation. We previously reported alternative DNA methylation of several genes in chicken epithelial ovarian cancers, which influenced changes in gene expression (Jeong et al., 2012; Lee et al., 2012; Lim et al., 2012b).

MicroRNAs (miRNA) are small non-coding segments of RNA that regulate post-transcriptional processing by binding to the 3’UTR region of a target gene, triggering down-regulation. miRNAs have been shown to play crucial roles in a wide range of biological and pathological processes. Many recent studies have revealed that miRNAs are down-regulated in various cancer types involved in human ovarian cancer (Dahiya et al., 2008; Laios et al., 2008; Nam et al., 2008; Zhang et al., 2008). The term epi-miRNA refers to a complex connection between an epigenetic
mechanism and an miRNA. This complex is affected by miRNA expression, which generates an epigenetic feedback mechanism (Valeri et al., 2009). Epigenetic regulation has been shown to mediate several miRNA-related instances of cancer development and disease.


3.1. Primate models

Ovarian cancer chemoprevention studies have been conducted on monkeys (cynomolgus macaques). In the studies, researchers combined a sex steroid hormone (estrogen and progesterone) and oral contraceptives, the influence of which increased apoptosis levels of ovarian surface epithelium (Rodriguez et al., 1998). Similarly, research in rhesus monkeys has validated primate animal models as appropriate parallels for human ovarian cancer research (Brewer et al., 2007). Resembling humans in pathologic anatomy and physiological characteristics including menstrual cycles, primate models are crucial for detailed ovarian cancer research efforts. However, primate models have some biological limitations, including non-spontaneous ovarian cancer development, a deficiency of surrogate biomarkers, and practical challenges due to specialized skills, and high cost (Lu et al., 2009).

3.2. Rodent models

Genetically modified mouse models for inducting ovarian cancer
provide efficiency and reproducibility with which to investigate functional interactions and specific pathways of cancer-related genes during in vivo tumor development.

Rodent models for ovarian cancer research were first developed by Orsulic, who systematically researched oncogenes in mouse ovarian cells, with Trp53+/+ or Trp53-/- insertions (Orsulic et al., 2002). Flesken-Nikitin generated ovarian cancer artificially in rodent models, by manipulating inactivation of Trp53 and Rb, in the mouse ovarian surface epithelium (Flesken-Nikitin et al., 2003).

Occasionally, ovarian tumors spontaneously develop with age in some abnormal mouse models and in Sprague-Dawley and Wistar rats (Gregson et al., 1984; Tillmann et al., 2000; Walsh and Poteracki, 1994). However, these models are not feasible for laboratory ovarian cancer research due to a wide variety of histological types, low incidence, and long durations needed for disease development.

3.3. **Avian models**

Frederickson first developed the laying hen as an ovarian cancer experimental model. Approximately 83% of avian species have genital tumor incidences including ovarian cancer, which is of considerable value compared to other species. In the chicken, after two years, a 45% chance of reproductive tract tumors developing exists. Fredrickson found that ovarian cancers develop age-dependently in laying hens, with 12% at age 3.9 years, 32% at age 4.2 years and more than 50% at age 6.1 years. The origin of the majority of ovarian tumors is the epithelium surface and
Furthermore, diagnostic molecular biomarkers for human ovarian cancer are usually expressed in chicken ovarian cancers. CA125 is an important biomarker for diagnosing human ovarian cancer and is expressed in spontaneous ovarian adenocarcinomas in chickens (Jackson et al., 2007). In addition, several growth factors and their receptors (TGF-a, EGFR), as well as human ovarian cancer signaling pathways (EGFR/PI3K, AKT/PI3K), and proliferation marker (Cytokeratin AE1/AE3, PCNA) expressions coincide with both human and chicken ovarian cancer (Liu et al., 2009; Liu et al., 2010; Rodriguez-Burford et al., 2001).

4. Cell cycle regulatory genes in ovarian cancer

The cell cycle in most eukaryotic cells includes a series of coordinated events consisting of cell growth, replication of genetic material, segregation of the duplicated chromosomes and cell division (Vermeulen et al., 2003). Also, cell cycles are classified under four phases and separated into G1, S, G2 and M. At the G1 and S phase, DNA synthesis occurs, while G2 and M phases control cell mitosis. In general, the cell division cycle in mammals is precisely and harmoniously regulated in a timely manner by different active heterodimeric complexes that include cyclin dependent kinases (CDKs) and their cognate cyclin partners, as well as CDK inhibitors (CDKIs) (D'Andrilli et al., 2004). Each cyclin type and CDK complex has different functions within each cell cycle phase, including cell growth, accumulation, process suspension, DNA repair, and division. Thus, tumor development frequently results when there is deregulation of the cell cycle control system including abnormal regulation of expression of cell cycle genes (Bovicelli et al., 2011). In human cancerous tissues, such as neoplasms, different families of cell cycle genes and regulators are frequently
mutated and dysfunctional (D'Andrilli et al., 2004). As well as recent insights gained from cancer mechanisms describe dysregulation of cell cycles, with abnormal regulation occurring in cancer development, including epithelial ovarian cancer.
CHAPTER 3

EPIGENETIC FUNCTIONS AND DNA METHYLTRANSFERASES IN THE CHICKEN OVARIAN CARCINOMAS
1. Abstract

DNA methyltransferases (DNMTs) are key regulators of DNA methylation and have crucial roles in carcinogenesis, embryogenesis and epigenetic modification. In general, DNMT1 has enzymatic activity affecting maintenance of DNA methylation, whereas DNMT3A and DNMT3B are involved in de novo methylation events. Although DNMT genes are well known in mammals including humans and mice, they are not well studied in avian species, especially laying hens which are the best animal model for research on human ovarian carcinogenesis.

Results of the present study demonstrated that expression of DNMT1, DNMT3A and DNMT3B genes was significantly increased, particularly in the glandular epithelia (GE) of cancerous ovaries, but not normal ovaries. Consistent with this result, immunoreactive 5-methylcytosine protein was very abundant in the nucleus of GE of cancerous ovaries, but barely detectable in GE of normal ovaries. Methylation-specific PCR analysis detected hypermethylation of the promoter regions of the tumor suppressor genes in the initiation and development of chicken ovarian cancer.

Further, several microRNAs, specifically miR-1741, miR-16c, and miR-222, and miR-1632 were discovered to influence expression of DNMT3A and DNMT3B, respectively, via their 3′-UTR which suggests post-transcriptional regulation of their expression in laying hens. Collectively, results of the present study demonstrated increased expression of DNMT genes in cancerous ovaries of laying hens and post-transcriptional regulation of those genes by specific microRNAs, as well
as control of hypermethylation of the promoters of tumor suppressor genes.

**Key World:** chicken, ovarian cancer, DNMT, microRNA, methylation
2. Introduction

Ovarian cancer is the most common malignancy in the female genital tract in the United States, and the fifth leading cause of cancer-related deaths among women. Of these, the surface epithelial-derived ovarian cancer accounts for 90% of all ovarian cancers (Siegel et al., 2011). Since the idea that the repeated rupture of the ovarian epithelium during the monthly ovulation event in women may contribute to accelerate the incidence of the epithelial ovarian cancer was coined by Fathalla about 40 years ago (Fathalla, 1971), the etiology of ovarian cancer is complicated and not fully understood. However, results of a number of epidemiological studies indicate that there is an increased ovarian cancer risk dependent on ovulation frequency and reproductive factors (Smith and Xu, 2008). Recently, the early diagnosis of epithelial ovarian cancer and prediction of prognosis for patient survival using specific biomarkers is increasingly recognized as a better approach to identify this disease. To overcome these limitations and obstacles and to elucidate the etiology and pathogenesis of epithelial ovarian cancer, various genetically engineered rodent models have been developed, but the artificial nature of the induced tumors in rodents limit their clinical relevance (Barua et al., 2009; Stakleff and Von Gruenigen, 2003; Vanderhyden et al., 2003). Meanwhile, the laying hen is known to be the only animal model that spontaneously develops ovarian surface epithelia-derived tumors, and is a unique and suitable model to develop novel biomarkers and anti-cancer drugs for patients with epithelial ovarian cancer (Barua et al., 2009; Stammer et al., 2008; Vanderhyden et al., 2003).

In higher organisms, DNA methylation plays pivotal roles in
normal growth/development and cellular differentiation and affects a variety of biochemical events such as genomic imprinting and X-chromosome inactivation (Jaenisch and Bird, 2003). In general, DNA methylation involves the addition of a methyl group to the carbon 5 position (5 meC) of the cytosine residue in the pyrimidine ring (Laird, 2010). Thereby this modification has the specific effect of reducing gene expression and can be inherited by offspring. DNA methylation events in mammalian cells are mainly carried out by two major classes of enzymatic activities; maintenance methylation via DNA methyltransferase 1 (DNMT1) and de novo methylation via DNMT3A and DNMT3B. In cancer biology, overexpression of DNMTs is a hallmark of cancer cells such as endometrioid carcinomas and prostate cancer (Lan et al., 2010; Robertson et al., 1999; Socha et al., 2009) and it is responsible for aberrant promoter hypermethylation of tumor suppressor genes in various human cancer cells (Jair et al., 2006; Jones and Baylin, 2002). Although expression and functional roles of DNMTs are well studied in mammalian species, including humans and mice, little is known about their expression and epigenetic regulation in avian species, especially laying hens that develop epithelial ovarian cancer spontaneously.

Therefore, the objectives of this study with laying hens were to determine: 1) the expression of DNMTs in normal and cancerous ovaries; and 2) whether DNMTs are regulated by post-transcriptional actions of specific microRNAs using a miRNA target validation assay. Our results confirm that the laying hen is a unique model for the research on human ovarian cancer and that DNMTs may play a key role in ovarian carcinogenesis.
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) laying hens were subjected to standard management practices at the University Animal Farm, Seoul National University, Republic of Korea. All hens were exposed to a light regimen of 15 h light and 9 h dark with *ad libitum* access to feed and water.

**Tissue Samples**

A total 136 mature hens (88 over 36 months and 48 over 24 months of age), which had stopped laying eggs, were euthanized for collection of cancerous ovarian tissues. We obtained cancerous ovarian tissues from 10 hens and normal ovarian tissues from 10 egg-laying hens of age. We evaluated tumor stage of 10 hens with cancerous ovaries according to characteristics features of chicken ovarian cancer (Barua et al., 2009; Lim et al., 2011b). Three hens had stage III disease because ovarian tumor cells had metastasized to the gastrointestinal (GI) tract and liver surface with profuse ascites in the abdominal cavity. Five hens had tumor cells spread to distant organs including liver parenchyma, lung, GI tract and oviduct with profuse ascites, indicating stage IV disease. Two hens had stage I disease as tumors were limited to their ovaries. 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). Paraffin-embedded tissues were sectioned at 5 µm and stained with hematoxylin and eosin. 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).

**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 analysis**

The expression of DNMTs mRNA in normal and cancerous ovaries of chicken was assessed using semi-quantitative RT-PCR as previously described (Song et al., 2007). The cDNA was synthesized from total cellular RNA (2 ug) using random hexamer (Invitrogen, Carlsbad, CA) and oligo (dT) primers and AccuPowerH RT PreMix (Bioneer, Daejeon, Korea). The cDNA was diluted (1:10) in sterile water before use in PCR. 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) 95uC for 3 min; (2) 95uC for 20 sec, 60uC for 40 sec (for *DNMT1, DNMT3A, DNMT3B* and *GAPDH*) and 72uC for 1 min for 35
cycles; and (3) 72\(^\text{oC}\) 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\textsuperscript{TM} XR+ system with Image Lab\textsuperscript{TM} software (Bio-Rad).

\textbf{Quantitative RT-PCR Analysis}

Total RNA was extracted from each normal and cancerous ovary tissues using TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen). Complementary DNA was synthesized using a Superscript\textsuperscript{®} III First-Strand Synthesis System (Invitrogen). Gene expression levels were measured using SYBR\textsuperscript{®} Green (Biotium, Hayward, CA, USA) and a StepOnePlus\textsuperscript{TM} 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. Using the standard curve method, we determined the expression quantities of the examined genes using the standard curves and Ct values, and normalized them using GAPDH expression quantities. The PCR conditions were 94°C for 3 min, followed by 40 cycles at 94°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 rose statistically above 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 of the normal ovary.

**In Situ Hybridization Analysis**

Total RNA was extracted from frozen tissues by Trizol reagent (Invitrogen, Carlsbad, CA) and cDNAs were synthesized using AccuPower® RT PreMix (Bioneer, Daejeon, Korea). 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, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5μm and sections placed 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 twice in DEPC-treated PBS. The sections were then digested in 5μg/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 [0.1M 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 with 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 overnight at 42°C in a humidified chamber. 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), the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche, Indianapolis, IN). 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 Chemical Co., St. Louis, MO).

**Immunofluorescence**

Immunocytochemical localization of 5-methylacytosine protein in normal and cancerous ovaries from chickens was performed using a mouse polyclonal antibody to 5-methylacytosine (Catalog number ab-10805; AbCam, CA, USA) at a final dilution of 1:200 (0.2µg/ml). Antigen retrieval was performed using the boiling citrate method. For immunostaining, Negative controls included substitution of the primary antibody (Antibody Diluent, Dako, Cat.No S0809) with purified non-immune mouse IgG at the same final concentration. For secondary antibody, Alexa-488 goat anti mouse (Catalog number A11001; Invitrogen,
USA) and, for mounting reagent was containing DAPI solution (Catalog number P36931; Invitrogen, USA).

**Immunohistochemical analysis**

Immunocytochemical localization of PTN protein in normal and cancerous ovaries from chickens was performed as described previously (Song et al., 2006) using a rabbit polyclonal antibody to 5-methylcytosine (Catalog number ab-10805; AbCam, CA, USA) at a final dilution of 1:200 (0.2µg/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 mouse IgG at the same final concentration.

