Original Research

Avian *SERPINB11* gene: a marker for ovarian endometrioid cancer in chickens

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Abstract

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

Keywords: SERPINB11, chicken, ovary, cancer

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Introduction

Although ovarian cancer accounts for only 4% of cancer cases in women, it is the most lethal gynecological malignancy and the fifth leading cause of cancer-related deaths among women in the USA.[1–3] Ovarian cancer is rarely diagnosed at an early stage[4] and over 75% of woman diagnosed are at an advanced stage of the disease, because it is generally asymptomatic[5] and there is no specific biomarker(s) for early detection.[6,7] There are three types of ovarian cancers: epithelial tumors, germ cell tumors and stromal tumors.[8,9] More than 90% of human ovarian cancers are thought to arise from the germinal epithelium of the ovary.[10] The rate of epithelial ovarian cancer is high because incessant ovulation causes genomic damage to the ovarian surface epithelium, increasing the possibility of gene mutations.[11,12] Even though genetically manipulated rodent models have been used to elucidate some aspects of the etiologies and pathogenesis of ovarian cancer, the non-spontaneous nature of their ovarian cancer limits their clinical relevance.[6,13,14] Chickens ovulate almost every day, whereas women ovulate only once each month. Epithelial-derived ovarian cancer in hens develops spontaneously at a high rate after they experience a severe depression in egg production when more than two years of age. Likewise, natural menopause in women usually occurs between 40 and 55 years of age when estrogen and progesterone production decreases progressively with advancing age of the ovaries. Given the prevalent hypothesis that the cause of ovarian cancer is incessant ovulation,[15] the chicken is the only animal that spontaneously develops ovarian cancer of the surface epithelium of the ovaries at a high rate, as also occurs in women.[16] Therefore, the laying hen is a unique model for research on human ovarian cancer aimed at development of biomarkers

and anticancer drugs for prevention, early diagnosis and therapies to treat the disease.

The serpin superfamily of serine/cysteine proteinase inhibitors is found in *Eukarya, Eubacteria, Archaea* and *Poxviridae.*[17] Most serpins are plasma proteins that have important roles in pivotal physiological events including inflammation, blood coagulation and fibronolysis.[18] Unlike the majority of serpins that are inhibitors of proteases, several serpins, such as SERPINA7 and SERPINH1, are not known inhibitors of proteases, but are involved in hormone transport and protein folding, respectively.[18–20] There are 13 clade B serpin (SERPINB) genes based on *in silico* analysis of human genomic DNA.[21] The SERPINBs are predominantly intracellular proteins that mainly inhibit target proteases.[22] However, there are two intracellular non-inhibitory SERPINBs, SERPINB5 and SERPINB11.[18,23] SERPINB5 is a class II tumor suppressor gene also known as maspin (mammary serine protease inhibitor). It was identified in patients with breast cancer and found to promote apoptosis of invasive breast and prostate cancer cells.[23,24] Additionally, down-regulation of SERPINB5 caused ovarian, colorectal, pancreatic and endometrioid adenocarcinoma.[25] However, a functional role(s) for SERPINB11 in carcinogenesis is unknown. Therefore, we investigated: (1) the expression of SERPINB11 in normal and cancerous ovaries from hens; (2) CpG methylation status of SERPINB11 between normal and cancerous ovarian cells *in vitro*; (3) SERPINB11 expression among chicken ovarian cells (normal and cancerous) compared with that in human epithelial-derived ovarian cancer cell lines, such as OVCAR-3 and SKOV-3, and human ovarian teratocarcinoma cell lines such as PA-1; and (4) whether SERPINB11 is regulated by post-transcriptional actions of specific microRNAs (miRNAs) using a miRNA target validation assay. We found that the laying hen is a unique model for the research on human ovarian cancer and that SERPINB11 has important functions in the development of epithelial ovarian cancer in hens, and should be useful for diagnosis and evaluation of therapies used to treat the disease.

Materials and methods

Experimental animals and animal care

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

Tissue samples

In this study, a total of 136 chickens (88 chickens over 36 months of age and 48 chickens over 24 months of age) which had completely stopped laying eggs were euthanized for biopsy and cancerous ($n = 10$) ovaries were collected. As a control, normal ($n = 5$) ovaries were also collected from egg-laying hens. We examined the tumor stage in 10 chickens with cancerous ovaries using characteristic features of chicken ovarian cancer.[6] In three hens, ovarian tumor cells were classified as stage III as they had metastasized to the gastrointestinal tract and superficial surface of the liver with profuse ascites in the abdominal cavity (Table 1). In five hens, the tumors had metastasized to distant organs such as the liver parenchyma, lung, gastrointestinal tract and oviduct with profuse ascites and were classified as stage IV tumors. The other two hens did not have tumors in any other organs and their ovarian tumors were classified as stage I. To include all cell types within the ovary, cross-sections were frozen or fixed in 4% paraformaldehyde for further analyses. Frozen tissue samples were cut into 5–7 mm pieces and frozen in liquid nitrogen. The other samples were cut into 10-mm pieces and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-embedded tissues were sectioned at 5 μm and stained with hematoxylin and eosin (please see Supplemental Figure 1 at http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2011.011250/-/DC1). 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.[6]

Cell culture

A total of five cell lines, including three human ovarian epithelial cancer and two chicken primary ovarian cells, were

