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TITLE: CYP1B1, Oxidative Stress, and Inflammation in the Etiology of Ovarian Epithelial Cancer Using an Avian Model of Ovarian Carcinoma

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14. ABSTRACT

Ovarian cancer is the most lethal of the gynecological malignancies due to its late stage of detection. Research in ovarian cancer has been hampered by a lack of suitable animal models. With the exception of the laying hen, no other animal gets ovarian epithelial cancer analogous to the human disease. The study further validates the hen model of ovarian cancer. The results of the study demonstrate that cancer markers in the human disease are valid in the hen including PCNA, E and N-cadherin, and cytokeratin; the histopathology of the human and chicken ovarian cancer are similar; that Cyclooxygenase-1 (COX-1) expression, an enzyme critical for inflammatory prostaglandin synthesis is up-regulated in both the chicken and human disease suggesting it may be molecular target for intervention; DNA microarray studies identified critical pathways that may be important to the understanding the disease. CYP1B1 demonstrates high basal expression which appears to increase with age in the chicken ovary with no apparent further increase observed in cancer, in contrast to what has been reported in human ovarian cancer. The hen model may provide a system for large scale therapeutic intervention studies.

15. SUBJECT TERMS
Ovarian cancer, chemoprevention, DNA microarray, Cyclooxygenase, cancer markers

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INTRODUCTION:

Ovarian cancer is the most lethal of the gynecological malignancies and the 5th leading cause of cancer death in women. Ovarian cancer is lethal due to the late stage at which it is usually detected. Research into ovarian cancer has been limited by lack of suitable animal models that replicate the human disease. Our studies utilize the aging White Leghorn hen as an animal model for the study of ovarian cancer. The hen is the only the only animal model in which spontaneous OSE cancer is known to arise, an analog to the human disease. One of the goals of our research is to further validate the use of the chicken model for the study of ovarian cancer. The underlying hypothesis of our studies is that inflammation and oxidative stress are important to the etiology of ovarian epithelial cancer and that enzymes involved in the production of reactive oxygen intermediates and inflammatory mediators may be aberrantly expressed in ovarian cancer and produce mediators important to the malignant transformation of ovary. We are examining enzymes such as CYP1B1 which metabolizes estrogens to genotoxic intermediates, and Cyclooxygenases which produce inflammatory prostaglandins. The long term goal of these studies is to identify molecular targets for therapeutic intervention, and use the chicken model in preclinical studies to evaluate the efficacy of the intervention.

BODY: The research accomplishments associated with each task outlined in the approved Statement of Work

Task 1: To obtain, develop and validate probes and reagents for analysis of chicken ovarian tissues.

A. CYP1B1
   i. The chicken CYP1B1 cDNA clone was purchased from Dr. Joan Burnside at the Delaware Biotechnology Institute (DBI) http://www.dbi.udel.edu/burnside.html. Primers for real time PCR were designed and validated using RNA extracted from chicken ovarian cancer.

B. Develop probes for in situ hybridization analysis of CYP1B1 in chicken tissues.
   ii. cDNA or CYP1B1 was cloned into a vector with T7 and SP6 promoters for generation of DIG labled probes

C. Develop and validate additional probes and reagents for analysis of chicken ovarian tissues

D. Obtain and validate other reagents to assess surrogate end points for cancer diagnosis and histological identification in chicken tissues
   i. In order to study changes in gene expression in the hen, we have cloned approximately 20 different chicken genes, for use with quantitative real-time PCR.
   ii. We have established the conditions for immunohistochemistry and validated the use of anti-human antibodies for these genes, including CYP1B1, COX-1, COX-2
   iii. Surrogate cancer endpoints which have been validated for use in chicken include PCNA, E-cadherin, N-cadherin, a-smooth muscle actin, cytokeratin 8, and TUNEL assay.
   iv. Prostaglandin biosyntheisis and metabolism
      a) Chicken probes for Cyclooxygenase-1 and 2 (PTGS 1/2), prostaglandin E synthase 1 and 2 (PTGES 1/2), prostaglandin 15-keto dehydrogenase (15-PGDH), were developed. cDNAs were cloned by TA cloning from a chicken cDNA library and probes designed for qRT-PCR and validated. Results are shown under Task 4 below
   v. Obtain 2 year old chickens and establish colony for cancer time course study (800 white leghorn hens).
   vi. Hens were obtained and the colony with more than 800 white leghorn hens was established, at the experimental poultry facility UIUC, in Urbana, IL

E. Tools and approaches were devised and validated under Task 1 to enable the subsequent tasks to be accomplished.

Task 2: To initiate time-course study, sacrifice first cohorts of chickens, perform necropsy and collect specimens for analyses.

A. Collect ovarian tissue from normal (disease free) and diseased (ovarian cancer). Separate oviduct involved cancers from purely ovarian cancers and other reproductive tract pathologies
   iii. Three collections of ovarian tissue were made in the first year of the study. On necropsy gross pathology was determined and hens were grouped into disease-free (control), ovary only carcinoma, ovary carcinoma with metastases to oviduct and peritoneal surfaces and the presence

of abdominal ascites fluid was documented. The incidence of ovarian cancer in hens as reported in the literature [1] was corroborated by the observed incidence in this study.

i. At each time point 20 to 40 hens were sacrificed, so that the initial time points 102 hens were sacrificed, ranging in age from 31 to 40 months of age.

B. Fix tissue for histological analysis and snap freeze for biochemical studies
   a. Ovarian tissues were collected for histology, RNA quantitation and biochemical analyses
C. This task will provide tissue samples for analyses and provided an estimation of cancer incidence in model colony

Task 3: Perform histological and biochemical analysis on initial time course samples.

Two manuscripts resulted from histological and biochemical analyses of initial time course samples.

i. **Hales, D.B**, Zhuge, Y., Lagman, JA, Ansenberger, K, Mahon, C, Barua, A., Luborsky, J., Bahr, JM. "Cyclooxygenase Expression and Distribution in the Normal Ovary and Their Role in Ovarian Cancer in Gallus Domesticus" Endocrine DOI: 10.1007/s12020-008-9800 (in press 2008). See appendix

ii. Zhuge, Y., Ansenberger, K., Mahon, C., Lagman, JA. J., Bahr, J., **Hales, D.B**. "Cyp1B1 Expression in Ovarian Cancer in the Laying Hen Gallus Dometicus." Submitted to Gynecological Oncology (2008). See appendix.

Components of Task 3 accomplished as described in the above referenced manuscripts:

a) Embed and section fixed tissue samples.
b) Perform gross histological analysis and immunohistochemical staining for surrogate cancer markers, markers of oxidative damage, and steroid hormone receptors with antisera validated for chicken tissue under studies described in Task one
c) Extract RNA from snap frozen tissue for real-time PCR analysis of CYP1B1

.Components of Task 3 completed and reported herein.

d) Fractionate snap frozen tissue for analysis of lipid peroxidative damage and LCMS/ MS analysis for DNA damage.

Results:

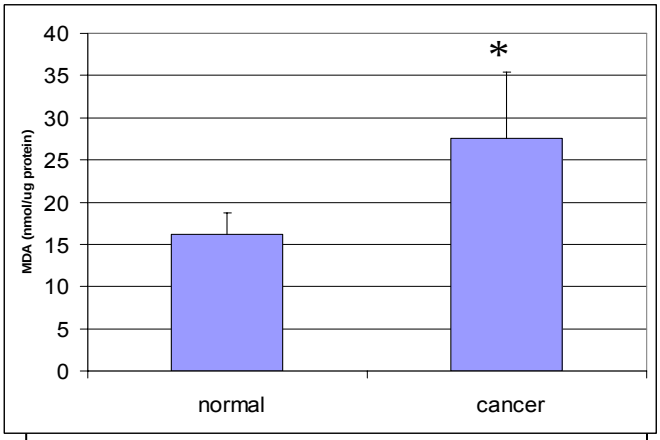


Figure 1) Analysis of MDA (lipid peroxidative damage) in normal vs. cancer samples. *, p<0.05.

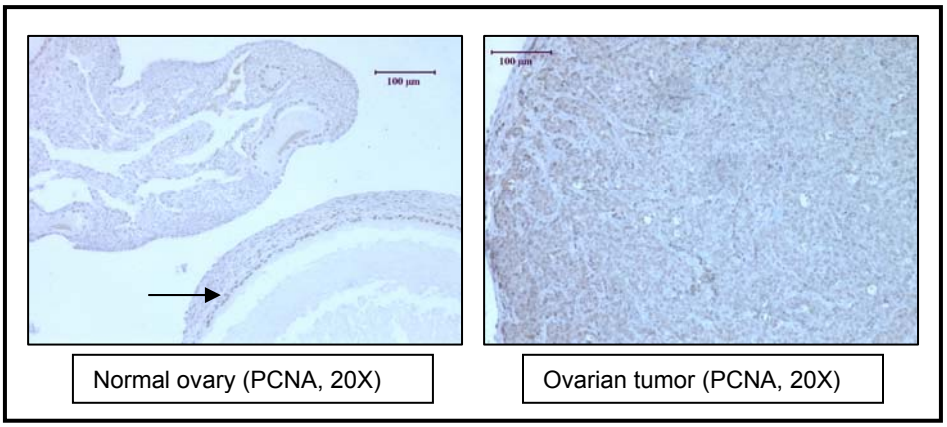
Analysis of lipid peroxidative damage was done with TBARS assay which measures malonaldehyde, a stable lipid peroxidation product. (Fig 1). Results indicate that cancer is associated with increased peroxidative damage suggesting that inflammation and oxidative stress may be important to the etiology of ovarian cancer, or may result from tissue responses to the cancer.

Analysis of DNA damage by LC MS/MS has not yet been completed.

