In-depth LC–MS/MS analysis of the chicken ovarian cancer proteome reveals conserved and novel differentially regulated…

Article *in* Analytical and Bioanalytical Chemistry · July 2015

DOI: 10.1007/s00216-015-8862-4 · Source: PubMed

CITATIONS
4

READS
720

9 authors, including:

David Muddiman
North Carolina State University
290 PUBLICATIONS **8,034** CITATIONS
SEE PROFILE

Adam M Hawkridge
Virginia Commonwealth University
53 PUBLICATIONS **1,874** CITATIONS
SEE PROFILE

Anal Bioanal Chem
DOI 10.1007/s00216-015-8862-4

RESEARCH PAPER

In-depth LC-MS/MS analysis of the chicken ovarian cancer proteome reveals conserved and novel differentially regulated proteins in humans

Angelito I. Nepomuceno[1] · Huanjie Shao[3] · Kai Jing[3] · Yibao Ma[3] · James N. Petitte[2] · Michael O. Idowu[4] · David C. Muddiman[1] · Xianjun Fang[3] · Adam M. Hawkridge[5,6]

Received: 11 May 2015 / Revised: 11 June 2015 / Accepted: 15 June 2015

Abstract Ovarian cancer (OVC) remains the most lethal gynecological malignancy in the world due to the combined lack of early-stage diagnostics and effective therapeutic strategies. The development and application of advanced proteomics technology and new experimental models has created unique opportunities for translational studies. In this study, we investigated the ovarian cancer proteome of the chicken, an emerging experimental model of OVC that develops ovarian tumors spontaneously. Matched plasma, ovary, and oviduct tissue biospecimens derived from healthy, early-stage OVC, and late-stage OVC birds were quantitatively characterized by label-free proteomics. Over 2600 proteins were identified in this study, 348 of which were differentially expressed by more than twofold ($p \leq 0.05$) in early- and late-stage ovarian tumor tissue specimens relative to healthy ovarian tissues. Several of the 348 proteins are known to be differentially regulated in human cancers including B2M, CLDN3, EPCAM, PIGR, S100A6, S100A9, S100A11, and TPD52. Of particular interest was ovostatin 2 (OVOS2), a novel 165-kDa protease inhibitor found to be strongly upregulated in chicken ovarian tumors ($p=0.0005$) and matched plasma ($p=0.003$). Indeed, RT-quantitative PCR and Western blot analysis demonstrated that OVOS2 mRNA and protein were also upregulated in multiple human OVC cell lines compared to normal ovarian epithelia (NOE) cells and immunohistochemical staining confirmed overexpression of OVOS2 in primary human ovarian cancers relative to non-cancerous tissues. Collectively, these data provide the first evidence for involvement of OVOS2 in the pathogenesis of both chicken and human ovarian cancer.

Keywords Mass spectrometry · Proteomics · Ovarian Cancer · Chicken · Ovostatin 2 · OVOS2

Electronic supplementary material The online version of this article (doi:10.1007/s00216-015-8862-4) contains supplementary material, which is available to authorized users.

✉ Adam M. Hawkridge
amhawkridge@vcu.edu

1 W.M. Keck FTMS Laboratory for Human Health Research, Department of Chemistry, North Carolina State University, 2620 Yarbrough Dr., Box 8204, Raleigh, NC 27695, USA

2 Prestage Department of Poultry Science, North Carolina State University, 2711 Founders Drive, Raleigh, NC 27695, USA

3 Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, 1101 E. Marshall Street, Richmond, VA 23298, USA

4 Department of Pathology, Virginia Commonwealth University, 1101 E. Marshall Street, Richmond, VA 23298, USA

5 Department of Pharmaceutics, Virginia Commonwealth University, 410 North 12th Street Smith Building, Richmond, VA 23298, USA

6 Department of Pharmacotherapy & Outcomes Science, Virginia Commonwealth University, 410 North 12th Street Smith Building, Richmond, VA 23298, USA

Introduction

Ovarian cancer (OVC) is the most lethal gynecological cancer in the Western World due to a combination of ineffective early-stage detection methods and late-stage treatment strategies [1]. Approximately 22,000 women in the USA were diagnosed with OVC in 2013, approximately 70 % of which presented with advanced stages of the disease (stages III and IV) when surgical intervention and chemotherapeutic treatment are least effective [2]. Currently, early detection is the single most important determinant for survival with 5-year mortality rates less than 10 % for women with stage I OVC. Thus, significant effort has been focused on identifying the

Published online: 10 July 2015

OVC-initiating molecular events that could serve as important early-stage markers for OVC such as cancer antigen-125 (CA-125), human epididymis protein 4 (HE-4), and transvaginal ultrasound [3] and novel therapeutic targets for improved patient outcomes.

Investigating the onset and progression of OVC has proven very challenging due to tumor heterogeneity, the paucity of early-stage primary tissues, and few natural animal models that faithfully recapitulate the pathophysiology of the human disease [4–6]. In recent years, the chicken has emerged as a unique experimental model that develops OVC spontaneously with morphological and molecular similarities to humans [7, 8]. The prevalence of spontaneous OVC in the chicken can exceed 35 % after 2 years of age depending on the genetic strain and ovulation rate (i.e., unfertilized egg production) [9], the latter being consistent with the incessant ovulation theory [10]. Important molecular-level similarities include CA-125 expression [11], frequency of p53 mutations [12], E-cadherin upregulation [13], and gene expression patterns [14]. Thus, the chicken provides a powerful experimental model for investigating the molecular events that lead to the onset and progression of spontaneous OVC with powerful bioanalytical tools such as mass spectrometry-based proteomics [15]. In a recent study, we measured the plasma proteome intra-individual variability of two chickens over a 1-year period: one bird was healthy with no visible neoplasms and the second bird developed late-stage OVC [16], followed by targeted SRM to quantify a candidate biomarker, ovostatin 2 (OVOS2) [17]. Although these early studies identified OVOS2, we were only able to identify approximately 120 proteins from plasma due to interference from high abundant proteins (e.g., albumin) and the lack of any standardized depletion reagents that are common in human and mouse plasma proteomics studies.

Herein, we report the first in-depth proteomics analysis of ovarian cancer in the chicken with matched plasma, ovary, and oviduct tissues from healthy, early-stage OVC, and late-stage OVC birds. A total of 248 proteins in plasma, 2680 proteins in the ovaries/ovarian tumors, and 1452 proteins in the oviduct tissues were identified with high confidence using high-performance mass spectrometry-based proteomics (LC-MS/MS). Among the 392 differentially regulated proteins in chicken OVC, over 70 of them have been associated with cancer in human studies. Within these 70 cancer-related proteins, we observed 5 known human OVC-related proteins including B2M, EPCAM, TPD52, CLDN3, and S100A6. In addition to these known OVC-related proteins, four members of the MEROPS I39 protease inhibitor family were identified including OVOS2 which was significantly upregulated in late-stage OVC plasma ($p<0.05$) and ovary ($p<0.01$). We then investigated the expression levels of human OVOS2 in primary tumor specimens and OVC cell lines. Strong OVOS2 immunohistochemical staining of stage III high-grade serous ovarian cancer tumors was observed for malignant epithelial cells compared to surrounding normal tissues. Furthermore, OVOS2 messenger RNA (mRNA) and protein were also overexpressed in multiple human OVC cell lines but not in normal ovarian epithelial (NOE). Collectively, these data provide convincing evidence for a conserved, yet undefined, role for OVOS2 in ovarian cancer of the chicken and human.

Experimental

Animal care, biospecimen collection, and pathology

All animals were cared for in accordance with North Carolina State University IACUC regulations. Details of the sample collection, sample storage, bird mortality, and pathology were described elsewhere [16]. Briefly, approximately 2 mL of blood was drawn from 150 age- and strain-matched Bovans White birds every 3 months for 1 year starting at 2.5 years of age into EDTA tubes followed by centrifugation at 3000×*g* for 3 min at 10 °C, plasma collection, and storage at −80 °C. After the final blood draw at 3.5 years of age, the remaining birds were euthanized followed by ovary and oviduct tissue collection. Tissues were preserved for both pathological (formalin fixation) and proteome (LN_2 snap frozen) characterization. Tissue slides of 5 μm were stained with hematoxylin and eosin (H&E) and assessed by a board-certified veterinary pathologist.

Chicken tissue lysate preparation

Frozen ovary and oviduct tissues were removed from −80 °C storage, weighed (frozen), and then placed on ice. Lysis buffer (50 mM Tris pH 7.8, 8 M urea, 2 M thiourea, 10 mM EDTA, 10 mM DTT, and 0.001 % sodium azide) was added to each frozen tissue sample at a concentration of 0.2 mg/mL. The samples were then homogenized using an OMNI TIP Homogenizing Kit (Omni International) for 1 min followed by the addition of 2 % (*w*/*v*) sodium dodecyl sulfate. Samples were shaken for 1 min, incubated on ice for 5 min, the cycle repeated twice more, and then centrifuged for 30 min at 14,000 rpm. The soluble fractions were removed from the centrifuge tube without disrupting the cellular debris pellet or the top lipid layer. The extracts were then stored at −80 °C until immediately prior to 1D SDS-PAGE.