Table 1 Lesions, stages and type of chicken ovarian cancer

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

used in this study. Human ovarian cancer cell lines (OVCAR-3, SKOV-3 and PA-1) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured according to the supplier's directions. Two different chicken ovarian surface epithelial cells (normal and cancerous cells) were isolated and cultured as previously described[26,27] with some modifications. Briefly, small yellow follicles (>8–12 mm) completely covered by the surface epithelial cells were received in a sterile vessel containing saline, washed and transferred to 5 mL prewarmed (37°C) collagenase (1 mg/mL; Sigma-Aldrich Inc, St Louis, MO, USA) in a six-well tissue culture dish. To obtain the ovarian epithelial cells, the dish was moved to a 37°C incubator and swirled every 10 min. After a 30-min incubation period, the follicle surface tissue was scraped gently with sterile forceps, and 5 mL of the used collagenase was transferred into a sterile 15 mL conical centrifuge tube containing complete Dulbelcco's modified Eagle's medium (DMEM)/F-12 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (HyClone, Waltham, MA, USA), 5% chicken serum (Sigma-Aldrich, Inc), 1% non-essential amino acids (Invitrogen), 1% sodium pyruvate (Invitrogen) and 1% antibiotics antimycotics (Abam; Gibco, Carlsbad, CA, USA) to inactivate the collagenase activity. The medium containing cells and ovarian tissue debris was filtered through the 0.45-μm filter. Diluted collagenase was washed by centrifugation at 1250 rpm for 10 min at room temperature. After removing the medium, a small pellet containing cells was re-suspended in 4 mL of complete DMEM/F-12 medium. Cells (4.0×10^5) were dispensed into each of the wells of a six-well tissue culture dish and incubated at 37°C, 5% CO_2. The medium was changed every other day until the cells reached 70–80% confluency. All chicken primary ovarian epithelial cells were placed in short-term culture and expanded (two to three passages). The purity of these cell cultures was confirmed based on their expression of cytokeratin (epithelial cells) or vimentin (mesenchymal cells) proteins.[27]

RNA isolation

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

Semi-quantitative reverse transcription polymerase chain reaction analysis

The expression of *SERPINB11* mRNA in normal and cancerous ovaries of chickens was assessed using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) as described previously.[28] The cDNA was synthesized from total cellular RNA (2 μg) using random hexamer (Invitrogen) 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 *SERPINB11*, the sense primer (5′ CGG AGA CCT GAG CAT GTT GG 3′) and antisense primer (5′ TAT CAC CCC TGT GGA GCC TG 3′) amplified a 337-bp product. For *GAPDH* (housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase), the sense primer (5′ TGC CAA CCC CCA ATG TCT CTG TTG 3′) and antisense primer (5′ TCC TTG GAT GCC ATG TGG ACC AT 3′) amplified a 301-bp product. The primers, PCR amplification and verification of their sequences were conducted as described previously.[28] PCR amplification was conducted using approximately 60 ng cDNA as follows: (1) 95°C for three minutes; (2) 95°C for 20 s, 60°C for 40 s (for *SERPINB11* and *GAPDH*) and 72°C for one minute for 33 cycles; and (3) 72°C for 10 min. After PCR, equal amounts of reaction product were analyzed using a 1% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a Gel Doc™ XR+ system with Image Lab™ software (Bio-Rad, Hercules, CA, USA).

Quantitative RT-PCR analysis

Total RNA was extracted from each sample of normal and cancerous ovarian tissue from hens using TRIzol (Invitrogen) and purified using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands). Complementary DNA was synthesized using AccuPower® RT PreMix (Bioneer). Gene expression levels were measured using SYBR® Green (Sigma-Aldrich) 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 GAPDH expression quantities. The PCR conditions were 95°C for three minutes, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s using a melting curve program (increasing the temperature from 55°C to 95°C at a rate of 0.5°C per 10 s) and continuous fluorescence measurement. ROX dye (Invitrogen) was used as a negative control for the fluorescence measurements. Sequence-specific products were identified by generating a melting curve in which the C_T value represented the cycle number at which a fluorescent signal was statistically greater than background, and relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method.[29] For the control, the relative quantification of gene expression was normalized to the C_T of the control oviduct.

In situ hybridization analysis

For hybridization probes, PCR products were generated from cDNA with the primers used for RT-PCR analysis. The products were gel-extracted and cloned into pGEM-T vector (Promega, Madison, WI, USA). 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′) and

then digoxigenin (DIG)-labeled RNA probes were transcribed using a DIG RNA labeling kit (Roche Applied Science, Indianapolis, IN, USA). Tissues were collected and fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 μm on 3-aminopropyltriethoxysilane-treated (silanized) slides. The sections were then deparaffinized in xylene and re-hydrated 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-Aldrich) in TE buffer (100 mmol/L Tris-HCl, 50 mmol/L ethylenediaminetetraacetic acid [EDTA], pH 8.0) at 37°C. After postfixation in 4% paraformaldehyde, sections were incubated twice for five minutes each in DEPC-treated PBS and incubated in triethanolamine buffer (0.1 mol/L triethanolamine) containing 0.25% (v/v) acetic anhydride. The sections were incubated in a prehybridization mixture containing 50% formamide and 4× standard saline citrate (SSC) for at least 10 min at room temperature. After prehybridization, the sections were incubated with a hybridization mixture containing 40% formamide, 4× SSC, 10% dextran sulfate sodium salt, 10 mmol/L dithiothreitol, 1 mg/mL yeast tRNA, 1 mg/mL salmon sperm DNA, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2 mg/mL RNase-free bovine serum albumin and denatured DIG-labeled cRNA probe overnight at 42°C in a humidified chamber. After hybridization, sections were washed for 15 min in 2× SSC at 37°C, 15 min in 1× SSC at 37°C, 30 min in NTE buffer (10 mmol/L Tris, 500 mmol/L NaCl and 1 mmol/L EDTA) at 37°C and 30 min in 0.1× SSC at 37°C. After blocking with a 2% normal sheep serum (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), the sections were incubated overnight with sheep anti-DIG antibody conjugated to alkaline phosphatase (Roche Applied Science). The signal was visualized by exposure to a solution containing 0.4 mmol/L 5-bromo-4-chloro-3-indolyl phosphate, 0.4 mmol/L nitroblue tetrazolium and 2 mmol/L levamisole (Sigma-Aldrich).