<u>Validation of surrogate cancer endpoints and validation of chicken model</u>

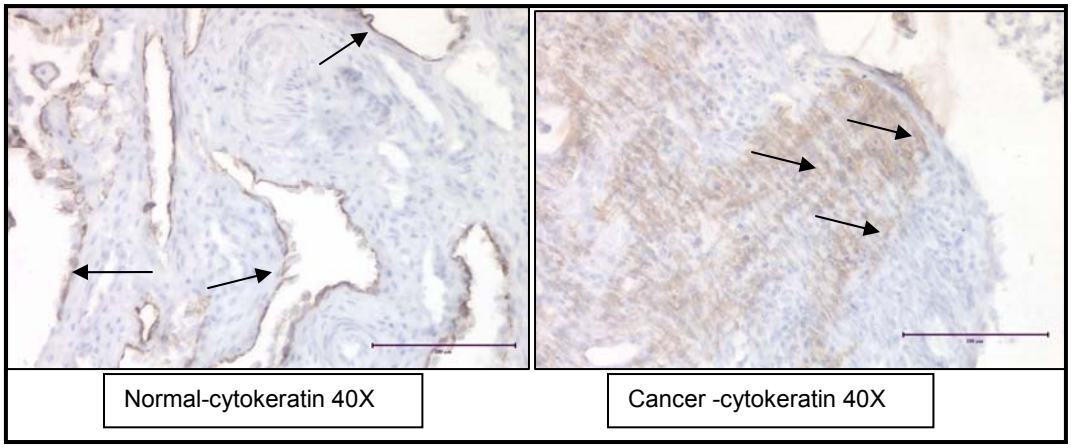
<u>Proliferating Cell Nuclear Antigen (PCNA)</u> immunohistochemistry (IHC) showing that in normal ovary only follicular granulosa cells are proliferating (arrow), in contrast to tumor in which most cells are positive for PCNA.

Cytokeratin IHC identifies cells of the epithelial lineage. In normal ovarian tissue cytokeratin is confined to the surface epithelium (arrows), whereas in ovarian cancer epithelial cells have involuted and present in a mixed pattern within the stroma of the tumor, indicative of epithelial-mesenchymal transition. Sections were subjected to antigen retrieval for cytokeratin (citrate buffer at 250 °C for 10 min), incubation with antibody, color developed with Vectastain peroxidase then counterstained with hematoxylin.



Normal ovary (PCNA, 20X)

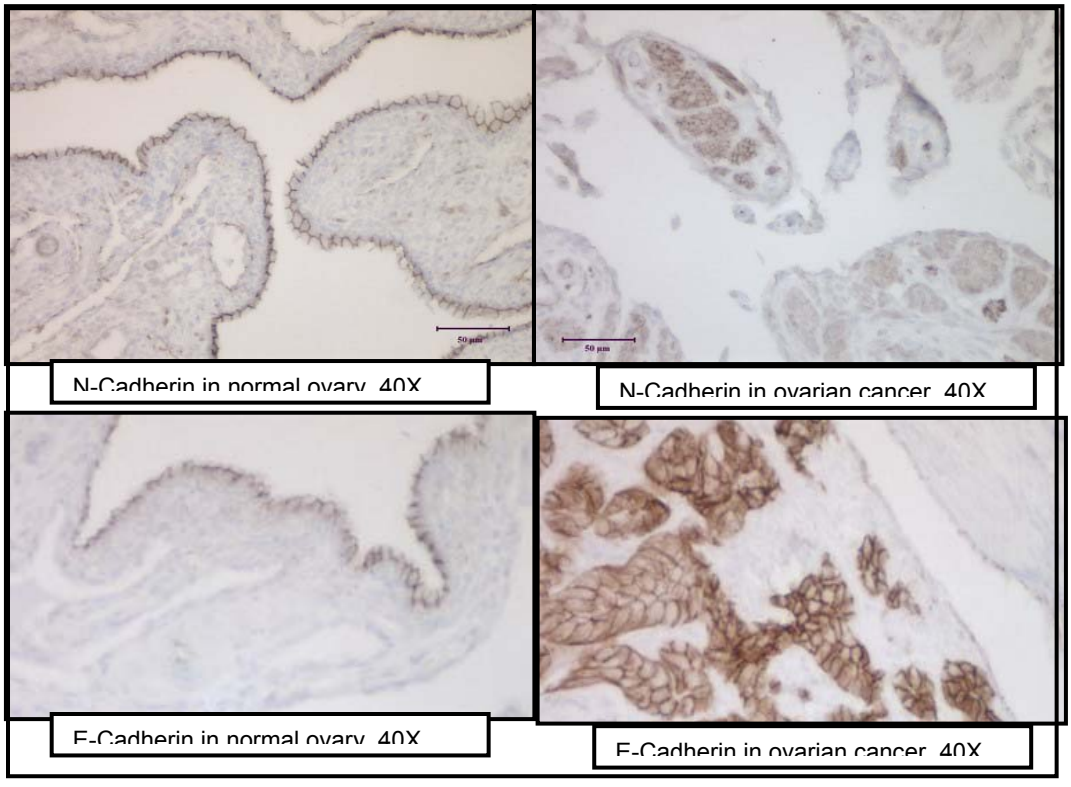
Ovarian tumor (PCNA, 20X)



Normal-cytokeratin 40X

Cancer -cytokeratin 40X

Cadherin: The ovarian surface epithelium (OSE) is a single layer of cells of squamous and cubodial cells that express both epithelial and mesenchymal characteristics (30). These cells are derived from the coelemic epithelia and are pleuripotent and less differentiated than other epithelium found elsewhere. After transformation, the epithelial cells migrate, and become more differentiated within the stroma of the tumor and more strong express epithelial cell markers such E and N Cadherin. As shown, both N and E cadherin expression is confined to the OSE, but in cancer, both N and E cadherin expression is localized to islands within the tumor stroma, with significant increase in E-cadherin immuno-reactivity. .



N-Cadherin in normal ovary, 40X

N-Cadherin in ovarian cancer, 40X

E-Cadherin in normal ovary, 40X

E-Cadherin in ovarian cancer, 40X

e) This task provides the first correlative data on CYP1B1 expression, oxidative and inflammatory damage vs. cancer end points and further validates the chicken model

Task 4: Continue time-course study, sacrifice subsequent cohorts of chickens, perform necropsy and collect specimens for analyses.

a) Sacrifice timed cohorts of chickens and perform necropsy. Collect ovarian tissue from normal (disease free) and diseased (ovarian cancer). Separate oviduct involved cancers from purely ovarian cancers and other reproductive tract pathologies. Collect tissue and fix and snap freeze.
   iv. One additional collection of ovarian tissues from normal and diseased hens was made in the second year of the study. On necropsy gross pathology was determined and hens were grouped into disease-free (control), ovary only carcinoma, ovary carcinoma with metastases to oviduct and peritoneal surfaces and the presence of abdominal ascites fluid was documented.
      i. An additional 25 hens, ranging in age from 28-40 months were sacrificed
b) Each six month time point will require sacrifice of approximately 100 to 200 chickens.
   a. Only 25 additional hens were required to complete the cancer time course in the 2nd year of study.
c) This task will completed the cancer time course and provided tissue samples for remainder of analyses

Task 5: Continue histological and biochemical analysis on all time course samples.

a. Perform analyses as described under Task 3
b. This task will complete histological and biochemical analyses of cancer time course samples.

Task 6: Obtain young chickens for developmental time course, sacrifice timed cohorts and perform histological and biochemical analyses.

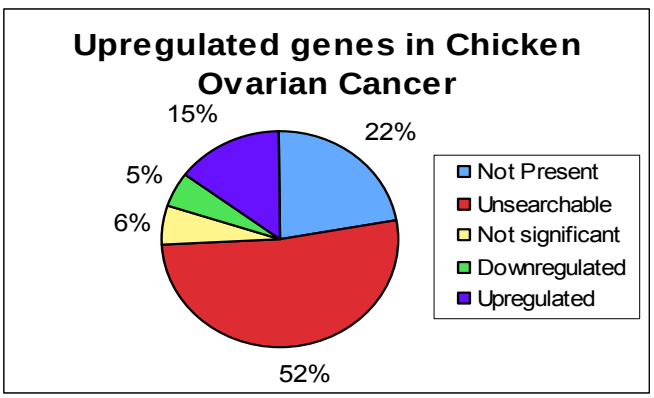
Not initiated.

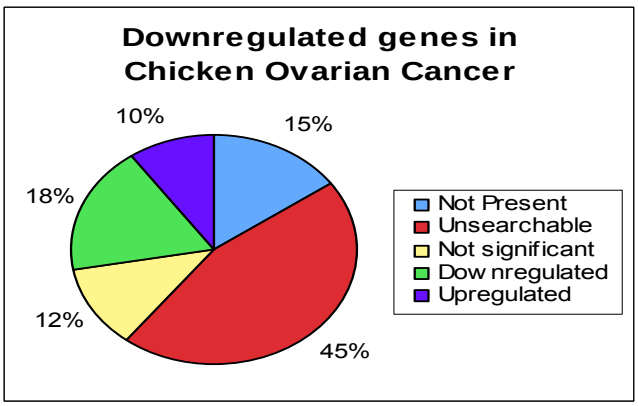
Task 7: Conduct DNA array analysis with RNA from tissue samples from aged timed cohort samples.

DNA microarray analysis was conducted using the 13K featured chicken array from the Fred Hutchinson Cancer Research Center: http://www.fhcrc.org/science/shared_resources/genomics/dna_array/spotted_arrays/chicken_array/).

To conduct DNA array analysis, 3-4 samples were pooled together and to assay 3 unique pools per condition (control vs. cancer). The RNA was prepared and sent to the Fred Hutchinson Cancer Center DNA array facility for analysis. The results determined that 318 genes were significantly upregulated, 165 genes were significantly down regulated. in chicken ovarian cancer. Genes present in the chicken arrays were entered into the Oncomine™ search engine and compared to human data. A P-value threshold of 0.01 was used to determine significance. Determination of significance was based on tumor grade and stage, but excluded histotype. Many of the genes that were significant in chicken ovarian cancer were not present in the Oncomine database. Some genes were unsearchable, i.e., when put into the search engine, there were no hits in the human database. Many genes were not actual genes, but cDNA clones and so could not be searched for in the database. Many genes were also present multiple times in the chicken array.

