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E-cadherin Expression in Ovarian Cancer in the Laying Hen, *Gallus Domesticus*, compared to Human Ovarian Cancer

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Abstract

Objective—Epithelial ovarian carcinoma (EOC) is a leading cause of cancer deaths in women. Until recently, a significant lack of an appropriate animal model has hindered the discovery of early detection markers for ovarian cancer. The aging hen serves as an animal model because it spontaneously develops ovarian adenocarcinomas similar in histological appearance to the human disease. E-cadherin is an adherens protein that is down-regulated in many cancers, but has been shown to be up-regulated in primary human ovarian cancer. Our objective was to evaluate E-cadherin expression in the hen ovary and compare its expression to human ovarian cancer.

Methods—White Leghorn hens aged 185 weeks (cancerous and normal) were used for sample collection. A human ovarian tumor tissue array was used for comparison to the human disease. E-cadherin mRNA and protein expression were analyzed in cancerous and normal hen ovaries by immunohistochemistry (IHC), Western blot, and quantitative real-time PCR (qRT-PCR). Tissue fixed in neutral buffered formalin was used for IHC. Protein from tissue frozen in liquid nitrogen was analyzed by Western blot. RNA was extracted from tissue preserved in RNAlater and analyzed by qRT-PCR. The human ovarian tumor tissue array was used for IHC.

Results—E-cadherin mRNA and protein expression were significantly increased in cancerous hen ovaries as compared to ovaries of normal hens by qRT-PCR and Western blot. Similar expression of E-cadherin was observed by IHC in both human and hen ovarian cancer tissues. Similar E-cadherin expression was also observed in primary ovarian tumor and peritoneal metastatic tissue from cancerous hens.

Conclusions—Our findings suggest that the up-regulation of E-cadherin is an early defining event in ovarian cancer and may play a significant role in the initial development of the primary ovarian tumor. E-cadherin also appears to be important in the development of secondary tumors within the peritoneal cavity. Our data suggest that E-cadherin may prove to be an important target in the preventative treatment of metastatic ovarian cancer and further confirm that the laying hen is a good model for the study of human epithelial ovarian carcinoma.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Keywords

Ovarian cancer; E-cadherin; *Gallus domesticus*; Epithelial-to-Mesenchymal Transition

Introduction

It was estimated that 21,650 women were diagnosed with ovarian cancer and 15,520 women would've died of ovarian cancer in 2008 [1]. 90% of ovarian cancers are epithelial in origin [2,3]. Stage 1 ovarian cancer is treatable 95% of the time, but due to a lack of symptoms and early detection markers, most cancers are not diagnosed until stage 3 or 4 [4]. Stages 3 and 4 involve metastasis to the abdomen and other peritoneal organs [5]. Women with peritoneal metastases have the greatest risk of dying from ovarian cancer [6]. There is a significant lack of experimental models because, with the exception of the aging hen, ovarian tumors in other species do not arise spontaneously from the ovarian surface epithelium (OSE) or are fundamentally different from epithelial ovarian carcinoma (EOC) [7]. Only hens spontaneously develop significant numbers of ovarian adenocarcinomas similar in histological appearance to common human epithelial carcinomas [8,9].

In 1971, Fathalla hypothesized that continuous ovulations, with successive rounds of rupture and wound healing, render the ovarian surface epithelial cells susceptible to malignant transformation [10]. The laying hen model supports this hypothesis because domestic hens ovulate almost daily and have a very high incidence of ovarian surface epithelial cancer [11]. Once a hen has entered her third year of egg laying, she has ovulated approximately as many times as a woman who is approaching menopause (about 500 times). The incidence of ovarian cancer in hens increases with age, approximately 4% incidence at 2 years of age, and rising to nearly 40% by 6 years of age [11]. At the site of ovulation, OSE cells suffer oxidative DNA damage [12,13]. Successive rounds of oxidative DNA damage render the OSE likely to evade endogenous DNA repair mechanisms. As a result of this constant remodeling, the ovarian surface becomes increasingly more convoluted, forming invaginations and crypts that form glandular cysts within the stroma [14,15]. These epithelial rearrangements are hypothesized to be the potential origin of many epithelial cancers [15].