Immunohistochemistry

Immunocytochemical localization of SERPINB11 protein in normal and cancerous ovaries from chickens was performed as described previously[30] and a goat anti-human SERPINB11 polyclonal antibody (Catalog number sc-85140; Santa Cruz Biotechnology, Inc) was used at a final dilution 1:100 (2 μg/mL). Antigen retrieval was performed using the boiling citrate method as described previously.[30] Negative controls included substitution of the primary antibody with purified non-immune goat IgG at the same final concentration.

Prediction of transcription factor-binding *cis*-elements

The presence of transcription factor-binding *cis*-elements within the SERPINB11 promoter region was predicted using a bioinformatics tool for orthologous sequences (TFSEARCH version 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 MethylEasy^{TM}Xceed (Human Genetic Signatures, North Ryde, NSW, Australia) according to the manufacturer's instructions. For amplifying the converted DNA, PCRs were performed with forward (5 TTA AAG TGT GTG TAT TTA TTA TT 3) and reverse (5 CCT AAA CAA ATT CTC TAA ATC CT 3) primers, which included the upstream region of the *SERPINB11* gene, as follows: 95°C for one minute and 50 s, 35 cycles at 94°C for 20 s, 52°C for 20 s, 72°C for 30 s and 72°C for 10 min for the final synthesis. The PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced using an ABI Prism 3730 XL DNA Analyzer (Applied Biosystems).

Immunofluorescence microscopy for detection of SERPINB11 activation

Ovarian cancer cells and normal ovarian cells obtained from laying hens, and three human ovarian cancer cell lines, including OVCAR-3, SKOV-3 and PA-1, were examined for SERPINB11 protein expression patterns by immunofluorescence microscopy. Each type of cell was seeded onto Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA). After 24 h, cells were fixed with −20°C methanol, and immunofluorescence staining was performed using a goat anti-human SERPINB11 polyclonal antibody (Catalog number sc-85140; Santa Cruz Biotechnology, Inc). Cells were then incubated with Alexa Fluor 488 rabbit anti-goat IgG secondary antibody (A21222; Invitrogen). Slides were overlayed with 4′,6-diamidino-2-phenylindole before images were captured. Images were captured using a Zeiss confocal microscope LSM710 (Carl Zeiss, Oberkochen, Germany) fitted with a digital microscope camera (AxioCam) using Zen 2009 software.

MicroRNA target validation assay

The 3′UTRs of *SERPINB11* was cloned and confirmed by sequencing. The 3′UTR was subcloned between the eGFP gene and the bovine growth hormone poly-A tail in pcDNA3eGFP (Clontech, Mountain View, CA, USA) to generate the eGFP-miRNA target 3′UTR (pcDNA-eGFP-3′UTR) fusion constructs. For the dual fluorescence reporter assay, the fusion constructs containing the *DsRed* gene and *miR-1582* were designed to be co-expressed under control of the CMV promoter (pcDNA-DsRed-miRNA). The pcDNA-eGFP-3′UTR and pcDNA-DsRed-miRNA (3 μg) were co-transfected into 293FT cells using the calcium phosphate method. When the DsRed-miRNA is expressed and binds to the target site of the 3′UTR downstream of the GFP transcript, green fluorescence intensity decreases due to degradation of the GFP transcript. At 48 h post-transfection, dual fluorescence was detected by fluorescence microscopy and calculated by FACSCalibur flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). For flow cytometry, the cells were fixed in 4% paraformaldehyde and analyzed using the FlowJo software (Tree Star Inc, Ashland, OR, USA).

Statistical analyses

Data presented for realtime PCR are expressed as mean ± 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.

Results

Differential expression of SERPINB11 in normal and cancerous ovaries of hens

We previously reported expression of cysteine protease cathepsins in ovarian tissue from hens with ovarian cancer.[31] Based on the morphological and immunohistochemical differences between normal and cancerous ovaries from hens (Table 1 and Supplemental Figure 1), we hypothesized that expression patterns for SERPINB11 would differ between normal and cancerous ovarian tissues from hens. Based on RT-PCR analysis, *SERPINB11* mRNA was predominantly found in endometrioid carcinoma, but there was little or no expression in serous, mucinous, or clear cell carcinomas and normal ovaries (Figures 1a and b). Further, the expression level of *SERPINB11* mRNA was greater ($P < 0.01$) in cancerous ovaries from hens (Figure 1c).

Localization of SERPINB11 mRNA and protein in cancerous ovaries of hens

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

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

The effect of CpG demethylation in the upstream region of a gene increases its transcriptional activity. Moreover, CpG methylation is closely associated with the development of cancers. We therefore investigated methylation patterns in the promoter region of the *SERPINB11* gene in normal ovarian epithelia and cancerous ovarian epithelia. Both normal and cancerous ovarian epithelial cells were extracted and cultured *in vitro* as previously reported.[32] Bisulfite sequencing results showed that CpG

