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Differential expression of secreted phosphoprotein 1 in response to estradiol-17β and in ovarian tumors in chickens

Whasun Lim [a], Wooyoung Jeong [a], Jinyoung Kim [a], Hakhyun Ka [b], Fuller W. Bazer [a,c], Jae Yong Han [a], Gwonhwa Song [a,*]

[a] WCU Biomodulation Major, Department of Agricultural Biotechnology, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151-921, Republic of Korea
[b] Division of Biological Science and Technology, Yonsei University, Wonju 220-710, Republic of Korea
[c] Center for Animal Biotechnology and Genomics and Department of Animal Science, Texas A&M University, College Station, TX 77843-2471, USA

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ABSTRACT

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

1. Introduction

Secreted phosphoprotein 1 (*SPP1*), also known as osteopontin, is a highly phosphorylated small integrin-binding ligand (SIBLING), *N*-linked glycoprotein originally isolated from bones of rats [1]. The *SPP1* gene includes seven exons and it is located on chromosome 4q13 in humans and chromosome 5 in mice, and it has a polyaspartic acid sequence and a highly conserved RGD motif [2]. *SPP1* plays essential roles in various biological events, such as cell–cell interactions, immune responses, carcinogenesis, wound healing, bone metabolism and calcification [2]. In chickens, *SPP1* participates in formation and calcification of the egg shell in the shell gland of the reproductive tract of laying hens [3]. Further, immunoreactive *SPP1* protein is detected mainly in the core of the non-mineralized shell membrane fibers, in the base of the mammillae and in the outermost part of the palisade of the egg shell [4]. Although the number of amino acid residues in mammalian and avian *SPP1* proteins are similar, *SPP1* exists as 44–75 kDa forms due to post-translational modifications [2] and proteolytic cleavage at its thrombin cleavage site. This indicates that *SPP1* is synthesized and secreted by epithelia of the shell gland and accumulated in the egg shell membrane as it becomes calcified during egg formation and oviposition.

The reproductive tract of the female chicken is a unilateral organ with one functional ovary and one oviduct. The chicken oviduct is well-known as an excellent research model studies of organ development, morphogenesis, and hormonal responsiveness [5]. It has four segments: infundibulum, magnum, isthmus and shell gland. Each segment has an important role in formation of the egg by secretion of egg proteins and formation of soft shell and calcified shell [6]. During development of the chicken oviduct, estrogen is essential as it stimulates proliferation and cytodifferentiation of epithelial to tubular gland cells and expression of oviduct-specific genes [7,8]. Therefore, this study was conducted to: (1) determine tissue- and cell-specific expression of the *SPP1* gene in various organs of the chicken; (2) examine effects of estrogen on expression of *SPP1* mRNA and protein during oviduct development in chicks; (3) determine whether *SPP1* is regulated by post-transcriptional action of specific microRNA and (4) compare differential expression of *SPP1* in normal and cancerous ovaries from hens. Results of this study provide novel insights into the chicken

* Corresponding author. Fax: +82 2 877 4906.
E-mail address: ghsong@snu.ac.kr (G. Song).

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SPP1 gene with respect to its tissue- and cell-specific expression and regulation of its expression by estrogen and microRNA during development of the chicken oviduct and in chicken ovarian carcinogenesis.

2. Materials and methods

2.1. Experimental animals and animal care

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

2.2. Tissue samples

2.2.1. Study one

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

2.2.2. Study two

Female chicks were identified by PCR analysis using W chromosome-specific primer sets [9]. Treatment with DES and recovery of the oviduct ($n = 5$) were conducted as reported previously [10,11].

2.2.3. Study three

A total 136 chickens (88 chickens aged over 36 months and 48 chickens aged over 24 months), which had completely stopped laying eggs were euthanized for biopsy and cancerous ($n = 10$) ovaries were collected. As a control, normal ($n = 5$) ovaries were also collected from egg-laying hens. We examined the tumor stage in 10 chickens with cancerous ovaries using characteristic features of chicken ovarian cancer [12,13].