The majority of both significantly up-regulated and downregulated genes in the chicken were unsearchable or not present in the Oncomine database.

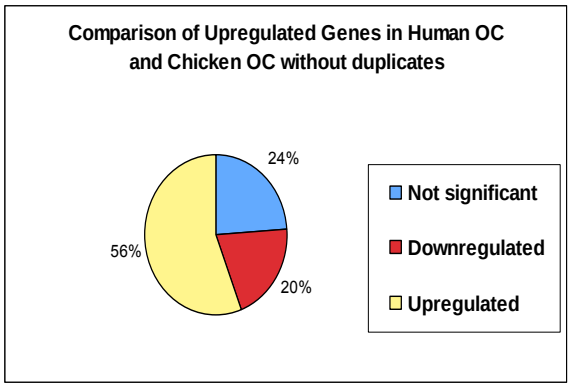


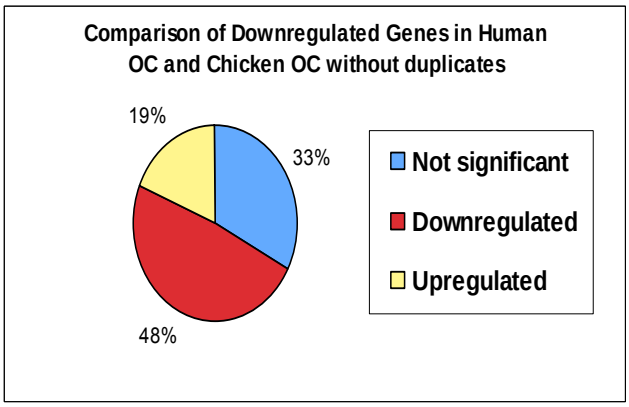


However, if you remove genes that were unsearchable or consisted of cDNA clones, there is a more realistic comparison of human to chicken ovarian cancer. A comparison of genes that were significantly upregulated in the chicken showed that 56% of these genes are also upregulated in human ovarian cancer. 48% of genes that were significantly downregulated in chicken ovarian cancer were also downregulated in human ovarian cancer

Several genes have been shown to be upregulated in both human and chicken ovarian cancer that are of great interest: MAP Kinase 8, TGF-b 3, MMP 15, Wnt-11, RAB1A, member of the Ras oncogene family. This analysis provides new evidence that the chicken is a good model for the human ovarian cancer and identifies new targets that may be important to the etiology of ovarian cancer. Candidate genes are being further evaluated.

a. Develop primers for candidate genes and validate results by real-time PCR.
b. Examine changes in expression of candidate genes revealed by array studies in cancer time course in RNA extracted from developmental time course.
c. This task with provide important new information about changes in gene expression during progression to ovarian cancer and may identify potential markers for early detection of ovarian cancer





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1. T.N. Fredrickson, Ovarian tumors of the hen. (1987). Environ Health Perspect 73, 35-51.

KEY RESEARCH ACCOMPLISHMENTS:

- Further validation of the chicken model of ovarian cancer for studies of the initiation and progression of the disease in humans.
- Demonstration that chicken ovarian tumors have similar histotypes and pathology as human ovarian tumors.
- Further validation of surrogate cancer endpoints for evaluation of the disease in chickens
- Establishment of conditions for immunohistochemical analyses of chicken ovarian tumors and validation of antibodies for these studies.
- Analysis of CYP1B1 mRNA and protein expression in the ovaries of young hens compared to old hens and age matched old hens with ovarian cancer, determined that CYP1B1 is highly expressed in the post-ovulatory follicle of the normal ovary. This finding suggests that CYP1B1 may be involved in production of genotoxic estrogen metabolites that may contribute to the initiation of ovarian cancer.
- COX-1 is significantly up-regulated in ovarian cancer and is a candidate molecular target for intervention.
- E-cadherin expression is significantly up-regulated in ovarian cancer similar to the human disease. The pattern of expression suggests that increases in E-cadherin may be an important early event in ovarian cancer.

REPORTABLE OUTCOMES

a) Published manuscripts (included in appendix)

1) Stammer, K., Edassery, S.L., Barua, A., Bitterman, P., Bahr, J.M., **Hales, D.B.,** Luborsky, J. "Selenium-Binding Protein 1 expression in ovaries and ovarian tumors of the laying hen, a spontaneous model of human ovarian cancer." Gynecologic Oncology (in press 2008).

2) Barua, A., Edassary, S.L., Bitterman, P., Abramowicz, J.S., Dirks, A., Bahr, J.M**., Hales, D.B.,** Bradaric, M.J., Luborsky, J.L. "Prevalence of anti-tumor antibodies in laying hen model of human ovarian cancer" Int J Gyn Cancer (in press 2008)

3) **Hales, D.B**, Zhuge, Y., Lagman, JA, Ansenberger, K, Mahon, C, Barua, A., Luborsky, J., Bahr, JM. "Cyclooxygenase Expression and Distribution in the Normal Ovary and Their Role in Ovarian Cancer in Gallus Domesticus" Endocrine DOI: 10.1007/s12020-008-9800 (in press 2008).

b) Abstracts published and presented (included in appendix)

1) Y Zhuge, C. Mahon, A. Barua, J. Luborsky, J. Bahr, **D.B. Hales** "Molecular Analysis Of Spontaneous Ovarian Cancer In The Laying Hen *Gallus Domesticus"* Presentated at the annual meeting of the Society for the Study of Reproduction, Omaha, NE 2006
2) Barua A, Bradaric MJ, Edassary SL, Sharma S, Rotmensch J, Bitterman P, **Hales DB**, Bahr JM, Luborsky JL 2006 "Anti-tumor antibodies and ovarian cancer in Women and Hens". First AACR International Conference on Molecular Diagnostics in Cancer Therapeutic Development, Sep 12-15, 2006, Chicago, IL, 2006, # A40
3) Luborsky, JL, Barua, A, Bitterman, P, Rotmensch, J, **Hales, DB** and Bahr, J "Anti-tumor and anti-ovarian antibodies associated with ovarian cancer in humans and hens" Presented at the 1st International Aegean conference on Ovarian Cancer, June 2006,Crete, Greece
4) Stammer;K., Edessary, S; Barua, A., Bahr, J**., Hales, DB**, Luborsky, J "Selenium Binding Protein 1 expression is altered in ovarian cancer in laying hens similar to women" Submitted for presentation American Society of Clinical Oncology, June 2007, Chicago, IL
5) Zhuge, Y., Lagman, JA, Mahon, C., Ansenberger, K., Bahr, J.M., **Hales, D.B** "Molecular characterization of ovarian cancer in the laying hen, Gallus domesticus "Presented at the Keystone Symposium "Mechanisms Linking Inflammation and Cancer" Santa Fe, NM, 2007
6) K. Ansenberger, Y Zhuge, JA Lagman, C Mahon, J Bahr, **DB Hales** "Epithelial lineage of ovarian carcinoma of the Laying Hen Gallus Domesticus" presented at the Annual Meeting of the Society for the Study of Reproduction, San Antonio, TX, 2007.
7) Ansenberger, K., Zhuge, Y., Mahon, C., Barua, A., Luborsky, J., Bahr, J. **Hales, D.B. "**Flaxseed/Omega-3 Fatty Acid Dietary Intervention in Ovarian Cancer in the Laying Hen, *Gallus Domesticus"* Submitted for presentation at the Annual Meeting of the Society for the Study of Reproduction, Kona, Hawaii, 2008.
8) Zhuge, Y. Ansenberger, K., Lagman, JA, Mahon, C., Bahr, J, **Hales, DB**. "Cyp1B1 Expression In Ovarian Cancer in the Laying Hen, *Gallus Domesticus"* Submitted for presentation at the Endocrine Society Annual Meeting, San Fransico, CA 2008.
9) **Hales, DB,** Zhuge, Y. Ansenberger, K., Lagman, JA, Mahon, C., Bahr, J, "Increased E-Cadherin Expression is a Hallmark of Epithelial Ovarian Cancer in the Laying Hen *Gallus Domesticus"* Submitted for presentation at the Endocrine Society Annual Meeting, San Francisco, CA 2008

c) Manuscripts submitted or in preparation

Submitted (included in appendix):
Zhuge, Y., Ansenberger, K., Mahon, C., Lagman, JA. J., Bahr, J., **Hales, D.B**. "Cyp1B1 Expression in Ovarian Cancer in the Laying Hen Gallus Dometicus." Submitted to Gynecological Oncology (2008)

Barua, A., Bitterman, P., **Hales, D.B**., Abramowicz, J.S., Bradaric, M.J., Edassary, S.L., Dirks, A., Bahr, J.M., Luborsky, J.L "Histopathology of malignant Ovarian Tumors in Laying Hens, a Preclinical Model of Human Ovarian Cancer' Submitted to *International Journal of Gynecological Cancer*:

In preparation

Ansenber, K., Zhuge, Y., Mahon, C., Lagman, JA., Bahr, JM, Hales, D.B. "Increased E-cadherin expression is an early defining event in ovarian epithelial cancer in the Laying Hen Gallus Dometicus ." Manuscript in preparation

Ansenber, K., Zhuge, Y., Mahon, C., Hales, D.B. "Characterization of a newly established chicken ovarian cancer cell line: COCA65." Manuscript in preparation

c) Research Opportunities applied for based on data from this study

Agency: NIH/NCCAM PA06-315 1 R21 AT004085-01A1
Title: Therapeutic Efficacy of Flax Seed in the Prevention of Ovarian Cancer
PI: Dale B Hales
Status: funded, 9/30/2007-10/1/2009