SDS-PAGE and in-gel digestion

The plasma samples from all nine birds were thawed, diluted 1:10 (*v*:*v*) with 50 mM Tris-HCl (pH 6.8), and then combined 1:1 (*v*:*v*) with freshly prepared 2-mercaptoethanol/Laemmli (Bio-Rad, Hercules, CA, USA) loading buffer. The ovary and oviduct lysates were thawed, vortexed, diluted 1:5 (*v*:*v*)

with 50 mM Tris-HCl (pH 6.8), and then combined 1:1 (*v*:*v*) with freshly prepared 2-mercaptoethanol/Laemmli (Bio-Rad, Hercules, CA, USA) loading buffer. The resulting solutions were then heated at 95 °C for 5 min, and then all three samples were analyzed on 12.5 % Tris-HCl precast gel (Bio-Rad Criterion 12+2 well format), run at 200 V for approximately 50 min, and then stained with Coomassie G-250 (Bio-Rad). The total protein concentrations for the plasma samples translated to gel loadings ranging from 20 to 32 μg based on Bradford assay. Attempts to quantify the total protein levels in the ovary and oviduct lysates were not successful due to the high levels of urea, thiourea, and SDS. Thus, each gel lane was loaded with 30 μL each of lysate proteome solution.

In-gel digestion was performed as previously described [16] in a laminar flow hood by cutting 36 even gel bands per lane (2 mm×7 mm) using a grid cutter (The Gel Company, San Francisco, CA). The 36 gel bands/lane were combined into 12 fractions (each fraction=3 neighboring bands) from the plasma and ovary gel lanes, and the top 12 gel bands were combined into 4 fractions from the oviduct gel lanes into individual vials for in-gel digestion (GeLC [16, 18]). At the conclusion of the trypsin digestion, the tryptic peptide solution was reduced to dryness in a Speedvac and stored at −80 °C.

LC-MS/MS

A nanoLC-2D (Eksigent Technologies, Dublin, CA) was coupled to a LTQ-FT-XL (Thermo Scientific, San Jose, CA) using a vented column configuration [19]. The vented column consisted of a 75 μm×5 cm trap (IntegraFrit: New Objectives, Woburn, MA) coupled to a 75 μm×15 cm column (PicoFrit: New Objectives, Woburn, MA). Both the trap and analytical column were self-packed with Magic C18AQ stationary phase (5 μm particle, 200 Å pore; Auburn, CA). Mobile phases A and B were composed of 98/2/0.2 and 2/98/0.2 water/acetonitrile/formic acid (*v*:*v*:*v*), respectively. The Speedvac-dried fractions were reconstituted in 65 μL of mobile phase A. Ten microliters of sample was loaded onto the trap at 1.5 μL/min with 2 % B. After washing the sample, the valve diverted the flow onto the column and the following gradient was applied at 350 nL/min: 2 % B (0–5 min), 2–60 % B (5–65 min), 40–90 % B (65–67 min), 90 % B (67–77 min), 90–2 % B (77–78 min), 2 % B (78–90 min). Data acquisition was initiated 5 min after the start of the nLC gradient. The sample analysis order for plasma and ovary tissues was randomized and run in triplicate with a blank injection every 13th LC-MS/MS run. The oviduct tissues were single LC-MS/MS runs.

Mass spectrometric analysis was performed on a 7T LTQ-FT Ultra (ThermoFisher) with a pulse sequence consisting of a broadband acquisition in profile mode followed by eight data-dependent MS/MS events in the ion trap. The LTQ-FT was mass calibrated with a target AGC limit of 1×10^6 every 40 LC-MS/MS runs (~2.5 days). The full scan was collected at 400–1600 *m*/*z* with a resolving power set at $100{,}000_{FWHM}$ at *m*/*z*=400. The dynamic exclusion time was set to 3 min.

Data analysis

LC-MS/MS data was searched against a concatenated target-reverse chicken database (IPI ver.3.74) with *Homo sapiens* keratin, keratin-related proteins, and porcine trypsin included. Mascot Distiller (ver. 2.3.2, Matrix Science, Boston, MA) was used to generate peaks lists, and then Mascot Daemon to perform the searches. Carbamidomethyl (C) was set as a fixed modification and deamidation (NQ), oxidation (M), and carbamyl (K and N-term) were set as variable modifications. Additional search parameters included a maximum of two missed cleavages, peptide tolerance of ±5 ppm, and MS/MS tolerance of ±0.6 Da. Protein grouping, statistical filtering (1 % false discovery rate), and quantification of Mascot DAT files were accomplished using ProteoIQ (NuSep, Athens, GA, ver 2.3.01), a spectral counting label-free software package that uses a combination of Peptide/Protein Prophet [20, 21] and PROVALT [22].

The spectral counts (SpCs) for each tissue type were first normalized within each sample (between technical replicates) followed by normalized across samples (between birds) to generate normalized SpC (NSpC) values for each protein. The NSpC data was then filtered to include proteins with a total of at least 5 NSpCs per sample for further quantitative analysis. Data imputation was implemented for the NSpC plasma and ovary data; normalized spectral abundance factors (NSAF) were subsequently calculated, natural log transformed (lnNSAF), and analyzed for goodness of fit [23]. The optimal imputement values were 1.0 and 0.7 for plasma and ovary datasets, respectively. Student's *t* test was used to determine statistical significance of differential protein expression.

Immunohistochemistry

The paraffin-embedded human ovarian cancer blocks were pre-existing specimens obtained from the VCU Tissue & Data Acquisition & Analysis Core. These samples had patient consent for use in scientific research but were not collected specifically for the present work. The use for OVOS2 study was approved by the Virginia Commonwealth University Human Subjects Committee. Immunohistochemical staining (IHC) of paraffin-embedded sections was conducted with a rabbit polyclonal antibody against OVOS2 N-term amino acid 99-128 (Abgent; San Diego, CA, USA) using a standard procedure as described previously [24].

Human cell lines

The sources and culture conditions of the 11 human ovarian cell lines (Caov-3, SKOV3, HEY, ES-2, Dov-13, OVCA420, OVCA429, OVCA432, OVCA433, A2780, and A2780CP)

and normal ovarian epithelial cell cultures (NOE001 and NOE002) were described previously [25, 26]. The cells in early passages were maintained in culture for less than 4 weeks before isolation of total cellular RNA with TRIzol following the protocol of the manufacturer (Invitrogen). OVOS2 expression in OVCA432 cells was knocked down by lentiviral-transduced short hairpin RNA (shRNA) (pGreenPuro, System Biosciences) carrying 5′-CAGGCCTGATATTAATAGCAAT-3′ (OVOS2-shRNA1), 5′-GTTCTCCTACCTAAGAAGGC A-3′ (OVOS2-shRNA2), or a non-targeting control sequence (ctrl-shRNA).

RT-qPCR

Expression levels of human OVOS2, A2M, A2ML1, and OVOS1 mRNA in each cell line were measured by RT-qPCR. Complementary DNA (cDNA) was synthesized from RNA (1 μg, random primers), using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. Relative expression levels of target genes were determined using the gene-specific probes for OVOS2 (Hs02384746), A2M (Hs00929971), A2ML1 (Hs00405670), and OVOS1 (Hs01393981), the TaqMan Universal PCR Master Mix, and the 7900HT Real-Time PCR System (Applied Biosystems). The results were normalized to the levels of GAPDH and presented as per mil of GAPDH. Student's *t* test was used to determine statistical significance of gene transcript levels in ovarian cancer cell lines relative to NOE002.

Western blot analysis

Whole-cell lysates were prepared with cell lysis buffer (Cell Signaling Technologies, MA, USA), separated on SDS-PAGE electrophoresis, and transferred to PVDF membrane (Bio-Rad, Hercules, CA). Blots were probed with rabbit anti-GAPDH (Cell Signaling) or a custom affinity purified rabbit polyclonal anti-OVOS2 antibody (GenScript) raised against the OVOS2 sequence EGAKASKQGVLDLP (AA 911-924). Immunocomplexes were visualized with the enhanced chemiluminescence detection kit from Amersham.

Results and discussion

Quantitative proteomics analysis of ovarian cancer in the chicken

A large-scale biorepository of longitudinal plasma samples and matched tissues from the chicken was established from 150 age-matched B-strain birds over a 1-year period (2.5 to 3.5 years of age) [16]. A total of 73 birds were still living at the conclusion of the study; following necropsy, we found 52 birds were healthy without any visible neoplastic lesions, three birds had tumors localized to the ovary, two birds had tumors on the ovary and neighboring tissues without oviductal involvement, and 13 birds had tumors on both the ovary and oviduct that had spread to neighboring tissues (note: three birds had tumors with origins other than the ovary). A total of nine birds were selected from this population including three random "healthy" birds with no visible lesions that were producing eggs (birds 1–3), three "early-stage OVC" birds (birds 4–6), and three "late-stage OVC" birds (birds 7–9) (Fig. 1a). Three sets of matched samples from these nine birds (Fig. 1a) were selected for analysis including plasma (drawn immediately prior to necropsy at 3.5 years of age), ovary, and oviduct tissues.