E-cadherin (CDH1) is a transmembrane protein that localizes to adherens junctions and is responsible for establishing intercellular junctions between neighboring cells [16]. E-cadherin binds cells by homophilic interactions and also interacts with the actin cytoskeleton intracellularly by binding to catenin molecules [16]. The expression of E-cadherin in ovarian cancer is distinct from other epithelial cancers. Very little E-cadherin is expressed in the normal ovary, but in ovarian cancer, E-cadherin expression is increased in 85% of primary tumors [17–20]. Increased E-cadherin in the primary tumor is one of the earliest identifying markers of human ovarian cancer [21]. In other epithelial cancers, E-cadherin is expressed in normal tissue and is lost as the tumor progresses. In these other cancers, there are clear changes of cellular phenotype from epithelioid to mesenchymal, especially in cells that have become invasive and initiated metastases [22]. The loss of E-cadherin is an important step in the process of epithelial-to-mesenchymal transition (EMT) in cancer. EMT is an embryonic process that is necessary for morphogenesis in multi-organ beings [23,24]. Cells lose E-cadherin and acquire a more mesenchymal, motile phenotype [6]. In contrast, in ovarian cancer, no clear progression to metastasis has yet been described, and primary ovarian tumors display increased, not decreased, expression of E-cadherin. However, once ovarian cancer has advanced to metastasis, metastatic ascites cells express significantly less E-cadherin than primary tumor cells [21]. The loss of E-cadherin in ovarian cancer is a later step which is associated with the metastatic phenotype, including peritoneal dissemination and ascites production [25]. Once

the metastases have established cancer at a distant site, E-cadherin is re-expressed and the secondary tumor re-acquires an epithelial phenotype [26].

The hen is an important animal model for developmental biology and much is known about the function of cadherins during development through the use of the chicken embryo [27]. However, little is known about E-cadherin expression in postnatal tissues and E-cadherin expression in the hen ovary has not been previously examined. The objective of this study was to compare E-cadherin expression in human ovarian cancer with ovarian cancer in the laying hen and to measure E-cadherin mRNA and protein expression in hen ovarian cancer. The results of this study demonstrate similarities between human and hen E-cadherin expression in ovarian carcinoma and show that E-cadherin is significantly increased in ovarian cancer in the laying hen. Our data further support the laying hen as a model for human ovarian cancer and suggest that E-cadherin is a marker of early neoplasia and a potential therapeutic target for ovarian cancer. Identification of factors that drive the increased expression of E-cadherin may lead to the discovery of early detection markers for ovarian carcinoma.

Materials and Methods

Animals and tissue collection

78 single-comb White Leghorn hens, aged 185 weeks (cancerous and normal), were used for sample collection. Hens were maintained three per cage, provided with feed and water ad libitum and exposed to a photoperiod of 17 h light: 7 h dark, with lights on at 05:00 h and lights off at 22:00 h. Animal management and procedures were reviewed and approved by the Institutional Animal Care and Use Committees at the University of Illinois at Urbana-Champaign and University of Illinois at Chicago.

Hens were euthanized by CO₂ asphyxiation. Upon necropsy, ovaries were removed and small yellow follicles (6–8mm) and pre-ovulatory follicles (F1-F5, 9–35mm) were removed from ovaries of the normal hens. Suspected abnormal ovarian tissues were noted and confirmed as cancerous or normal ovarian tissue by histology. The ovarian tissue consisting of the stroma, cortical (<1mm), small and large white follicles (1–5mm), was cut into smaller portions and divided for three analyses: 1) snap frozen and stored at -80°C for protein analysis; 2) placed into RNeasy (Applied Biosystems, CA) and stored at 4°C for RNA isolation; and 3) fixed in neutral-buffered formalin (NBF) (Sigma-Aldrich, MO) for histological and immunohistochemical analysis. Of the 78 hens necropsied, 23 hens presented with varying stages of ovarian cancer. Cancers were staged according to the TNM system based on the presentation of disease and pathology: T1=microscopic tumor, T2= localized tumor, T3 and T4= locally advanced tumor; N1=lymph node metastasis, N0= no lymph node involvement; M1= distant metastasis, M0= no metastases [28]. Metastasis to the lymph nodes was not determined. Of the 23 cancerous hens necropsied, 13 presented with T3NXM1 or T4NXM1 ovarian cancer. Samples from these hens were used for further analysis. Metastatic tissue from these hens was taken from the peritoneal wall, intestine, and oviduct and used for IHC.