Agency: NIH/NCI PAR 06-313 Cancer prevention research small grants program (RO-3)
Title: Dietary Intervention in the Gallus Domesticus Model of Ovarian Cancer
PI: Dale B Hales
Status: funded; 5/2/2008-5/1/2010

Agency: Department of Defense Ovarian Cancer Department of Defense (DOD) Ovarian Cancer Research Program (OCRP) OC080092 Funding Opportunity Number W81XWH-08-OCRP-IDA Idea Development Award (pre-application)
Title: Mechanism and significance of increased E-cadherin expression, an early defining event in ovarian epithelial cancer
PI: Dale B Hales
Status: pending

CONCLUSION:

Previous studies suggested that the laying hen may serve as a suitable model for ovarian cancer research. Our results have further validated the use of the hen as a model system by demonstrating that several surrogate cancer endpoints important to the human disease are valid as markers in the chicken. We have determined that increased expression of E cadherin, Cyclooxygenase 1 (COX-1), and CYP1B1 are early defining events in ovarian epithelial cancer. These data help to identify molecular targets for therapeutic intervention. In particular, the demonstration that COX-1 is highly up-regulated in ovarian cancer indicates that anti-prostaglandin oriented therapy maybe effect in preventing or suppressing ovarian cancer. The demonstration that CYP1B1 is up-regulated in the post-ovulatory follicle may provide insight into the molecular events that mediate the initiation of the disease. Unlike other animal models of ovarian cancer, the hen gets ovarian cancer spontaneously and our data demonstrates that it is epithelial in nature, and analogous to the human disease, E-cadherin is significantly increased in the primary tumor. The chicken model which faithfully reproduces the human disease, provides the opportunity for conducting large scale pre-clinical studies of targeted therapeutics at relatively low cost.

REFERENCES:

1. Fredrickson TN. Ovarian tumors of the hen. Environ Health Perspect 1987; 73: 35-51.

APPENDICIES:

Abstracts published and presented**:**

Page 10

1) Y Zhuge, C. Mahon, A. Barua, J. Luborsky, J. Bahr, **D.B. Hales** "Molecular Analysis Of Spontaneous Ovarian Cancer In The Laying Hen *Gallus Domesticus"* Presentated at the annual meeting of the Society for the Study of Reproduction, Omaha, NE 2006

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2) Barua A, Bradaric MJ, Edassary SL, Sharma S, Rotmensch J, Bitterman P, **Hales DB**, Bahr JM, Luborsky JL 2006 "Anti-tumor antibodies and ovarian cancer in Women and Hens". First AACR International Conference on Molecular Diagnostics in Cancer Therapeutic Development, Sep 12-15, 2006, Chicago, IL, 2006, # A40

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3) Luborsky, JL, Barua, A, Bitterman, P, Rotmensch, J, **Hales, DB** and Bahr, J "Anti-tumor and anti-ovarian antibodies associated with ovarian cancer in humans and hens" Presented at the 1st International Aegean conference on Ovarian Cancer, June 2006,Crete, Greece.

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4) Stammer;K., Edessary, S; Barua, A., Bahr, J**., Hales, DB**, Luborsky, J "Selenium Binding Protein 1 expression is altered in ovarian cancer in laying hens similar to women" Submitted for presentation American Society of Clinical Oncology, June 2007, Chicago, IL

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5) Zhuge, Y., Lagman, JA, Mahon, C., Ansenberger, K., Bahr, J.M., **Hales, D.B** "Molecular characterization of ovarian cancer in the laying hen, Gallus domesticus "Presented at the Keystone Symposium "Mechanisms Linking Inflammation and Cancer" Santa Fe, NM, 2007

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6) K. Ansenberger, Y Zhuge, JA Lagman, C Mahon, J Bahr, **DB Hales** "Epithelial lineage of ovarian carcinoma of the Laying Hen Gallus Domesticus" presented at the Annual Meeting of the Society for the Study of Reproduction, San Antonio, TX, 2007

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7) Ansenberger, K., Zhuge, Y., Mahon, C., Barua, A., Luborsky, J., Bahr, J. **Hales, D.B. "**Flaxseed/Omega-3 Fatty Acid Dietary Intervention in Ovarian Cancer in the Laying Hen, *Gallus Domesticus"* Submitted for presentation at the Annual Meeting of the Society for the Study of Reproduction, Kona, Hawaii, 2008

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8) Zhuge, Y. Ansenberger, K., Lagman, JA, Mahon, C., Bahr, J, **Hales, DB**. "Cyp1B1 Expression In Ovarian Cancer in the Laying Hen, *Gallus Domesticus"* Submitted for presentation at the Endocrine Society Annual Meeting, San Fransico, CA 2008

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9) **Hales, DB,** Zhuge, Y. Ansenberger, K., Lagman, JA, Mahon, C., Bahr, J, "Increased E-Cadherin Expression is a Hallmark of Epithelial Ovarian Cancer in the Laying Hen *Gallus Domesticus"* Submitted for presentation at the Endocrine Society Annual Meeting, San Francisco, CA 2008

Manuscripts published, submitted or in press

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Zhuge, Y., Ansenberger, K., Mahon, C., Lagman, JA. J., Bahr, J., **Hales, D.B**. "Cyp1B1 Expression in Ovarian Cancer in the Laying Hen Gallus Dometicus." Submitted to Gynecological Oncology (2008)

Pages 37-71

Barua, A., Bitterman, P., **Hales, D.B**., Abramowicz, J.S., Bradaric, M.J., Edassary, S.L., Dirks, A., Bahr, J.M., Luborsky, J.L "Histopathology of malignant Ovarian Tumors in Laying Hens, a Preclinical Model of Human Ovarian Cancer' Submitted to *International Journal of Gynecological Cancer*: (2008)

Pages 72-80

Stammer, K., Edassery, S.L., Barua, A., Bitterman, P., Bahr, J.M., **Hales, D.B.,** Luborsky, J. "Selenium-Binding Protein 1 expression in ovaries and ovarian tumors of the laying hen, a spontaneous model of human ovarian cancer." Gynecologic Oncology (in press 2008).

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Barua, A., Edassary, S.L., Bitterman, P., Abramowicz, J.S., Dirks, A., Bahr, J.M**., Hales, D.B.,** Bradaric, M.J., Luborsky, J.L. "Prevalence of anti-tumor antibodies in laying hen model of human ovarian cancer" Int J Gyn Cancer (in press 2008)

Pages 112-122

Hales, D.B, Zhuge, Y., Lagman, JA, Ansenberger, K, Mahon, C, Barua, A., Luborsky, J., Bahr, JM. "Cyclooxygenase Expression and Distribution in the Normal Ovary and Their Role in Ovarian Cancer in Gallus Domesticus" Endocrine DOI: 10.1007/s12020-008-9800 (in press 2008).

MOLECULAR ANALYSIS OF SPONTANEOUS OVARIAN CANCER IN THE LAYING HEN *GALLUS DOMESTICUS*

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Ovarian carcinoma is the most lethal gynecological malignancy and the leading cause of cancer death in women. There is a lack of experimental animal models for the study of this cancer, *with the exception of the aging hen.* The purpose of this study was to characterize the molecular and histopathological phenotype of ovarian carcinoma in the aging White Leghorn hen *(Gallus domesticus)* in order to identify factors important to the etiology and early detection of the disease. Age vs. disease-dependent expression of cytochrome P4501B1 (CYP1B1), cyclooxygenase (COX)-1, markers of inflammation, oxidative stress and proliferation were examined in disease-free hens aged 9months to 2.5 years, and in hens with cancer. Upon necropsy and gross examination, the presence of metastatic carcinoma, or non-metastatic tumors in the oviduct and ovary, or ovary only was determined. Tissue was either fixed in neutral buffered formalin, dehydrated, paraffin embedded, and sectioned at 5 um; preserved in *RNAlater*, or flash frozen in liquid nitrogen. RNA was isolated by *Trizol*, treated with RQ1 DNase, and analyzed by end-point RT-PCR, and real-time PCR. Oligonucleotide probes were designed by interrogating the *Gallus gallus* genome. Real-time PCR was performed using the ABI 7900HT. Tumors presented with endometrioid, clear cell, poorly differentiated and mixed histology in H&E stained sections. Immunohistochemistry for demonstrated that only the granulosa cell layer surrounding the follicle was strongly positive for proliferative cell nuclear antigen (PCNA)b Immunohistochemical staining in the normal ovary, whereas the majority of nuclei in ovarian tumors were strongly positive, consistent with the proliferative phenotype. COX-1 reactivity paralleled PCNA expression. Analysis of CYP1B1 mRNA levels demonstrated that there was high constitutive expression which increased in an age-dependent manner. Oxidative stress markers were elevated in tumor tissue. These results further support the use of the hen as a model of human ovarian cancer. [supported by CDMRP OC050091]

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Anti-tumor antibodies and ovarian cancer in Women and Hens

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Abstract A40

Background: The high mortality rate of OVCA reflects a lack of early detection tests. Anti-tumor antibodies (aTAB) are a potential diagnostic for early detection of OVCA. Since there is no method to identify patients with early stage disease, it is difficult to validate aTAB as an early marker of OVCA. Laying hens are the only animal that spontaneously develops OVCA. We evaluated the association of anti-tumor antibodies with OVCA in humans and hens, in order to determine the feasibility of (a) aTAB as a diagnostic and (b) pre-clinical validation studies in hens.Methods: Sera were obtained from patients (ages 35-85, n=80) undergoing examination for gynecologic tumors and control women without ovarian pathology (ages 25-60, n=24). Normal ovarian and tumor tissues were obtained at surgery. Serum aTAB were detected by immunoassay described previously (Luborsky et al, 1990; Barua et al 2001). Two dimensional Western blots (2D-WB) were performed to identify potential antigens by standard methods (Barua et al, 2006). The aTAB were assessed similarly in sera from laying hens (2.5 years old, 16 hens with ovarian cancer, 18 normal control hens, 6 young hens for immunoassay control). Sections of hen ovary and tumors were stained with hematoxylin & eosin to evaluate tumor histology.Results: Significantly more patients with malignant ovarian cancer had aTABs than patients with malignant endometrial cancer (78% and 44%, respectively) or patients with benign ovarian tumors (29%) and controls (25%) ($p \leq 0.001$). The molecular weight and pI of immunoreactive spots in 2D-WB had major similarities and minor differences among patients. Significantly more hens with ovarian cancer had aTABs than control hens (65% and 22%, respectively) ($p \leq 0.001$). Serous, endometrioid, clear cell and poorly differentiated tumors were observed in hens and the histological appearances of these tumors were microscopically similar to humans. The primary reaction in 2D-WB was a common group at 50kD (pI 6-8) against proteins from both normal ovary and tumors suggesting that antigens are similar in humans and hens. Conclusion: The results suggest that anti-tumor antibodies are more prevalent in women and hens with malignant OVCA. Further study of the time course of anti-tumor antibody appearance in the hen will facilitate the validation of anti-tumor antibodies as a diagnostic marker for OVCA. Support: NIH R01-AI055060 (to JLL), CDMRP OC050091 (to DBH) and Rice Foundation.