Representative one-dimensional (1D) gels for plasma, ovary, and oviduct tissue lysates are shown in Fig. 1b including molecular weight markers (left) and excised band fractions for each sample type (right). Excised bands were processed for in-gel digestion and analyzed by LC-MS/MS (GeLC), and the number of unique proteins identified in each sample plotted as a function of bird ID (Fig. 1c). Importantly, the entire gel lane for both plasma and ovary samples was processed for triplicate LC-MS/MS analysis whereas only the top four gel fractions (high molecular weight) were processed and analyzed by LC-MS/MS as single runs. The oviduct samples were processed and analyzed differently to rapidly assay the high molecular weight bands to establish the presence or absence of the MEROPS I39 family of protease inhibitors which are potentially important in human OVC (*vide infra*). The GeLC analysis from all plasma, ovary, and oviduct samples identified 248, 2680, and 1452 proteins, respectively, with proteome overlap for the plasma, ovary, and oviduct proteins shown in Fig. 1d. The 1D gels for plasma, ovary, and oviduct samples from all nine birds, proteins identified per gel lane, and proteome overlap as a function of phenotype are given in Fig. S1 in the Electronic Supplementary Material (ESM). A complete list of all proteins identified in this study is given in Table S1 in the ESM.

The raw plasma and ovary proteomics datasets were normalized prior to quantitative analysis and biological interpretation. The oviduct gel fraction samples were analyzed once by LC-MS/MS rather than in triplicate; thus, quantitative analysis was not performed. The spectral count (SpC) data [27] were initially normalized within each sample and then normalized between samples (NSpC) to account for sampling differences that are typical for heterogeneous biospecimens. After normalization, we filtered the plasma and ovary datasets by including only those proteins with 5 NSpCs in at least one sample which was previously shown to be the limit of SpC quantitation [28]. The 5 NSpC filtering reduced the number of quantifiable proteins in plasma from 248 to 237 and in the ovarian tissue data from 2680 to 2307. We then empirically determined the optimum imputement values (i.e., those that gave the most normal distributions) for plasma and ovary

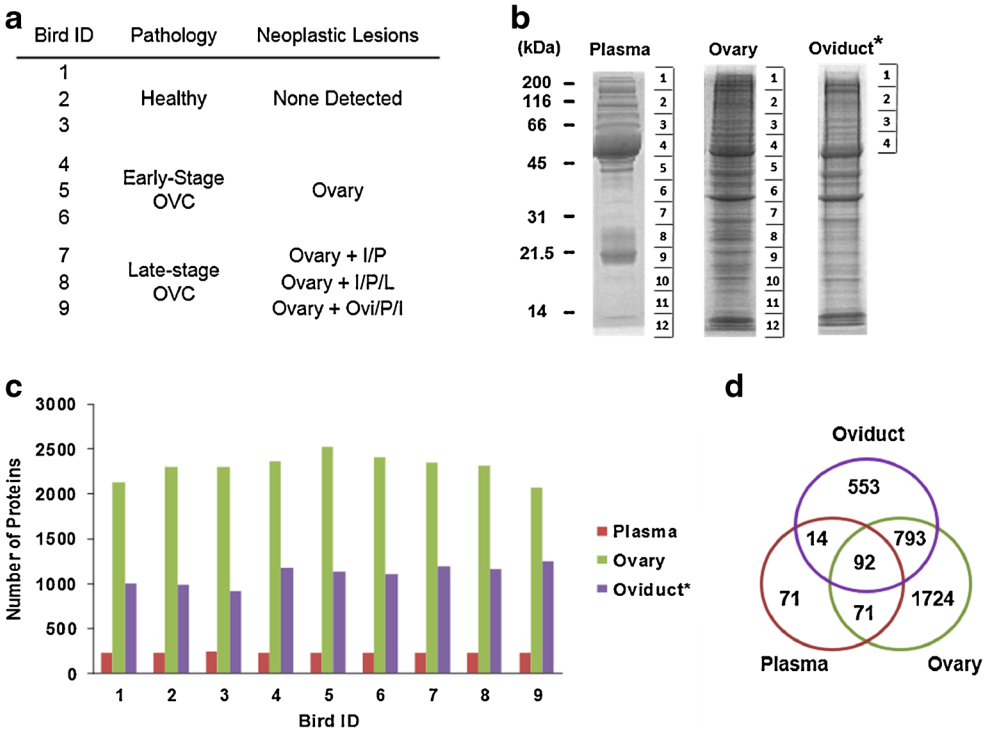


Fig. 1 Overview of proteomics study for nine birds including matched plasma, ovary, and oviduct tissue. (**a**) A total of nine birds were selected for this study including three healthy, three with tumors localized to the ovary (*Early-Stage OVC*), and three with tumors on the ovary and neighboring tissues (*Late-Stage OVC*: *Ovi*=oviduct, *I*=intestine, *P*=pancreas, and *L*=liver). (**b**) Representative 1D gels for the plasma, ovary, and oviduct with molecular weight markers and digested band size/*numbering* shown on the *left* and *right sides* of the lanes, respectively. (**c**) The total number of proteins identified from each tissue type as a function of the animal. (**d**) Venn diagram showing the overlap between all identified proteins from the plasma, ovary, and oviduct samples. *Note: only the top four bands (i.e., high molecular weight) for the oviduct samples were analyzed in single LC-MS/MS runs

datasets by varying the NSpC values by 0.1 from 0.5 to 1.5 followed by abundance factor normalization (NSAF) and natural log transformation (lnNSAF) [23]. The lnNSAF data with different imputement values were then fitted and analyzed by goodness of fit in JMP 10.0. The minimal imputement values that gave the best fit were 1.0 and 0.7 for plasma and ovary, respectively. LnNSAF datasets for plasma and ovary with different imputement values are given in ESM Fig. S2 and ESM Fig. S3, respectively.

Student's *t* test was then used to determine the statistical significance of each protein expression level in early- and late-stage OVC relative to healthy birds. Volcano plots for plasma (Fig. 2a) and ovary (Fig. 2b) show the $-\log_{10} p$ values for each protein vs. the respective $\log_2$ early OVC NSAF/healthy NSAF (blue) and $\log_2$ late OVC NSAF/healthy NSAF (red) expression levels. We used a twofold (p value≤0.05) cutoff to segregate significant differentially regulated proteins in OVC vs. healthy birds. The number of proteins in each cutoff region for early- and late-stage OVC is denoted in the top left and right corners of Fig. 2a, b. For example, 70 and 121 proteins were significantly down- and upregulated, respectively, in early-stage OVC ovarian tumor tissues relative to healthy ovary tissues. Furthermore, 144 and 89 proteins were significantly down- and upregulated, respectively, in late-stage OVC ovarian tumor tissues relative to healthy ovary tissues. Overall, 44 proteins in OVC plasma (44/237=19 %) and 348 proteins in ovarian tumors (348/2307=15 %) were found to be ≥2-fold differentially expressed (p value≤0.05). The Venn diagram in Fig. 2c shows the overlap between the 44 differentially expressed proteins in early- vs. late-stage OVC plasma with 19 proteins significantly up- or downregulated in both stages of OVC. The second Venn diagram in Fig. 2d shows the overlap between the 348 differentially expressed proteins in early- vs. late-stage ovarian tumor tissues with 76 proteins significantly up- or downregulated in both stages of the tumor. A full list of differentially expressed proteins (p value≤0.05) in both OVC plasma and ovarian tumor tissues is given in Table S1 in the ESM.

Cancer-related candidate protein biomarkers in chicken ovarian cancer

There have been many candidate protein biomarkers identified for cancers [29] that are conveniently catalogued in the interactive The Human Protein Atlas [30]. There are 1703 cancer-related genes listed in The Human Protein Atlas, of

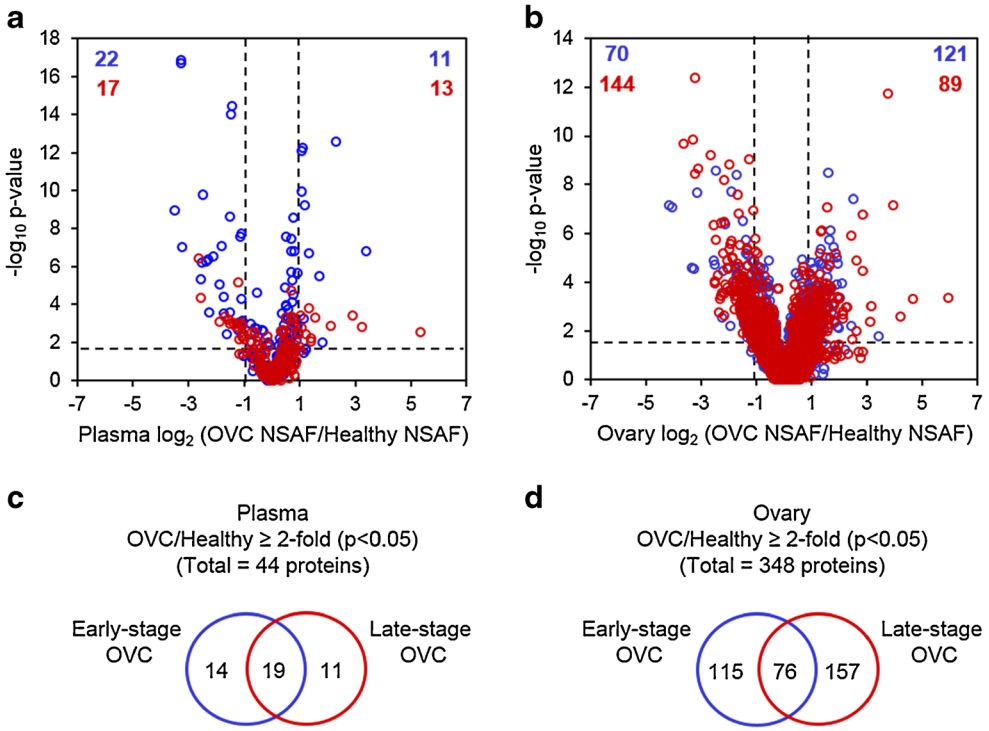


Fig. 2 Quantitative proteomics data analysis for plasma and ovary. Volcano plot of the $-\log_{10} p$ value vs. the $\log_2$ expression levels for ovarian cancer protein levels relative to healthy protein levels for (**a**) plasma and (**b**) ovary tissue. Protein expression is reported as a normalized spectral abundance factor (NSAF) for early-stage OVC (*blue*) and late-stage OVC (*red*). *Vertical dashed lines* represent ≥2-fold change in protein expression, and the *horizontal dashed lines* indicate a p value ≤0.05. Proteins that fall outside of these ranges are considered candidate biomarkers. The *colored numbers* in the *top left*/*right corners* of the volcano plots represent the number of proteins that are both ≥2-fold ($p \leq 0.05$) for reference. An overview for the unique and shared significantly up- or downregulated proteins in plasma (**c**) and ovary tissues (**d**) for early- and late-stage OVC is given in the Venn diagrams. For example, a total of 33 proteins were differentially present in early-stage OVC, of which 14 were unique to early-stage and 19 were shared with late-stage OVC