2.3. RNA isolation

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

2.4. Semi-quantitative and quantitative RT-PCR analysis

The level of expression of *SPP1* mRNA in various organs from chickens, including the oviduct, was assessed using semi-quantitative RT-PCR as described previously [14].

2.5. In situ hybridization analysis

Location of *SPP1* mRNA in sections (5 μm) of chicken oviduct was determined by radioactive *in situ* hybridization analysis as described previously [14].

2.6. Immunohistochemistry

Immunocytochemical localization of *SPP1* protein in the chicken oviduct was performed as described previously [14].

2.7. MicroRNA target validation assay

The 3′-UTR of *SPP1* was cloned and confirmed by sequencing. The 3′-UTR was subcloned between the eGFP gene and the bovine growth hormone poly-A tail in pcDNA3eGFP (Clontech, Mountain View, CA) to generate the eGFP-miRNA target 3′-UTR (pcDNA-eGFP-3′UTR) fusion constructs as described previously [15]. For the dual fluorescence reporter assay, the fusion constructs containing the DsRed gene and *miR-140* were designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miRNA). At 48 h post-transfection, dual fluorescence was detected by fluorescence microscopy and calculated by FACSCalibur flow cytometry (BD Biosciences). For flow cytometry, the cells were fixed in 4% paraformaldehyde and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

2.8. Statistical analyses

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

3. Results

3.1. Analysis of SPP1 expression in adult chicken organs (study one)

To determine tissue-specific expression of *SPP1* mRNA in various organs of male and female chickens, RT-PCR analysis was performed. The analysis revealed that *SPP1* mRNA was abundant in kidneys from male and female chickens and also gizzard and oviduct of females (Fig. 1A and B). *SPP1* mRNA was also detected in small intestines from both sexes, and, to a lesser extent, testis of males and livers of both sexes. In oviduct of hens, *SPP1* is involved in eggshell formation and calcification [3]. Therefore, subsequent aspects of this study focused on expression and potential roles of *SPP1* in the oviduct and ovary of hens.

3.2. Localization of SPP1 mRNA and protein expression in adult chicken oviduct (study one)

We first determined cell-specific expression of *SPP1* mRNA in each segment of chicken oviduct; infundibulum (site of fertilization), magnum (production of components of egg-white), isthmus (formation of the shell membrane), and shell gland (formation of the egg shell) using *in situ* hybridization analysis (Fig. 1C). *SPP1* mRNA was most abundant in the glandular epithelium (GE) of the shell gland, and also at a lower abundance in luminal epithelium (LE) of the infundibulum and magnum, and GE of the isthmus. Little or no mRNA was detected in stromal cells, blood vessels or immune cells of the oviduct. Next, immunohistochemistry was used to determine the localization of immunoreactive SPP1 protein in the chicken oviduct (Fig. 1D). Interestingly, SPP1 protein is present in the LE of all segments of the oviduct, but less abundant in GE of the isthmus and shell gland. The mouse IgG used as a negative control did not detect SPP1.

3.3. Effects of DES on SPP1 expression in the chick oviduct (study two)

Next we examined the effects of DES on *SPP1* expression in the chicken oviduct. As illustrated in Fig. 2A and B, RT-PCR and real-time PCR analyses revealed that DES decreased expression of *SPP1* mRNA in the oviduct as compared to control chicks ($P < 0.001$). Consistent with these results, *in situ* hybridization analyses revealed abundant expression of *SPP1* mRNA in control chick oviducts (Fig. 2C). However, *SPP1* mRNA was also localized in GE of the shell gland of DES-treated chick oviducts and present at a lower abundance in LE and GE of the infundibulum, magnum, and