Anti-tumor and anti-ovarian antibodies associated with ovarian cancer in humans and hens

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We established that women with unexplained infertility or premature ovarian failure, and hens with reduced ovarian function (egg production,) have ovarian autoimmune disease associated with ovarian autoantibodies (Luborsky, 2002; Barua, 2001). Based on epidemiologic risk factors in humans and observation that hens with ovarian antibodies spontaneously develop ovarian cancer (OVCA), we hypothesized that ovarian autoimmunity may be a risk factor for OVCA. The objective of this study was to evaluate autoantibodies as a potential biomarker for early detection of OVCA. We evaluated anti-ovarian and anti-tumor antibodies in women and hens with OVCA by immunoassay and two-dimensional Western blot. Sera and tumors were obtained from patients (35-85 years old, n=80) with malignant OVCA, borderline or benign ovarian tumors or endometrial cancer. Normal ovaries and sera of women (age = 25-60 years, n=24) without ovarian pathology were used as controls. Sera, normal ovary and ovarian tumors were obtained from White Leghorn laying hens (n=20). Four normal hens with full follicular hierarchy were used as negative controls. Anti-ovarian (HOVab) and anti-tumor (TOVab) antibodies were more frequent in OVCA patients (HOVab 74%; TOVab, 78%, n=27) than in patients with benign (HOVab 24%; TOVab, 29%, n=38) or borderline ovarian tumors (HOVab 33%; TOVab, 0%, n=3), endometrial cancer (HOVab 37%; TOVab, 44%, n=9) and controls (HOVab 29%; TOVab, 25%, n=24) (p=0.0009 (HOVab); p=0.0002 (TOVab)). Similarly, anti-ovarian and anti-tumor antibodies were detected in hens with OVCA (65%). Western blot patterns obtained with human or hen sera had significant commonalties. The primary reaction was a prominent and common group at 50kD (pI 6-8) against proteins from both normal ovary and tumors. These unique observations support the concept of a link between ovarian autoimmunity and ovarian cancer and suggest that ovarian antibodies may be a very early predictor of ovarian cancer. The results also support use of the hen as an animal model for further studies of immunologic reactions associated with tumor development. Support (JL): NIH/ NIAID R01 grant, Rice Foundation. (DBH) CDMRP-OC050091

Selenium Binding Protein 1 expression is altered in ovarian cancer in laying hens similar to women

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BACKGROUND: Ovarian cancer mortality represents 2.5% of cancer deaths and is the most lethal gynecological cancer in the United States. Selenium binding protein 1 (SBP1) is down-regulated in ovarian, lung and gastrointestinal tumors and its down-regulation is associated with poor prognosis. There is relatively little information on its regulation except that steroid hormones and selenium regulate SBP1. Dietary selenium has anti-cancer and immune-modulatory effects. However, a primary obstacle to study of early events associated with down-regulation of SBP1 is the difficulty of obtaining early stage ovarian cancer serum or tissue. An alternative to human studies is to examine SBP1 expression and regulation in an animal model. Ovarian cancer occurs spontaneously in the laying hen and has biochemical and histological features that are strikingly similar to human ovarian cancer. Therefore, our objective was to determine if SBP1 is present in normal ovary and if its expression is altered in ovarian cancer in the laying hen.

METHODS: The expression of SBP1 was evaluated in normal ovary (n=15) and ovarian tumors (n=20) from laying hens (2-3 years old) by RT-PCR for mRNA and Western Blot for protein by standard procedures. Formalin fixed tissue was processed for immunohistochemistry and sections stained with a commercial anti-human SBP1 antibody to determine the localization of SBP1.

RESULTS: SBP1 mRNA and protein was expressed in 100% of normal ovary and ovarian tumors in the hen. SBP1 expression was decreased in some (n=5) but not all tumors similar to observations in human ovarian tumors. SBP1 was localized in the surface epithelial cells of normal ovary, but was found in the stroma of tumors.

CONCLUSIONS: Our study demonstrates for the first time that SBP1 is expressed in normal ovary and ovarian tumors in the hen. SBP1 localization appears to shift from normal surface epithelium to tumor stroma. Thus, it will be possible to examine the regulation of SBP1 in ovarian tumor development using the laying hen to determine the role of SBP1 in tumor development, and the possible anti-cancer effect of selenium.

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Molecular characterization of ovarian cancer in the laying hen, *gallus domesticus*

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Ovarian cancer is the most lethal gynecologic malignancy due to the late stage detection. With the exception of the laying hen there are no other animal models of spontaneous ovarian surface epithelial (OSE) cancer that replicate the human disease. OSE cancer is believed to be due to incessant ovulation where mutations and subsequent neoplasia arise during constant remodeling of the OSE in the inflammatory milieu that accompanies ovulation. Ovarian cancer in hens and humans share many features including tumor histotype, severe ascites and peritoneal metastases. Similar to the human, cyclooxyegenase (COX)- 1, not COX-2 is aberrantly upregulated in OSE cancer. Cytokeratin, E and N cadherin are confined to OSE of normal ovary, but tumors show expression these epithelial lineage markers suggesting that the inflammation that accompanies ovulation may promote epithelial-mesenchymal transition (EMT) and subsequent mesenchymal-epithelial transition (MET). Analysis of specific transcription factors associated with EMT/MET provides insight into the etiology of OSE cancer and may identify potential molecular targets for dietary and/or therapeutic intervention.

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"Epithelial lineage of ovarian carcinoma of the Laying Hen Gallus Domesticus"

Kristine Ansenberger, Yan Zhuge, Jo Ann Joecn Lagman, Cassie Mahon, Janice Bahr, Dale Buck Hales

More than 24,000 women in the United States are diagnosed with ovarian cancer each year and half of these women die from their disease. Ninety percent of these cancers are epithelial in origin, arising mainly from the ovarian surface epithelium (OSE). This is believed to be a function of incessant ovulation, resulting in mutations and subsequent neoplasia due to the constant remodeling in an inflammatory milieu. With the exception of the laying hen, there are no other animal models of spontaneous OSE cancer that replicate the human disease. Ovarian cancer in hens and humans share many features including tumor histotype, severe ascites and peritoneal metastases. The objective of this study was to determine genes and factors important in the development of OSE cancer and determine the mechanism through which OSE cells acquire a metastatic and invasive phenotype. Tissue samples were collected from 3 and 4 year old hens and subjected to immunohistochemical staining and quantitative gene analysis. Ascitic cancer cells were also collected and used for gene analysis. Our findings indicate that in normal ovary tissue, cytokeratin, E-cadherin, and N-cadherin are confined to OSE, but tumors show mixed expression of these epithelial lineage markers. In solid tumors, there is significant up-regulation of E-cadherin expression in the ovarian stroma, consistent with the MET process resulting in re-epithelialization. This suggests that the inflammation that accompanies ovulation may promote epithelial-mesenchymal transition (EMT) and subsequent mesenchymal-epithelial transition (MET). In the OSE, both E-caherin and N-cadherin are expressed, indicating that these cells are more pluripotent than strictly committed to the epithelial lineage. The observation that cTwist is significantly upregulated in cancer, in parallel to increased E-cadherin, is in marked contrast to what has been observed in other epithelial carcinomas, and suggests that secondary events are mediating the dominant increase in E-cadherin. In contrast, cSlug expression is not significantly changed in cancer, while N-cadherin is most highly expressed in the OSE. The establishment of the ascitic cell line, COCA 65, will provide a homologous cell model to determine the mechanism through which OSE cells acquire a metastatic and invasive phenotype. Similar to OSE, N-cadherin is highly expressed in the COCA 65, whereas there is no detectable E-cadherin. However, these cells have cTwist expression similar to the cancer phenotype. Analysis of surrogate cancer markers, such as ER alpha/beta, PR, and COX2, in COCA 65 compared to human ovarian cancer cells will demonstrate that these cells have a similar molecular phenotype and validates these cells as a model for ovarian cancer. Analysis of specific transcription factors associated with EMT/MET provides insight into the etiology of OSE cancer and may identify potential molecular targets for dietary and/or therapeutic intervention.
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Flaxseed/Omega-3 Fatty Acid Dietary Intervention in Ovarian Cancer in the Laying Hen, *Gallus Domesticus*