OSE Collection

Pre-ovulatory follicles (12–35mm) were removed from normal ovaries for OSE collection. Ovarian surface epithelial cells were carefully removed by scraping the follicular surface with a sterile cell scraper. Samples were then placed directly into RNeasy and stored at 4°C for RNA isolation. OSE was only obtained from healthy hens as hens with ovarian cancer rarely have pre-ovulatory follicles.

Human Ovary Tumor Tissue Array

The human ovarian cancer tissue array was obtained from BioChain (Hayward, CA). Twelve cases of human ovarian cancer of differing histotypes from stages 1 and 2 were arrayed. Histotypes include serous cystadenocarcinoma, endometrioid adenocarcinoma, poorly differentiated adenocarcinoma, serous papillary adenocarcinoma, and mucinous cystadenocarcinoma. Corresponding uninvolved tissues from the same patients were used as controls. All the tissues were obtained from surgical resection. Tissues were fixed in 10% neutral buffered formalin for 24 hours and processed using identical standard operating procedures. Sections were mounted onto Superfrost Plus or APES coated Superfrost slides.

Histology, immunohistochemistry and immunofluorescence

Laying hen ovarian tissues fixed in NBF solution were processed and paraffin embedded. 5µm sections were cut and mounted on SuperFrost Plus microscope slides (Fisher Scientific, IL). Slides were deparaffinized and rehydrated through xylene and graded ethanol solutions (Fisher Scientific). Hematoxylin and eosin (H&E) staining was performed as described [29]. Confirmed cancerous tissues were characterized by histotype. Of the 78 hens necropsied, 23 hens were confirmed as having ovarian cancer of varying histologies. Of the 23 hens with ovarian cancer, the majority of them (13 hens) presented with endometrioid type tumors. Other histologies seen were clear cell and serious-papillary type tumors, and also mixed tumors. Immunohistochemistry was performed using the Vectastain Elite ABC kit (Vector Laboratories, CA). Antigen retrieval was achieved using Antigen Unmasking Solution (Vector Laboratories) and heated under pressure at 20 psi for 5 min in a Decloaking Chamber electric pressure cooker (Biocare Medical, CA). Slides were cooled and quenched in 0.3% H₂O₂ (Sigma-Aldrich) in methanol for 15 min. Slides were blocked with normal serum and incubated in anti-human E-cadherin monoclonal antibody (BD Transduction Laboratories, NJ) at a dilution of 1:1000 overnight at 4°C. Humans and hens share about 75% homology in the nucleotide and amino acid sequences for E-cadherin. After rinsing in Tris-buffered saline, sections were incubated with anti-mouse secondary antibody and avidin-biotin complex (Vector Laboratories). Specific binding was visualized using diaminobenzidine (DAB) (Sigma-Aldrich) in the presence of H₂O₂ and sections were counter-stained with Gills hematoxylin (Sigma-Aldrich), and mounted with Histomount (Fisher Scientific). For immunofluorescence, sections were incubated with either Texas Red fluorescent anti-mouse secondary antibody (Vector Laboratories) and mounted with Vector Shield Hard Set mounting media (Vector Laboratories). Slides were then examined on a Nikon ECLIPSE E400 microscope and were documented using SPOT Advanced version 4.0.1 software (Diagnostic Instruments, MI).

Western Blot

Hen ovarian tissue homogenates were prepared from snap frozen samples, pulverized on dry ice, re-suspended in ice-cold lysis buffer (PBS/0.1% sodium dodecyl sulfate (SDS), supplemented with protease inhibitor cocktail HALT), and homogenized using an Ultra-Turrax (Jenke and Kunkel, Staufenh, Germany). Protein concentrations were determined by BCA protein assay (Pierce). Ten micrograms of total protein were separated by SDS-PAGE using 12.5% acrylamide/SDS separating gels and transferred to nitrocellulose membranes as described previously [30–32]. Monoclonal anti-human E-cadherin (BD Transduction Laboratories) was used for detection of E-cadherin and data were normalized to monoclonal anti-chicken β-Actin (Santa Cruz, CA). Detection of bound antibody on the blot was assessed with a horseradish peroxidase-conjugated, goat anti-mouse IgG antibody (Promega), visualized by chemiluminescent detection (SuperSignal West Pico Chemiluminescent Substrate, ThermoScientific, IL), and quantified after densitometry using Imagequant software (Molecular Dynamics, CA). Data for protein are represented as integrated OD.