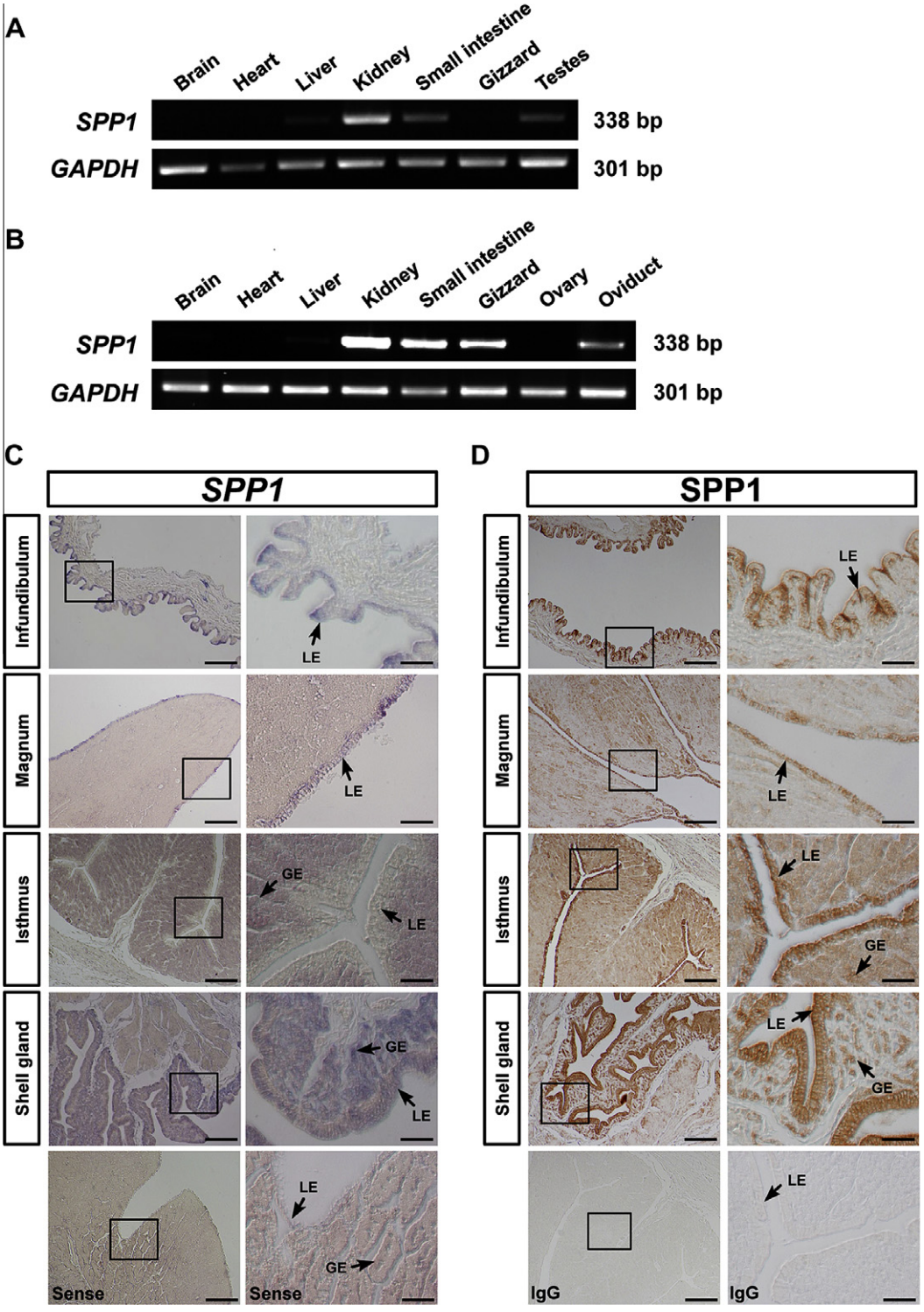

Fig. 1. Expression of *SPP1* in various organs of male and female of chickens. Results of RT-PCR analysis using cDNA templates from different organs of male (A) and female (B) chickens with chicken *SPP1* and chicken *GAPDH*-specific primers. (C) *In situ* hybridization analyses of *SPP1* mRNAs in the chicken oviduct. Cross-sections of the four components of the chicken oviduct (infundibulum, magnum, isthmus and shell gland) were hybridized with antisense or sense chicken *SPP1* cRNA probes. (D) Immunoreactive SPP1 protein in the chicken oviduct. For the IgG control, normal mouse IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium; Scale bar represents 200 μm (the first columnar panels) and 50 μm (the second columnar panels).