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With the exception of the laying hen, there are no other animal models of spontaneous ovarian surface epithelial (OSE) cancer that replicate the human disease. Diet has been shown to play an important role in colon cancer, and may also affect the progression and severity of ovarian cancer. Diets with a higher ratio of omega-3 to omega-6 fatty acids are considered to be protective against cancer. Omega-3 fatty acids have been implicated as chemopreventative of breast cancer and may also be important in the suppression of ovarian cancer. One mechanism through which omega-3 fatty acids are believed to act is through substrate level modulation of cyclooxygenase (COX) enzyme activity. COX enzymes catalyze the rate limiting step in prostaglandin synthesis, which are implicated in many carcinomas. Omega-6 metabolites are catalyzed by COX enzymes to form inflammatory prostaglandins, whereas omega-3 metabolites are less inflammatory. Flaxseed is the most abundant plant source of omega-3 fatty acids. The objectives of this study were to determine if there was chemoprevention or chemosuppression of ovarian cancer in hens fed a diet enriched with omega-3 fatty acids compared to hens fed a control diet and to determine the effects of omega-3 fatty acids on the COX enzymes. 200 2.5 year-old hens were fed a 10% flaxseed enriched or standard diet for one year. Tissue samples were subjected to immunohistochemical staining and RNA was extracted and analyzed by real time PCR. Eggs yolks were assayed for total omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), by gas chromatography. Our findings show that hens on the flaxseed diet had a significantly decreased mortality rate compared to hens fed the control diet. During the course of the study, 37.5% of the control hens died, whereas only 21.5% of the flax-fed hens died. There was an initial reduction body weight on the flax diet, but as the hens aged, control hens had a precipitous loss of weight due to failing health. The overall cancer rate decreased in the flax hens (32% control vs. 24% flax), with a nominal decrease in ovarian cancer (24% control vs. 22% flax). However, there was a decrease in the severity and progression of ovarian cancer in the flax-fed hens; 61% of the control hens with cancer presented with stage 3 or 4 cancer, whereas only 43% of the flax hens developed late stage ovarian cancer. These data suggest that omega-3 fatty acids may act as a chemosuppressant of ovarian cancer. There was a significant increase in omega-3 metabolites, DHA and EPA, in flax egg yolks compared to control egg yolks. The flax group had significant decreases in COX-1 and COX-2 mRNA in total ovarian homogenate and OSE compared to control. Immunohistochemical staining for COX-1 and COX-2 also revealed a reduction of COX enzymes in the flax hens. COX enzymes are necessary for the production of prostaglandins that are important in egg shell formation in the hen uterus/shell gland. Even though COX mRNA and protein were decreased in the flaxseed fed hens, there was no effect on egg laying frequency in these hens when compared to control. These findings may provide the basis for a clinical trial that evaluates the efficacy of flaxseed as a chemosuppressant of ovarian cancer in women.

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CYP1B1 EXPRESSION IN OVARIAN CANCER IN THE LAYING HEN *GALLUS DOMESTICUS*

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Abstract

Ovarian carcinoma is the most lethal gynecological malignancy and the leading cause of cancer death in women. There is a lack of experimental animal models for the study of ovarian cancer, with the exception of the aging hen. CYP1B1 is a gene which can activate environmental procarcinogens and catalyze the conversion of estrogens to genotoxic catechol estrogens. CYP1B1 may cause cell mutations and/or contribute to oxidative stress which could contribute to ovarian epithelial cancer. The objective of this study was to evaluate CYP1B1 expression, distribution and localization in normal and cancerous hen ovaries. We compared CYP1B1 mRNA and protein expression from the ovaries of young, cancerous and age-matched normal hens by qRT-PCR, *In Situ* Hybridization (ISH) and Immunohistochemistry (IHC). RNA was extracted from *RNAlater* preserved tissue and analyzed later by qRT- PCR; fresh tissue frozen in liquid nitrogen was used for *In Situ* Hybridization, tissue fixed in neutral buffered formalin was subjected to Immunohistochemistry. High expression of CYP1B1 mRNA was seen in the cancerous ovaries as compared with age-matched normal and young by qRT- PCR (7.63 +/- 1.36, n=17; 3.46 +/- 0.76, n=10; 2.95 +/- 0.23, n=5, copy number CYP1B1 mRNA/ GAPDH +/- SEM for cancer, age-matched normal and young respectively). ISH also revealed that the expression of CYP1B1 was much higher in tumor samples compared with age-matched normal ovary. Extensive CYP1B1 mRNA was distributed throughout the whole carcinoma tissue. IHC revealed that CYP1B1 protein is mostly localized to the granulosa cell layer surrounding the follicle in the young and age-matched normal hen ovaries. There was extensive CYP1B1 protein expression throughout the ovarian carcinoma of the hens. IHC also revealed nuclear localization of CYP1B1. Our data further confirm that the laying hen is a good model for human ovarian cancer and that the unique pattern of CYP1B1 expression in the hen ovary may provide possible insights into the etiology of ovarian cancer.

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Increased E-Cadherin Expression Is a Hallmark of Epithelial Ovarian Cancer in the Laying Hen *Gallus Domesticus*

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Ovarian cancer is the most lethal gynecological malignancy due to its late stage of detection. 90% of ovarian carcinomas arise from the ovarian surface epithelium (OSE), a highly specialized mesothelium which expresses both E- and N-type cadherins. Research into ovarian cancer has been hampered by the lack of suitable animal models. Only the laying hen is afflicted with spontaneous epithelial ovarian cancer which recapitulates the human disease. The objective of this study was to analyze E and N-cadherins and associated regulatory factors in normal and cancerous ovaries from the laying hen. Normal and cancerous ovarian tissues and peritoneal metastases were collected on necropsy from 2.5 yr old white leghorn hens. Tissue was fixed in formalin and processed for histology and immunohistochemistry (IHC) or preserved in *RNAlater* for RNA extraction and qRT-PCR analysis. IHC demonstrated that in the normal ovary E-cadherin was confined to the OSE and granulosa cells, but in the carcinoma, its expression was markedly increased and distributed throughout the tumor. Pre-neoplastic lesions in adjacent normal regions of the ovary displayed evidence of metaplastic OSE with markedly increased E-cadherin staining. In the metastases, the pattern of E-cadherin expression was identical to the ovarian tumor. N-cadherin was seen only in the OSE of the normal ovary. In cancerous ovaries, N-cadherin was widely but diffusely expressed in the tumor. Quantitation of E and N-cadherin mRNA by qRT-PCR demonstrated that there was a significant increase in E-cadherin in the carcinoma compared to normal ovary or OSE, while N-cadherin expression was not significantly different (1.46+/- 0.17, n=8 ; 4.27 +/- 1.47, n=14; 141.47 +/- 50.56, n=15, E-cadherin; 316.38+/- 31.70, n=8 ; 178.31 +/- 40.65, n=9; 146.21 +/- 20.10, n=16, N-cadherin--copy number mRNA/ GAPDH +/- SEM for OSE, normal and cancer respectively). The transcription factors *twist, snail* and *slug* were quantitated by qRT-PCR and found to be expressed in a reciprocal pattern relative to E-cadherin but not N-cadherin (9.89+/- 1.04, n=8 ; 5.32 +/- 1.08, n=14; 4.46 +/- 0.86, n=15, *twist*; 15.52+/- 2.17, n=8 ; 23.31 +/- 4.60, n=14; 13.69 +/- 2.07, n=16, *snail*; 88.85+/- 7.88, n=8 ; 57.79 +/- 22.46, n=14; 11.74 +/- 2.07, n=16, *slug*--copy number mRNA/ GAPDH +/- SEM for OSE, normal and cancer respectively). These data suggest that up-regulation of E-cadherin is an early defining event in the progression of ovarian cancer.

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CYP1B1 Expression in Ovarian Cancer in the Laying Hen *Gallus domesticus*

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Abstract

Objective. Ovarian carcinoma is the most lethal gynecological malignancy and a leading cause of cancer death in women. There is a lack of suitable experimental animal models for the study of ovarian cancer, with the exception of the laying hen. CYP1B1 is a cytochrome P450 enzyme and can catalyze the conversion of estrogens to genotoxic catechol estrogens which may cause DNA mutations and initiate ovarian epithelial cancer. Our objective was to evaluate CYP1B1 expression, distribution and localization in cancerous, age-matched normal and young hen ovaries in order to better understand CYP1B1's role in ovarian cancer.

Methods. CYP1B1 mRNA and protein expression were analyzed in the ovaries of cancerous, age-matched normal and/or young hens by quantitative real-time PCR (qRT-PCR), *in situ* hybridization (ISH) and immunohistochemistry (IHC). RNA was extracted from tissue preserved in *RNAlater* and analyzed by qRT-PCR; tissue frozen in liquid nitrogen was used for ISH, and tissue fixed in neutral buffered formalin was subjected to IHC.

Results. Higher expression of CYP1B1 mRNA was seen in the cancerous ovaries as compared to age-matched normal and young ovaries by qRT-PCR. ISH and IHC revealed that the expression of CYP1B1 was much higher in tumor samples compared to age-matched normal or young ovaries. CYP1B1 mRNA and protein were distributed extensively throughout the whole carcinoma; however, they are mostly localized to the granulosa cell layer surrounding the follicle in the age-matched normal or young hen ovaries. IHC also demonstrated nuclear localization of CYP1B1. We revealed for the first time that CYP1B1 is expressed in normal hens' pre- and post-ovulatory follicles (POF). qRT - PCR analysis indicated that CYP1B1 mRNA was highly expressed in ovarian surface epithelium (OSE) and POF-3 from both young and age-matched normal hen as compared to POF-1 and POF-2. No significant difference was found in the expression of CYP1B1 between the top and bottom parts of POF-1 from both young and age-matched normal hens.

Conclusions. Our data further confirm that the laying hen is a good model for human ovarian cancer. The unique pattern of CYP1B1 expression in the hen ovary may be important to the etiology of the disease. Moreover, our data suggests that CYP1B1 may prove to be a target for the prevention or treatment of ovarian cancer.