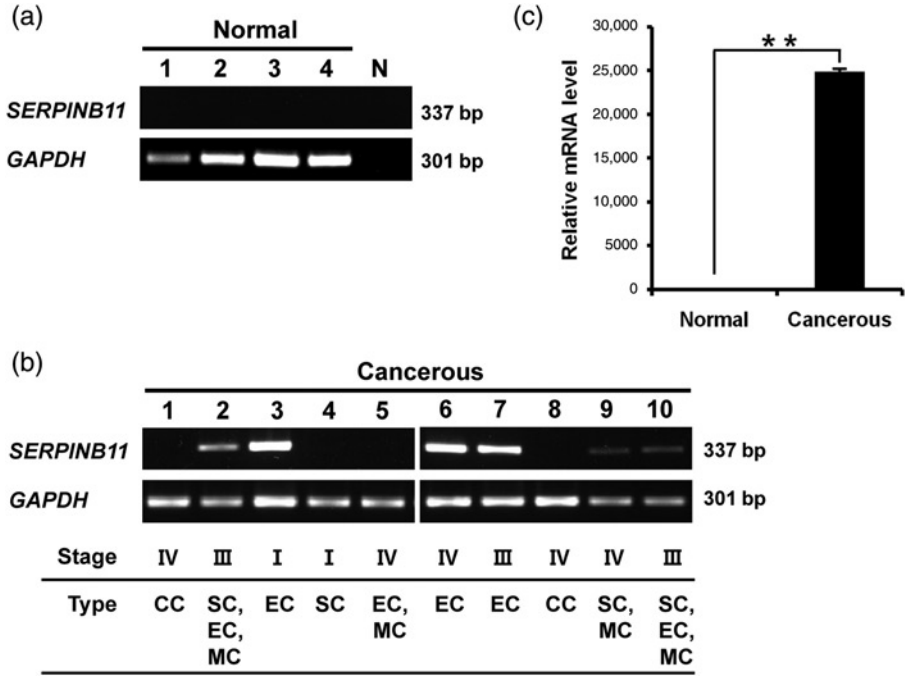

Figure 1 Expression and quantitation of *SERPINB11* mRNA levels in normal and cancerous ovaries from hens. (a and b) Reverse transcription polymerase chain reaction (PCR) analyses were performed using cDNA templates from normal and cancerous ovaries of chickens with chicken *SERPINB11* and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*-specific primers. Lanes 1–4 show four different normal ovaries and N is negative control (a). Lanes 1–10 in (b) show 10 different cancerous ovaries. *SERPINB11* mRNA was predominantly found in endometrioid carcinoma, but there was little or no expression in serous, mucinous or clear cell carcinomas or normal ovaries. (c) The quantitative realtime PCR analysis was performed using cDNA templates from normal and cancerous ovaries of chickens (mean ± SEM; $P < 0.01$). Please see Materials and methods for a complete description of the methods. (b) Lane 1, clear cell carcinoma (stage IV); lane 2, endometrioid/serous/mucinous carcinoma (stage III); lane 3, endometrioid carcinoma (stage I); lane 4, serous carcinoma (stage I); lane 5, mucinous/endometrioid carcinoma (stage IV); lane 6, endometrioid carcinoma (stage IV); lane 7, endometrioid carcinoma (stage III); lane 8, clear cell carcinoma (stage IV); lane 9, serous/mucinous carcinoma (stage IV); and lane 10, serous/mucinous/endometrioid carcinoma (stage III)

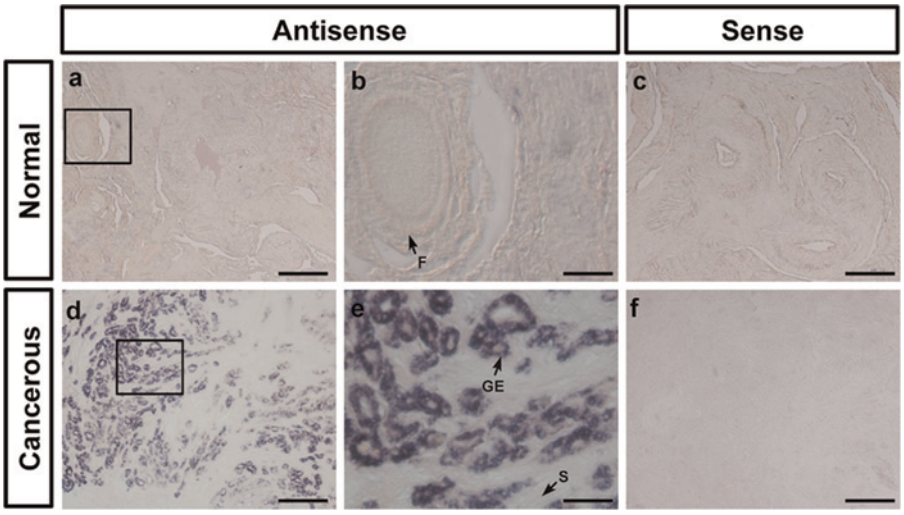

Figure 2 *In situ* hybridization analyses of *SERPINB11* mRNAs in normal and endometrioid cancerous ovaries of hens. Cross-sections of normal and cancerous ovaries of hens were hybridized with antisense or sense chicken *SERPINB11* cRNA probes. Please see Materials and methods for a complete description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

sites at −54, −329 and −346 CpG from the transcriptional start site maintained methylation status in both cells. However, about 30% of the CpG site at −110 was methylated in ovarian cancer cells, whereas no CpG sites at −110 were methylated in normal ovarian epithelial cells (Figure 4).

Post-transcriptional action of miRNAs on SERPINB11

Based on the possibility that SERPINB11 expression is regulated at the post-transcriptional level by miRNAs, we performed a miRNA target validation assay. Analysis of potential miRNA binding sites within the 3′UTR for *SERPINB11* using a miRNA target prediction database (miRDB; http://mirdb.org/miRDB/) revealed one putative binding site for *miR-1582*. Therefore, we examined whether *miR-1582* influenced *SERPINB11* expression via its 3′UTR. A fragment of the *SERPINB11* 3′UTR harboring the *miR-1582* binding site was cloned downstream of the green fluorescent protein reading frame, thereby creating a fluorescent reporter for the function of the 3′UTR region. In the presence of *miR-1582*, there was no significant decrease in green fluorescence from the reporter containing the *SERPINB11* 3′UTR compared with the control. This result indicates that *miR-1582* does not directly target the 3′UTR of *SERPINB11* mRNA (please see Supplemental Figure 2 at http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2011.011250/-/DC1). Thus, post-transcriptional regulation of *SERPINB11* gene expression is highly unlikely.