RNA extraction and analysis

Total RNA was extracted from OSE and ovaries using Trizol reagent (Invitrogen, CA) and was quantified by determination of absorbance at 260nm. RNA was collected from whole ovarian homogenates which were heterogeneous. RNA samples were then treated with RQ1 RNase-free DNase (Promega, WI) prior to the reverse transcription reaction. Synthesis of single-stranded cDNA was performed using a high capacity cDNA Archive Kit (Applied Biosystems) and cDNA was quantified by Quant-iT fluorescent reagent (Invitrogen). Equal amounts of cDNA from all samples were subjected to quantitative real-time PCR.

Quantitative Real-Time PCR (qRT-PCR)

Hen specific primers were designed to amplify target gene transcripts using Primer Express software (Applied Biosystems) and obtained from Sigma-Genosys (Sigma-Aldrich). The qRT-PCR primer pairs were designed so that one spanned an intron. E-cadherin (NM_001039258.1) forward primer: 5' GCAGAAGATCACGTACCGCAT 3'; reverse primer: 5' AAGGACCTGCCCCACATA 3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_204305) was used as an internal control gene. GAPDH forward primer: 5' GATGGGTGTCAACCATGAGAAA 3'; reverse primer: 5' CAATGCCAAAGTTGTCATGGA 3'. Cytokeratin 8 (XM_424502) was also used as an internal control for epithelial cells. Cytokeratin 8 forward primer: 5' GCGACCTACAGGAAGCTGCT 3'; reverse primer: 5' CCCAAATCCTCCTGAGTACCC 3'. Plasmid standards for E-cadherin, GAPDH, and cytokeratin 8 were used for absolute quantification. To clone the GAPDH plasmid, total RNA was extracted from hen ovarian tissue, pooled, and reversed transcribed into cDNA with the Reverse Transcription System Kit (Promega). The GAPDH cDNA fragments were amplified by Taq DNA Polymerase (Qiagen, CA) and the following primer sequences were used: forward: 5' GCAGATGCAGGTGCTGAGTATG 3'; reverse: 5' GGCAGGTCAGGTCAACAACAGA 3'. The GAPDH fragments were then cloned using TOPO TA cloning Kit (Invitrogen) and verified by sequencing. E-cadherin (pg11n.pk014.k6) and cytokeratin 8 (pg11n.pk005.a13) clones were obtained from the Delaware Biotechnology Institute (Newark, DE) and verified by DNA sequencing. The corresponding copy numbers of plasmids were calculated using the formula: $1\mu\text{g of } 1000\text{bp of DNA} = 9.1 \times 10^{11} \text{ molecules}$. qRT-PCR was conducted by amplifying cDNA with SYBR Green Master Mix (Applied Biosystems) on ABI 7900HT using a 384 well plate and analyzed with ABI Prism software. Control reactions lacking the template were run for each gene. Reactions were 10 μl in total volume and 200nM of each primer was applied. The plasmid standards and cDNA were simultaneously assayed in duplicate reactions. The amplification conditions were as follows: 50°C 2 min, 95°C 10 min, then 40 cycles of: 95°C 15 sec, 60°C 1 min.

Statistical analysis

Statistical analysis was performed with GraphPad InStat by using One-way ANOVA with Student-Newman-Keuls comparison. A value of ($P < 0.05$) was considered significant whereas a value of ($P < 0.01$) was considered highly significant.

Results

Histology of Laying Hen and Human Ovaries

Figure 1A is a picture of a normal hen ovary. The large yellow follicles are arranged in a hierarchy with the largest follicle F1 (arrow) being the next to ovulate. The normal hen ovary is not encapsulated. Figure 1B shows a cancerous ovary in the hen. The cancerous ovary is much larger than the normal ovary, with several surface lesions (white arrow). There are also many atretic follicles (black arrow), indicating that this is a non-functional ovary. H&E staining

of the normal hen ovary (Fig. 1C) shows many distinct cortical follicles, a sign of a healthy ovary. H&E staining of uninvolved normal tissue from the human ovarian cancer tissue array lacks follicular ovarian structures (Fig. 1E). The formation of glandular structures are one of many structural changes seen in human endometrioid type ovarian cancer tissue (Fig. 1F). These structural changes are also seen in the hen cancerous ovarian tissue (Fig. 1D). This tissue was typed as an endometrioid type tumor based on the presence of cells that resemble the glandular cells of the endometrium [33].