Keywords: ovarian cancer, CYP1B1, *Gallus domesticus*, etiology, estrogens, pre- and post-ovulatory

Introduction

Ovarian carcinoma is the fifth leading cause of cancer death in women and the most lethal of the gynecological malignancies [1].The high mortality rate is due to the late stage of detection when approximately 75% of ovarian cancers are diagnosed. More than 90% of human ovarian cancers are epithelial in origin and are thought to arise from invaginations of the specialized layer of tissue that covers the ovary [2]. Though considerable progress has been made in the development of therapies for ovarian cancer, the genetic and molecular mechanisms that cause ovarian cancer still remain largely unknown. The lack of a reliable and specific method for early detection results in clinical presentation at later stages when treatment is less effective [3]. Research into the cause of ovarian cancer has been hampered by lack of suitable animal models. Ovarian cancer is rarely observed in most species with the notable exception of the domestic hen, which, like humans, spontaneously develops ovarian cancer. Therefore, the hen model is of possible value for etiological studies and for establishing new therapeutic modalities for the prevention and treatment of ovarian cancer [4, 5] .

Cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1) is an enzyme that is expressed in estrogen target tissues including mammary, ovary and uterus and specifically catalyzes the hydroxylation of estradiol to 4-hydroxy estradiol (4-OHE_2) [6]. 4-OHE_2 is a highly reactive catechol estrogen which is further oxidized to the estrogen-3,4-semiquinone or quinone that can react with DNA to form depurinating adducts. These adducts are released from DNA to generate apurinic sites. Error-prone base excision repair of this damage may lead to DNA mutations and may possibly result in carcinogenesis [7-10]. Higher expression of CYP1B1 has been observed in several types of cancer, including ovary, breast, colon and lung, compared to the adjacent normal tissues [11-15]. CYP1B1 expression in various cancers and its role in the metabolic activation of endogenous pro-carcinogens implicate it in the cause of these cancers.

There are several proposed hypotheses about the etiology of ovarian cancer [16]. The “incessant ovulation hypothesis” was first proposed by Fathalla in 1971 [17] and revisited by Fleming in 2006 [18]. In support of this hypothesis, multiparity, duration of lactation and use of birth control pills all decrease the risk of epithelial ovarian cancer (EOC). Other hypotheses concerning the origin of ovarian cancer have been proposed, as reviewed by Landen, et al. 2008 [16]. The hen model strongly supports the incessant ovulation hypothesis because domestic hens lay eggs intensively and frequently develop ovarian cancer. The incidence of ovarian cancer in hens rises dramatically with age, with a 4% incidence at 2 years of age, increasing to as high as 40% by 6 years [19]. After each ovulation, the rapid growth and reconstruction of the ovarian surface epithelium (OSE) may cause the formation of inclusion cysts where the surface cells become trapped. In these cysts, cells are exposed to high concentrations of growth factors, steroid hormones and inflammatory mediators, which may promote the malignant transformation of the trapped surface cells [20, 21]. Estrogen concentrations are high in the ovary. The high estrogen in the ovary may provide stimuli for proliferation of the

OSE via estrogen receptor mediated actions. In addition, estradiol may also be a substrate for CYP1B1 which could convert it to form directly genotoxic intermediates. These intermediates may cause mutations of trapped OSE cells and possibly lead to the initiation of cancer.

CYP1B1 expression in the hen ovary has not been examined. The objectives of this investigation were to characterize the expression, distribution and localization of CYP1B1 in the ovaries of cancerous, age-matched normal and young hens, and to determine if CYP1B1 expression and distribution is correlated with ovarian cancer. The results of this study demonstrate that CYP1B1 is significantly increased in ovarian cancer of the hen and the pattern of CYP1B1 expression may be important to the etiology of the disease. Our data further support the laying hen model for ovarian cancer and suggest that CYP1B1 may prove to be a target for intervention.

Materials and Methods

Animals and tissue collection

Single-comb White Leghorn hens, 40-50 weeks (young) and 165 weeks (cancer or age-matched normal), were used for sample collection. The three largest pre-ovulatory follicles (F1, F2 and F3) which were used for OSE collection were taken from hens that were 100 weeks old. 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. Oviposition was monitored daily at 1 h intervals between 08:00 h and 12:00 h. Animal management and procedures were reviewed and approved by the Division of Animal Research of the University of Illinois at Urbana-Champaign and the Animal Care Committee, University of Illinois at Chicago.

Hens were sacrificed by CO_2 asphyxiation. Ovarian tissues were collected immediately after dissection and portions were snap frozen and later stored at -80 for biochemical analysis; or put into RNA*later* (Applied Biosystems, Foster City, CA) and stored at 4^{o}C for RNA isolation; or fixed in neutral-buffered formalin (NBF) (Sigma-Aldrich, St Louis, MO) for routine histological and immunohistochemical analysis. In normal ovaries, the three largest pre-ovulatory follicles (F1, F2 and F3) and the three post-ovulatory follicles (POF-1, POF-2 and POF-3) were collected. The ovarian surface epithelium (OSE) was isolated from F1-F3 by scrapping off the outer thin layer of the follicles (F1-F3) using a sterile cell scraper (Biologix Research Corp, Lenexa, KS). In order to compare the difference between the site of follicular rupture and the point of follicular attachment, POF-1 was cut in half into “top” (site of rupture) and “bottom” (site of attachment) pieces. OSE and POFs dissected from ovaries were placed in RNA*later* for RNA isolation. The cancerous ovary lacks discernable or separable pre-ovulatory and post-ovulatory follicles.

Histology and immunohistochemistry

Ovary tissues fixed in NBF solution were processed and paraffin embedded. 5μm sections were cut and mounted on SuperFrost Plus microscope slides (Fisher Scientific, Itasca, IL). Slides were deparaffinized and rehydrated through xylene and graded ethanol solutions (Fisher Scientific). Hematoxylin and Eosin (H.&E.) staining was done as described [22].

Immunohistochemistry was performed by using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) and 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, Walnut Creek, CA). Slides were cooled and quenched in 0.3% H_2O_2 (Sigma-Aldrich) in methanol for 15 min. Slides were blocked in normal serum, and incubated in anti-human CYP1B1 polyclonal antibody (a generous gift from Dr. Craig Marcus, University of New Mexico) at a dilution of 1:1000 overnight at 4^{o}C. After rinsing in Tris-buffered saline, sections were incubated with anti-rabbit secondary antibody and avidin-biotin complex (Vector Laboratories). Specific binding was visualized using Diaminobenzidine (DAB) (Sigma-Aldrich) in the presence of H_2O_2 and sections were counter-stained with Gils hematoxylin (Sigma-Aldrich), mounted

with Histomount (Fisher Scientific), examined on a Nikon ECLIPSE E400 microscope and were documented using SPOT Advanced version 4.0.1 software (Diagnostic Instruments, Inc., Sterling Heights, MI).

RNA extraction and analysis

Total RNA was extracted from OSE, POF1-3 and ovaries using Trizol reagent (Invitrogen, Carlsbad, CA) and was quantified by determination of Absorbance at A260. All RNA samples used in this study had a 260:280 ratio between1.9-2.1. RNA samples were then treated with RQ1 RNase-free DNase (Promega, Madison, WI) prior to reverse transcription reaction. Synthesis of first stand of cDNA was performed using the 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.

In situ hybridization

In situ hybridization followed the protocol as previously described [23]. In brief, frozen sections (10 μm) were mounted onto poly-L-lysine-coated slides (Sigma-Aldrich) and fixed in cold 4% paraformaldehyde (Polysciences, INC) in PBS. Sections were prehybridized and then hybridized at 45°C for 4 hours in 50% formamide hybridization buffer containing ^{35}S-labeled antisense or sense cRNA probes. RNase A–resistant hybrids were detected by autoradiography. Sections were post-stained with H&E. Sections hybridized with sense probes did not exhibit positive signals and served as negative controls.

Hybridization probes

The cDNA clone for CYP1B1 (pgn1c.pk007. j13) was obtained from Delaware Biotechnology Institute (Newark, DE) and verified by DNA sequence. The plasmid was linearized by using Not1 (Invitrogen) for the sense and Kpn1 (Invitrogen) for the antisense probe. For *in situ* hybridization, sense and antisense ^{35}S-labeled *cRNA* probes were generated using Sp6 and T7 polymerases (New England Biolabs), respectively. Probes had specific activities of 2 x 10^9 dpm/μg.

Quantitative Real-Time PCR (qRT-PCR)

Chicken specific primers were designed to recognize target genes using Primer Express software (Applied Biosystems) and obtained from Sigma-Genosys (Sigma-Aldrich). The q-PCR primer pairs were designed so that at least one of them spanned an intron. CYP1B1 (XM_419515) forward primer: 5’ GCATCCCAAAGCAAAATCCC 3’; reverse primer: 5’ CGGCAGGTTCCAGTAGAATGA 3’. Glyceraldehyde-3-phosphate dehydrogenase(GAPDH) (NM_204305) was used as internal control gene. GAPDH forward primer: 5’ GATGGGTGTCAACCATGAGAAA 3’; reverse primer: 5’ CAATGCCAAAGTTGTCATGGA 3’. Plasmid standards for CYP1B1 and GAPDH 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, Valencia, CA) and primer sequences used are: forward: 5' GCAGATGCAGGTGCTGAGTATG 3'; reverse: 5' GGCAGGTCAGGTCAACAACAGA 3'. The GAPDH fragments were then cloned by using TOPO TA cloning Kit (Invitrogen) and verified by sequencing. CYP1B1 plasmid was as described under I*n situ* hybridization. Plasmid concentrations were measured by spectrophotometer and the corresponding copy numbers were calculated based on the formula that 1µg of 1000bp of DNA=$9.1X10^{11}$ molecules. Real-time PCR was conducted by amplifying cDNA with SYBR Green Master Mix (Applied Biosystems) on ABI 7900HT using a 384 well plate and analyzed with AB1 Prism software. Control reactions lacking the template were run for each gene. Reactions were 10µl 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^{o}C 2 min, 95^{o}C 10 min, then 40 cycles of: 95^{o}C 15 sec, 60^{o} 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; a value of $P<0.01$ was considered as highly significant.