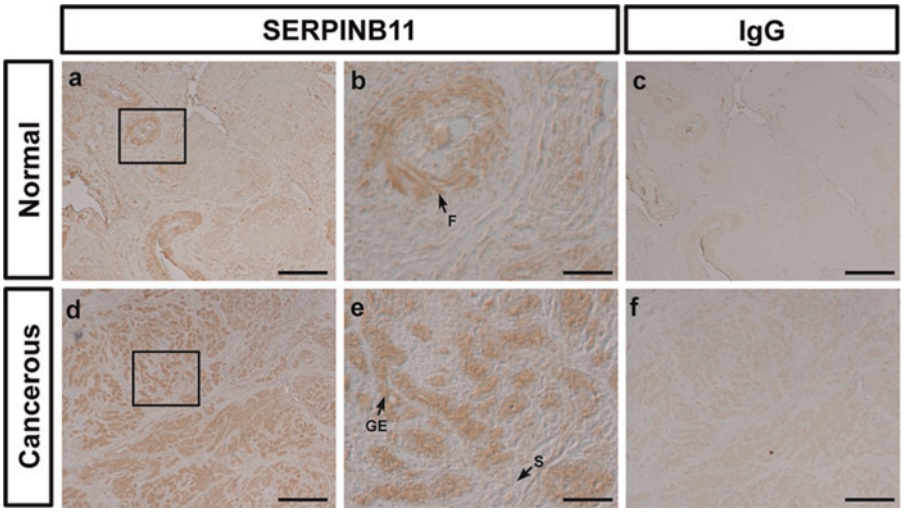

Figure 3 Immunoreactive SERPINB11 protein in normal and endometrioid cancerous ovaries of hens. For the IgG control, normal goat IgG was substituted for the primary antibody. Sections were not counterstained. Please see Materials and methods for a complete description of the methods. F, follicle; GE,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

Immunofluorescence detection of SERPINB11 protein in human immortalized ovarian carcinoma cells

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

Discussion

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

We reported that *lysosomal cysteine cathepsin B* (*CTSB*), *CTSC* and *CTSS* genes are highly expressed in the cancerous ovaries of hens.[31] These and other proteases, such as metalloproteinase, serine, threonine, cysteine and aspartic proteases, occur naturally in all tissues to affect protein catabolism by hydrolysis of the specific peptide bonds.[33,34] Indeed, proteases play fundamental roles in a wide variety of biological events including cancer progression and metastasis due to their capacity to degrade extracellular matrix proteins.[33] Protease inhibitors can be classified based on the protease they block or by their inhibitory mechanism. Of these, serpins (serine protease inhibitors) are the largest and most broadly distributed superfamily of protease inhibitors, and they play pivotal roles in many important physiological cascades such as inflammation and blood coagulation pathways.[35,36] At present, there are two intracellular non-inhibitory SERPINBs, i.e. SERPINB5 and -11 in mammals, but a functional role(s) for SERPINB11 in cancer biology has not been established. Therefore, in the present study, we used the unique chicken ovarian cancer model,[6,31,32] as it is the only animal that spontaneously develops ovarian cancer from the ovarian epithelia with a high incidence as occurs in women. As illustrated in Figures 2 and 3, results of the present study show that

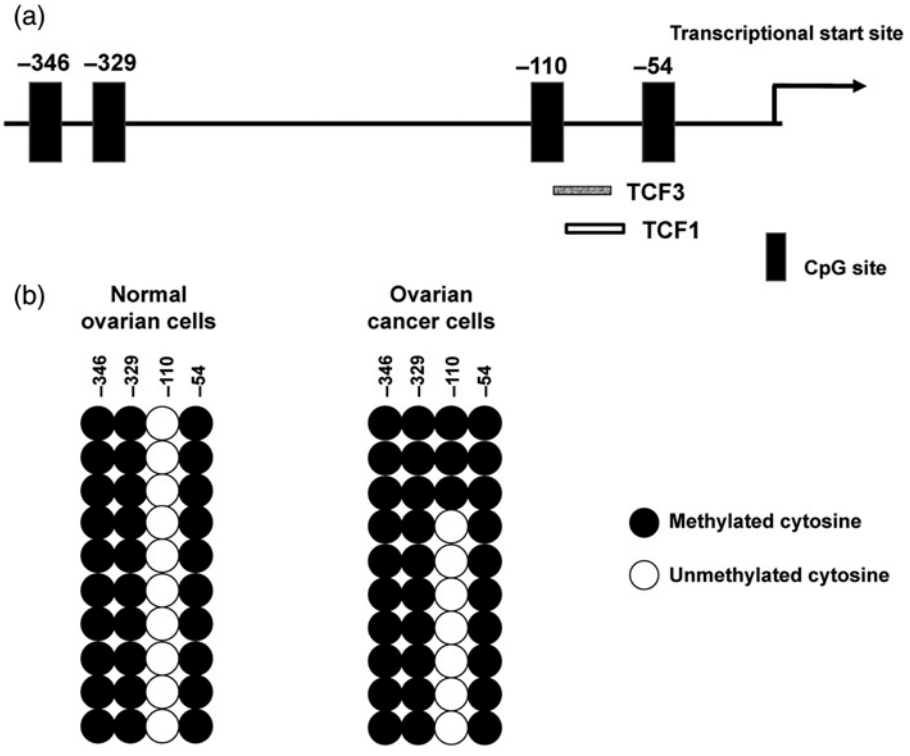

Figure 4 Bisulfite sequencing of CpG sites in the upstream region of the *SERPINB11* gene. (a) Schematic of the four CpG sites in the promoter region of the *SERPINB11* gene are indicated by the heavy black vertical lines. The numbers on the line indicate positions relative to the transcription start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