Histology of Laying Hen Ovarian Cancer Metastatic Tissue

H&E staining of peritoneal metastatic tissue from hens with ovarian cancer revealed similar morphology to the primary ovarian tumor (Fig. 4A and 4B). Glandular structures were seen in the metastatic tissue, similar to the pattern seen in the primary ovarian tumor.

Immunohistochemistry of E-cadherin in the Laying Hen and Human Ovaries

Expression of E-cadherin protein in hen and human ovaries was examined by immunohistochemistry. In the laying hen, there was very little E-cadherin expression in the ovarian surface epithelial cell layer and in the granulosa cell layer (arrow) of follicles (Fig. 2A). In the uninvolved normal human tissue, there was no positive E-cadherin staining (Fig. 2D). In another section of normal human ovarian tissue, there was an increase in E-cadherin staining in the OSE similar to what was seen in the hen, suggesting the tissue may not be normal (Fig. 2B and Fig. 2E).

In the primary human ovarian tumor, E-cadherin was expressed throughout the tissue (Fig. 2F). E-cadherin was expressed in human ovarian cancer cells in large glandular structures, similar to what was seen in the hen (Fig. 2C). In the hen, E-cadherin was expressed intensely in the cells of these glandular structures throughout the tumor tissue. The hen ovarian cancer metastatic tissue also expressed strong E-cadherin staining in a similar pattern to the primary tumor (Fig. 4C and Fig. 4D).

Immunofluorescence of E-cadherin in the Laying Hen ovary

Expression of E-cadherin in normal and cancerous hen ovaries was examined by immunofluorescence. Positive E-cadherin staining is seen in the granulosa cell layer of a periovulatory follicle (Fig. 3A). In an early stage cancer, a small gland expressing high E-cadherin is seen, but the rest of the tissue has little expression (Fig. 3B). In the cancerous ovary, E-cadherin is expressed throughout the tissue, and is strongly positive in the membranes of the cells making up glands (Fig. 3C).

E-cadherin protein expression in Hen Ovaries by Western blot

E-cadherin protein expression was significantly increased ($P < 0.0003$) in hen ovarian cancer as compared to normal ovarian tissue when compared by Western blot (Fig. 5A). In the normal ovarian tissue, a faint band was seen at 130kDa. In the cancerous ovarian tissue, a doublet was expressed around 120–130kDa. Data were normalized to β -Actin (43kDa) (Fig. 5B).

E-cadherin mRNA in Hen Ovaries by qRT-PCR

E-cadherin mRNA expression was measured in hen ovarian cancerous tissue as compared to OSE or normal ovarian tissue (Fig. 6A and Fig. 6B) by qRT-PCR. Data were normalized to GAPDH, which is expressed in all cell types, or cytokeratin, which is expressed only in epithelial cells [29]. There was a significant increase in E-cadherin mRNA expression in the cancerous ovarian tissue as compared to the normal ovarian tissue and OSE when data were normalized to GAPDH (Fig. 6A) ($P < 0.02$). The increase in E-cadherin mRNA expression in

the cancerous ovarian tissue as compared to the normal tissue and OSE was more highly significant ($P < 0.001$) when data were normalized to cytokeratin (Fig. 6B).

Discussion

The goal of this study was to compare E-cadherin expression in laying hen ovarian cancer with human ovarian cancer and to measure E-cadherin mRNA and protein expression in ovarian cancer in the laying hen. We found similar patterns of E-cadherin protein expression in the human ovarian tissue and the hen tissue including increased expression of E-cadherin in early neoplastic ovarian tissue. These data support the theory that ovarian cancer arises from transformed OSE as these early neoplastic cells stain intensely for epithelial marker E-cadherin with subsequent E-cadherin expression seen throughout the primary tumor.