isthmus from oviducts of DES-treated chicks. In contrast, immunoreactive SPP1 protein was detected in LE of oviducts of control chicks and the infundibulum of the oviducts of chicks treated with DES (Fig. 2D). In addition, SPP1 protein was abundant in GE of the magnum, isthmus and shell gland of DES-treated oviducts and, to a lesser extent, in LE of each segment of the oviduct.

3.4. Post-transcriptional regulation of microRNA affecting SPP1

To investigate the possibility that *SPP1* expression is regulated at the post-transcriptional level by miRNAs, we performed a miRNA target validation assay. Analysis of potential miRNA binding sites within the 3′-UTR for *SPP1* using a miRNA target prediction

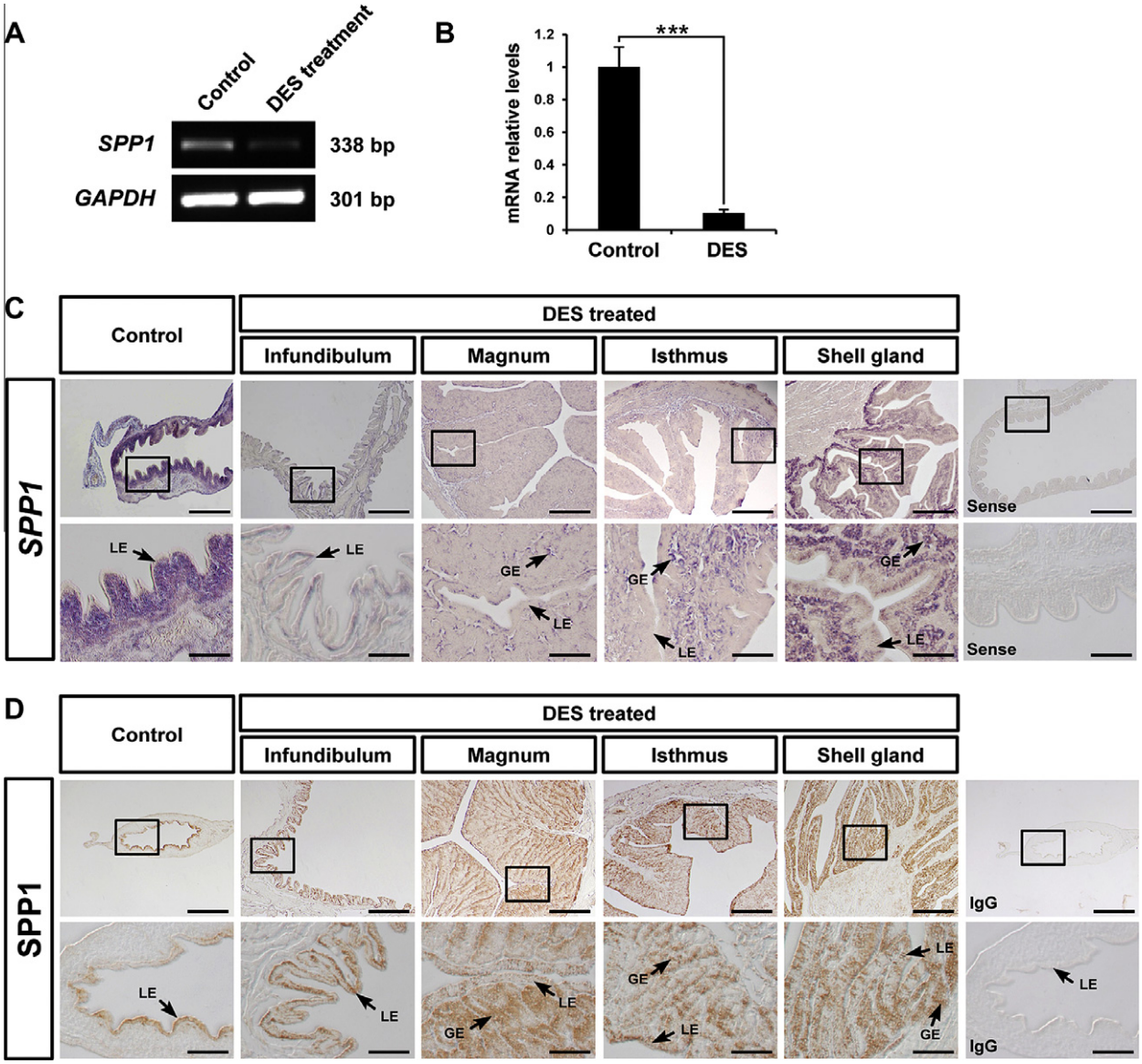

Fig. 2. Effect of DES on tissue- and cell-specific expression of *SPP1* in the chicken oviduct. Both RT-PCR (A) and quantitative-PCR (B) analyses were performed using cDNA templates from DES-treated and control chicken oviducts (mean ± SEM; $P < 0.001$). These experiments were was conducted in triplicate and normalized to control *GAPDH* expression. (C) *In situ* hybridization analyses of *SPP1* mRNA in oviducts of DES-treated and control chicks. Cross-sections of the four segments of chicken oviduct (infundibulum, magnum, isthmus, and shell gland) treated with DES or vehicle were hybridized with antisense or sense chicken *SPP1* cRNA probes. (D) Immunoreactive SPP1 protein in oviducts of DES-treated and control chicks. For the IgG control, normal mouse IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium. Scale bar represents 200 μm (the first horizontal panels) and 50 μm (the second horizontal panels).

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

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

To examine if *SPP1* is up- and down-regulated in ovarian cancer cells of chickens, because the chicken is the only animal that spontaneously develops ovarian cancer of the surface epithelium of the ovaries at a high rate as occurs in women [16], we performed RT-PCR and quantitative PCR analyses. Results of RT-PCR analyses revealed that expression of *SPP1* mRNA was predominantly found in five ovarian endometrioid carcinomas, but there was little or no expression in serous, mucinous or clear cell carcinomas and normal ovaries (Fig. 4A, B). Further, quantitative PCR showed that expression of *SPP1* mRNA was greater ($P < 0.05$) in endometrioid cancerous ovaries from hens (Fig. 4C). To determine cell-specific expression of *SPP1* mRNA and protein, we performed *in situ*