which 1053 are identified as candidate biomarkers. We compared the differentially regulated chicken OVC proteins to the 1053 candidate biomarker genes to assess the translational potential of the chicken model. Within the 392 differentially regulated proteins from the chicken in both OVC plasma and ovarian tumors, we identified 73 cancer-related protein biomarkers common between the chicken and human (Table 1). Of the 73 proteins, 49 were upregulated and 24 were downregulated in OVC of the chicken. Within the 49 upregulated proteins, seven were found exclusively in plasma (bold) and two were found in both plasma and ovarian tissues (bold and underlined). Within the 24 downregulated proteins, two were found exclusively in plasma (bold) and one was found in both plasma and ovarian tissues (bold and underlined).

A number of proteins listed in Table 1 are associated with human ovarian cancer. We generated a list of OVC-specific genes in humans that were known to be differentially expressed from three sources: TCGA study [31], Polanski et al. [29], and Emmanuel et al. [32]. Five genes significantly upregulated in Table 1 are also upregulated in human OVC including B2M, CLDN, EPCAM, S100A6, and TPD52. B2M (beta-2 microglobulin) is a 12-kDa protein that is part of major histocompatibility complex I [33]. Altered levels of B2M have been observed in a number of cancers [34–36] including OVC where it is measured as part of the OVA1 test for prognostic evaluation of OVC patients following surgical removal of tumors and chemotherapeutic treatment [37, 38]. CLDN3 is a 23-kDa tight junction protein that forms the extracellular interface between healthy epithelial cells. CLDN3 is frequently upregulated in primary ovarian cancer tumors and cell lines [39, 40]. The epithelial cell adhesion molecule (EPCAM) is a 34-kDa cell surface protein that maintains undifferentiated stem cells and contributes to cancer cell proliferation [41–43]. S100A6 is a 12-kDa calcium-binding protein that is involved in several types of inflammation responses. S100A6 levels in plasma were recently shown to predict tumor burden in a xenograft mouse model of ovarian cancer [44]. Tumor protein D52 (TPD52) is a 25-kDa protein that is consistently upregulated in many cancers, yet its function is not well understood. Increased TPD52 protein expression in NIH3T3 cells and LnCaP cells showed increases in cell proliferation, anchorage independence, and tumorigenicity [45].

Cancer antigen 125 (CA-125) and human epididymis protein 4 (HE-4) are both Food and Drug Administration (FDA)-approved biomarkers for ovarian cancer [1]. Given the importance of CA-125 and HE-4 in human OVC, we searched the

Table 1 Co-differentially expressed proteins in chicken OVC and human cancers. Proteins (gene ID) listed were differentially expressed exclusively in ovary, exclusively in plasma (bold), and in both ovary plasma (bold and underlined)

Expression (OVC/H)	Genes (*Gallus gallus*)
Up	ACPP, **B2M**, BLMH, **C6**, CASP1, CAT, CDC37, CDH1, CDH11, CLDN3, CLU, CST3, CSTA, CTNNB1, CTNND1, CTSB, CTSD, DIAPH1, EPCAM, EZR, **FBLN1**, HIP1, IGF2R, ITGA1, ITGA3, ITGA6, LCP1, LGALS3, LGMN, NGFR, **NME2**, NONO, NUCB2, **PIGR**, PTPRC, RBBP4, S100A6, S100A9, S10DA11, **SERPINA5**, STAT5B, THBS1, TPD52, TRAP1, TSPO, TPMT, UBE2N, VAMP3, **VWF**
Down	A2M, AHSG, ALDOB, AOC3, CD74, CDKN2C, CFH, COL4A5, DAB2, DDX6, **F13A1**, **F13B**, FGA, FGF2, FKBP5, HPGD, IGF2BP1, LRP1, MCAM, NCAM1, POSTN, SERPINA1, SMAD2, TGM4

chicken proteomics data for these proteins. Human CA-125 is a 2-MDa mucin expressed from the MUC16 gene that is overexpressed and then shed from the membrane surface of tumor cells into the bloodstream. We did not detect protein(s) in this study that aligned with human CA-125 although we did detect two mucins: MUC ("mucin-like") and MUC5B. Ovomucin α-subunit (MUC5B), an abundant protein in egg [46], was found to be upregulated in the late-stage OVC tumor tissues, detected with 49 % sequence coverage in oviduct tissues and not detected in any of the plasma samples. Chicken MUC5B was BLAST searched against the Human UniProt database and resulted in a 45 % sequence identity to MUC5AC (P98088), a gel-forming protein found in gastric and respiratory tract epithelia [47]. Abherrant regulation of MUC5AC has been observed in several cancers [48–51] including ovarian neoplasms [51, 52]. Thus, the upregulation of MUC5B in the late-stage OVC tissues of the chicken agrees with earlier OVC studies in humans. We did not find evidence for the chicken orthologue to HE-4 (WFDC2) in the chicken proteomics dataset.

Additional markers that have been evaluated alone or in combination with CA-125 and HE-4 include CA-19-9, CA-15-3, CEA, B2M, ApoA1, ApoC3, TTR, TF, IGF2, EGFR, CRP, MYO, OPN, IL-6, and IL-18 [38]. Among this group of potential biomarkers, ApoA1, B2M, TTR, TF, and CRP were identified in the chicken. Apolipoprotein A1 (ApoA1), B2M, transthyretin (TTR), and transferrin (TF) along with CA-125 are measured in the multiplexed OVA1 test for monitoring women after OVC diagnosis. In the OVA1 test, plasma levels for ApoA1, TTR, and TF are lower whereas B2M and CA-125 levels are increased in women with OVC [37]. Log_2 OVC/healthy expression levels for B2M in plasma were 0.44 (p=0.1055) for early-stage birds and 1.32 (p=0.0002) for late-stage birds which correlates with the OVA-1 test. TF levels in plasma are lower in both early- and late-stage OVC plasma but not statistically significant (log_2 early OVC/healthy=−0.11, p=0.2350; log_2 late OVC/healthy=−0.14, p=0.2619). ApoA1 and TTR were upregulated in OVC plasma samples which is opposite the OVA1 values for human OVC. However, the levels for both ApoA1 (log_2 early OVC/healthy=0.67, p=1.8E−6; log_2 late OVC/healthy=0.32, p=0.0025) and TTR (log_2 early OVC/healthy=0.34, p=0.0300; log_2 late OVC/healthy=0.45, p=0.0425) were less than two-fold increases. C-reactive protein (CRP) is a 23-kDa acute-phase protein synthesized in the liver which increases in concentration by as much as 1000-fold in response to inflammation [53]. The upregulation of CRP in cancer is well established [54, 55] including OVC [56, 57]; thus, we would expect CRP to increase in the chicken with the OVC stage. The label-free data for plasma supports this acute-phase response in the chicken with increased severity of the disease where CRP is 1.4-fold higher (p=0.0101) in early-stage OVC and 1.7-fold higher (p=0.0005) in late-stage OVC relative to healthy plasma levels. Overall, the quantitative proteomics analysis of chicken OVC agrees with previously observed human protein expression levels.

MEROPS I39 protein family expression in chicken ovarian cancer

OVOS2 is a predicted member of the MEROPS I39 family of protease inhibitors [58] that includes alpha-2 macroglobulin (A2M), pregnancy zone protein (PZP), alpha-2 macroglobulin like 1 and 2 (A2ML1 and A2ML2), and ovostatin 1 (OVOS1) which is one of the most abundant proteins in egg white [59–63]. The elevated levels of OVOS2 in our previous studies [16, 17] and a recent report on elevated levels of A2M in chicken OVC at the transcript level [64] led us to evaluate the expression levels of all MEROPS I39 protease inhibitors in healthy and neoplastic plasma, ovary, and oviductal tissues. The six members of the MEROPS I39 protease inhibitor family in the chicken clustered on the q-arm of chromosome 1 including A2ML1, A2ML2, A2M, LOC425756, OVOS1 (OVST), and OVOS2 (OVSTL) (Fig. 3a). These proteins exhibit broad substrate specificity for all four classes of proteases and in some human studies have been implicated in tumorigenesis. We detected each protein in at least one tissue type with the exception of LOC425756 (Table 2). A2ML1 and OVOS1 were only detected in the oviduct with high sequence coverages (~50 %) and SpCs (>60 SpCs/sample) in birds 1–3 and 7 (Table 2 and ESM Table S1). A2M and A2ML2 were detected in all three tissue types yet their levels were

drastically different for birds 1–3 and 7. A2M levels were high (>100 SpCs/sample) in those birds and low in birds 4–6, 8, and 9 whereas the inverse was true for A2ML2. These observations can be explained given that birds 1–3 and 7 were producing eggs (i.e., ovulating) at the time of necropsy (birds 4–6, 8, and 9 were out of production, i.e., anovulatory) which is in agreement with the proteomics characterization of chicken egg white [63].