This is the first report that E-cadherin mRNA and protein are highly expressed in ovarian cancer in the laying hen, *Gallus domesticus*. Using immunohistochemistry, we observed similarities in the expression of E-cadherin between the laying hen and human ovarian cancer and between the primary ovarian tumor and secondary metastases in the laying hen. qRT-PCR analysis indicated that E-cadherin mRNA was significantly increased in the hen ovarian tumor compared to normal ovarian tissue and OSE. Western blot analysis showed that E-cadherin protein expression was also significantly increased in the hen ovarian tumor compared to normal ovary. Immunohistochemistry of E-cadherin showed light staining in cells found in the granulosa cell layer and ovarian surface epithelium in the normal hen ovary, whereas in the hen ovarian tumor, E-cadherin was highly expressed throughout the tissue as was reported in human ovarian cancer [25,34]. Similar to human ovarian cancer, the secondary tumor in the hen also showed increased E-cadherin expression throughout the tissue [26].

Due to the common late stage presentation of epithelial ovarian cancer, clinicians have not been able to identify uniform early changes in ovarian tissue that would aid in staging and prognosis. In later stages of ovarian cancer we see a very prominent up-regulation of E-cadherin in both laying hen and human tissue. In both hen and human early neoplastic tissue, we see a shape change in the OSE, from cuboidal to columnar cells with displaced nuclei, similar to what has been seen in prostate cancer. The ovarian microenvironment is rich in inflammatory factors and ovulation itself is an inflammatory event [35,36]. In prostate cancer, loss of caretaker genes like glutathione S-transferase P1-1 (GSTP1) lead to genomic damage by inflammatory carcinogens and reactive oxygen species. Cells undergo proliferative inflammatory atrophy as a response to this damage and enter into prostatic intraepithelial neoplasia resulting in a shape change [37]. These data suggest that a similar phenomenon may be occurring in ovarian cancer. If, after several rounds of ovulation, caretaker and tumor suppressor genes were no longer able to repair damage caused by reactive oxygen species or inflammatory mediators, these cells would be the likely source of the cancer [13].

In both the laying hen and human ovarian cancer, the primary tumor tissue stained intensely for E-cadherin. These data show the epithelialization of the primary tumor and support the theory that ovarian cancer arises from the OSE. Large glandular structures were seen in the primary tumors. It has been reported that down-regulation of E-cadherin is necessary for peritoneal dissemination and metastasis to secondary sites [38], but not much is known about what happens once these cells form secondary tumors within the peritoneal cavity. We show here, for the first time in the laying hen, that peritoneal metastases form similar morphological structures to that of the primary tumor. Glandular structures were seen by basic histology in the metastases and the primary tumor. The metastatic tissue expressed E-cadherin in similar patterns to the primary tumor. This finding suggests that the disseminated ovarian cancer cells from the primary tumor undergo a mesenchymal to epithelial (MET) transition at the secondary site and that E-cadherin is needed to form adherens complexes to structure and strengthen the

secondary tumor. Future studies will examine E-cadherin expression in human ovarian cancer cell lines to determine if an EMT/MET event occurs. Identification of the factors and mechanisms that drive the up-regulation of E-cadherin may also prove to be useful therapeutic targets to prevent metastases formation.

The laying hen provides an excellent animal model for the investigation of ovarian cancer as hens frequently develop ovarian cancer that is similar histopathologically to the human disease. Laying hen and human ovarian cancer share very similar patterns of expression for several different proteins including cytokeratin, proliferating cell nuclear antigen (PCNA) [39], selenium binding protein (SELENBP1)[40] and anti-tumor antibodies [41]. Our laboratory and others have shown that laying hen ovarian cancer tissue expresses increased cyclooxygenase enzyme-1 (COX-1) [42,43], similar to the human disease and also CYP1B1[44], an enzyme that can metabolize estrogen into genotoxic substances. We report here that both laying hens and women develop glandular structures in the disease state that stain intensely for E-cadherin, further validating the laying hen as an important animal model for human ovarian cancer. We also show here, for the first time, that laying hen metastatic tissue recapitulates primary tumor morphology and protein expression, which suggests that E-cadherin expression may be necessary to form stable secondary tumor sites. Our findings suggest that up-regulation of E-cadherin is an early defining event in ovarian cancer and may play a significant role in the initial development of the primary tumor. E-cadherin is also important in the development of secondary tumors within the peritoneal cavity. E-cadherin may prove to be an important target in the treatment of metastatic ovarian cancer.