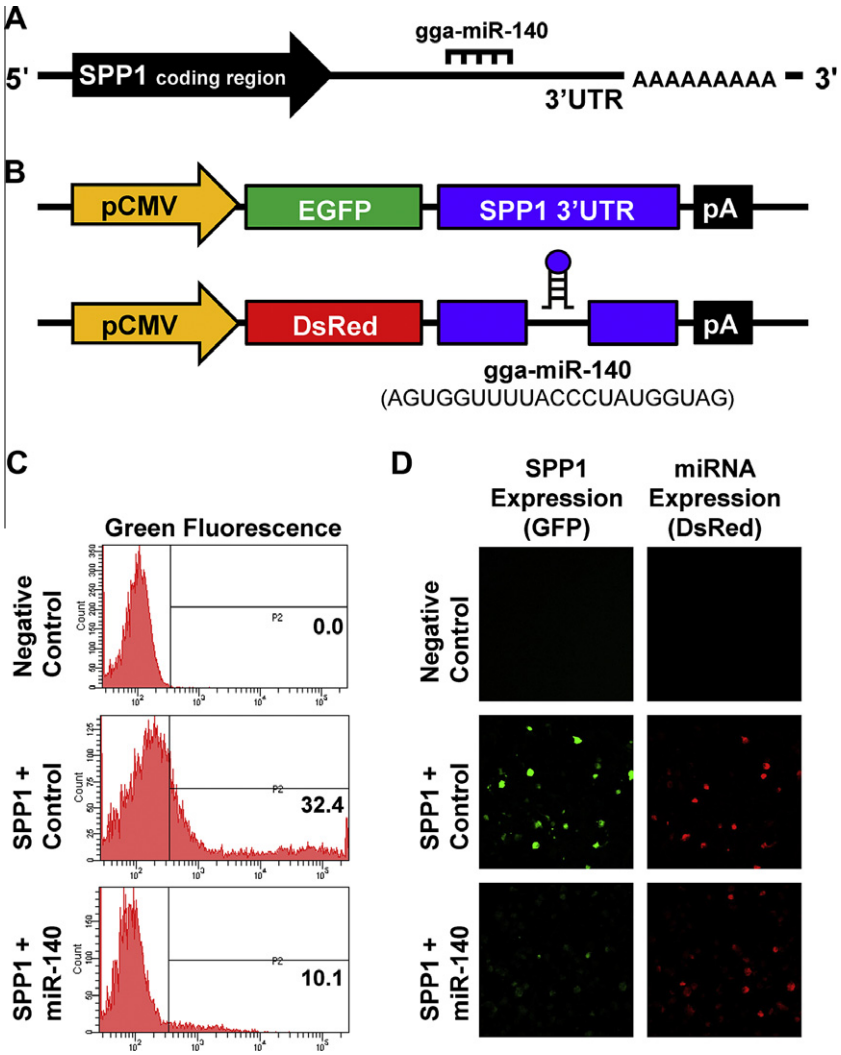

Fig. 3. *In vitro* target assay of microRNAs on the *SPP1* transcript. (A) Diagram of *miR-140* binding site in *SPP1* 3′-UTR. (B) Expression vector maps for eGFP with *SPP1* 3′-UTR and Ds-Red with *miR-140*. The 3′-UTR of the *SPP1* transcript was subcloned between the eGFP gene and the polyA tail to 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 *miR-140* (pcDNA-DsRed-miRNA) (lower panel). (C and D) After co-transfection of pcDNA-eGFP-3′UTR for the *SPP1* transcript and pcDNA-DsRed-miRNA for the *miR-140*, the fluorescence signals of GFP and DsRed were detected using FACS (C) and fluorescent microscopy (D).

hybridization analysis and immunohistochemistry. There was abundant *SPP1* mRNA localized predominantly in GE of cancerous ovaries, but not in LE, stroma or blood vessels (Fig. 4D). Consistent with this result, immunoreactive SPP1 protein was abundant in GE of cancerous ovaries, but not in any other cell types in the same tissues (Fig. 4E).

4. Discussion

Results of the present study demonstrate tissue- and cell-specific expression of *SPP1* in normal chickens. Our results also revealed that *SPP1* gene expression is post-transcriptionally regulated by *miR-140* critical to development of the chick oviduct in response to estrogen. Furthermore, these results are the first to identify high levels of expression of *SPP1* gene in ovarian endometrioid carcinoma as compared to normal ovaries of hens. In chickens, *SPP1* isolated from chicken bone exists as two phosphorylated proteins of approximately 66 kDa and 60 kDa [17] and has seven of nine consecutive residues of aspartic acid, an RGD (Arg-Gly-Asp) integrin recognition motif and four recognition sequences for phosphorylation with the greatest similarity to mammalian *SPP1* [18]. Expression of *SPP1* in a variety of tissues from different organisms suggests that it has important roles in multiple functions such as inflammation responses, calcification, organ development, and carcinogenesis [2]. In this study, chicken *SPP1* gene expression was detected in various organs from male and female chickens. Although the expression of *SPP1* gene in the oviduct and eggshell of chickens has been reported [3,4,19], results of the present study indicate that *SPP1* was not only detected only in the shell gland, but also expressed at lower abundance in LE of the infundibulum and magnum, and GE of the isthmus. Moreover, immunoreactive *SPP1* protein is readily detectable in LE of all segments of the hen oviduct.