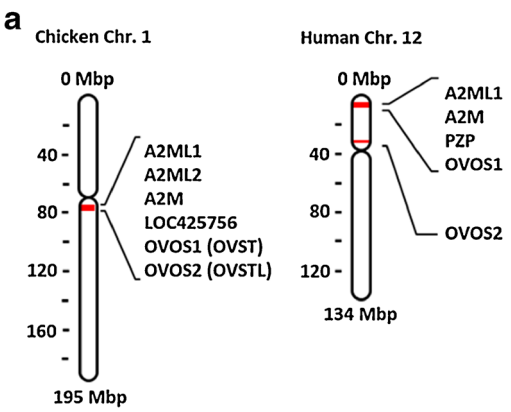
OVOS2 is a 165-kDa protein identified and validated in our earlier proteomics studies of ovarian cancer in the chicken [16, 17]. In the present study with a higher level of protein fractionation and matched tissue analysis, we were able to detect OVOS2 with an overall sequence coverage >30 % (Table 2 and ESM Table S1). OVOS2 levels were upregulated by more than 60-fold in late-stage OVC plasma (p=0.003) and tumor tissues (p=0.0005). Unlike the other I39 protease inhibitors in Table 2, OVOS2 levels appear to correlate with the stage of OVC rather than the egg production status. Furthermore, OVOS2 was only detected in late-stage OVC oviduct tissues. Although we were unable to detect OVOS2 in early-stage OVC samples using label-free proteomics which would strengthen its potential as an early-stage OVC marker, we were interested in pursuing this candidate marker in human OVC. Interestingly, the MEROPS I39 family in chickens on chromosome 1 shows a high degree of conserved synteny with human chromosome 12 (Fig. 3a and ESM Fig. S4) [65]. Chicken OVOS2 sequence BLAST analysis against the human protein database showed human OVOS2 (Q6IE36) gave the highest sequence identity at 45 % (Fig. 3b). Based on these results, we sought to establish the expression levels for OVOS2 in human ovarian cancer to determine translational potential as a diagnostic biomarker.

OVOS2 is upregulated in human ovarian cancer

To assess the presence of OVOS2 in human OVC, we first measured OVOS2 mRNA in 11 human OVC cell lines and NOE cells by RT-qPCR. The qPCR results are shown in Fig. 4a with the OVOS2 mRNA levels normalized to Ct values of GAPDH. Importantly, the qPCR product for OVOS2 mRNA analysis spanned an exon to prevent the detection of genomic DNA. OVOS2 mRNA levels in all OVC cell lines were significantly higher (p<0.05) than those in NOE cells. OVOS2 mRNA levels in NOE001 and NOE002 were statistically the same, but the mean OVOS2/GAPDH for NOE002 was slightly higher (0.062 vs. 0.061). Fold increases in expression of OVOS2 relative to the NOE002 cells ranged from 3- (A2780) to 35-fold (OVCA432). We also measured the mRNA levels for A2M, A2ML1, and OVOS1 in the OVC and NOE cells (ESM Fig. S5) and did not observe consistently up- or downregulation for these genes across the cell types. OVOS2 protein expression in these cell lines was examined by Western blot analysis which confirmed the presence of high levels of OVOS2 protein at the predicted molecular weight of ~165 kDa in all OVC cell lines but not in NOE cells (Fig. 4b). The identity of the ~165-kDa protein band as OVOS2 was verified by shRNA downregulation of OVOS2 expression in OVCA432 cells using two target specific sequences only found in OVOS2 mRNA (Fig. 4c). We next used immunohistochemical staining to analyze the expression of OVOS2 in paraffin-embedded sections of stage III high-grade serous ovarian adenocarcinomas. As shown in Fig. 5, all primary ovarian adenocarcinomas displayed intense OVOS2 staining in malignant epithelial cells while the surrounding non-cancerous tissues were not or only weakly stained

OVOS2 in chicken and human ovarian cancer

The interplay between proteases and endogenous inhibitors has been actively studied in relation to cancer for many years. The differential regulation of several protease inhibitors has been observed in ovarian cancer including serpins (i.e., SERPINA1/alpha 1-antitrypsin [66], SERPINE1/plasminogen activator inhibitor-1 [67], SERPINB2/plasminogen activator inhibitor-2 [68], SERPINA5/plasminogen activator inhibitor-3 [69], SERPINB5/maspin [70]), tissue



b

BLAST Results for Chicken OVOS2 (F1NEW8)

Human Gene	Accession (UniProt)	BLAST Identity (% AA match)
OVOS2	Q6IE36	45%
A2M	P01023	43%
PZP	P20742	42%
A2ML1	A8K240	37%
OVOS1	Q6IE37	36%

Fig. 3 MEROPS I39 protease inhibitor genes in chickens and humans. (**a**) The chicken MEROPS I39 protease inhibitor family is clustered at the top of the q-arm of chromosome 1 whereas the human orthologues are clustered in the p-arm of chromosome 12. (**b**) Chicken OVOS2/OVSTL has the highest amino acid sequence identity to human OVOS2

Table 2 MEROPS I39 protease inhibitor family in the chicken. Relative expression levels for six chicken I39 protease inhibitors in early and late OVC plasma and ovary tissues relative to healthy birds. Expression levels in the oviduct are given as percent sequence coverage (% Cov) detected in LC-MS/MS analyses of the four gel bands

Gene (*Gallus*)	Protein	Plasma				Ovary				Oviduct
		Early		Late		Early		Late		
		Log_2 OVC/H	*p* value	Log_2 OVC/H	*p* value	Log_2 OVC/H	*p* value	*p* value	Log_2 OVC/H	% Cov
A2ML1	Alpha-2-macroglobulin like 1	ND	ND	ND	ND	ND	ND	ND	ND	49.6
A2ML2	Alpha-2-macroglobulin like 2	0.71	3.23E−08	0.43	0.272	−0.40	0.094	−1.34	0.001	12 %
A2M	Alpha-2-macroglobulin	−0.04	0.700	−0.08	0.427	−1.09	0.001	−1.34	0.001	37 %
LOC425756	Alpha-2-macroglobulin like	ND		ND		ND		ND		ND
OVOS1	Ovostatin	ND		ND		ND		ND		47 %
OVOS2	Ovostatin2	ND		5.32	0.003	ND		5.97	0.0005	31 %

ND not detected

inhibitors of metalloproteases (TIMP) [71, 72], secretory leukocyte protease inhibitor (SLPI) [73, 74], and now OVOS2. The conventional view of cancer progression would suggest that upregulation of extracellular protease inhibitors like OVOS2 is a beneficial endogenous response to limiting the proteolysis of extracellular matrix proteins which contributes to metastasis. However, functional studies have shown that many endogenous protease inhibitors can actually promote cancer cell growth and metastasis through complex interactions or as indirect products from key regulators (e.g., colony stimulating factor 1 [67, 68]). Finally, a recent study by Perets et al. [75] using a mouse model for fallopian tube-initiated OVC showed the "A2M region" of the mouse chromosome 6 was upregulated. This study adds to a growing literature that supports oviductal involvement in serous OVC [76–78] which runs counter to the established hypothesis that OVC originates from the ovarian surface epithelium (OSE) [79]. Thus, the