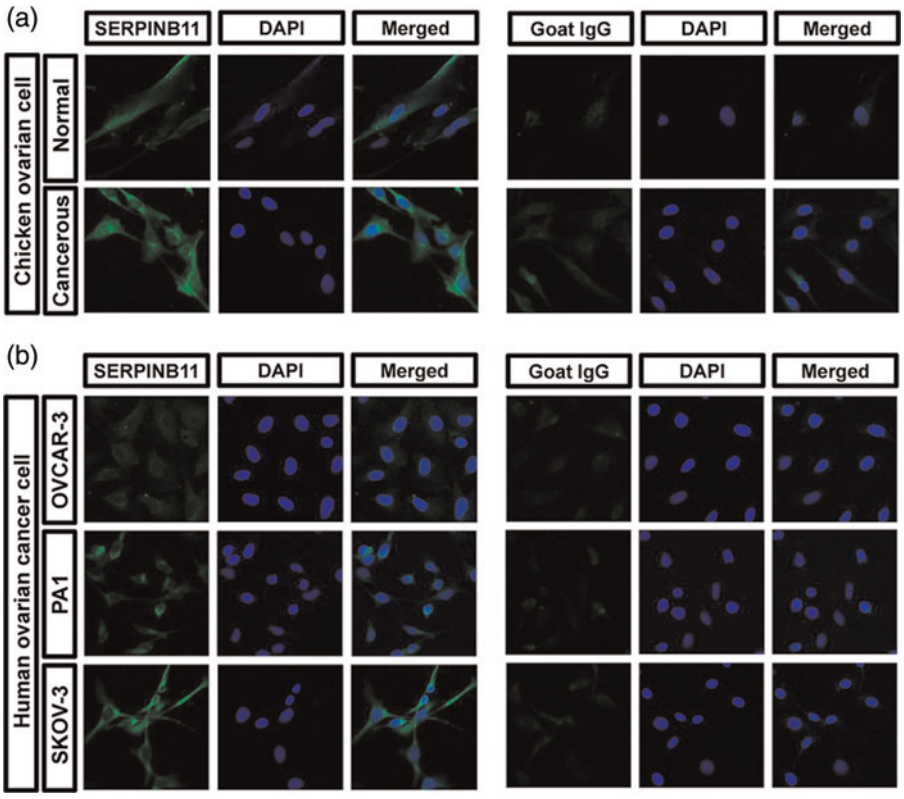

Figure 5 Differential expression of SERPINB11 protein in normal and cancerous ovarian cells from hens (a) and human immortalized ovarian carcinoma cells (b). (a) SERPINB11 protein was barely detectable in normal cells, but abundant in the cytoplasm of cancer cells. (b) SERPINB11 was expressed in SKOV-3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

SERPINB11 is expressed predominantly in the glandular epithelium of cancerous ovaries of chickens. Unlike SERPINB5, SERPINB11 is an effective inhibitor of angiogenesis by inhibiting migration of endothelial cells and limiting mitogenesis and tube formation.[37] In laying hens, SERPINB11 may be involved in gland morphogenesis and angiogenesis. Alternatively, it is possible that SERPINB11 interacts reciprocally with other types of proteases in cancerous ovaries of chickens as it exhibits some inhibitory activity against several cysteine protease cathepsins.[18]

In cancer cells, a variety of genes are either up-regulated or down-regulated by an epigenetic mechanism such as DNA methylation and histone modification (methylation and/or deacetylation).[38] The epigenetic regulatory mechanism mainly involves carcinogenesis cascades that, for instance, increase the rate of tumor growth by inactivation of tumor suppressor genes, and established oncogenes by hypermethylation of promoter regions of genes and gene silencing.[39,40] Indeed, the methylation of the promoter region of the *SERPINB5* gene leads to gene silencing in various cancers, such as thyroid, colorectal and skin cancers.[40–43] In the present study, our bisulfite sequencing results revealed that about 30% of −110 CpG sites were methylated in ovarian cancer cells, but not in normal ovarian epithelial cells (Figure 4). This difference in methylation status between normal and cancerous ovarian cells is likely important in the development of cancer phenotypes. Expression of SERPINB11 may be epigenetically regulated, and its cell-type specific expression closely associated with DNA methylation. The results of this study also showed that the position of the −110 CpG site is located within putative binding elements for transcription factor 3 (TCF3; E2A immunoglobulin enhancer-binding factors E12/E47) and transcription factor 1 (TCF1) encoding hepatocyte nuclear factor 1 alpha. Furthermore, we compared the 5′ flanking region (about 2 kb) of mouse and human SERPINB11 with that of chicken SERPINB11 to identify differences in sequences around CpG sites among these species. We identified eight and ten CpG sites in mouse and human, respectively. In addition, the position of the −110 CpG site is located only within putative binding elements for TCF3 and TCF1, which are highly conserved in each of the three species (please see Supplemental Figure 3 at http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2011.011250/-/DC1). In humans, TCF3 is related to development of acute lymphoblastic leukemia,[44] whereas TCF1 is mutated in 50% of liver cell adenomas and its mutation causes endometrial carcinomas.[45] Further research is required for elucidation of the relationship between SERPINB11 and TCF signaling cascades in cancerous ovaries of hens.

MicroRNAs (miRNAs), non-coding RNAs of 18–23 nucleotides in length, regulate gene expression and are capable of defining and altering cell fate by inducing or inhibiting translation or cleavage of their target mRNAs through base pairing at partially or fully complementary sites.[46] By regulating gene expression, miRNAs affect the function of a number of cellular processes in development,

differentiation and oncogenesis.[47–49] About 500–1000 miRNAs are present in the mammalian genome, and up to 30% of human mRNAs are thought to be under the regulation of one or more miRNAs, suggesting functions of miRNAs in post-transcriptional regulation.[50,51] In the present study, analysis of potential miRNA binding sites within 3′UTR for *SERPINB11* using the miRNA target prediction database (miRDB; http://mirdb.org/miRDB/) revealed one putative binding site for *miR-1582*. Thus, we investigated the effect of *miR-1582* on *SERPINB11* gene expression via its 3′UTR, but *miR-1582* did not directly target the 3′UTR of *SERPINB11* mRNA (Supplemental Figure 2). These results indicate that post-transcriptional regulation does not likely influence *SERPINB11* expression and that an alternative mechanism(s) is involved in regulation of its expression. Clearly, further research on the mechanism for post-transcriptional regulation of *SERPINB11* expression is needed.