**Methylation Specific PCR (MSP) analysis**

To investigate differential methylation patterns of selected tumor suppressor genes including APC gene (APC), cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as p16), phosphatase and tensin homolog (PTEN), brac2 gene (BRCA2), and rb1 gene (RB1) between normal and cancerous ovaries, we performed methylation-specific PCR analysis. For PCR template, DNA samples were prepared using an AccuPrep Genomic DNA Extraction Kit (Bioneer) and converted using Epitect Bisulfite kit (QIAGEN, Doncaster, Australia) according to the manufacturer’s instructions. For amplifying the converted DNA, PCRs were performed with both a methylation-specific primer and an unmethylation-specific primer for each gene with forward and reverse primers, which information was described in Table1, as follows: (1) 95°C
for 3 min; (2) 95°C for 20 sec, 60°C for 40 sec and 72°C for 1 min for 35 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 DocTM XR+ system with Image LabTM software (Bio-Rad).

**MicroRNA Target Validation Assay**

The 3’-UTRs of DNMTs were cloned and confirmed by sequencing. Each 3’-UTR was subcloned between the eGFP gene and the bovine growth hormone poly-A tail in 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., 2012). For the dual fluorescence reporter assay, the fusion contained the DsRed gene and either miR-148a or miR-1612 for DNMT1; miR-1596, miR-1687, miR-1741, or miR-1749 for DNMT3A; and miR-16c, miR-222, or miR-1632 for DNMT3B, and each was designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miRNA). At 48h 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 for quantitative PCR were subjected to analysis of variance
(ANOVA) according to the general linear model (PROC-GLM) of the SAS program (SAS Institute, Cary, NC) to determine whether effects time post-ovulation were significant. Data presented are mean ± SEM unless otherwise stated. Differences in the variance between Normal Ovaries and Cancerous ovaries for each group were analyzed using the $F$ test, and differences in the means were subjected to Student’s $t$ test. Differences were considered significant at $P < 0.05$. 
### Table 1. Information of primers for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'→3'): forward and reverse</th>
<th>Gene Bank accession no.</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT1</td>
<td>TTCAGCGTCTACGACAAACG GAGCGAATCCTCTGTGAAGC</td>
<td>NM_206952.1</td>
<td>423</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>CACGACCAGGAGTTTGATCC CTTTGGCATCAATCATGACG</td>
<td>NM_001024832.1</td>
<td>514</td>
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<tr>
<td>DNMT3B</td>
<td>CAAGAGGCTGAAGAGCAACC GAGCACTCTGATGGGTCTCC</td>
<td>NM_001024828.1</td>
<td>550</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGCCAACCCCCCAATGTCTCTGT TCCTTGGATGCCATGTTGACCA</td>
<td>NM_204305.1</td>
<td>301</td>
</tr>
</tbody>
</table>

### Table 2. Information of primers for quantitative Real Time PCR

<table>
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<tr>
<th>Gene</th>
<th>Sequence (5'→3'): forward and reverse</th>
<th>Gene Bank accession no.</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT1</td>
<td>GTACATCAAGGGCAGCAACC GCGGTAGAACTTCCAGATGC</td>
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<td>136</td>
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<tr>
<td>DNMT3A</td>
<td>CTGTCCCTGCTGAAAAGAGG TGATAGAGTCCTCGCACACC</td>
<td>NM_001024832.1</td>
<td>138</td>
</tr>
<tr>
<td>DNMT3B</td>
<td>ACCCAAAAGCTCAGAGAACG GCAGCTGTCCTCCAGACTCC</td>
<td>NM_001024828.1</td>
<td>196</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ACACAGAAGACGGTGGATGG GGCAGGTTCAGTCAACAAACA</td>
<td>NM_204305.1</td>
<td>193</td>
</tr>
</tbody>
</table>
Table 3. Information of primers for Methylation Specific PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'→3'): forward and reverse</th>
<th>Gene Bank accession no.</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC-M</td>
<td>ATAAGAATTTTATTATAAAAAAGTTTGTTCGT TTTATTCAATCATACACAAAAACGAT</td>
<td>NC_006127.3</td>
<td>232</td>
</tr>
<tr>
<td>APC-U</td>
<td>TATAAGAATTTTATTATAAAAAAGTTTGTTCGT TTTATTCAATCATACACAAAAACGAT</td>
<td>NC_006127.3</td>
<td>233</td>
</tr>
<tr>
<td>P16-M</td>
<td>TTTTGTATCGAATAACGG AAACCAACTTTTTAAAACACGATACGT</td>
<td>NC_006127.3</td>
<td>233</td>
</tr>
<tr>
<td>P16-U</td>
<td>TTTTGTATCGAATAACGG AAACCAACTTTTTAAAACACGATACGT</td>
<td>NC_006127.3</td>
<td>233</td>
</tr>
<tr>
<td>PTEN-M</td>
<td>ATTTAAGGTGTATGGAGAGAACGT AAAATAAAAAACACAAACTCGTA</td>
<td>NC_006093.3</td>
<td>163</td>
</tr>
<tr>
<td>PTEN-U</td>
<td>ATTTAAGGTGTATGGAGAGAACGT AAAATAAAAAACACAAACTCGTA</td>
<td>NC_006093.3</td>
<td>163</td>
</tr>
<tr>
<td>RBCA2-M</td>
<td>TGTTTTTGTATGGAGAGAACGT AAAATAAAAAACACAAACTCGTA</td>
<td>NC_006088.3</td>
<td>141</td>
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<tr>
<td>RBCA2-U</td>
<td>TTGGTATAGAATGGAGAGAACGT AAAATAAAAAACACAAACTCGTA</td>
<td>NC_006088.3</td>
<td>140</td>
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<tr>
<td>RB1-M</td>
<td>GGTTGTTATAGAAGGAGAAAAGTC ATACCATTGCACAAATAAAAAACG</td>
<td>NC_006088.3</td>
<td>175</td>
</tr>
<tr>
<td>RB1-U</td>
<td>GGTTGTTATAGAAGGAGAAAAGTC ATACCATTGCACAAATAAAAAACG</td>
<td>NC_006088.3</td>
<td>172</td>
</tr>
</tbody>
</table>
4. Results

*Patterns of expression and cell-specific localization of DNMT1, DNMT3A and DNMT3B mRNAs in normal and cancerous ovaries of laying hens*

Laying hens are the best relevant animal model for research on epithelial ovarian cancer in women because their persistent daily ovulations lead to ovarian cancers resulting from a gene(s) mutations caused by genomic damage to the surface epithelium of the ovary. We have reported differential patterns of gene expression between normal and cancerous ovaries which are similar to those in women with ovarian cancers (Ahn et al., 2010; Jeong et al., 2012; Lee et al., 2012; Lim et al., 2012a; Lim et al., 2011a; Lim et al., 2012b). To determine if DNMTs are up- or down-regulated in ovarian cancer cells of our laying hen model, we performed RT-PCR and quantitative RT-PCR analyses. Results of the present study identified three DNMT mRNAs that are unique to ovarian carcinomas in laying hens (Figure 1A to -C). Further, quantitative PCR revealed that expression of DNMT1, DNMT3A and DNMT3B mRNAs increased 13.8- \((P < 0.01)\), 9.1- \((P < 0.05)\), and 2.7-fold \((P < 0.05)\) in the cancerous ovaries, respectively, as compared with normal ovaries of laying hens (Figure 1D to -F). Further, *in situ* hybridization analysis revealed that the three DNMT mRNAs were abundantly expressed in glandular epithelium (GE) of cancerous ovaries, but not in stroma and blood vessels (Figure 1G to -I). Consistent with results of PCR analyses, expression of DNMT mRNAs in GE of normal ovaries was extremely weak.
DNA methylation patterns in normal and cancerous ovaries of hens

To compare general methylation patterns in normal and cancerous ovaries from laying hens, we performed immunohistochemical analysis and immunofluorescence staining using an antibody to 5-methylcytocine (5 meC). As shown in Figure 2A and 2B, immunoreactive 5 meC protein was detected in nuclei of GE cells of both normal and cancerous ovaries. In addition, 5 meC was more abundant in cancerous ovaries compared with normal. This indicates that GE cells in cancerous ovaries have patterns with hypermethylation than normal ovaries but, Global DNA methylation patterns are existed both normal and cancerous ovaries.

DNA methylation pattern of promoter regions of DNMTs and tumor suppressor genes

To investigate the DNA methylation status of the promoter regions of selected tumor suppressor genes such as APC, CDKN2A, PTEN, BRCA2, and RB1, we performed methylation-specific PCR analysis. As illustrated in Figure 2C, the unmethylation status of APC, CDKN2A, and RB1 is higher than their methylation status in normal ovaries, whereas those genes are highly methylated in cancerous ovaries. Similarly, the product band intensity of the methylation or unmethylation statuses of the PTEN and BRCA2 promoter regions are equivalent in normal ovaries; but both regions are predominantly methylated in cancerous ovaries. This result indicates that the hypermethylation of the promoter regions of the tumor suppressor genes is likely responsible for the initiation and development of ovarian cancer in laying hens.
Post-transcriptional regulation of microRNA affecting DNMTs

Based on the possibility that expression of chicken DNMT genes is regulated at the post-transcriptional level by microRNAs (miRNAs), we performed a miRNA target validation assay. Analysis of potential miRNA binding sites within the 3′-UTR of the each DNMT gene using the miRNA target prediction database (miRDB; http://mirdb.org/miRDB/) revealed putative binding sites for miR-148a and miR-1612 (for DNMT1); miR-1596, miR-1687, miR-1741, and miR-1749 (for DNMT3A); and miR-16c, miR-222, and miR-1632 (for DNMT3B). Therefore, we determined whether these miRNAs influenced expressions of each DNMT gene via its 3′-UTR. A fragment of the 3′-UTR of each gene harboring binding sites for the miRNAs were cloned in downstream of the green fluorescent protein (GFP) reading frame, thereby creating a fluorescent reporter for function of the 3′-UTR region. After co-transfection of eGFP-3′-UTR of each gene and DsRed-miRNA, the intensity of GFP expression and percentage of GFP-expressing cells were analyzed by fluorescence microscopy and fluorescence activated cell sorting (FACS). As illustrated in Figure 3, in the presence of miR-1741 for DNMT3A, the intensity and percentage of GFP-expressing cells (100% in control vs. 73.15% in miR-1741) decreased (P < 0.01). However, in the presence of miR-1596, miR-1687, or miR-1749, neither the intensity nor percentage of GFP-expressing cells changed (data now shown). In addition, as shown in Figure 4, in the presence of miR-16c, miR-222, or miR-1632 for DNMT3B, there was a decrease (P < 0.01) in the percentage of GFP-expressing cells (100% in control vs. 85.3% in miR-16c, 40.3% in miR-222, and 25.9% in miR-1632). In the presence of miR-148a or miR-1612 for DNMT1, neither the intensity
nor percentage of GFP-expressing cells changed (data now shown). These results indicate that miR-1741, miR-16c, miR-222, or miR-1632 directly bind to DNMT3A or DNMT3B transcripts, respectively, and post-transcriptionally regulate expression of the DNMT3A and DNMT3B genes.
Figure 1. Expression, quantitation and localization of DNMTs in normal and cancerous ovaries from laying hens.

[A-C] RT-PCR and [D-F] q-PCR analyses were performed using cDNA templates from normal and cancerous ovaries of laying hens using chicken DNMT1, DNMT3A, DNMT3B and GAPDH primers. The asterisks denote statistically significant differences (**P < 0.01 and *P < 0.05). [G-I] In situ hybridization analyses of DNMT mRNAs in normal and cancerous ovaries of hens. Cross-sections of normal and cancerous ovaries of hens hybridized with antisense or sense chicken DNMT cRNA probes. Legend: GE, glandular epithelium. See Materials and Methods for a complete description of the methods.
Figure 2. Methylation patterns of DNMTs and tumor suppressor genes in normal and cancerous ovaries from laying hens.

Figure 3. *In vitro* target assay for microRNAs of the *DNMT3A* transcript.

[A] Diagram of *miR-1741* binding site in the *DNMT3A* 3’-UTR. [B] Expression vector map for eGFP with *DNMT3A* 3’-UTR and Ds-Red with *miR-1741*. [C and D] After co-transfection of pcDNA-eGFP-3’-UTR for the *DNMT3A* transcript and pcDNA-DsRed-miRNA for the *miR-1741*, the fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D].
Figure 4. *In vitro target assay of microRNAs on the DNMT3B transcript.*

[A] Diagram of *miR*-16c, *miR*-222, and *miR*-1632 binding sites in the DNMT3B 3’-UTR. [B] Expression vector map for eGFP with DNMT3B 3’-UTR and Ds-Red with each miRNA. [C and D] After co-transfection of pcDNA-eGFP-3’-UTR for the DNMT3B transcript and pcDNA-DsRed-miRNA for the *miR*-16c, *miR*-222, and *miR*-1632, the fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D].
5. Discussion

Key findings of the present study were that expression of the \textit{DNMT1}, \textit{DNMT3A} and \textit{DNMT3B} genes are abundantly expressed only in GE of cancerous ovaries as compared to normal ovaries of laying hens, and that expression of \textit{DNMT3A} and \textit{DNMT3B} genes are post-transcriptionally regulated by \textit{miR-1741}, \textit{miR-16c}, \textit{miR-222}, or \textit{miR-1632}, respectively. These results support our hypothesis that DNMTs are critical regulators of initiation, growth and development of epithelial-derived ovarian cancer in hens. Generally, tumorigenesis is associated with accumulation of genetic changes such as mutation, rearrangement, deletion and translocations in genes. However, these classical theories alone were unable to clarify the basis for carcinogenesis, and it is now understood that epigenetic events involving multiple interactions with DNMTs, small non-coding RNAs and tumor suppressor genes likely lead to ovarian carcinogenesis. In the present study, we mainly focused on multiple epigenetic mechanisms involved in the regulation of DNMT genes in normal and cancerous ovaries of laying hens, which are the most relevant animal model to identify biomarkers of human ovarian cancer such as CA125, cytokeratin, EGFR, Lewis Y, and erbB-2 and also expressed in carcinoma cells, but not normal cells in ovaries of laying hens (Anderson et al., 2010; Jackson et al., 2007; Johnson, 2009; Rodriguez-Burford et al., 2001).