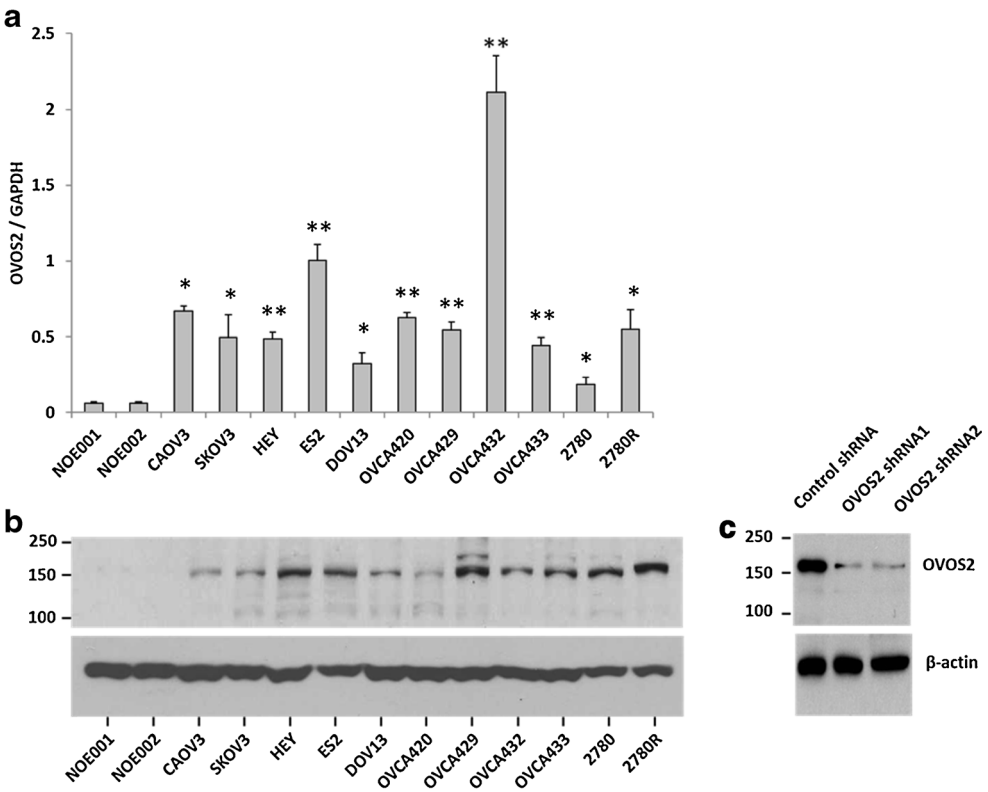


Fig. 4 OVOS2 expression analysis in ovarian cancer cells relative to primary cultures of normal ovarian surface epithelium. (**a**) RT-qPCR and (**b**) Western blot analysis of OVOS2 expression in human ovarian cancer cell lines and normal ovarian epithelial cells. The OVOS2 mRNA results were normalized with GAPDH as internal control and presented as per mil of *Ct* values of NADPH. The statistical significances between each of the ovarian cancer cell lines (Caov-3, SKOV3, HEY, ES-2, Dov-13, OVCA420, OVCA429, OVCA432, OVCA433, 2780, and 2780R) and NOE002 cells were determined with Student's *t* test. **p* value<0.05; ***p* value<0.01. (**c**) Western blot analysis of OVCA432 lysates following shRNA knockdown of OVOS2 expression showing OVOS2-911 antibody specificity toward OVOS2

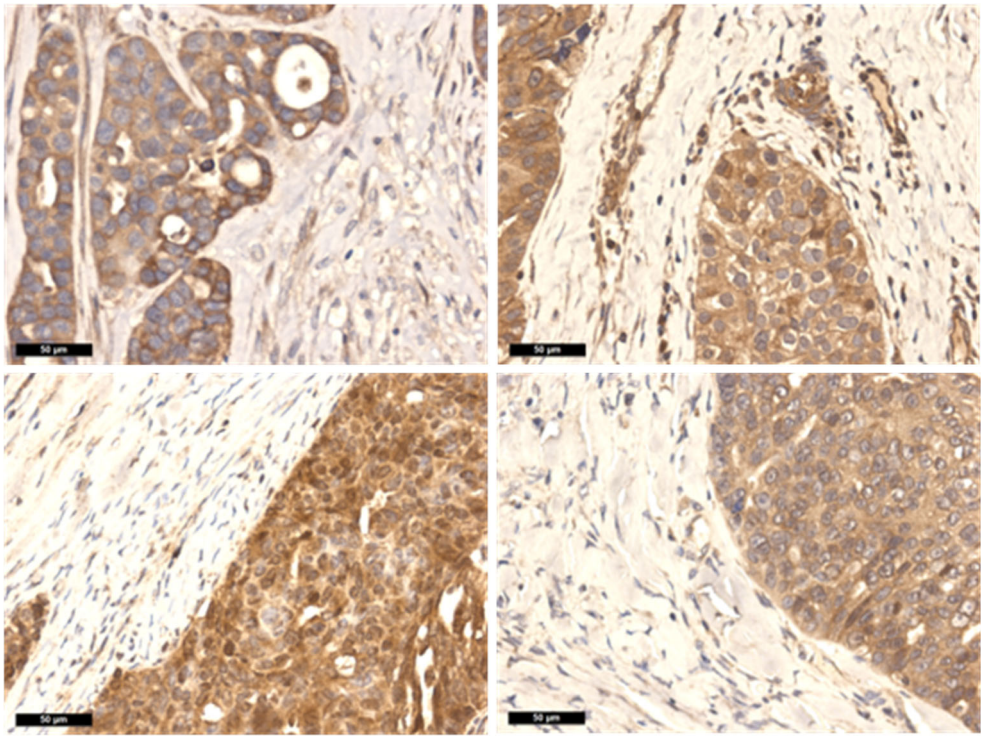


Fig. 5 OVOS2 immunohistochemical staining of primary human ovarian cancer tissues. Shown are positively stained malignant epithelial cells relative to the neighboring non-cancerous tissues from four patients with stage III high-grade serous ovarian adenocarcinomas. *Bar*=50 μm

upregulation of OVOS2 in chicken and human OVC suggests it plays a conserved role in OVC pathogenesis and possesses translational potential.

Conclusion

An in-depth label-free proteomics study of matched plasma, ovary, and oviduct tissues in the hen resulted in the quantification of over 2300 unique proteins, representing the most comprehensive proteomics study of ovarian cancer in the chicken to date. We identified 392 differentially regulated proteins in OVC plasma and ovarian tumor tissues including B2M, EPCAM, TPD52, CLDN3, and S100A6 which are established candidate biomarkers in human OVC. A 165-kDa protease inhibitor and candidate biomarker, OVOS2, a newly discovered protein in our laboratory, was detected at elevated levels in the plasma, ovaries, and oviduct of all late-stage OVC birds in this study. Because OVOS2 is found in plasma and has biological relevance to cancer as a protease inhibitor, it was a candidate for translational studies in human OVC. Human OVOS2 expression at both mRNA and protein levels was found to be significantly upregulated in human OVC cell lines. OVOS2 immunohistochemical staining in primary stage III high-grade serous OVC tissue specimens showed strong staining in regions containing malignant epithelial cells while surrounding non-cancerous tissues were negative or only weakly stained. The combined in-depth proteomics study of OVC in the chicken with targeted expression analysis of OVOS2 in human OVC biospecimens illustrates the power of this model for identifying novel differentially regulated proteins with translational potential.

Acknowledgments This work was financially supported by NIH-NCI through award K25CA128666 and VCU (AMH) and R01GM087964 (D.C.M. and A.I.N for tissue proteomics). H.S and X.F were supported, in part, by NIH-NCI R21CA161478. Human ovarian cancer tissues were provided by the VCU Tissue and Data Acquisition and Analysis Core (TDAAC) Facility, supported, in part, with the funding from NIH-NCI Cancer Center Core Support Grant P30 CA016059, as well as through the Dept. of Pathology, School of Medicine, and Massey Cancer Center of Virginia Commonwealth University.

References

1. Bast RC, Hennessy B, Mills GB (2009) The biology of ovarian cancer: new opportunities for translation. Nat Rev Cancer 9:415–428
2. American Cancer Society (2015) Cancer Facts & Figures 2015. American Cancer Society, Atlanta
3. Williams TI, Toups KL, Saggese DA, Kalli KR, Cliby WA, Muddiman DC (2007) Epithelial ovarian cancer: disease etiology, treatment, detection, and investigational gene, metabolite, and protein biomarkers. J Proteome Res 6:2936–2962
4. Cho KR, Shih Ie M (2009) Ovarian cancer. Annu Rev Pathol 4: 287–313
5. Vanderhyden BC, Shaw TJ, Ethier JF (2003) Animal models of ovarian cancer. Reprod Biol Endocrinol 1:1–11
6. Garson K, Shaw TJ, Clark KV, Yao DS, Vanderhyden BC (2005) Models of ovarian cancer—are we there yet? Mol Cell Endocrinol 239:15–26