In this study, we compared expression patterns of SERPINB11 protein in chicken and human ovarian cancer cells. Immunoreactive SERPINB11 protein was localized predominately to the cytoplasmic compartment in chicken cancer cells, as well as human SKOV-3 cells and, to a lesser extent, in human PA-1 cells. However, it was not detected in human OVCAR-3 cells (Figure 5). This result indicates that chicken and human SERPINB11, a member of clade-B serpins characteristically detected in cytoplasmic compartment,[21,52] may be involved in carcinogenesis although it is not known how expression of SERPINB11 proteins is regulated by epigenetic programming.

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

Author contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript. WL, J-HK and JK conducted the experiments; GS and FWB wrote the manuscript; JYH contributed to providing all animals for tissue sampling; and GS designed the experiments and analyzed experimental data.

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REFERENCES

1 Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007;**57**:43–66
2 Wong AS, Auersperg N. Ovarian surface epithelium: family history and early events in ovarian cancer. *Reprod Biol Endocrinol* 2003;**1**:70
3 Cvetkovic D. Early events in ovarian oncogenesis. *Reprod Biol Endocrinol* 2003;**1**:68
4 Goodman MT, Correa CN, Tung KH, Roffers SD, Cheng Wu X, Young JL Jr, Wilkens LR, Carney ME, Howe HL. Stage at diagnosis of ovarian cancer in the United States, 1992–1997. *Cancer* 2003;**97**:2648–59
5 Bast RC Jr, Urban N, Shridhar V, Smith D, Zhang Z, Skates S, Lu K, Liu J, Fishman D, Mills G. Early detection of ovarian cancer: promise and reality. *Cancer Treat Res* 2002;**107**:61–97
6 Barua A, Bitterman P, Abramowicz JS, Dirks AL, Bahr JM, Hales DB, Bradaric MJ, Edassery SL, Rotmensch J, Luborsky JL. Histopathology of ovarian tumors in laying hens: a preclinical model of human ovarian cancer. *Int J Gynecol Cancer* 2009;**19**:531–9
7 Pepe MS, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, Winget M, Yasui Y. Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst* 2001;**93**:1054–61
8 Kurman RJ, Visvanathan K, Roden R, Wu TC, Shih Ie M. Early detection and treatment of ovarian cancer: shifting from early stage to minimal volume of disease based on a new model of carcinogenesis. *Am J Obstet Gynecol* 2008;**198**:351–6
9 Kurman RJ, Shih Ie M. Pathogenesis of ovarian cancer: lessons from morphology and molecular biology and their clinical implications. *Int J Gynecol Pathol* 2008;**27**:151–60
10 Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC. Ovarian surface epithelium: biology, endocrinology, and pathology. *Endocr Rev* 2001;**22**:255–88
11 Murdoch WJ, Van Kirk EA, Alexander BM. DNA damages in ovarian surface epithelial cells of ovulatory hens. *Exp Biol Med (Maywood)* 2005;**230**:429–33
12 Auersperg N, Edelson MI, Mok SC, Johnson SW, Hamilton TC. The biology of ovarian cancer. *Semin Oncol* 1998;**25**:281–304
13 Vanderhyden BC, Shaw TJ, Ethier JF. Animal models of ovarian cancer. *Reprod Biol Endocrinol* 2003;**1**:67
14 Stakleff KD, Von Gruenigen VE. Rodent models for ovarian cancer research. *Int J Gynecol Cancer* 2003;**13**:405–12
15 Fathalla MF. Incessant ovulation – a factor in ovarian neoplasia? *Lancet* 1971;**2**:163
16 Stammer K, Edassery SL, Barua A, Bitterman P, Bahr JM, Hales DB, Luborsky JL. Selenium-binding protein 1 expression in ovaries and ovarian tumors in the laying hen, a spontaneous model of human ovarian cancer. *Gynecol Oncol* 2008;**109**:115–21
17 Gettins PG. The F-helix of serpins plays an essential, active role in the proteinase inhibition mechanism. *FEBS Lett* 2002;**523**:2–6
18 Askew DJ, Cataltepe S, Kumar V, Edwards C, Pace SM, Howarth RN, Pak SC, Askew YS, Bromme D, Luke CJ, Whisstock JC, Silverman GA. SERPINB11 is a new noninhibitory intracellular serpin. Common single nucleotide polymorphisms in the scaffold impair conformational change. *J Biol Chem* 2007;**282**:24948–60
19 Zhou A, Wei Z, Read RJ, Carrell RW. Structural mechanism for the carriage and release of thyroxine in the blood. *Proc Natl Acad Sci USA* 2006;**103**:13321–6
20 Dafforn TR, Della M, Miller AD. The molecular interactions of heat shock protein 47 (Hsp47) and their implications for collagen biosynthesis. *J Biol Chem* 2001;**276**:49310–9
21 Silverman GA, Whisstock JC, Askew DJ, Pak SC, Luke CJ, Cataltepe S, Irving JA, Bird PI. Human clade B serpins (ov-serpins) belong to a cohort of evolutionarily dispersed intracellular proteinase inhibitor clades that protect cells from promiscuous proteolysis. *Cell Mol Life Sci* 2004;**61**:301–25
22 Bird CH, Blink EJ, Hirst CE, Buzza MS, Steele PM, Sun J, Jans DA, Bird PI. Nucleocytoplasmic distribution of the ovalbumin serpin PI-9 requires