As a major epigenetic modification, DNA methylation affects various biochemical processes such as regulation of gene transcription, maintenance of genomic stability and imprinting, and X-chromosome inactivation in mammals (Jones and Takai, 2001). In fact, all DNMTs have functional roles in regulation of DNA methylation. DNMT1, as a member
of the maintenance-type methyltransferase family, consists of an N-terminal regulatory domain, glycine-lysine repeat and C-terminal catalytic domains and is predominantly responsible for hemimethylated CpG dinucleotides in the mammalian genome (Robertson, 2002). Indeed, appropriate expression of DNMT1 is essential for the preservation of parental imprinting (Kurihara et al., 2008). For instance, in mice, although \textit{Dnmt1}^{-/-} embryonic stem cells are viable, have no obvious abnormalities related to growth rate or morphology and contain a small percentage of methylated DNA and methyltransferase activity, the \textit{Dnmt1}^{-/-} embryos are stunted in development and die during mid-gestation (Li et al., 1992). Furthermore, overexpression of DNMT1 is a hallmark of endometrioid carcinomas and prostate cancer (Lan et al., 2010) and it is also responsible for both \textit{de novo} and maintenance methylation of tumor suppressor genes in various human cancer cells (Jair et al., 2006). On the other hand, DNMT3A and DNMT3B could methylate hemimethylated or unmethylate CpG islands at the same rate. Although the general architecture of DNMT3 enzymes is very similar to that of DNMT1, their total length is shorter than DNMT1 and they have an unique tetrapeptide of proline-tryptophan-tryptophan-proline (PWWP) motif (Stec et al., 2000). Likewise \textit{Dnmt1}^{-/-}, \textit{Dnmt3a}^{-/-} and \textit{Dnmt3b}^{-/-} mice experience embryonic lethality during gestation or early in the neonatal period due to hypometylation of pericentromeric repeats (Okano et al., 1999). In addition, overexpression of either DNMT3A or DNMT3B is associated with tumorigenesis depending on cancer types in humans (Robertson et al., 1999; Socha et al., 2009). These results indicate that both DNMT3A and DNMT3B function as \textit{de novo} methyltransferases that play important roles in normal development and disease.

Consistent with previous reports, results of the present study demonstrate that expression levels of \textit{DNMT1}, \textit{DNMT3A} and \textit{DNMT3B}
genes are significantly increased in cancerous as compared with normal ovaries (Figure 1). Furthermore, all DNMT mRNAs were predominantly abundant in GE of cancerous ovaries. In fact, 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. This is especially true for ovaries of both avian and mammalian species, as these glandular structures are mainly detected in the endometrioid-type tumors with characteristics such as nuclear atypia, cribriform foci and atresia of stromal follicles (Barua et al., 2009). In addition, as illustrated in Figure 2A and 2B, immunoreactive 5-methylcytosine protein was predominantly detected in GE of normal and cancerous ovaries which indicates that these GE cells are undergoing DNA methylation in response to increased expression of DNMTs. Further, methylation-specific PCR data demonstrated that there was a significant increase in methylation patterns of the promoter regions of APC, CDKN2A, PTEN, BRCA2, and RB1 which are tumor suppressor genes. These results support the idea that epigenetic silencing of tumor suppressor genes by promoter CpG island hypermethylation is one of the most important regulatory mechanisms leading to the generation and proliferation of carcinomas (Esteller, 2007). Recently, Socha and colleagues reported that secreted protein acidic and rich in cysteine (SPARC) is down-regulated in ovarian cancer through aberrant promoter hypermethylation (Socha et al., 2009). Likewise, results of the present study indicate that hypermethylation of tumor suppressor genes in their promoter regions may initiate and sustain development of ovarian cancer in laying hens.

MicroRNAs (miRNAs) are small and non-coding RNAs of 18-23 nucleotides in length that regulate gene expression post-transcriptionally and alter cell fate by controlling translation of target mRNAs in diverse
tissues and cell types. Thus, miRNAs play crucial roles in various biological processes including vertebrate growth, development, differentiation and oncogenesis by regulating gene expression (Garzon et al., 2006). In the present study, our miRNA target validation assay demonstrated that in the presence of miR-1741 for DNMT3A, the intensity and percentage of GFP-expressing cells decreased ($P < 0.01$), but this did not occur in the presence of miR-1596, miR-1687, or miR-1749. Similarly, the presence of miR-16c, miR-222, or miR-1632 for DNMT3B, the percentage of GFP-expressing cells was decreased ($P < 0.01$). These results indicate that miR-1741, miR-16c, miR-222, or miR-1632 directly bind to the DNMT3A or DNMT3B transcript, respectively, and post-transcriptionally regulate expression of those genes.

Collectively, results of the present study are the first to demonstrate distinct cell-specific expression patterns for DNMTs genes and determine the methylation status of CpG islands of promoter regions of tumor suppressor genes between normal and cancerous ovaries of laying hens. Further, our results revealed that DNMT gene expression is post-transcriptionally regulated by several miRNAs critical to ovarian carcinogenesis of laying hens. DNA methylation is required for normal embryonic development, X-chromosome inactivation and gene imprinting in mammalian species, and its aberrant effects leading to promoter hypermethylation of tumor suppressor genes by inappropriate expression of DNMTs contributes to development of ovarian cancer. Therefore, results of the present study provide new insights into DNMTs with respect to epigenetic regulation and functional roles in ovarian carcinogenesis in laying hens that are likely highly relevant to the development of therapies for treatment of ovarian cancers in humans.
CHAPTER 4

DISTINCT EXPRESSION PATTERN
AND POST-TRANSCRIPTIONAL
REGULATION OF CELL CYCLE
GENES IN THE GLANDULAR
EPITHELIA OF AVIAN OVARIAN
CARCINOMAS
1. Abstract

The cell cycle system is controlled in a timely manner by three groups of cyclins, cyclin dependent kinases and cyclin dependent kinase inhibitors. Abnormal alterations of cell cycle regulatory mechanisms are a common feature of many diseases including numerous tumor types such as ovarian cancer. Although a variety of cell cycle regulatory genes are well known in mammalian species including human and mice, they are not well studied in avian species, especially in laying hens which are known to be the best animal model for human ovarian carcinogenesis. Therefore, in the present study, we focused on comparative expression and regulation of expression of candidate genes which might be involved in the cell cycle program in surface epithelial ovarian cancer in laying hens.

Our current results indicate that expression levels of cell cycle gene transcripts are greater in cancerous as compared to normal ovaries. In particular, cyclin A2 (CCNA2), CCND1, CCND2, CCND3, CCNE2, cyclin dependent kinase 1 (CDK1), CDK3, CDK5, cyclin dependent kinases inhibitor 1A (CDKN1A) and CDKN1B were upregulated predominantly in the glandular epithelia of cancerous ovaries from laying hens. Further, several microRNAs (miRs), specifically miR-1798, miR-1699, miR-223 and miR-1744 were discovered to influence expression of CCND1, CCNE2, CDK1, and CDK3 mRNAs, respectively, via their 3′-UTR which suggests that post-transcriptional regulation of gene expression influences their expression in laying hens. Moreover, miR-1626 influenced CDKN1A expression and miR-222, miR-1787 and miR-1812 regulated CDKN1B expression via their 3′-UTR regions. Collectively, results of the present study demonstrate increased expression
of cell cycle-related genes in cancerous ovaries of laying hens and indicate that expression of these genes is post-transcriptionally regulated by specific microRNAs.

**Keywords:** cell cycle, ovary, cancer, chicken, microRNA
2. Introduction

Epithelial ovarian cancer (EOC), the most lethal gynecological malignancy, claims the lives of over 15,000 women and 22,000 are diagnosed with the disease in the US each year (Bovicelli et al., 2011). However, over 75% of women diagnosed are at an advanced stage of EOC, because it is generally asymptomatic and there is no specific biomarker(s) for early detection (Bast et al., 2002). Therefore, to prevent and cure this lethal disease and to improve the long-term survival of patients with EOC, the most promising approach is to identify markers for early diagnosis. To overcome the problem that EOC is rarely detected at an early stage, many animal models have been developed, but they have not proven to be successful. For instance, genetically manipulated rodent models have been used to elucidate some aspects of the pathogenesis and etiologies of EOC; however, 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). In fact, the laying hen 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). Thus, the laying hen is a unique animal model for human EOC research aimed at development of a biomarker(s) for detection and early diagnosis, as well as for discovery of anti-cancer drugs/biomaterials for prevention and treatment of this deadly disease.

The cell cycle in most eukaryotic cells includes a series of coordinated events: cell proliferation/growth, replication of genetic material, segregation of the duplicated chromosomes and cell division (Vermeulen et al., 2003). In general, the cell division cycle in mammals
is precisely and harmoniously regulated in a timely manner by different active heterodimeric complexes that include cyclin dependent kinases (CDKs) and their cognate cyclin partners, as well as CDK inhibitors (CDKIs) (D'Andrilli et al., 2004). Thus, tumor development frequently results when there is deregulation of the cell cycle control system including abnormal regulation of expression of cell cycle genes (Bovicelli et al., 2011). In human cancerous tissues, such as neoplasms, different families of cell cycle genes and regulators are frequently mutated and dysfunctional (D'Andrilli et al., 2004). Although expression and functional roles of many CDKs, cyclins and CDKIs are well studied in mammalian species, including humans and mice, little is known about their expression and regulation in avian species, especially laying hens. Therefore, the objectives of this study with laying hens were to determine: 1) the expression of cyclins, CDKs and CDKIs in normal and cancerous ovaries; and 2) whether cyclins, CDKs and CDKIs are regulated by post-transcriptional actions of specific microRNAs (miRs) using a miR target validation assay. Our results confirm that the laying hen is a unique model for the research on human ovarian cancer and cell cycle-related genes and those regulatory factors for cell cycle-related genes play a key role in ovarian carcinomas. These cell cycle-related genes may be important targets for discovery of a biomarker(s) for diagnosis and evaluation of therapeutics designed to treat EOC in women.
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) laying hens were subjected to standard management practices at the Seoul National University Animal Farm, Seoul, Republic of 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, as well as standard management practices for feeding and husbandry.

Tissue Samples

In this study, a total of 136 laying hens (88 over 36 months of age and 48 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 laying hens. We examined tumor stage in 10 hens with cancerous ovaries based on characteristic features of chicken ovarian cancers. 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 in liquid nitrogen or fixed in 4%
paraformaldehyde for further analyses. Frozen tissue samples were cut into 5- to 7-mm pieces before being 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 μm and stained with hematoxylin and eosin. Epithelial ovarian cancers in laying hens were classified based on their cellular subtypes and patterns of cellular differentiation with reference to ovarian malignant tumor types in humans.

**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.

**Semiquantitative RT-PCR analysis**

The expression of mRNAs for *cell cycle genes* in normal and cancerous ovaries of laying hens was assessed using semi-quantitative RT-PCR as described previously (Song et al., 2007). Information on the primer sets is provided in Table 1. The cDNA was synthesized from total cellular RNA (2 ug) using random hexamer (Invitrogen, Carlsbad, CA) and oligo (dT) primers and AccuPower® RT PreMix (Bioneer, Daejeon, Korea). The cDNA was diluted (1:10) in sterile water before use in PCR.
The primers, PCR amplification and verification of their sequences were conducted as described previously (Song et al., 2007). 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™ XR+ system with Image Lab™ software (Bio-Rad).

**Quantitative RT-PCR Analysis**

Total RNA was extracted from normal and cancerous ovarian tissue 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 (Biotium, Hayward, CA, 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. Each target gene and GAPDH were analyzed in triplicate. Using the standard curve method, we determined the expression quantities of the examined genes using the standard curves and \( C_t \) values, and normalized them to GAPDH expression values. 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 rose statistically above 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\textsubscript{t} of the control ovaries. Information on the primer sets is provided in Table 2.

**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 μm 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 in TE buffer (100 mMTris-HCl, 50 mM EDTA, pH 8.0) containing 5 μg/ml Proteinase K (Sigma Chemical Co., St. Louis, MO) 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 with 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 overnight at 42°C in a humidified chamber. 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 a 2% normal sheep serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche, Indianapolis, IN). The signal was visualized by exposure to a solution containing 0.4 mM 5-bromo-4-chloro-3-indolylphosphate, 0.4 mM nitrobluetetrazolium, and 2 mM levamisole (Sigma Chemical Co., St. Louis, MO).

**MicroRNA Target Validation Assay**

The 3’-UTR of selected genes 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 pcDNA3eGFP (Clontech, Mountain View, CA) 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 each microRNA were designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miRNA). The pcDNA-eGFP-3’UTR and pcDNA-DsRed-miRNA (4µg) 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).

**Statistical Analyses**

Data for quantitative PCR were subjected to analysis of variance (ANOVA) according to the general linear model (PROC-GLM) of the SAS program (SAS Institute, Cary, NC). Data are presented as mean ± SEM unless otherwise stated. Differences in the variance between normal and each classification of cancerous ovary group were analyzed using the $F$ test, and differences in the means were subjected to Student’s $t$ test. Differences were considered significant at $P < 0.05$. 
Table 1. Information on primers for RT-PCR analyses

<table>
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<tr>
<th>Gene</th>
<th>Sequence (5’→3’): forward and reverse</th>
<th>GenBank accession no.</th>
<th>Product Size (bp)</th>
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<td>CCND1</td>
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<td>CCNE2</td>
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<td>NM_204305.1</td>
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4. Results

Comparative expression of cyclin genes in normal and cancerous ovaries from laying hens

To determine tissue-specific expression patterns of the cell cycle-related genes in normal (n=5) and cancerous (n=10) ovaries from laying hens, we performed RT-PCR, and quantitative PCR analyses. As illustrated in Figure 1, expression of cyclin A2 (CCNA2), CCND1, CCND2, CCND3 and CCNE2 mRNAs was 3.42- (P < 0.01), 1.32- (P < 0.05), 2.41- (P < 0.01), 3.31- (P < 0.05) and 2.36-fold (P < 0.001) greater in cancerous ovaries from hens. Next, cell-specific localization of these genes in the normal and cancerous ovaries was determined using in situ hybridization analysis. The mRNAs for CCNA2, CCND1, CCND2, CCND3 and CCNE2 were predominantly in the glandular epithelium (GE), but there was very little or weak expression of these genes in the luminal epithelium (LE), stromal cells and blood vessels in the both normal and cancerous ovaries. Moreover, consistent with the results from the quantitative PCR analyses, mRNA levels for all genes were significantly more abundant in GE of cancerous as compared with normal ovaries.