7. Johnson PA, Giles JR (2013) The hen as a model of ovarian cancer. Nat Rev Cancer 13:432–436
8. Hawkridge AM (2014) The chicken model of spontaneous ovarian cancer. Proteomics Clin Appl 8:689–699
9. Fredrickson TN (1987) Ovarian tumors of the hen. Environ Health Perspect 73:35–51
10. Fathalla MF (1971) Incessant ovulation—a factor in ovarian neoplasia? Lancet 2:163
11. Jackson E, Anderson K, Ashwell C, Petitte J, Mozdziak PE (2007) CA125 expression in spontaneous ovarian adenocarcinomas from laying hens. Gynecol Oncol 104:192–198
12. Hakim AA, Barry CP, Barnes HJ, Anderson KE, Petitte J, Whitaker R, Lancaster JM, Wenham RM, Carver DK, Turbov J, Berchuck A, Kopelovich L, Rodriguez GC (2009) Ovarian adenocarcinomas in the laying hen and women share similar alterations in p53, ras, and HER-2/neu. Cancer Prev Res 2:114–121
13. Ansenberger K, Zhuge Y, Lagman JA, Richards C, Barua A, Bahr JM, Hales DB (2009) E-cadherin expression in ovarian cancer in the laying hen, Gallus domesticus, compared to human ovarian cancer. Gynecol Oncol 113:362–369
14. Gonzalez Bosquet J, Peedicayil A, Maguire J, Chien J, Rodriguez GC, Whitaker R, Petitte JN, Anderson KE, Barnes HJ, Shridhar V, Cliby WA (2011) Comparison of gene expression patterns between avian and human ovarian cancers. Gynecol Oncol 120:256–264
15. Hawkridge AM, Muddiman DC (2009) Mass spectrometry–based biomarker discovery: toward a global proteome index of individuality. Annu Rev Anal Chem 2:265–277
16. Hawkridge AM, Wysocky RB, Petitte JN, Anderson KE, Mozdziak PE, Fletcher OJ, Horowitz JM, Muddiman DC (2010) Measuring the intra-individual variability of the plasma proteome in the chicken model of spontaneous ovarian adenocarcinoma. Anal Bioanal Chem 398:737–749
17. Andrews Kingon GL, Petitte JN, Muddiman DC, Hawkridge AM (2013) Multi-peptide nLC-PC-IDMS-SRM-based assay for the quantification of biomarkers in the chicken ovarian cancer model. Methods 61:323–330
18. Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins from silver-staining polyacrylamide gels. Anal Chem 68:850–858
19. Andrews GL, Shuford CM, Burnett JC Jr, Hawkridge AM, Muddiman DC (2009) Coupling of a vented column with splitless nanoRPLC-ESI-MS for the improved separation and detection of brain natriuretic peptide-32 and its proteolytic peptides. J Chromatogr B Anal Technol Biomed Life Sci 877:948–954
20. Nesvizhskii AI, Keller A, Kolker REA (2003) A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem 75:4646–4658
21. Keller A, Nesvizhskii AI, Kolker E, Aebersold R (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem 74:5383–5392
22. Weatherly DB, Atwood JA 3rd, Minning TA, Cavola C, Tarleton RL, Orlando R (2005) A Heuristic method for assigning a false-discovery rate for protein identifications from Mascot database search results. Mol Cell Proteomics 4:762–772
23. Zybailov B, Mosley AL, Sardiu ME, Coleman MK, Florens L, Washburn MP (2006) Statistical analysis of membrane proteome expression changes in Saccharomyces cerevisiae research articles. J Proteome Res 5:2339–2347
24. Teves ME, Zhang Z, Costanzo RM, Henderson SC, Corwin FD, Zweit J, Sundaresan G, Subler M, Salloum FN, Rubin BK, Strauss JF 3rd (2013) Sperm-associated antigen-17 gene is essential for motile cilia function and neonatal survival. Am J Respir Cell Mol Biol 48:765–772
25. Wu J, Mukherjee A, Lebman DA, Fang X (2012) Gene expression of the lysophosphatidic acid receptor 1 is a target of transforming growth factor beta. Oncogene 32(26):3198–3206
26. Fang X, Yu S, Bast RC, Liu S, Xu HJ, Hu SX, LaPushin R, Claret FX, Aggarwal BB, Lu Y, Mills GB (2004) Mechanisms for lysophosphatidic acid-induced cytokine production in ovarian cancer cells. J Biol Chem 279:9653–9661
27. Liu HB, Sadygov RG, Yates JR (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem 76:4193–4201
28. Gokce E, Shuford CM, Franck WL, Dean RA, Muddiman DC (2011) Evaluation of normalization methods on GeLC-MS/MS label-free spectral counting data to correct for variation during proteomic workflows. J Am Soc Mass Spectrom 22(12):2199–2208
29. Polanski M, Anderson NL (2007) A list of candidate cancer biomarkers for targeted proteomics. Biomark Insights 1:1–48
30. The human protein atlas. http://www.proteinatlas.org/.
31. Cancer Genome Atlas Research N (2011) Integrated genomic analyses of ovarian carcinoma. Nature 474:609–615
32. Emmanuel C, Gava N, Kennedy C, Balleine RL, Sharma R, Wain G, Brand A, Hogg R, Etemadmoghadam D, George J, Birrer MJ, Clarke CL, Chenevix-Trench G, Bowtell DDL, Harnett PR, deFazio A (2011) Comparison of expression profiles in ovarian epithelium in vivo and ovarian cancer identifies novel candidate genes involved in disease pathogenesis. PLoS One 6(3):e17617
33. Abbas AK, Lichtman AH (2003) Cellular and Molecular Immunology, 5 ed. Elsevier Saunders, Philadelphia, PA, p 576
34. Li K, Du H, Lian X, Yang S, Chai D, Wang C, Yang R, Chen X (2014) Characterization of β2-microglobulin expression in different types of breast cancer. BMC Cancer 14:750
35. Restifo NP, Marincola FM, Kawakami Y, Taubenberger J, Yannelli JF, Rosenberg SA (1996) Loss of functional B2-microglobulin in metastatic melanomas from five different patients receiving immunotherapy. J Natl Cancer Inst 88(2):100–108
36. Tsimberidou AM, Kantarjian HM, Wen S, O'Brien S, Cortes J, Wierda WG, Koller C, Pierce S, Brandt M, Freireich EJ, Keating MJ, Estey EH (2008) The prognostic significance of serum β2 microglobulin levels in acute myeloid leukemia and prognostic scores predicting survival: analysis of 1,180 patients. Clin Cancer Res 14:721–730
37. Ware Miller R, Smith A, DeSimone CP, Seamon L, Goodrich S, Podzielinski I, Sokoll L, van Nagell JR, Zhang Z, Ueland FR (2011) Performance of the American College of Obstetricians and Gynecologists' ovarian tumor referral guidelines with a multivariate index assay. Obstet Gynecol 117:1298–1306
38. Yurkovetsky Z, Skates S, Lomakin A, Nolen B, Pulsipher T, Modugno F, Marks J, Godwin A, Gorelik E, Jacobs I, Menon U, Lu K, Badgwell D, Bast RC, Lokshin AE (2010) Development of a multimarker assay for early detection of ovarian cancer. J Clin Oncol 28:2159–2166
39. Honda H, Pazin MJ, D'Souza T, Ji H, Morin PJ (2007) Regulation of the CLDN3 gene in ovarian cancer cells. Cancer Biol Ther 6: 1733–1742
40. Rangel LB, Agarwal R, Souza TD, Pizer ES, Alo PL, Lancaster WD, Gregoire L, Schwartz DR, Cho KR (2003) Tight junction proteins claudin-3 and claudin-4 are frequently overexpressed in ovarian cancer but not in ovarian cystadenomas. Clin Cancer Res 9(7):2567–2575
41. Ng VY, Ang SN, Chan JX, Choo ABH (2010) Characterization of epithelial cell adhesion molecule as a surface marker on undifferentiated human embryonic stem cells. Stem Cells 28:29–35
42. Lu T-Y, Lu R-M, Liao M-Y, Yu J, Chung C-H, Kao C-F, Wu H-C (2010) Epithelial cell adhesion molecule regulation is associated with the maintenance of the undifferentiated phenotype of human embryonic stem cells. J Biol Chem 285:8719–8732
43. Münz M, Kieu C, Mack B, Schmitt B, Zeidler R, Gires O (2004) The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. Oncogene 23:5748–5758