a nonconventional nuclear import pathway and the export factor Crm1. *Mol Cell Biol* 2001;**21**:5396–407
23 Luo JL, Tan W, Ricono JM, Korchynskyi O, Zhang M, Gonias SL, Cheresh DA, Karin M. Nuclear cytokine-activated IKKalpha controls prostate cancer metastasis by repressing Maspin. *Nature* 2007;**446**: 690–4
24 Zou Z, Anisowicz A, Hendrix MJ, Thor A, Neveu M, Sheng S, Rafidi K, Seftor E, Sager R. Maspin, a serpin with tumor-suppressing activity in human mammary epithelial cells. *Science* 1994;**263**:526–9
25 Li HW, Leung SW, Chan CS, Yu MM, Wong YF. Expression of maspin in endometrioid adenocarcinoma of endometrium. *Oncol Rep* 2007;**17**:393–8
26 Shepherd TG, Theriault BL, Campbell EJ, Nachtigal MW. Primary culture of ovarian surface epithelial cells and ascites-derived ovarian cancer cells from patients. *Nat Protoc* 2006;**1**:2643–9
27 Giles JR, Olson LM, Johnson PA. Characterization of ovarian surface epithelial cells from the hen: a unique model for ovarian cancer. *Exp Biol Med (Maywood)* 2006;**231**:1718–25
28 Song G, Bazer FW, Spencer TE. Pregnancy and interferon tau regulate RSAD2 and IFIH1 expression in the ovine uterus. *Reproduction* 2007;**133**:285–95
29 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;**25**:402–8
30 Song G, Spencer TE, Bazer FW. Progesterone and interferon-tau regulate cystatin C in the endometrium. *Endocrinology* 2006;**147**:3478–83
31 Ahn SE, Choi JW, Rengaraj D, Seo HW, Lim W, Han JY, Song G. Increased expression of cysteine cathepsins in ovarian tissue from chickens with ovarian cancer. *Reprod Biol Endocrinol* 2010;**8**:100
32 Giles JR, Shivaprasad HL, Johnson PA. Ovarian tumor expression of an oviductal protein in the hen: a model for human serous ovarian adenocarcinoma. *Gynecol Oncol* 2004;**95**:530–3
33 Lopez-Otin C, Matrisian LM. Emerging roles of proteases in tumour suppression. *Nat Rev Cancer* 2007;**7**:800–8
34 Puente XS, Sanchez LM, Overall CM, Lopez-Otin C. Human and mouse proteases: a comparative genomic approach. *Nat Rev Genet* 2003;**4**:544–58
35 Irving JA, Pike RN, Lesk AM, Whisstock JC. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Res* 2000;**10**:1845–64
36 Law RH, Zhang Q, McGowan S, Buckle AM, Silverman GA, Wong W, Rosado CJ, Langendorf CG, Pike RN, Bird PI, Whisstock JC. An overview of the serpin superfamily. *Genome Biol* 2006;**7**:216
37 Zhang M, Volpert O, Shi YH, Bouck N. Maspin is an angiogenesis inhibitor. *Nat Med* 2000;**6**:196–9
38 Suzuki K, Oneyama C, Kimura H, Tajima S, Okada M. Down-regulation of the tumor suppressor C-terminal Src kinase (Csk)-binding protein (Cbp)/PAG1 is mediated by epigenetic histone modifications via the mitogen-activated protein kinase (MAPK)/phosphatidylinositol 3-kinase (PI3K) pathway. *J Biol Chem* 2011;**286**:15698–706
39 Laird PW, Jaenisch R. DNA methylation and cancer. *Hum Mol Genet* 1994;3 Spec No:1487–95
40 Khalkhali-Ellis Z. Maspin: the new frontier. *Clin Cancer Res* 2006;**12**:7279–83
41 Boltze C, Schneider-Stock R, Quednow C, Hinze R, Mawrin C, Hribaschek A, Roessner A, Hoang-Vu C. Silencing of the maspin gene by promoter hypermethylation in thyroid cancer. *Int J Mol Med* 2003;**12**:479–84
42 Bettstetter M, Woenckhaus M, Wild PJ, Rummele P, Blaszyk H, Hartmann A, Hofstadter F, Dietmaier W. Elevated nuclear maspin expression is associated with microsatellite instability and high tumour grade in colorectal cancer. *J Pathol* 2005;**205**:606–14
43 Reis-Filho JS, Torio B, Albergaria A, Schmitt FC. Maspin expression in normal skin and usual cutaneous carcinomas. *Virchows Arch* 2002;**441**:551–8
44 Barber KE, Harrison CJ, Broadfield ZJ, Stewart AR, Wright SL, Martineau M, Strefford JC, Moorman AV. Molecular cytogenetic characterization of TCF3 (E2A)/19p13.3 rearrangements in B-cell precursor acute lymphoblastic leukemia. *Genes Chromosomes Cancer* 2007;**46**:478–86
45 Rebouissou S, Rosty C, Lecuru F, Boisselier S, Bui H, Le Frere-Belfa MA, Sastre X, Laurent-Puig P, Zucman-Rossi J. Mutation of TCF1 encoding hepatocyte nuclear factor 1alpha in gynecological cancer. *Oncogene* 2004;**23**:7588–92
46 Garzon R, Fabbri M, Cimmino A, Calin GA, Croce CM. MicroRNA expression and function in cancer. *Trends Mol Med* 2006;**12**:580–7
47 Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;**116**:281–97
48 Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. *Nature* 2005;**435**:834–8
49 Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 2005;**123**:631–40
50 Aravin A, Tuschl T. Identification and characterization of small RNAs involved in RNA silencing. *FEBS Lett* 2005;**579**:5830–40
51 Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 2005;**120**:15–20
52 Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PG, Irving JA, Lomas DA, Luke CJ, Moyer RW, Pemberton PA, Remold-O'Donnell E, Salvesen GS, Travis J, Whisstock JC. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J Biol Chem* 2001;**276**: 33293–6

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