Comparative expression of cyclin dependent kinase and cyclin dependent kinase inhibitor genes in normal and cancerous ovaries of laying hens

As shown in Figure 2, the results from RT-PCR and quantitative PCR analyses showed that expression of mRNAs for cyclin dependent
kinase 1 (CDK1), CDK3 and CDK5 were 2.87- ($P < 0.01$), 5.18- ($P < 0.01$) and 3.66-fold ($P < 0.01$) greater in cancerous ovaries from hens, respectively. Interestingly, cyclin dependent kinase inhibitor 1A (CDKN1A) and CDKN1B mRNAs were 5.62- ($P < 0.01$) and 2.31-fold ($P < 0.05$) more abundant in cancerous as compared with normal ovaries. Compatible with these results, in situ hybridization analyses demonstrated increased expression of CDK1, CDK3, CDK5, CDKN1A and CDKN1B genes in the GE of cancerous as compared with normal ovaries from laying hens.

**Post-transcriptional regulation of specific cell cycle regulatory genes by microRNAs in ovaries from laying hens.**

A microRNA (miR) target validation assay was used to test the hypothesis that expression of cell cycle genes is regulated at the post-transcriptional level by specific miRNAs. Analysis of potential miRNA binding sites within the 3′-UTR of the six cell cycle regulatory genes was performed using the miRNA target prediction database (miRDB; http://mirdb.org/miRDB/). This analysis revealed putative binding sites for several chicken miRNAs (miR-1798 for CCND1; miR-1699 for CCNE2; miR-223 for CDK1; miR-1744 for CDK3; miR-1626 for CDKN1A; and miR-222, miR-1787 and miR-1812 for CDKN1B), but not for the other four genes of interest. Therefore, we determined whether these miRNAs influenced expressions of cell cycle regulatory genes via the 3′-UTR. A fragment of the 3′-UTR of each gene harboring binding sites for the miRs was cloned in downstream of the green fluorescent protein (GFP) reading frame, thereby creating a fluorescent reporter for function of the 3′-UTR region. After co-transfection of eGFP-3′-UTR of
each gene and DsRed-miRNA, the intensity of GFP expression and percentage of GFP-expressing cells were analyzed by fluorescence microscopy and FACS. As illustrated in Figures 3 and 4, in the presence of miR-1798 and miR-1699, the intensity and percentage of GFP-CCND1-expressing cells (12.7% in control vs. 4.2% in miR-1798) and GFP-CCNE2-expressing cells (96.4% in control vs. 71.4% in miR-1699) decreased ($P < 0.01$). In addition, as shown in Figures 5 and 6, in the presence of miR-223 and miR-1744, the intensity and percentage of GFP-CDK1-expressing cells (17.2% in control vs. 1.3% in miR-223) and GFP-CDK3-expressing cells (16.1% in control vs. 6.8% in miR-1744) were decreased ($P < 0.01$). Moreover, in the presence of miR-1626, the intensity and percentage of GFP-CDKN1A-expressing cells (54.6% in control vs. 34.7% in miR-1626) were decreased ($P < 0.01$) (Figure 7). In addition, for CDKN1B, in the presence of miR-222, miR-1787 and miR-1812, the intensity and percentage of GFP-CDKN1B-expressing cells (29.0% in control vs. 15.6% in miR-1787, 12.6% in miR-1812, 9.8% in miR-222) were decreased ($P < 0.01$) (Figure 8). These results indicate that at least one to three miRNAs bind directly to the cell cycle-related gene transcripts to regulate expression.
Figure 1. Comparative expression patterns for CCNA2, CCND1, CCND2, CCND3 and CCNE2 mRNAs in normal and cancerous ovaries from laying hens.

RT-PCR and quantitative RT-PCR analysis were conducted using cDNA templates from normal and cancerous ovaries of laying hens using chicken CCNA2, CCND1, CCND2, CCND3 and CCNE2 and chicken GAPDH specific primers (left panel). These experiments were conducted in triplicate and values normalized to those for GAPDH. In situ hybridization analysis indicates cell specific expression patterns for CCNA2, CCND1, CCND2, CCND3 and CCNE2 mRNAs in both normal and cancerous ovaries from laying hens (right panel).
Figure 2. Comparative expression patterns for CDK1, CDK3, CDK5, CDKN1A and CDKN1B mRNAs in normal and cancerous ovaries from laying hens

RT-PCR and quantitative RT-PCR analyses were conducted using cDNA templates from normal and cancerous ovaries of laying hens with chicken CDK1, CDK3, CDK5, CDKN1A and CDKN1B and chicken GAPDH specific primers (left panel). These experiments were conducted in triplicate and normalized to values for GAPDH. *In situ* hybridization analysis indicates cell specific expression patterns of CDK1, CDK3, CDK5, CDKN1A and CDKN1B mRNAs both normal and cancerous ovaries from laying hens (right panel).
Figure 3. *In vitro* target assay for microRNAs on the *CCND1* transcript.

[A] Diagram of miR-1798 binding sites in the 3’-UTR of the *CCND1* gene. [B] Expression vector maps for eGFP within the 3’-UTR of the *CCND1* gene and Ds-Red within each miRNA. The 3’-UTR of the *CCND1* 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 the miRNA expression vector was designed to co-express DsRed and each miRNA (pcDNA-DsRed-miRNA) (lower panel). [C and D] After co-transfection of pcDNA-eGFP-3’UTR for the *CCND1* transcript and pcDNA-DsRed-miRNA for miR-1798, the fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D]. See Materials and Methods for complete description.
Figure 4. *In vitro* target assay for microRNAs on the CCNE2 transcript

[A] Diagram of miR-1699 binding sites in the 3’-UTR of the CCNE2 gene.  
[B] Expression vector maps for eGFP within the 3’-UTR of the CCNE2 gene and Ds-Red within each miRNA. The 3’-UTR of the CCNE2 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 the miRNA expression vector was designed to co-express DsRed and each miRNA (pcDNA-DsRed-miRNA) (lower panel).  
[C and D] After co-transfection of pcDNA-eGFP-3’UTR for the CCNE2 transcript and pcDNA-DsRed-miRNA for the miR-1699, the fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D]. See *Materials and Methods* for complete description.
Figure 5. *In vitro* target assay for microRNAs on the CDK1 transcript

[A] Diagram of miR-223 binding sites in the 3’-UTR of the CDK1 gene.  
[B] Expression vector maps for eGFP within the 3’-UTR of the CDK1 gene and Ds-Red within each miRNA. The 3’-UTR of the CDK1 transcript was subcloned between the eGFP gene and the polyA tail to generate the fusion construct for the GFP transcript following the miRNA target 3’-UTR (pcDNA-eGFP-3’UTR) (upper panel) and the miRNA expression vector was designed to co-express DsRed and each miRNA (pcDNA-DsRed-miRNA) (lower panel).  
[C and D] After co-transfection of pcDNA-eGFP-3’UTR for the CDK1 transcript and pcDNA-DsRed-miRNA for the miR-223, the fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D]. See Materials and Methods for complete description.
Figure 6. *In vitro* target assay for microRNAs on the CDK3 transcript

[A] Diagram of miR-1744 binding sites in the 3’-UTR of the CDK3 gene. 
[B] Expression vector maps for eGFP within the 3’-UTR of the CDK3 gene and Ds-Red within each miRNA. The 3’-UTR of the CDK3 transcript was subcloned between the eGFP gene and the polyA tail to generate the fusion construct for the GFP transcript following the miRNA target 3’-UTR (pcDNA-eGFP-3’UTR) (upper panel) and the miRNA expression vector was designed to co-express DsRed and each miRNA (pcDNA-DsRed-miRNA) (lower panel). [C and D] After co-transfection of pcDNA-eGFP-3’UTR for the CDK3 transcript and pcDNA-DsRed-miRNA for the miR-1744, the fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D]. See *Materials and Methods* for complete description.
Figure 7. *In vitro* target assay for *microRNAs* in the *CDKN1A* transcript

[A] Diagram of *miR-1626* binding sites in 3’-UTR of the *CDKN1A* gene.

[B] Expression vector maps for eGFP within the 3’-UTR of the *CDKN1A* gene and Ds-Red within each miRNA. The 3’-UTR of the *CDKN1A* 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 the miRNA expression vector was designed to co-express DsRed and each miRNA (pcDNA-DsRed-miRNA) (lower panel).

[C and D] After co-transfection of pcDNA-eGFP-3’UTR for the *CDKN1A* transcript and pcDNA-DsRed-miRNA for the *miR-1626*, the fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D]. See *Materials and Methods* for complete description.
Figure 8. *In vitro* target assay for *microRNAs* in the *CDKN1B* transcript

[A] Diagram of miR-222, miR-1787 and miR-1812 binding sites in the 3’-UTR of the *CDKN1B* gene.  [B] Expression vector maps for eGFP within the 3’-UTR of the *CDKN1B* gene and Ds-Red within each miRNA. The 3’-UTR of the *CDKN1B* 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 the miRNA expression vector was designed to co-express DsRed and each miRNA (pcDNA-DsRed-miRNA) (lower panel).  [C and D] After co-transfection of pcDNA-eGFP-3’UTR for the *CDKN1B* transcript and pcDNA-DsRed-miRNA for the miR-222, miR-1787 and miR-1812, the fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D]. See Materials and Methods for complete description.
5. Discussion

Results of the present study provide the first evidence of significant differences in expression of \textit{CCNA2, CCND1, CCND2, CCND3, CCNE2, CDK1, CDK 3, CDK5, CDKN1A} and \textit{CDKN1B} genes in cancerous as compared to normal ovaries of laying hens. In addition, our results indicate that several microRNAs (miRs), specifically \textit{miR-222, miR-223, miR-1626, miR-1699, miR-1744, miR-1787, miR-1798} and \textit{miR-1812} interact with sites in the 3'-UTR of the cell cycle genes and regulatory factors affecting cell cycle genes including \textit{CCND1, CCNE2, CDK1, CDK3, CDKN1A} and \textit{CDKN1B} to influence post-transcriptional regulation of its expression in laying hens. These results support our hypothesis that cell cycle genes are critical regulators for growth and developmental aspects of epithelial cells of the ovaries of hens and that there is dysregulation of their level of expression as ovaries of laying hens transition from a normal to a cancerous state.

Cyclins are a family of proteins that control the cell cycle by binding and activating cyclin-dependent kinases. As illustrated in Figures 1, D-type cyclins, which are G\textsubscript{1} phase regulators of the cell cycle (Bovicelli et al., 2011; D'Andrilli et al., 2004), such as \textit{CCND1, CCND2} and \textit{CCND3} are predominantly found in cancerous ovaries, but there was weak or little expression in normal ovaries of laying hens in the present study. In humans, \textit{CCND1} is frequently overexpressed in a variety of tumor types and is associated with carcinogenesis and metastasis (Masamha and Benbrook, 2009). Dhar and colleagues reported that expression of \textit{CCND1} was up-regulated in about 90% of patients with EOC and expressed mainly in both borderline and invasive tumours.
without any association between immunoreactive protein overexpression and stage of tumor differentiation or grade of tumor (Dhar et al., 1999). In the present study, we found CCND1, CCND2 and CCND3 mRNAs in the nucleus and the cytoplasm of epithelial cells of normal ovaries, but exclusively in the cytoplasmic compartment of the epithelial cells in cancerous ovaries of laying hens. This result is consistent with deregulation of CCND1 expression leading to localization of the protein in both the cytoplasmic and nuclear compartments of cells from cancerous ovaries (Dhar et al., 1999). Interestingly, although CCND2 and CCND3 are not overexpressed in human ovarian cancer (Courjal et al., 1996), messenger RNA expression levels for both CCND2 and CCND3 were significantly upregulated in cancerous ovaries of laying hens. The difference in expression of these genes between humans and laying hens should be elucidated. Expression of CCNE2 mRNA, a G1-S phase regulator (Vermeulen et al., 2003), in cancerous ovaries from hens was 2.36-fold \((P < 0.001)\) greater than in normal ovaries, and mainly detected in the glandular epithelium (GE). This result supports the idea that CCNE protein is valuable prognostic factors for EOC patients because amplification and over-expression of the CCNE1 gene occurs in many cases with a gradual increase from benign to borderline to malignant tumors (Courjal et al., 1996; Sui et al., 2001). In addition, CCNA2 mRNA, an S phase regulators (Bovicelli et al., 2011; Vermeulen et al., 2003), was found predominantly in GE and its expression was 3.42-fold \((P < 0.01)\) greater in cancerous ovaries of laying hens. In humans, CCNA expression increased in the ovarian carcinoma cell line compared with normal cells (Barboule et al., 1998) and CCNA protein was detected mainly in serous and endometrioid carcinomas, but not in mucinous and clear cell carcinomas (Shimizu et al., 1999).
Cyclin dependent kinases (CDKs) are the catalytic subunits of a large family of heterodimeric serine/threonine protein kinases and play essential roles in controlling progression through the cell cycle (Malumbres and Barbacid, 2005). In the present study, we also found that CDK1, CDK3 and CDK5 mRNAs were up-regulated predominantly in the GE of cancerous ovaries, but there was weak or no expression in normal ovaries of laying hens. In humans, overexpression of CDK2 was found in only 6% of EOC patients, but its level of expression was positively correlated with CCNE abundance, suggesting that overexpression of both CDK2 and CCNE is significantly associated with development of malignant ovarian tumors (Marone et al., 1998; Sui et al., 2001). In addition, Masciullo and colleagues found that CDK4 was overexpressed in 14% to 15% of ovarian tumors (Masciullo et al., 1997) and that its activity in malignant ovarian tumors was significantly greater than in benign tumors (Sui et al., 2001). These results suggest that CDK4 activity may play an important role in ovarian carcinogenesis. Collectively, results of the present study strongly indicate that CDK activity is regulated by cyclin synthesis and degradation, and that orderly progression through the cell cycle requires coordinated activation of the CDK proteins by binding to the cyclin partner (D'Andrilli et al., 2004). Furthermore, the results confirm that the laying hen is an appropriate animal model for identifying and developing biomarkers for early diagnosis and evaluation of therapeutics for treatment of ovarian cancer (Ahn et al., 2010; Barua et al., 2009; Giles et al., 2006; Giles et al., 2004; Lee et al., 2012; Lim et al., 2012a; Lim et al., 2011a; Lim et al., 2012b; Stammer et al., 2008).