44. Wei BR, Hoover SB, Ross MM, Zhou W, Meani F, Edwards JB, Spehalski EI, Risinger JI, Alvord WG, Quinones OA, Belluco C, Martella L, Campagnutta E, Ravaggi A, Dai RM, Goldsmith PK, Woolard KD, Pecorelli S, Liotta LA, Petricoin EF, Simpson RM (2009) Serum S100A6 concentration predicts peritoneal tumor burden in mice with epithelial ovarian cancer and is associated with advanced stage in patients. PLoS One 4, e7670
45. Byrne J, Frost S, Chen Y, Bright RK (2014) Tumor protein D52 (TPD52) and cancer-oncogene understudy or understudied oncogene? Tumour Biol 52:7369–7382
46. Robinson DS, Monsey JB (1971) Studies on the composition of egg-white ovomucin. Biochem J 121:537–547
47. Reid CJ, Harris A (1998) Developmental expression of mucin genes in the human gastrointestinal system. Gut 42:220–226
48. Berger JT, Voynow JA, Peters KW, Rose MC (1999) Respiratory carcinoma cell lines. MUC genes and glycoconjugates. Am J Respir Cell Mol Biol 20:500–510
49. Sylvester PA, Myerscough N, Warren BF, Carlstedt I, Corfield AP, Durdey P, Thomas MG (2001) Differential expression of the chromosome 11 mucin genes in colorectal cancer. J Pathol 195:327–335
50. Tsukashita S, Kushima R, Bamba M, Sugihara H, Hattori T (2001) MUC gene expression and histogenesis of adenocarcinoma of the stomach. Int J Cancer 94:166–170
51. Stewart CJ, Tsukamoto T, Cooke B, Leung YC, Hammond IG (2006) Ovarian mucinous tumour arising in mature cystic teratoma and associated with pseudomyxoma peritonei: report of two cases and comparison with ovarian involvement by low-grade appendiceal mucinous tumour. Pathology 38:534–538
52. Fazzari C, Fedele F, Pizzi G, Crisafulli C, Parisi A, Caruso RA (2008) Krukenberg tumour of the ovary: a case report with light microscopy, immunohistochemistry and electron microscopy study. Anticancer Res 28:1417–1420
53. Black S, Kushner I, Samols D (2004) C-reactive protein. J Biol Chem 279:48487–48490
54. Coussens LM, Werb Z (2002) Inflammation and cancer. Nature 420:860–867
55. Mantovani A, Allavena P, Sica A, Balkwill F (2008) Cancer-related inflammation. Nature 454:436–444
56. Ogata Y, Heppelmann CJ, Charlesworth MC, Madden BJ, Miller MN, Kalli KR, Cilby WA, Bergen HR 3rd, Saggese DA, Muddiman DC (2006) Elevated levels of phosphorylated fibrinogen-alpha-isoforms and differential expression of other post-translationally modified proteins in the plasma of ovarian cancer patients. J Proteome Res 5:3318–3325
57. Williams DK, Muddiman DC (2009) Absolute quantification of C-reactive protein in human plasma derived from patients with epithelial ovarian cancer utilizing protein cleavage isotope dilution mass spectrometry. J Proteome Res 8:1085–1090
58. Rawlings ND, Waller M, Barrett AJ, Bateman A (2014) MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res 42:D503–D509
59. Miller Feeney REHT (1966) The physical and chemical properties of an immunologically cross-reactive protein from avian egg whites. Biochemistry 5:952–958
60. Nagase H, Harris ED, Woessner JF, Brew K (1983) Ovostatin—a novel proteinase-inhibitor from chicken egg-white 1. purification, physicochemical properties, and tissue distribution of ovostatin. J Biol Chem 258:7481–7489
61. Nielsen KL, Sottrup-Jensen L (1993) Evidence from sequence analysis that hen egg-white ovomacroglobulin (ovostatin) is devoid of an internal beta-Cys-gamma-Glu thiol ester. Biochim Biophys Acta 1162:230–232
62. Nielsen KL, Sottrup-Jensen L, Nagase H, Thogersen HC, Etzerodt M (1994) Amino acid sequence of hen ovomacroglobulin (ovostatin) deduced from cloned cDNA. DNA Seq 5:111–119
63. Mann K (2007) The chicken egg white proteome. Proteomics 7: 3558–3568
64. Lim W, Jeong W, Kim JH, Lee JY, Kim J, Bazer FW, Han JY, Song G (2011) Differential expression of alpha 2 macroglobulin in response to diethylstilbestrol and in ovarian carcinomas in chickens. Reprod Biol Endocrinol 9:137–146
65. Scherer SE, Muzny DM, Buhay CJ, Chen R, Cree A, Ding Y, Dugan-Rocha S, Gill R, Gunaratne P, Harris RA, Hawes AC, Hernandez J, Hodgson AV, Hume J, Jackson A, Khan ZM, Kovar-Smith C, Lewis LR, Lozado RJ, Metzker ML, Milosavljevic A, Miner GR, Montgomery KT, Morgan MB, Nazareth LV, Scott G, Sodergren E, Song XZ, Steffen D, Lovering RC, Wheeler DA, Worley KC, Yuan Y, Zhang Z, Adams CQ, Ansari-Lari MA, Ayele M, Brown MJ, Chen G, Chen Z, Clerc-Blankenburg KP, Davis C, Delgado O, Dinh HH, Draper H, Gonzalez-Garay ML, Havlak P, Jackson LR, Jacob LS, Kelly SH, Li L, Li Z, Liu J, Liu W, Lu J, Maheshwari M, Nguyen BV, Okwuonu GO, Pasternak S, Perez LM, Plopper FJ, Santibanez J, Shen H, Tabor PE, Verduzco D, Waldron L, Wang Q, Williams GA, Zhang J, Zhou J, Allen CC, Amin AG, Anyalebechi V, Bailey M, Barbaria JA, Bimage KE, Bryant NP, Burch PE, Burkett CE, Burrell KL, Calderon E, Cardenas V, Carter K, Casias K, Cavazos I, Cavazos SR, Ceasar H, Chacko J, Chan SN, Chavez D, Christopoulos C, Chu J, Cockrell R, Cox CD, Dang M, Dathorne SR, David R, Davis CM, Davy-Carroll L, Deshazo DR, Donlin JE, D'Souza L, Eaves KA, Egan A, Emery-Cohen AJ, Escotto M, Flagg N, Forbes LD, Gabisi AM, Garza M, Hamilton C, Henderson N, Hernandez O, Hines S, Hogues ME, Huang M, Idlebird DG, Johnson R, Jolivet A, Jones S, Kagan R, King LM, Leal B, Lebow H, Lee S, LeVan JM, Lewis LC, London P, Lorensuhewa LM, Loulseged H, Lovett DA, Lucier A, Lucier RL, Ma J, Madu RC, Mapua P, Martindale AD, Martinez E, Massey E, Mawhiney S, Meador MG, Mendez S, Mercado C, Mercado IC, Merritt CE, Miner ZL, Minja E, Mitchell T, Mohabbat F, Mohabbat K, Montgomery B, Moore N, Morris S, Flagg N, Forbes LD, Gabisi AM, Garza M, Hamilton C, Henderson N, Hernandez O, Hines S, Hogues ME, Huang M, Idlebird DG, Johnson R, Jolivet A, Jones S, Kagan R, King LM, Leal B, Lebow H, Lee S, LeVan JM, Lewis LC, London P, Lorensuhewa LM, Loulseged H, Lovett DA, Lucier A, Lucier RL, Ma J, Madu RC, Mapua P, Martindale AD, Martinez E, Massey E, Mawhiney S, Meador MG, Mendez S, Mercado C, Mercado IC, Merritt CE, Miner ZL, Minja E, Mitchell T, Mohabbat F, Mohabbat K, Montgomery B, Moore N, Morris S, Munidasa M, Ngo RN, Nguyen NB, Nickerson E, Nwaokelemeh OO, Nwokenkwo S, Obregon M, Oguh M, Oragunye N, Oviedo RJ, Parish BJ, Parker DN, Parrish J, Parks KL, Paul HA, Payton BA, Perez A, Perrin W, Pickens A, Primus EL, Pu LL, Puazo M, Quiles MM, Quiroz JB, Rabata D, Reeves K, Ruiz SJ, Shao H, Sisson I, Sonaike T, Sorelle RP, Sutton AE, Svatek AF, Svetz LA, Tamerisa KS, Taylor TR, Teague B, Thomas N, Thorn RD, Trejos ZY, Trevino BK, Ukegbu ON, Urban JB, Vasquez LI, Vera VA, Villasana DM, Wang L, Ward-Moore S, Warren JT, Wei X, White F, Williamson AL, Wleczyk R, Wooden HS, Wooden SH, Yen J, Yoon L, Yoon V, Zorrilla SE, Nelson D, Kucherlapati R, Weinstock G, Gibbs RA, Baylor College of Medicine Human Genome Sequencing Center Sequence Production T (2006) The finished DNA sequence of human chromosome 12. Nature 440:346–351
66. Normandin K, Peant B, Le Page C, de Ladurantaye M, Ouellet V, Tonin PN, Provencher DM, Mes-Masson AM (2010) Protease inhibitor SERPINA1 expression in epithelial ovarian cancer. Clin Exp Metastasis 27:55–69
67. Chambers SK, Gertz RE Jr, Ivins CM, Kacinski BM (1995) The significance of urokinase-type plasminogen activator, its inhibitors, and its receptor in ascites of patients with epithelial ovarian cancer. Cancer 75:1627–1633
68. Chambers SK, Ivins CM, Carcangiu ML (1997) Expression of plasminogen activator inhibitor-2 in epithelial ovarian cancer: a

favorable prognostic factor related to the actions of CSF-1. Int J Cancer 74:571–575
69. Sieben NL, Oosting J, Flanagan AM, Prat J, Roemen GM, Kolkman-Uljee SM, van Eijk R, Cornelisse CJ, Fleuren GJ, van Engeland M (2005) Differential gene expression in ovarian tumors reveals Dusp 4 and Serpina 5 as key regulators for benign behavior of serous borderline tumors. J Clin Oncol 23:7257–7264
70. Bauerschlag DO, Habermann M, Weimer J, Meinhold-Heerlein I, Hilpert F, Weigel M, Bauer M, Mundhenke C, Jonat W, Maass N, Schem C (2010) Heterogeneous expression of serine protease inhibitor maspin in ovarian cancer. Anticancer Res 30:2739–2744
71. Okamoto T, Niu R, Yamada S (2003) Increased expression of tissue inhibitor of metalloproteinase-2 in clear cell carcinoma of the ovary. Mol Hum Reprod 9:569–575
72. Ripley D, Tunuguntla R, Susi L, Chegini N (2006) Expression of matrix metalloproteinase-26 and tissue inhibitors of metalloproteinase-3 and -4 in normal ovary and ovarian carcinoma. Int J Gynecol Cancer 16:1794–1800
73. Devoogdt N, Hassanzadeh Ghassabeh G, Zhang J, Brys L, De Baetselier P, Revets H (2003) Secretory leukocyte protease inhibitor promotes the tumorigenic and metastatic potential of cancer cells. Proc Natl Acad Sci U S A 100:5778–5782
74. Rasool N, LaRochelle W, Zhong H, Ara G, Cohen J, Kohn EC (2010) Secretory leukocyte protease inhibitor antagonizes paclitaxel in ovarian cancer cells. Clin Cancer Res 16:600–609
75. Perets R, Wyant GA, Muto KW, Bijron JG, Poole BB, Chin KT, Chen JYH, Ohman AW, Stepule CD, Kwak S, Karst AM, Hirsch MS, Setlur SR, Crum CP, Dinulescu DM, Drapkin R (2013) Transformation of the fallopian tube secretory epithelium leads to high-grade serous ovarian cancer in Brca, Tp53, Pten models. Cancer Cell 24:751–765
76. Dubeau L (2008) The cell of origin of ovarian epithelial tumours. Lancet Oncol 9:1191–1197
77. Karst AM, Levanon K, Drapkin R (2011) Modeling high-grade serous ovarian carcinogenesis from the fallopian tube. Proc Natl Acad Sci U S A 108:7547–7552
78. Kim J, Coffey DM, Creighton CJ, Yu Z, Hawkins SM, Matzuk MM (2012) High-grade serous ovarian cancer arises from fallopian tube in a mouse model. Proc Natl Acad Sci U S A 109:3921–3926
79. Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC (2001) Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr Rev 22:255–288