Since the discovery of *SPP1*, a number of comparative studies have offered insights into the potential hormonal and molecular mechanisms underlying *SPP1*-mediated cell adhesion, organ remodeling, and cell-extracellular matrix interactions within the reproductive tract such as uterus and embryo/placenta. For examples, in the ovine uterus, *SPP1* mRNA is expressed by endometrial GE during pregnancy [20]. These results suggest that ovarian steroid hormone such as progesterone and/or interferon tau, maternal

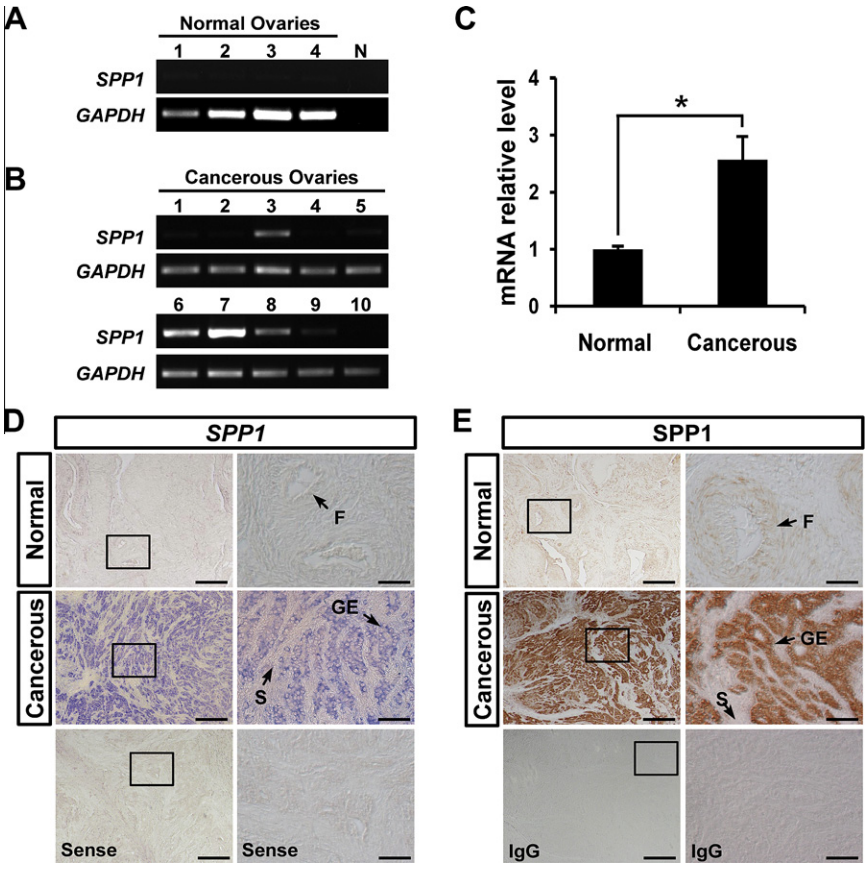

Fig. 4. Expression and quantitation of *SPP1* in normal and cancerous ovaries from hens. (A) and (B) RT-PCR analyses were performed using cDNA templates from normal and cancerous ovaries of laying hens using chicken *SPP1* and *GAPDH*-specific primers. (A) Lanes 1 to 4 show results of analyses of four normal ovaries with N as a negative control. (B) Lanes 1–10 are from analyses of 10 different cancerous ovaries from laying hens. Legend for panel B: 1, clear cell carcinoma (Stage IV); 2, serous carcinoma (Stage III); 3, endometrioid carcinoma (Stage I); 4, serous carcinoma (Stage I); 5, mucinous carcinoma (Stage IV); 6, endometrioid carcinoma (Stage IV); 7, endometrioid carcinoma (Stage III); 8, endometrioid/clear cell carcinoma (Stage IV); 9, serous/mucinous carcinoma (Stage IV); 10, serous carcinoma (Stage III). (C) The q-PCR analysis for *SPP1* mRNA was performed using cDNA templates from normal and cancerous ovaries of laying hens (mean ± SEM; $P < 0.001$). (D) *In situ* hybridization analyses of *SPP1* mRNA in normal and cancerous ovaries of hens. Cross-sections of normal and cancerous ovaries of hens hybridized with antisense or sense chicken *SPP1* cRNA probes. (E) Immunoreactive SPP1 protein in normal and cancerous ovaries of hens. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: GE, glandular epithelium. Scale bar represents 200 μm (the first horizontal panels, sense and IgG) or 50 μm (the second horizontal panels, sense and IgG).