MicroRNAs, short and noncoding RNAs of 18 to 23 nucleotides in length, regulate complex patterns of gene expression post-transcriptionally and are capable of defining and altering cell fate by
silencing translational of gene transcripts through cleavage of their target mRNAs through base pairing at partially or fully complementary sites (Garzon et al., 2006). As shown in Figures 3-6, miR-1798 and miR-1699 influence the expression of CCND1 and CCNE2, respectively, while miR-223 and miR-1744 regulate expression of CDK1 and CDK3, respectively. By regulating post-transcriptional events, miRs affect function of a number of cellular processes in development, differentiation and oncogenesis (Bartel, 2004; Gregory et al., 2005; Lu et al., 2005). Results of the present study demonstrated that miR-1798 inhibits expression of CCND1 in laying hens. In human ovarian cancer, deregulation of CCND1 expression mainly occurs without any gene amplification (Dhar et al., 1999). Thus, we suggest dysfunction of miR-1787 leads to the overexpression of CCND1 in cancerous ovaries of laying hens. In fact, deregulation of miRs is generally considered to be a prerequisite for initiation and progression of carcinogenesis in humans. For instance, functional overexpression of miR-31 inhibits proliferation and induces apoptosis in a variety of serous-type ovarian cancer cell lines, such as SKOV3, with a dysfunctional p53 pathway (Creighton et al., 2010). In addition, miR expression of transcriptional targets of p53 (i.e. miR-34b and miR-34c) is markedly down-regulated in human EOC tissues (Zhang et al., 2008).

These results indicated that miRs may be useful in predicting outcomes of many diverse carcinomas, including EOC. On the other hand, as shown in Figure 2, there is overexpression of cyclin dependent kinase inhibitor 1A (CDKN1A) and CDKN1B mRNAs in cancerous ovaries of laying hens. CDKN1A and CDKN1B (also known as p21/WAF1 and p27, respectively) are potent CDK inhibitors and act as regulators in G1 phase of cell cycle. In addition, our miR target validation assay demonstrated
that miR-1626 regulates CDKN1A expression and miR-222, miR-1787 and miR-1812 influence post-transcriptional modification of transcripts of the CDKN1B gene. These results suggest that down-regulation of these miRs might contribute to the overexpression of cell cycle genes and regulatory factors in chicken ovarian cancer and to transcriptional deregulation of many genes in the genome, that may lead to uncontrolled carcinogenesis.

Collectively, results of the present study demonstrate that cyclins, their associated kinases and inhibitors play a key role in controlling the cell cycle and that disregulation of these molecules can potentially lead to uncontrolled cell proliferation, growth and loss of function in cells that leads to ovarian tumorigenesis in laying hens. Furthermore, post-transcriptional regulation of the specific miRs that influence expression of cell cycle genes likely leads to an alternative mechanism(s) for regulation of their expression. Although results of this study indicate that various miRs might be involved in many different oncogenic/carcinogenic pathways, details of altered expression patterns and their relevance to EOC remain to be elucidated. Thus, further research is clearly required to unravel the mechanism(s) for post-transcriptional regulation of cell cycle-dependent gene expression and different oncogenic pathways leading to ovarian carcinogenesis in women and in laying hens.
CHAPTER 5

CHICKEN PLEIOTROPHIN GENE: IDENTIFICATION AND REGULATIONS OF TISSUE SPECIFIC EXPRESSION BY ESTROGEN IN THE OVIDUCT AND DISTINCT EXPRESSION PATTERN IN THE GLANDULAR EPITHELIA OF OVARIAN CARCINOMAS
1. Abstract

Pleiotrophin (PTN) is a developmentally-regulated growth factor which is widely distributed in various tissues and also detected in many kinds of carcinomas. However, little is known about the PTN gene in chickens. In the present study, we found chicken PTN to be highly conserved with respect to mammalian PTN genes (91-92.6%) and its mRNA was most abundant in brain, heart and oviduct. This study focused on the PTN gene in the oviduct where it was detected in the glandular (GE) and luminal (LE) epithelial cells. Treatment of young chicks with diethylstilbesterol induced PTN mRNA and protein in GE and LE, but not in other cell types of the oviduct. Further, several microRNAs, specifically miR-499 and miR-1709 were discovered to influence PTN expression via its 3′-UTR which suggests that post-transcriptional regulation influences PTN expression in chickens. We also compared expression patterns and CpG methylation status of the PTN gene in normal and cancerous ovaries from chickens. Our results indicated that PTN is most abundant in the GE of adenocarcinoma of cancerous, but not normal ovaries of hens. Bisulfite sequencing revealed that 30- and 40% of -1311 and -1339 CpG sites are demethylated in ovarian cancer cells, respectively. Collectively, these results indicate that chicken PTN is a novel estrogen-induced gene expressed mainly in the oviductal epithelia implicating PTN regulation of oviduct development and egg formation, and also suggest that PTN is a biomarker for epithelial ovarian carcinoma that could be used for diagnosis and monitoring effects of therapies for the disease.

Keywords: PTN, chicken, oviduct, ovary, cancer
2. Introduction

Pleiotrophin (PTN), also known as heparin-binding growth factor 8 (HBGF-8) and heparin-binding growth associated molecule (HB-GAM), is a low molecular weight protein (about 18 kDa) which was originally isolated from the bovine uterus (Milner et al., 1989). As a member of developmentally regulated cytokine/growth factor family, it is widely distributed in various tissues and especially plays pivotal roles in neurogenesis and epithelial mesenchymal interactions through promoting cell growth and migration during early embryo differentiation and morphogenesis (Li et al., 1990; Muramatsu, 2002; Perez-Pinera et al., 2007). PTN binds to its cognate receptor protein tyrosine phosphatase beta/zeta (RPTP β/ζ) (Meng et al., 2000) and then activates several cytosolic proteins such as CTNNB1 (beta-catenin), ADD2 (beta-adducin), FYN (Fyn oncogene related to SRC, FGR, YES) and ALK (anaplastic lymphoma receptor tyrosine kinase) for many different cellular functions and systems (Perez-Pinera et al., 2007). In addition, PTN is a proto-oncogene (Chauhan et al., 1993) expressed in malignant tumors and cell lines of various organs such as breast, prostate, colon, lung and skin and is thought to be involved in tumor angiogenesis and metastasis (Fang et al., 1992; Jager et al., 1997; Perez-Pinera et al., 2007; Vacherot et al., 1999; Wu et al., 2005). In spite of the fact that PTN is involved in the regulation of cellular development and differentiation, and the etiology of carcinogenesis in many vertebrates, little is known about its expression and regulation by steroid hormones in the oviduct or its expression in normal and cancerous ovaries of chickens.

The chicken oviduct is one of best animal models for studies of
organ development and differentiation, and biological actions and signaling pathways for steroid hormones such as estrogen (Dougherty and Sanders, 2005). As a representative sex hormone in female reproductive organs, estrogen not only regulates reproductive behavior but also stimulates epithelial cells within the immature oviduct to transform into tubular gland cells via proliferation and cytodifferentiation, as well as transactivation of oviduct-specific genes such as ovalbumin during development of the chicken oviduct (Palmiter and Wrenn, 1971; Socher and Omalley, 1973). In addition, estrogen plays a pivotal role in calcium metabolism and calcification of the eggshell prior to oviposition in laying hens (Bar, 2009; Hincke et al., 2010), and exogenous estrogen administration to postnatal chicks induces cellular hyperplasia and hypertrophy of the oviduct resulting in its rapid growth rate and maturation (Dougherty and Sanders, 2005; Song et al., 2011b).

The laying hen is a unique animal model for research on human ovarian cancer because it spontaneously develops epithelial cell-derived ovarian cancer as in women (Ahn et al., 2010; Ansenberger et al., 2009; Barua et al., 2009; Lim et al., 2011b; Stammer et al., 2008). Indeed, ovarian cancer is the most lethal gynecological disease as well as the 5th leading cause of cancer-derived deaths among women in the U.S.A. (Cvetkovic, 2003; Jemal et al., 2007; Wong and Auersperg, 2003), because it is rarely diagnosed at an early stage due to the lack of a specific biomarker(s) for early detection and it is generally asymptomatic (Barua et al., 2009; Goodman et al., 2003; Pepe et al., 2001). Among three types of ovarian cancers, i.e., epithelia-, germ cell-and stroma-derived malignant tumors (Kurman and Shih Ie, 2008; Kurman et al., 2008), germinal epithelia-derived ovarian cancer (EOC) accounts for over 90% of ovarian cancer incidences in women (Auersperg et al., 2001). This high rate of
EOC incidence likely results from incessant ovulation and menstrual cycles that lead to genomic damage and mutations in genes in the ovarian surface epithelium (Auersperg et al., 1998; Murdoch et al., 2005). To investigate and elucidate the etiological and pathological aspects of EOC, several rodent models have been developed through biotechnological manipulation, but they have many limitations and obstacle associated with clinical relevance because of the non-spontaneous nature and physiologically distinct differences in their EOC (Barua et al., 2009; Stakleff and Von Gruenigen, 2003; Vanderhyden et al., 2003). Meanwhile, the chicken spontaneously develops EOC at a high rate as occurs in women and shows very similar morphological characteristics to that of EOC in women (Ahn et al., 2010; Ansenberger et al., 2009; Barua et al., 2009; Lim et al., 2011b; Stammer et al., 2008). Therefore, the chicken EOC could be used to develop anti-cancer drugs and biomarkers for early diagnosis and therapies to prevent adverse outcomes of EOC in women.

We reported that the avian homolog of the human PTN transcript is highly expressed in chicks treated with the synthetic estrogen agonist diethylstilbestrol (DES) (Song et al., 2011a). However, little is known about the expression and function of PTN in most species except humans and mice (Askew et al., 2007). Therefore, the objectives of the present study were to: 1) investigate tissue- and cell-specific expression of the PTN gene in chickens; 2) determine whether estrogen regulates expression of PTN during oviduct development in chicks; 3) determine whether PTN is regulated by post-transcriptional actions of specific microRNAs; 4) compare differential expression of PTN in normal and cancerous ovaries from hens and 5) examine CpG methylation status in the upstream promoter region of the PTN gene in normal and cancerous ovarian cells from hens. Results of current study indicate that PTN is a novel estrogen-
stimulated gene during development of the chicken oviduct and that it may be a candidate gene for further research on its role in tumorigenesis leading to EOC in the laying hen.
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) laying hens and chicks 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 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- 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 μm.

**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 μm 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 the manufacturer’s recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

**Semiquantitative RT-PCR analysis**

The expression of *PTN* mRNA in normal and cancerous ovaries of chickens 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® RT PreMix (Bioneer, Daejeon, Korea). The cDNA was diluted (1:10) in sterile water before use in PCR. For *PTN*, the sense primer (5′-TGC TCT CCT GGC ACT TGT CT-3′) and
antisense primer (5′-CTT GGA TTC TTG AGG TTT GGG-3′) amplified a 414-bp product. For ACTB (housekeeping gene; beta-actin), the sense primer (5′-GTG TGA TGG TTG GTA TGG GC-3′) and antisense primer primer (5′-TTT CTC TCT CGG CTG TGG TG-3′) amplified a 498-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, 60°C for 40 sec (for PTN and ACTB) and 72°C for 1 min for 35 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™ XR+ system with Image Lab™ software (Bio-Rad).

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). 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 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_T values,
and normalized them using \textit{GAPDH} expression quantities. The 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 a melting curve program (increasing the temperature from 55°C to 95°C at a rate of 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\textsubscript{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\textsubscript{T} of the control oviduct.

\textbf{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 TOPO® vector (invitrogen). After verification of the sequences, plasmids containing the correct gene sequences were amplified with T7- and SP6-specific primers (T7:5’-TGT AAT ACG ACT CAC TAT AGG G-3’; SP6:5’-CTA TTT AGG TGA CAC TAT AGA AT-3’) 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, embedded in paraffin and sectioned at 5 μm 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, they were digested with 5 μg/ml proteinase K (Sigma) in TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) at 37°C. After postfixation 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 with 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, 0.2 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 2X SSC at 37°C, 15 min 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 a 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).

**Immunohistochemistry**

Immunocytochemical localization of PTN protein in normal and cancerous ovaries from chickens was performed as described previously (Song et al., 2006) using a rabbit polyclonal antibody to PTN (Catalog
number ab-95391; AbCam, CA, USA) at a final dilution of 1:200 (0.2µg/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 rabbit IgG at the same final concentration.

**Prediction of Transcription Factor-Binding cis-Elements**

The presence of transcription factor-binding *cis*-elements within the PTN promoter region was predicted using a bioinformatics tool for orthologous sequences (TFSEARCH ver. 1.3; 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 Epitect Bisulfite kit (QIAGEN, Doncaster, Australia) according to the manufacturer’s instructions. For amplifying the converted DNA, PCRs were performed with forward (5'-GGA TTT TTG TGT AAA TTT GGA GTA G-3') and reverse (5'-TTC CAA AAT CCA AAC AAT TTC TAT C-3') primers, which included the upstream region of the *PTN* gene, as follows: 95°C for 3 min, 35 cycles at 95°C for 1min, 56°C for 1min, 72°C for 2min, and 72°C for 5 min for the final synthesis. The PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI) and sequenced using an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA).
**MicroRNA Target Validation Assay**

The 3’-UTR of *PTN* 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 pcDNA3eGFP (Clontech, Mountain View, CA) 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 either *miR-499, miR-1555, miR-1632, miR-1709, miR-1787* or *miR-1815* were designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miRNA). The pcDNA-eGFP-3’UTR and pcDNA-DsRed-miRNA (4µg) 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).