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Figure 1. H&E staining of hen and human normal and cancerous ovary

Hematoxylin and eosin staining of laying hen and human ovaries. A-B Pictures of normal and cancerous hen ovary. Normal ovaries have a hierarchy of follicles (arrow) (1A). Cancerous ovary has surface tumor lesions (white arrow) and atretic follicles (black arrow) signifying that it is non-functioning (1B). C-F: Hematoxylin and eosin staining of normal and cancerous hen and human ovary (bar=100 μ m), C-D: Normal ovarian tissue from a healthy hen (1C); ovarian tissue taken from a hen with metastatic ovarian cancer typed as endometrioid adenocarcinoma (1D). E-F: Uninvolved normal human ovarian tissue (1E); human endometrioid type tumor [40] (1F). The morphology of this tissue is similar to the hen ovarian cancer tissue in 1C.

Figure 2. Immunohistochemistry of E-cadherin in hen and human normal and cancerous ovary
 Immunohistochemical localization of E-cadherin expression in laying hen and human normal ovarian and cancerous tissue. Immunohistochemistry for E-cadherin (bar=50µm). Hen IHC A-C. Normal hen ovarian tissue shows little E-cadherin staining in the ovarian surface epithelium and granulosa cell layer (arrow) of follicles (2A). Transformation of ovarian surface epithelial cells (black arrows) marked by increased E-cadherin expression in an early stage ovarian tumor (2B). Expression is further increased in the primary tumor, with distinct glandular structures staining intensely for E-cadherin throughout the tissue (2C). Human IHC D-F. Uninvolved normal human ovarian tissue shows no positive stain for E-cadherin (2D). Tissue labeled as normal shows early neoplastic transformation (black arrow) of ovarian surface epithelial cells in an early stage tumor similar to hen tissue in Figure 1B. (2E). White arrows in Figure 1B and 1E indicated areas of mild to moderate dysplastic cells. Human Endometrioid type tumor expresses high E-cadherin in distinct patterns throughout the tissue (2F).

Figure 3. Immunofluorescence of E-cadherin in normal and cancerous hen ovary

Immunofluorescence of E-cadherin in normal and cancerous laying hen ovary (bar=100 μ m). Positive E-cadherin staining is seen in the granulosa cell layer of a periovulatory follicle (3A). A small gland expressing E-cadherin in otherwise normal ovary (3B). In the cancerous ovary, E-cadherin is expressed throughout the tissue, and is strongly positive in the membranes of the cells making up glands (3C). (E-cadherin Red, DAPI Blue)

Figure 4. H&E staining and E-cadherin immunohistochemistry in the hen metastatic tissue
 Laying hen metastatic tissue shares morphology and protein expression with the primary ovarian tumor. A-B: H and E staining of metastatic tissue (bar=100µm), C-D: Immunohistochemistry for E-cadherin (bar=50µm). Metastatic tissue taken from the peritoneal wall reveals glandular-like structures similar to the primary tumor (4A and 4B). Metastatic tissue expresses high E-cadherin in a similar pattern to the primary tumor, suggesting that secondary tumors mimic primary tumor morphology and protein expression (4C and 4D).

Figure 5. E-cadherin protein expression in normal and cancerous hen ovary

E-cadherin protein expression is significantly higher in laying hen ovarian cancer. Protein homogenates from eight different hen ovaries (four normal, four cancerous) were used to compare E-cadherin protein expression by Western blot using anti-human E-cadherin antibody. In the normal ovarian tissue, a faint band was seen at 130kDa. Western blot revealed a doublet around 130kDa in the cancerous ovarian tissue (5A). Data were normalized to β -Actin (5B). There was a significant increase (** $P < 0.0003$) in protein expression in the cancer tissue compared to the normal ovarian tissue (5C). There was no statistical difference between the normal and cancerous tissues for β -Actin expression.

A. E-cadherin mRNA expression normalized by GAPDH

B. E-cadherin mRNA expression normalized by cytokeratin

Figure 6. E-cadherin mRNA expression in hen OSE, normal ovary, and cancerous ovary

E-cadherin mRNA expression is significantly increased in the laying hen cancerous ovary. E-cadherin mRNA expression in ovarian surface epithelium, cancerous and age-matched normal ovarian tissue was compared by qRT-PCR. There was a significant increase in E-cadherin expression in the ovarian cancer tissue when normalized to GAPDH (*P<0.02) (6A) and cytokeratin (**P<0.001) (6B). Bars indicate standard error.