recognition signal from developing trophectoderm in ruminant species, induce expression and secretion of *SPP1* by uterine glands during the peri-implantation period and that *SPP1* induces cell–cell adhesion between LE of the uterus and trophectoderm of the embryo to facilitate superficial implantation in ruminants [20,21]. *SPP1* also has a critical role in the reproductive tract of chickens. Pines et al. reported that *SPP1* is synthesized and secreted by LE cells of the shell gland and accumulates in the egg shell for calcification [22]. However, little is known about the effect of steroid hormones on *SPP1* gene expression in the chicken oviduct. Therefore, we investigated effects of estrogen on expression of *SPP1* mRNA and protein during oviductal development in chicks. In this study, a synthetic nonsteroidal estrogen, diethylstilbestrol (DES) was used. McKnight in 1978 and Kohler in 1969 reported that the developmental pattern of the neonatal chick oviduct in response to DES implants was similar to that in response to natural estrogen based on confirmation of differentiation of tubular glands and ciliated cell, and the expression of egg white protein in the magnum [23,24]. Indeed, the developmental pattern of chick oviduct in response to DES treatment in our study is very similar to the previous reports [25–28]. As illustrated in Fig. 2, DES decreased expression of *SPP1* mRNA and protein in the oviduct as compared to control chicks. These results are consistent with our previous differential gene profiling data on the chicken oviduct treated with the synthetic estrogen agonist DES [29]. Therefore, these results indicate that estrogen down-regulates *SPP1* gene expression during development of the chicken oviduct; however, the underlying mechanisms for DES-induced down-regulated *SPP1* expression are not known.

In a wide variety of fundamental processes and biological events in vertebrates, such as cellular survival, growth, development and differentiation, microRNAs (miRs) play pivotal roles in post-transcriptional regulation and pathways [30]. As shown in Fig. 3, co-transfection of eGFP-*SPP1* 3′-UTR and DsRed-miRNA decreased the percentage of GFP-positive cells and GFP fluorescence density in *miR-140* transfected cells, when compared to controls. These results indicate that *miR-140* bind directly to the 3′-UTR of the *SPP1* transcript and post-transcriptionally regulate *SPP1* gene transcription. Thus, we propose that the *miR-140* is closely related to the regulatory pathways of oviduct development and differentiation in chickens; however, this requires further investigation.

The chicken is a unique animal model for research on human ovarian cancer, because it spontaneously develops epithelial cell-derived ovarian cancer as occurs in women [12]. In general, natural menopause in women occurs between 40 and 55 years of age when estrogen and progesterone production decreases progressively with advancing age of the ovaries. Likewise, epithelial-derived ovarian cancer in hens develops spontaneously at a high rate after they experience a severe depression in egg production when more than 2 years of age. Therefore, the laying hen is a unique model for

research on human ovarian cancer aimed at development of biomarkers and anti-cancer drugs for prevention, early diagnosis, and therapies to treat the disease. In the present study, *SPP1* mRNA and protein are most abundant in GE of ovarian endometrioid carcinoma, but not normal ovaries of hens. These results suggest that *SPP1* increases only in chicken ovarian endometrioid cancer cells that could be used for monitoring effects of therapies for the disease.

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

Acknowledgments

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