**Statistical Analyses**

Data presented for real-time PCR 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. Excel (Microsoft, Redmond, WA, USA) was used for statistical analyses.
## Table 1. Pairwise comparison of PTN between chicken and other species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Symbol</th>
<th>Identity (%)</th>
</tr>
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<tbody>
<tr>
<td>Chicken (\textit{Gallus gallus})</td>
<td>PTN</td>
<td></td>
</tr>
<tr>
<td>vs. Human (\textit{Homo sapiens})</td>
<td>PTN</td>
<td>91.4</td>
</tr>
<tr>
<td>vs. Rhesus monkey (\textit{Macaca mulatta})</td>
<td>PTN</td>
<td>91.0</td>
</tr>
<tr>
<td>vs. Mouse (\textit{Mus musculus})</td>
<td>Ptn</td>
<td>92.6</td>
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<tr>
<td>vs. Rat (\textit{Rattus norvegicus})</td>
<td>Ptn</td>
<td>92.6</td>
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<tr>
<td>vs. Cattle (\textit{Bos taurus})</td>
<td>PTN</td>
<td>90.9</td>
</tr>
<tr>
<td>vs. Dog (\textit{Canis lupus familiaris})</td>
<td>PTN</td>
<td>91.4</td>
</tr>
<tr>
<td>vs. Zebrafish (\textit{Danio rerio})</td>
<td>ptn</td>
<td>65.4</td>
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4. Results

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

The *PTN* gene was found to span 66,234 bp on chicken chromosome 1 and consists of three exons. *PTN* mRNA has 2,551 bp encoding a protein with 165 amino acid residues (Figure 1A). The amino acid sequence of chicken *PTN* was compared to those of eight other species. The pair-wise comparisons of *PTN* orthologs revealed that chicken *PTN* protein is well conserved with high homology relative to other mammalian *PTN* proteins (90.9 to 92.6%, Table 1). The phylogenetic tree constructed with the neighbor-joining method is presented in Figure 1B. The human and rhesus monkey *PTN* genes clustered together and formed a larger cluster with cattle and dog, and an even larger cluster with sister groups was detected for mouse and rat. However, chicken *PTN* is in a separate branch, but closer to zebrafish than to other species. These results indicate that chicken *PTN* diverged from mammalian *PTNs* at very early stage in its evolution.

*Expression pattern of *PTN* mRNA in various organs from chickens*

Tissue specific expression of *PTN* mRNA in brain, heart, liver, kidney, small intestine, gizzard, ovary, oviduct and testis of 1- to 2-year-old male (n = 3) and female (n = 3) chickens was determined by RT-PCR analyses. Results indicated high levels of expression of *PTN* mRNA in
brain and heart from male and brain, heart, gizzard and oviduct from female chickens (Figures 1C and 1D), and lower expression in kidney, gizzard and testis from males and kidney and ovary from females. However, expression of PTN mRNA was not detected in other organs analyzed. We reported differential gene profiling of the chicken oviduct (Song et al., 2011b) and found that the avian homolog of the human PTN transcript is highly expressed in chicks treated with diethylstilbestrol (DES, a synthetic estrogen agonist). However, little is known about expression and function of PTN in the oviduct of any species, so this study focused on the chicken oviduct.

**Localization of chicken PTN mRNA and protein in chicken oviduct**

Structurally, 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). Results of RT-PCR analysis showed abundant level of PTN mRNA in isthmus and shell gland compared to infundibulum and magnum (Figure 2A). To determine cell-specific localization of PTN mRNA in the chicken oviduct, *in situ* hybridization analysis was performed. As illustrated in Figure 2B, PTN mRNA was most abundant in the glandular (GE) and luminal (LE) epithelia of the isthmus and shell gland, and it was expressed at lower abundance in GE of the magnum. Little or no mRNA was detected in stromal cells, blood vessels, immune cells or myometrium of the oviduct. In addition, results of immunohistochemical analysis (Figure 2C) were consistent with results from *in situ* hybridization analyses in that PTN protein was abundant in
LE of the isthmus and shell gland and also detected, to a lesser extent, in the infundibulum and magnum. The nonspecific rabbit IgG, used as a negative control, did not detect any PTN protein.

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

Cell-type specific expression of PTN in the oviductal segments of mature hens suggested regulation by estrogen during development of the chick oviduct. We reported that administration of exogenous DES stimulates growth, development and cytodifferentiation of the postnatal chick oviduct and found several candidate genes and pathways related to the regulation of oviduct development (Song et al., 2011b). Therefore, we examined effects of DES on PTN expression in the chick oviduct. As illustrated in Figures 3A and 3B, semi-quantitative RT-PCR analysis indicated that DES treatment increased PTN mRNA levels in all segments of the chick oviduct. Further results from quantitative PCR revealed that DES induced an 8.1-fold increase (P < 0.001) in oviductal PTN mRNA as compared to control chicks (Figure 3C). In addition, DES stimulated 3.6-, 51.1-, and 6.9-fold increases (P < 0.001) in PTN mRNA in the infundibulum, magnum and isthmus, respectively (Figure 3D). As shown in Figure 3E, in situ hybridization analyses revealed that PTN mRNA is expressed specifically in GE and LE of the magnum and isthmus of chick oviducts treated with DES and, at lower abundance in the shell gland and infundibulum. Consistent with these results, immunoreactive PTN protein was detected predominantly in GE and LE of magnum and isthmus, and to a lesser extent, in infundibulum and shell gland (Figure 3F).
Post-transcriptional regulation of microRNAs affecting PTN

To investigate the possibility that PTN 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 PTN using a miRNA target prediction database (miRDB; http://mirdb.org/miRDB/) revealed six putative binding site for miR-499, miR-1555, miR-1632, miR-1709, miR-1787 and miR-1815. Therefore, we determined if these six miRNAs influenced PTN expression via its 3′-UTR. A fragment of the PTN 3′-UTR harboring binding sites for the miRNAs were cloned downstream of the green fluorescent protein (GFP) reading frame, thereby creating a fluorescent reporter for function of the 3′-UTR region. After co-transfection of eGFP-PTN 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 4C and 4D, in the presence of miR-499 and miR-1709, the intensity and percentage of GFP-expressing cells (21.7% in control vs. 14.1% in miR-499, 16.8% in miR-1709) decreased (p < 0.01). On the other hand, in the presence of miR-1555, miR-1632, miR-1787 and miR-1815, there was no significant decrease in green fluorescence as compared to the control (data not shown). These results indicate that at least two miRNAs directly bind to the PTN transcript and post-transcriptionally regulate PTN gene expression.

Differential expression of PTN in normal and cancerous ovaries of hens
Chickens are considered the most relevant animal model to identify and develop biomarkers for patients with epithelial ovarian cancer because their incessant ovulation increases the possibility of gene mutations by genomic damage to the ovarian surface epithelium which can lead to ovarian cancer (Murdoch et al., 2005). We previously reported expression of cysteine protease cathepin B (CTSB), serpin peptidase inhibitor, clade B, member 11 (SERPINB11) and alpha 2 macroglobulin (A2M) in ovarian tissue from hens with ovarian cancer (Ahn et al., 2010; Lim et al., 2011a; Lim et al., 2011b). Based on similarities among these genes in expression patterns and estrogen-mediated regulation in the oviduct, we hypothesized that expression patterns for PTN would differ between normal and cancerous ovarian tissues from hens. Based on RT-PCR analysis, *PTN* mRNA was found in all carcinomas, but there was little or no expression in normal ovaries (Figure 5A and 5B). Further, the level of expression of PTN mRNA was greater (P < 0.001) in cancerous ovaries from hens (Figure 5C).

**Localization of PTN mRNA and protein in cancerous ovaries of hens**

To determine cell-specific expression of PTN mRNA and protein, *in situ* hybridization analysis and immunohistochemistry were performed. As illustrated in Figure 5D, there was abundant *PTN* mRNA localized predominantly in GE of cancerous ovaries, but not in LE, stroma or blood vessels. Consistent with this result, immunoreactive PTN protein was detected in GE of cancerous ovaries, but not in any other cell types of the ovaries (Figure 5E). Furthermore, we determined if the increased expression of PTN in cancerous tissue was correlated with changes in
circulating concentrations of estradiol in plasma and expression of estrogen receptor alpha (ESR1) in normal and cancerous ovaries. Even though in our preliminary experiment we could not detect any differences in concentrations of estradiol in serum between cancerous and non-cancerous groups (0.1860.1 ng/ml vs. 0.1760.1 ng/ml), quantitative PCR analysis revealed that ESR1 mRNA levels increased 51-fold (P,0.001) in cancerous ovaries, but not in normal ovaries (Figure 5F). In addition, immunohistochemical analysis showed that immunoreactive ESR1 was abundant in the GE of cancerous ovaries (Figure 5G). Little or no ESR1 was detected in normal ovaries. These results indicate that PTN abundance increases in response to estrogen in cancerous ovaries of hens.

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

The CpG methylation status in the upstream region of genes regulates its transcriptional activity and it is closely associated with the initiation and growth of carcinomas. Therefore, we investigated methylation patterns in the promoter region of the PTN gene in normal and cancerous ovarian epithelial cells. Both normal and cancerous epithelial cells were extracted and cultured in vitro as previously reported (Giles et al., 2004). Results of bisulfite sequencing indicated that CpG sites at -1,353 and -1,355 CpG from the transcriptional start site remained methylated in both normal and cancerous cells. However, 30- and 40 % of -1,339 and -1,311 CpG sites were demethylated in ovarian cancer cells, but not in normal ovarian epithelial cells (Figure 6A and 6B).
Figure 1. Multiple sequence alignment and tissue-specific expression of *PTN* in chickens.

[A] The amino acid sequences of PTN from each of seven species (chicken, human, rhesus monkey, mouse, rat, cattle, dog and zebrafish) 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 PTN and dashes indicate gaps in the sequences. [B] The phylogenetic tree of PTN generated from alignments of primary sequences of chicken, human, rhesus monkey, mouse, rat, cattle, dog and zebrafish PTN proteins using bootstrap analysis with 1,000 replicates. [C and D] Expression of PTN in various organs of male and female of chickens. Results of RT-PCR analysis using cDNA templates from different organs of male [C] and female [D] chickens with chicken PTN and chicken *ACTB*-specific primers. See Materials and Methods for complete description.
[A]

<table>
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<th>PTN</th>
<th>ACTB</th>
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<td>414 bp</td>
<td>498 bp</td>
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[B]

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<th>Infundibulum</th>
<th>Magnum</th>
<th>Isthmus</th>
<th>Shell gland</th>
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[C]

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<tr>
<th>Infundibulum</th>
<th>Magnum</th>
<th>Isthmus</th>
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Figure 2. Expression of PTN in the chicken oviduct.

[A] Results of RT-PCR analysis using cDNA templates from each segment of the chicken oviduct with chicken PTN and chicken ACTB-specific primers. [B] In situ hybridization analyses of PTN mRNAs in the chicken oviduct. Cross-sections of the infundibulum, magnum, isthmus and shell gland of the chicken oviduct were hybridized with antisense or sense chicken PTN cRNA probes. [C] Immunoreactive PTN protein in the chicken oviduct. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium. Scale bar represents 100 μm. See Materials and Methods for complete description.
Figure 3. Effect of DES on tissue- and cell-specific expression of PTN in the chicken oviduct.

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 (mean ± SEM; P < 0.001). These experiments were conducted in triplicate and normalized to control GAPDH expression. [E] In situ hybridization analyses indicate cell-specific expression of PTN mRNA in oviducts of DES-treated and control chicks. Cross-sections of the infundibulum, magnum, isthmus, and shell gland of oviducts from chicks treated with DES or vehicle were hybridized with antisense or sense chicken PTN cRNA probes. [F] Immunoreactive PTN protein in oviducts of DES-treated and control chicks. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium. Scale bar represents 100 μm. See Materials and Methods for complete description.
Figure 4. *In vitro* target assay of miR-499, miR-1555, miR-1632, miR-1709, miR-1787 and miR-1815 on the PTN transcript.

[A] Diagram of miR-499 and miR-1709 binding sites in PTN 3’-UTR. [B] Expression vector maps for eGFP with PTN 3’-UTR and Ds-Red with each miRNA. The 3’-UTR of the PTN 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 the miRNA expression vector was designed to co-express DsRed and each miRNA (pcDNA-DsRed-miRNA) (lower panel). [C and D] After co-transfection of pcDNA-eGFP-3’UTR for the PTN transcript and pcDNA-DsRed-miRNA for the miR-499 and miR-1709, the fluorescence signals of GFP and DsRed were detected using FACS [C] and fluorescent microscopy [D]. See Materials and Methods for complete description.
Figure 5. Quantitation of expression of PTN and ESR1 in normal and cancerous ovaries from hens

[A] RT-PCR analyses were performed using cDNA templates from normal and cancerous ovaries of laying hens using chicken PTN and ACTB-specific primers. Lanes 1 to 4 show results of analyses of four normal ovaries. [B] Lanes 1-9 are from analyses of 9 different cancerous ovaries from laying hens. Expression of PTN mRNA was abundant in all carcinomas, but not in normal ovaries. Legend for panel B: Lane 1, endometrioid/serous/mucinous carcinoma (Stage III); Lane 2, endometrioid carcinoma (Stage I); Lane 3, serous carcinoma (Stage I); Lane 4, mucinous/endometrioid carcinoma (Stage IV); Lane 5, endometrioid carcinoma (Stage IV); Lane 6, endometrioid carcinoma (Stage III); Lane 7, clear cell carcinoma (Stage IV); Lane 8, serous/mucinous carcinoma (Stage IV); and Lane 9, serous/mucinous/endometrioid carcinoma (Stage III) [21]. [C] The q-PCR analysis was performed using cDNA templates from normal and cancerous ovaries of laying hens (mean ± SEM; P < 0.001). [D] In situ hybridization analyses of PTN mRNA in normal and cancerous ovaries of hens. Cross-sections of normal and cancerous ovaries of hens hybridized with antisense or sense chicken PTN cRNA probes demonstrated abundant PTN mRNA predominantly in GE of cancerous ovaries, but not in LE, stroma or blood vessels. [E] Immunoreactive PTN 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 μm (the first horizontal panels, sense and IgG) or 50 μm (the second horizontal panels, sense and IgG).
[F] The q-PCR analysis for expression of estrogen receptor alpha (ESR1) was performed using cDNA templates from normal and cancerous ovaries of laying hens (mean 6 SEM; P<0.001). [G] Immunoreactive ESR1 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 mm (the first horizontal panels, sense and IgG) or 50 mm (the second horizontal panels, sense and IgG). See Materials and Methods for a complete description of the methods.
[A] - Transcriptional start site (TSS)

[B] - Normal ovarian cell
Normal ovarian cell

[C] - Chicken
- Transcriptional start site

Mouse
- Transcriptional start site

Human
- Transcriptional start site

[Image] - CpG site
[Image] - Putative protein binding site
[Image] - Methylated cytosine
[Image] - Unmethylated cytosine

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Figure 6. Bisulfite sequencing of CpG sites in the upstream region of the PTN gene.

[A] Schematic of the four CpG sites in the promoter region of the PTN gene are indicated by the heavy black vertical lines. The numbers on the line indicate positions relative to the transcription start site. Legend: a, complex contains E2F and p107 (E2F+p107); b, upstream stimulatory factor (USF also known as gamma-factor). [B] The CpG methylation status in the upstream region of the PTN gene was analyzed in normal and ovarian cancer cells from 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. [C] Comparison of the sequences around CpG regions of PTN genes of chicken, mouse, and human. The 5’ flanking region (about 2.2 kb) of mouse and human PTN was compared to that of chicken PTN to identify differences in sequences around each CpG sites among those species.
5. Discussion

Results of the current study are novel in providing the first comparisons among chicken and mammalian PTN genes with respect to structure, phylogenetic evolution, tissue specific expression of PTN mRNA and protein, and regulation of expression by estrogen in the chicken oviduct. Our results also revealed that PTN gene expression is post-transcriptionally regulated by several miRNAs critical to development of the chick oviduct in response to estrogen. These results support our hypothesis that PTN is required for growth, development and functional aspects of the mature oviduct of hens in response to estrogen during their reproductive cycle. Our previous report on differential gene profiling of the chick oviduct indicated that the avian homolog of the human PTN transcript is highly expressed in chicks treated with DES (Song et al., 2011a).

The PTN and midkine (MK) proteins are members of a family of proteins regulating growth and differentiation which share over 50% amino acid sequence identity and their genes are located on human chromosomes 7q.33 and 11p.11.2, respectively (Muramatsu, 1993, 2002). In chickens, MK (also called retinoic acid-inducible heparin binding protein) purified from 11-day-old chicken embryos is predominantly localized within the basement membranes in embryonic tissues and it stimulates neurite outgrowth and proliferation of PC12 cells (Raulais et al., 1991; Vigny et al., 1989). However, little is known about the expression and function of PTN in chickens. In the present study, multiple gene sequence alignment showed that the PTN gene in the chicken genome spans 66,234 bp on chromosome 1 and consists of 3 exons (2,551 bp
mRNA) encoding a protein with 165 amino acid residues (Figure 1A). The human PTN gene is also about 68 kb (Lai et al., 1992), although the human MK genes is only 2 kb (Uehara et al., 1992). In addition, results of the present study indicated that chicken PTN protein is highly conserved with respect to mammalian PTN proteins (90.9 to 92.6%) and that it diverged from mammalian PTN very early in its evolution.

As illustrated in Figure 1D, PTN mRNA is abundantly expressed in the chicken oviduct. In mice, the Ptn gene is detected in a number of tissues and co-localizes with the Mk gene in many cases during embryogenesis (Mitsiadis et al., 1995). Although PTN has important roles in various biological events such as differentiation of renal tubular epithelial cells and dopaminergic neurons (Jung et al., 2004; Sakurai et al., 2001), Ptn−/− mice are fertile and exhibit no gross anatomical abnormalities except for abnormal structure and function of components of the nervous system (Amet et al., 2001; Hienola et al., 2004). Recently, Muramatsu and colleagues [56] reported that mice deficient in both Ptn and Mk were infertile and had abnormal estrous cycles with long periods of proestrus and diestrus and short periods of estrus (Muramatsu et al., 2006). In addition, the presence of PTN in the porcine uterus and uterine flushings during early pregnancy may be a member of the regulators of implantation and conceptus development (Brigstock et al., 1996a; Brigstock et al., 1996b). In the present study, PTN mRNA and protein were most abundant in the LE of the isthmus and shell gland, but detectable at lower abundance in GE in each segment. As shown in Figure 3, q-PCR analyses revealed that DES induced oviductal PTN mRNA to a 51.1-fold increase as compared to control chicks (P < 0.001) in the magnum. In chickens, estrogen stimulates proliferation and cytodifferentiation of immature oviductal epithelial cells to mature tubular
gland cells to activate egg white protein genes during oviduct development (Palmiter and Wrenn, 1971; Socher and Omalley, 1973). Of particular note, the fully differentiated tubular gland cells of the magnum produce and secrete several egg-white proteins such as ovalbumin, ovomucoid, ovo transferrin and avidin during egg formation and oviposition (Kohler et al., 1968). Therefore, the magnum is the most estrogen-responsive portion of the chicken oviduct that affects the quality of the egg. These results suggest that the functional role(s) of PTN in the LE of the magnum is similar with those in the porcine uterus during early pregnancy.

In a wide variety of fundamental processes and biological events in vertebrates, such as cellular survival, growth, development and differentiation, microRNAs (miRNAs) play pivotal roles in post-transcriptional regulation and pathways (Bartel, 2009). As shown in Figure 4, co-transfection of eGFP-PTN 3’-UTR and DsRed-miRNA decreased the percentage of GFP-positive cells and GFP fluorescence density in miR-499 and miR-1709 transfected cells, but not in cell transfected with miR-1555, miR-1632, miR-1787 and miR-1815 when compared to controls. These results indicate that miR-499 and miR-1709 bind directly to the 3’-UTR of the PTN transcript and post-transcriptionally regulate PTN gene transcription. Thus, we propose that these two miRNAs are closely related to the regulatory pathways of oviduct development and differentiation in chickens; however, this requires further investigation.

Results of the present study are the first to identify a high level of expression of the PTN gene in GE of ovarian carcinoma in laying hens. Indeed, cancerous ovaries of hens show very similar patterns of expression of tumor-related genes compared with those in women (Hakim et al., 2007).
2009), and high cross-reactivity and expression of biomarkers such as CA125, EGFR, and ERBB-2 for human ovarian cancer (Anderson et al., 2010; Jackson et al., 2007; Johnson, 2009; Rodriguez-Burford et al., 2001). Therefore, laying hens are the most relevant animal model to identify biomarkers for patients with epithelial ovarian cancer. Indeed, we found that cathepin B (CTSB) (Ahn et al., 2010), serpin peptidase inhibitor, clade B, member 11 (SERPINB11) (Lim et al., 2011b) and alpha 2 macroglobulin (A2M) (Lim et al., 2011a) genes are most abundant in GE of chicken adenocarcinoma. Likewise, we now report that the PTN gene is expressed predominantly in GE of the cancerous ovaries from hens. Moreover, we recently reported that SERPINB3 is a biomarker for chicken ovarian endometrioid carcinoma and that it can serve as a prognostic factor for platinum resistance and poor survival in patients with epithelial ovarian cancer (Kim et al., 2011). On the other hand, results of the the present study revealed differences in the methylation status of CpG sites in the promoter region of the PTN gene in surface epithelial cells of cancerous ovaries. In general, a number of genes are up- and down-regulated in cancer cells of various origins via DNA methylation and histone modification (Suzuki et al., 2011). These epigenetic regulatory mechanisms stimulate rates of tumor growth and metastasis by activation of oncogenes and inactivation of tumor suppressor genes through differential methylation of genes in the promoter region (Khalkhali-Ellis, 2006; Laird and Jaenisch, 1994). In the present study, results of bisulfite sequencing revealed that 30- and 40 % of -1,339 and -1,311 CpG sites were demethylated in ovarian cancer cells, but not in normal ovarian epithelial cells (Figure 6). This different methylation status from between normal and cancerous ovarian cells likely affects development of cancer phenotypes. In addition, expression of the PTN gene may be epigenetically regulated, and its cell type specific expression closely associated with
DNA methylation. As illustrated in Figure 6C, the position of the -1,339 CpG site is located within the putative binding elements for the upstream stimulatory factor (USF also known as gamma-factor). Furthermore, we compared the 5’ flanking region (about 2.2 kb) of mouse and human PTN with that of chicken PTN to identify differences in sequences around CpG sites and found one CpG site in mouse, but not in human. However, further research is required to elucidate the relationship between PTN and USF signaling cascades in cancerous ovaries of hens. These results support our hypothesis that PTN is a critical regulator for growth and developmental aspects of epithelial cells of the ovaries of laying hens as they transition from a normal to a cancerous state. In humans, the PTN gene is involved in carcinogenesis including mitogenesis, metastasis and angiogenesis, and it is expressed in a variety of cancers, such as lung (Jager et al., 2002; Jager et al., 1997), colon (Souttou et al., 1998), prostate (Vacherot et al., 1999), breast (Fang et al., 1992) and pancreas (Klomp et al., 2002) as well as melanomas (Wu et al., 2005), neuroblastomas (Soulie et al., 2004) and many carcinoma cell lines. It is thought that PTN is an angiogenic factor that stimulates tumor growth and metastasis. For instance, nude mice implanted with breast cancer cells and treated with dominant negative PTN showed a significant decrease in the rates of tumor growth and angiogenesis (Zhang et al., 1997), whereas mice overexpressing PTN in breast cancer cells showed increased levels of tumor growth, microvessel density and endothelial cell proliferation (Choudhuri et al., 1997). These results indicate that PTN secreted from tumor cells stimulate proliferation and tube formation of endothelial cells (Perez-Pinera et al., 2007).

Collectively, results of the present study indicate that PTN is a novel estrogen-stimulated gene during growth, development and...
differentiation of the chicken oviduct and that it is likely a critical regulator of abnormal growth and functional aspects of ovarian surface epithelial cells as they transition from normal to a cancerous state in laying hens. These results also provide important insight into future research to investigate the precise role(s) and signal transduction cascades involving PTN. Research will be directed toward understanding mechanisms responsible for estrogen-mediated development and cytodifferentiation of cells of the chicken oviduct and the significance of PTN as a biomarker of epithelial ovarian cancer of laying hens to elucidate the etiologies and pathogenesis of the disease.
CHAPTER 6

GENERAL DISCUSSION
In the present study, to better understand research on ovarian carcinogenesis, we focused on both genetic and epigenetic mechanisms. There are several scientifically developed animal models in human ovarian cancer research. However, primate models are biologically limited, with problems including non-spontaneous ovarian cancer development, lack of practicality due to specialized skills, and high cost. Rodents are also established with genetic manipulations as animal models. However, these models are not feasible for laboratory ovarian cancer research due to a wide variety of histological types, low incidence, and a requirement for a long period before tumor appearance.

We confirm the laying hen model as the most appropriate and practical for human ovarian cancer research. There are several advantages to other species. First, approximately 83% of avian species have genital tumors including ovarian cancer, and ovarian cancers develop in an age-dependent manner in laying hens over the ages of 2 and spontaneously without any genetic manipulation. Furthermore, morphological and physiological characteristics are in accordance with human ovarian cancer. Most human ovarian diagnosis markers, such as CA125, TGF-a, and EGFR in chickens are also expressed in cancer tissue.

In order to better understand ovarian carcinogenesis in avian species we conducted several studies. Recently, cancer researchers have focused on epigenetic mechanisms such as DNA methylation, histone acetylation and post-transcriptional regulation via microRNA. We discovered multiple regulations of the DNMT gene, a key regulator in DNA methylation. The notable overexpression of DNMTs suggests that distinct epigenetic pathways are involved in ovarian carcinogenesis compared with normal ovaries. DNMTs directly mediate hypomethylation
of tumor suppressor genes, in addition to methylation patterns of DNMTs themselves, in ovarian cancer development. Moreover, we determined that DNMTs are mediated by post-transcriptional regulation. These results provide evidence for multiple epigenetic regulatory mechanisms involved in ovarian carcinogenesis.

We performed a series of experiments to investigate genetic alterations related to cell cycle regulatory genes in chicken epithelial ovarian cancer. Abnormal cell cycles are a common feature of various cancer types, and are mechanistically regulated by cell cycle regulatory genes. Alterations of these genes have been previously detected in various cancer types. The results of our study demonstrate that cyclins and their associated kinases and inhibitors play a key role in controlling the cell cycle, and that dysregulation of these molecules can potentially lead to uncontrolled cell proliferation, growth, and loss of function in cells, and ovarian tumorigenesis in laying hens. Furthermore, post-transcriptional regulation of the specific miRNAs that influence expression of cell cycle genes likely leads to an alternative mechanism(s) for regulation of expression. Although the results of this study indicate that various miRNAs may be involved in many different oncogenic/carcinogenic pathways, details of altered expression patterns and their relevance to EOC require further study.

We identified the avian pleiotrophin (PTN) gene expressed in the female genital tract, estrogen-stimulated oviduct and cancer ovaries. PTN is widely distributed in various tissues and plays pivotal roles in neurogenesis and epithelial mesenchymal interactions through the promotion of cell growth and migration during early embryonic differentiation and morphogenesis. This study indicates that PTN is a
novel estrogen-stimulated gene during growth, development and differentiation of the chicken oviduct and is likely a critical regulator of abnormal growth and functional aspects of ovarian surface epithelial cells, as they transition from a normal to a cancerous state in laying hens.

Consequently, we demonstrated that the chicken is a crucial model system for human ovarian cancer research. We suggest the potential for multiple novel ways to diagnose and treat human ovarian cancer, such as the manipulation of cell cycle regulatory genes and epigenetic modification of factors like miRNAs and DNMTs. These may be further developed for clinical applications. In addition, PTN could be a potential biomarker for epithelial ovarian cancer in laying hens, for further elucidation of the etiology and pathogenesis of this deadly disease.
REFERENCES


Choudhuri, R., Zhang, H. T., Donnini, S., Ziche, M., and Bicknell, R.


D'Andrilli, G., Kumar, C., Scambia, G., and Giordano, A. (2004). Cell cycle genes in ovarian cancer: steps toward earlier diagnosis and


Jair, K. W., Bachman, K. E., Suzuki, H., Ting, A. H., Rhee, I., Yen, R. W.,

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T., and Thun, M. J.


Jeong, W., Kim, H. S., Kim, Y. B., Kim, M. A., Lim, W., Kim, J., Jang, H.
J., Suh, D. H., Kim, K., Chung, H. H., Bazer, F. W., Song, Y. S.,
Han, J. Y., and Song, G. (2012). Paradoxical expression of
AHCYL1 affecting ovarian carcinogenesis between chickens and

Johnson, K. A. (2009). The standard of perfection: thoughts about the
laying hen model of ovarian cancer. Cancer Prev Res (Phila) 2, 97-
9.


mammalian epigenetics. Science 293, 1068-70.


Lim, W., Jeong, W., Kim, J. H., Lee, J. Y., Kim, J., Bazer, F. W., Han, J. Y., and Song, G. (2011a). Differential expression of alpha 2
macroglobulin in response to diethylstilbestrol and in ovarian carcinomas in chickens. Reprod Biol Endocrinol 9, 137.


Masamha, C. P., and Benbrook, D. M. (2009). Cyclin D1 degradation is sufficient to induce G1 cell cycle arrest despite constitutive


of the heparin-binding cytokines, midkine (MK) and HB-GAM (pleiotrophin) is associated with epithelial-mesenchymal interactions during fetal development and organogenesis. *Development* **121**, 37-51.


angiogenesis, remodeling of the tumor microenvironment, and activation of stromal fibroblasts. *Cell Cycle* 6, 2877-83.


Song, G., Seo, H. W., Choi, J. W., Rengaraj, D., Kim, T. M., Lee, B. R.,


Soulie, P., Heroult, M., Bernard-Pierrot, I., Caruelle, D., Oglobine, J.,

Souttou, B., Juhl, H., Hackenbruck, J., Rockseisen, M., Klomp, H. J.,


Stammer, K., Edassery, S. L., Barua, A., Bitterman, P., Bahr, J. M., Hales,


Tillmann, T., Kamino, K., and Mohr, U. (2000). Incidence and spectrum of


난소암은 여성의 난소에서 발병하는 질병으로 주기적인 배란으로 인한 상피세포의 반복적인 자극과 이로 인한 유전자 변이로부터 발생한다.

또한 난소암은 여성의 암에 의한 사망률에 있어 5번째로 높은 사망률이 보고되었으며 난소암 진단 후 5년이상 생존률이 30% 정도로 극히 낮은 생존률을 보이는 질병이다. 이처럼 생존률이 낮은 이유로 난소암은 조기 진단이 어려울 뿐만 아니라 조기난소암 진단을 위한 바이오마커 또한 전무하기 때문이다. 알기 난소암은 진단시 대부분 다른 조직이나 기관으로의 암세포의 전이와 청병이 진행된 상태로 예후가 좋지 않은 것이 일반적이다. 하지만 난소암의 조기 발견시 생존률은 70% 이상으로 상승한다는 연구결과가 있음에 따라 조기진단을 위한 바이오마커 발굴은 임상적으로 아주 유용할 것으로 기대된다.

난소암에 관한 보다 심도있는 연구를 위한 적합한 동물모델 개발이 필요하다. 난소암 연구를 위한 동물모델로 영장류나 설치류는 여러 연구에서 적용된 바 있으나 대부분 유전자 조작을 통해 인위적으로 난소암을 발생시킨 경우로 이는 자연적으로 발생하는 인간의 난소암과 생리학적 특성 및 발생 메커니즘 이 동일하다고 볼 수 없기에 연구동물모델로서의 한계가 있다. 반면에 닭은 산란 후 2-3년이 지나면 자연발생적으로 난소암이 발병하며 상피세포 유래라는 점에서 인간의 난소암과 형태학적, 생리학적 특징이 상당히 유사한 것으로 보고되고 있다. 실제로 본 연구를 통해 인간 난소암의 바이오마커가 닭의 난
소암 조직에서도 유사한 패턴으로 발현하는 것을 확인하였다.

특히 닭은 거의 매일 산란하기 때문에 포유동물의 배란주기와 비교할 때 매우 빠른 산란 사이클을 가지고 있으며, 따라서 호르몬에 의한 난소상피세포의 자극으로 발생하는 난소암의 발생비율이 월등히 높은 것으로 추측된다. 또한 닭을 비롯한 조류는 유전자 구조 및 클러스터링이 인간과 매우 유사하여 실험모델로서의 중요성이 점차적으로 부각되고 있음에 따라 본 연구를 통한 조류 난소암 관련 유전자의 후생유전학적 분석 및 전사 후 조절 기작 규명은 향후 인간 난소암의 분자생리학적 기작 규명에 매우 유용한 정보를 제공할 수 있을 것으로 사료된다.

우선 난소암의 후생유전학적 메커니즘 연구를 수행하였다. 후생유전학이란 유전자의 염기서열의 변화 없이도 유전자의 발현이 조절되는 메커니즘으로 최근에 여러 분야에 걸쳐 후생유전학적 연구가 많이 시도되고 있다. 후생유전학적 주요 메커니즘인 메틸레이션 분석을 위해 메틸레이션을 유도하는 유전자인 DNA Methyltransferase (DNMT)의 발현패턴을 분석한 결과, 정상난소에 비해 난소암에서 발현이 유의적으로 증가하는 것을 확인하였으며, 종양억제 유전자와의 메틸레이션패턴을 분석한 결과, 정상난소에 비해 난소암에서 고메틸화 되어있는 것을 알 수 있었다. 따라서 종양억제유전자가 메틸레이션에 의해 전사되지 못하고 그 기능을 하지 못함으로써 난소암의 진행이 촉진되는 것임을 알 수 있었다. 또한 유전자의 전사 후 조절에 관여하는 microRNA의 작용에 대해 분석한 결과 DNMT가 microRNA에 의해 조절되고 있으며 DNMT 관련
microRNA가 직간접적으로 난소암의 발생 메커니즘에 관여하고 있음을 확인할 수 있었다.

또한 난소암에서 유전학적 메커니즘 분석을 위해 정상난소와 난소암에서의 세포주기 조절 유전자들의 발현을 분석하였다. 세포주기에 의해 세포의 성장 및 사멸이 적절하게 조절됨으로써 정상적인 세포상태를 유지할 수 있는 것이 다. 이에 세포주기에 이상이 발생하면 세포는 적절하게 제어되지 못함에 따라 무한증식을 거듭하게 되고 암세포화가 진행되는 것은 이미 많은 연구를 통해 보고된 바 있으며 이러한 세포주기는 Cyclin, Cyclin Dpendent Kinase, 그리고 Cyclin Dependent Kinase Inhibitor 등 세포주기 조절 유전자에 의해 조절된다. 따라서 세포주기조절 유전자에 이상이 발생하면 세포주기는 제 기능을 하지 못하게 되며 암세포화가 진행되는 것으로 이에 본 연구에서는 닭의 난소암조직에서 세포주기 조절 유전자의 비교적적인 과다 발현을 확인함으로써 닭의 난소에 암세포화의 발달이 세포주기의 조절 유전자의 직접적인 영향을 받는 것임을 확인할 수 있었다. 그리고 이러한 세포주기 조절 유전자들 또한 micro RNA 에 의해 전사 후 조절되는 것을 밝혔으며 이를 통해 닭 난소암의 발달에 있어 유전학적 그리고 후생유전학적 메커니즘이 복합적으로 관여하는 것임을 밝혔다.

앞서 언급한 바와 같이 난소암 조기진단이 가능한 바이오마커 발굴 연구는 동물을 이용한 난소암 연구분야에서 사람에게 임상적용이 가능한 유용하고 필수적인 분야이다. 이에 본 연구에서는 Pleiotrophin (PTN) 유전자의 바이오마크로
커로서의 가능성을 확인하기 위해 닭의 정상난소와 난소암 조직에서 유전자의 차등적인 발현을 확인하였다. 정상난소에서는 유전자의 발현이 거의 확인되지 않았던 반면에 난소암 조직에서는 초기, 중기, 말기의 모든 난소암 조직에서 PTN 유전자의 발현이 유의적으로 증가하는 것이 확인되었다. 또한 닭의 난관에서 여성호르몬인 에스트로겐 처리에 의한 유전자의 조절을 분석한 결과, 에스트로겐에 의해 PTN 유전자의 발현이 유의적으로 증가하는 것으로 보아 PTN 유전자의 발현에 에스트로겐과 상관관계가 있음을 알 수 있었고 이를 통해 에스트로겐과 같은 호르몬에 암세포화가 촉진된다고 알려진 난소암에서 PTN 유전자의 발현이 연관성이 있음을 확인하였다. 따라서 PTN 유전자는 인간의 조기난소암을 진단하는데 있어 바이오마커로 사용이 가능할 것으로 추측되며 항후 세포신흐전달 등의 구체적인 분석을 통해 난소암진단을 위한 바이오마커로 정립할 수 있을 것으로 사료된다.

본 연구를 통해 조류 난소암의 유전적 분석뿐만 아니라 후생유전학적 분석과 전사 후 조절작용 등 다양한 메커니즘을 분석 하였다. 또한 닭을 이용한 난소암 연구에 있어서 본 연구 결과가 상당한 의미가 있을 것으로 생각되며, 난소암 진단마커발굴 및 동물모델 개발에 중요한 가치가 있을 것으로 사료된다.
ACKNOWLEDGEMENTS

학위논문을 완성하기까지 지난 2년간은 여러 가지로 소중한 시간들이었습니다. 석사학위라는 학문적 성과뿐만 아니라 인생을 살아가는 중요한 가르침을 배웠고 앞으로 과학자로서의 길을 함께 할 소중한 인연들을 만났습니다. 그 동안 도움을 주셨던 많은 분들께 감사의 마음을 전하고자 합니다.

가장 먼저 지도교수님이신 송권화 교수님께 깊은 존경과 감사의 마음을 올립니다. 항상 큰 꿈을 심어주셨고 제 자신보다 더 저를 믿어주시고 따뜻한 마음과 애정으로 지도해 주셔서 진심으로 감사드립니다. 교수님과 함께했기 때문에 모든 과정을 마무리 할 수 있었습니다.

또한 학위논문심사와 논문작성에 아낌없는 격려와 많은 조언을 해주신 안용준 교수님과 서정용 교수님께도 깊이 감사드리며 바이오모듈레이션 전공 교수님들께도 감사드립니다. 그리고 항상 인자한 미소로 열정을 다해 지도해 주신 고줄거운 금요일을 함께 해주셨던 Texas A&M University 의 Fuller W. Bazer 교수님께도 깊은 감사의 말씀을 올립니다. Prof. Bazer, I really appreciate your patience and support.

무엇보다 학위과정 동안 가장 큰 추억이 있는 분자내분비학 및 세포신호전달 연구실 식구들께 고마움을 전합니다. 실험을 가르쳐 주실 때마다 항상 차근차근 원리를 설명해 주시며 정신 없는 저를 정리해 주셨던 김진영박사님께 감사드립니다. 석사 첫 학기, 스파르타로 아낌없는 조언을 해주었던 은서 언
네 고향입니다. 대학원에 입학하여 처음으로 실험을 가르쳐 주었고 학위기간 내내 여러모로 많은 의지가 되어준 화선후배. 항상 힘을 되어 줄서 진심으로 감사합니다. 많은 시간 향배하며 실험실 생활에 큰 웃음을 주었던 우영 선배, 어른스럽고 강한 모습에 많은 것을 배웠습니다. 항상 이해해 줄서 고맙기도 하고 미안하기도 합니다. 티격태격했지만 믿음직했던 동기 창호이, 저에게 처음 실험을 배우며 고생을 많이 한 후배 승민이에게도 깊은 고마움을 전합니다.
또한 학위논문심사준비에 많은 도움이 되어 준 실험실에 새로운 식구들 효선 언니, 청이, 은희, 승연이, 안나 많은 시간을 향배하지 못해서 아쉽지만 우리 연구실에서 보다 큰 꿈을 가지고 열정적인 실험실 생활이 되시길 바랍니다.

그리고 언제나 힘이 되어주신 조부모님, 지금까지 공부 할 수 있도록 마음을 다해 키워주신 부모님, 닮고 싶은 연구자 선영 언니, 동적인 동생 재호에 게도 깊은 감사의 마음을 전합니다.

"20년이 지나면 당신은 당신이 한 일보다는 하지 않은 일들 때문에 더 후회할 것이다. 그러니 낯을 옮려 안전한 포구를 떠나라. 당신의 끝에 무역풍을 가득 안고 출발하여 탐험하라. 꿈꾸라. 그리고 발견하라." 마크 트웨인의 이 글귀처럼 더 큰 꿈을 안고 목표를 향해 나아가라 합니다. 앞으로 과학자로 성장하는데 어떠한 길이 제 앞에 나타나더라도 저에게 힘을 주셨던 분들이 있기 에 이제는 그 길을 해쳐나갈 수 있는 용기가 생겼습니다. 서울대학교 분자내 분비학 및 세포신호전달 연구실에서 보고 배우고 느낀 것들을 디딤돌 삼아 더 큰 과학자로 성장해 세상에 꼭 필요한 연구를 하겠습니다.