Results

Immunohistochemistry

H&E staining shows a normal and healthy ovary with cortical follicles containing an oocyte surrounded by prominent granulosa and interstitial cell layers (Fig. 1A). Fig. 1B shows H&E staining of a poorly differentiated ovarian tumor with endometrioid-like glands. Expression of CYP1B1 protein from the ovaries of cancerous and normal hens was examined by IHC. Higher expression of CYP1B1 protein was observed in cancerous ovaries (Fig. 1D) as compared to ovaries from normal hens (Fig. 1C). In the normal hen ovaries, CYP1B1 was localized primarily to the granulosa cell layer surrounding the follicle, with some staining observed in the OSE and cortical stroma adjacent to the follicle. Extensive CYP1B1 protein expression was found throughout the ovarian carcinoma. IHC also showed nuclear localization of CYP1B1 in the hen ovary (1D).

In situ hybridization

Expression of CYP1B1 mRNA from the ovaries of cancerous and normal hens was analyzed by ISH. Fig. 2A-B shows H & E staining of the hybridized sections. The expression of CYP1B1 was considerably higher in cancerous ovarian tissue as compared to the ovary of age-matched normal hens (Fig. 2C-D). CYP1B1 was localized to the granulosa cell layer surrounding the follicle in the ovary from age-matched normal hens (Fig.

2C), while extensive CYP1B1 mRNA expression was observed throughout the whole carcinoma tissue (Fig. 2D). No signal above background was observed when section was hybridized to sense probe (Fig. 2E-F).

qRT-PCR

CYP1B1 mRNA expression from the ovaries of cancerous, age-matched normal and young hens was compared by qRT-PCR. There was a significant ($p<0.05$) increase of CYP1B1 mRNA in cancerous ovaries as compared to age-matched normal and young ovaries after normalization to GAPDH expression. No significant difference of CYP1B1 mRNA expression was found between ovaries from age-matched normal and young hens (Fig. 3).

CYP1B1 mRNA in OSE from 100 week old hen and POFs from age-matched normal and young hens was analyzed by qRT- PCR. There was significantly higher expression of CYP1B1 mRNA in OSE and POF-3 as compared to POF-1 and POF-2 from both young hens (Fig. 4A) and age-matched normal hens (Fig. 4B). No significant difference was detected in CYP1B1 expression between POF-1 top and bottom from both young and age-matched normal hens.

Discussion

The present results demonstrated that CYP1B1 mRNA and protein are expressed in cancerous, age-matched normal and young ovaries of the hen. qRT-PCR analysis indicated that CYP1B1 mRNA was elevated in the hen ovarian tumor as compared to age-matched normal and young hen ovaries. This is in agreement with the *In situ* hybridization and Immunohistochemistry results. Both the CYP1B1 mRNA and protein were found to be highly expressed in cancerous ovaries as compared to the normal ovaries of the hens, which is consistent with previous studies in the human [11, 13]. CYP1B1 mRNA and protein are localized to the granulosa cell layer in normal hen ovaries, while extensive CYP1B1 mRNA and protein were distributed throughout the entire cancerous ovarian tissue. CYP1B1 protein appeared to be nuclear, as was reported in the human ovary [24].

Furthermore, we demonstrated that CYP1B1 is expressed in normal hen's pre- and post-ovulatory follicles. qRT-PCR analysis compared the expression of CYP1B1 mRNA between the top and bottom parts of POF-1 from both young and age-matched normal hens. Ovulation takes place along an avascular band (stigma line) that extends over the apical surface of the follicle. More oxidative damage was detected in the top surface of postovulatory follicles (POF1-2) as compared to the bottom [21, 25, 26]. However, the results of the present study indicated that there was not a significant difference in the expression of CYP1B1 between the top and bottom parts of POF-1 from both young and age-matched normal hens. qRT-PCR analysis also demonstrated that CYP1B1 mRNA was highly expressed in OSE and POF-3 from normal hen as compared to POF-1 and POF-2. This is the first report that shows CYP1B1's expression is involved in pre- and post-ovulatory follicles of hens. The observed high level of CYP1B1 in OSE and POF-3 could be an important phenomenon and may help to understand the origin of ovarian cancer.

The hen ovary differs morphologically from the human ovary. It has three POFs and four to six pre-ovulatory follicles which are arranged in a distinct hierarchy [27]. The pre- and post-ovulatory follicles consist of granulosa cells, interstitial theca cells and OSE cells. Post-ovulatory follicles, unlike those in mammals, do not form a corpus luteum but rather regress and are assimilated into the ovary stroma [25]. POF-3 is the last remnant of the POFs and is resorbed into the ovarian stroma. In hen ovary, no clear-cut ingrowths of these cells into the ovarian stroma to form inclusion cysts have been observed. The rupture of the follicle and its rapid postovulatory atrophy make it difficult to see infolding; but there is a moderate degree of structural similarity to the human [19]. Remodeling of the surface epithelium after ovulation is a dynamic process and we speculate that the rapidly growing OSE and POF-3 during resorption might become trapped and result in the formation of the inclusion cysts. Highly elevated levels of CYP1B1 were detected in the OSE and POF-3. In the inclusion cyst, high levels of CYP1B1 in the OSE and/or POF-3, together with the high concentrations of estradiol in the microenvironment, may cause the production of genotoxic metabolites. These metabolites

could potentially cause the malignant transformation of the ovarian surface epithelial cells and result in the initiation of ovarian cancer.

Whereas OSE and POF-3 from normal hen ovaries all have a high CYP1B1 expression, not every hen will develop ovarian cancer. As mentioned in the introduction, estrogens can be converted to 4-OHE_2 by CYP1B1, further oxidized to the catechol estrogen-3, 4-quinones (CE-3, 4-Q), which can react with DNA to form depurinating adducts that may further cause mutations and initiate cancer. However, formation of the CE-3, 4-quinones could be prevented by methylation of the 4-OHE_2 via the enzyme catechol-O-methyltransferase (COMT). In addition, CE-3, 4-quinones can be reduced back to catechol estrogens by NADPH quinone oxidoreductase 1 (NQO1) and/or are coupled with glutathione, preventing reaction with DNA [7, 28]. These protective and repair mechanisms likely add to the variable extent to which estrogen metabolites might contribute to ovarian carcinogenesis. Furthermore, individual heterogeneity, loss of tumor suppressor genes, amplification of growth stimulatory and/or suppression of death signaling pathway, may cause certain individuals to be more sensitive to developing ovarian cancer [29].

The laying hen model shows marked similarities to human ovarian cancer from both histologically and in the pattern of protein expression, for example, cytokeratin, PCNA [5], COX-1 and COX-2 [30, 31], SELENBP1 [32] and anti-tumor antibodies [33]. The results of this study have further demonstrated that ovarian cancer in the hen shares features with human ovarian carcinoma, in particular, elevated CYP1B1 expression, thus further supporting the laying hen as an important model for the human disease. Moreover, our results show for the first time that CYP1B1 mRNA is highly expressed in the OSE and POF-3. During the dynamics process of ovarian surface remodeling, OSE and/or POF-3 may invaginate to form inclusion cysts rendering the cells susceptible to subsequent malignant transformation in the presence of both high concentrations of estrogens and CYP1B1. Our findings suggest that CYP1B1 may play an important role in the initiation of ovarian cancer and may prove to be a target for intervention.

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References

[1] Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin; 2007;57: 43-66.

[2] Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC. Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr Rev; 2001;22: 255-88.

[3] Vanderhyden BC, Shaw TJ, Ethier JF. Animal models of ovarian cancer. Reprod Biol Endocrinol; 2003;1: 67.

[4] Giles JR, Shivaprasad HL, Johnson PA. Ovarian tumor expression of an oviductal protein in the hen: a model for human serous ovarian adenocarcinoma. Gynecologic Oncology; 2004;95: 530.

[5] Rodriguez-Burford C, Barnes MN, Berry W, Partridge EE, Grizzle WE. Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention. Gynecol Oncol; 2001;81: 373-9.

[6] Tsuchiya Y, Nakajima M, Kyo S, Kanaya T, Inoue M, Yokoi T. Human CYP1B1 is regulated by estradiol via estrogen receptor. Cancer Res; 2004;64: 3119-25.

[7] Cavalieri E, Chakravarti D, Guttenplan J, Hart E, Ingle J, Jankowiak R, Muti P, Rogan E, Russo J, Santen R, Sutter T. Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. Biochim Biophys Acta; 2006;1766: 63-78.

[8] Tsuchiya Y, Nakajima M, Yokoi T. Cytochrome P450-mediated metabolism of estrogens and its regulation in human. Cancer Lett; 2005;227: 115-24.

[9] Hayes CL, Spink DC, Spink BC, Cao JQ, Walker NJ, Sutter TR. 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1B1. Proc Natl Acad Sci U S A; 1996;93: 9776-81.

[10] Zhu BT, Conney AH. Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis; 1998;19: 1-27.

[11] Murray GI, Taylor MC, McFadyen MC, McKay JA, Greenlee WF, Burke MD, Melvin WT. Tumor-specific expression of cytochrome P450 CYP1B1. Cancer Res; 1997;57: 3026-31.

[12] McFadyen MC, Breeman S, Payne S, Stirk C, Miller ID, Melvin WT, Murray GI. Immunohistochemical localization of cytochrome P450 CYP1B1 in breast cancer with monoclonal antibodies specific for CYP1B1. J Histochem Cytochem; 1999;47: 1457-64.

[13] McFadyen MC, Cruickshank ME, Miller ID, McLeod HL, Melvin WT, Haites NE, Parkin D, Murray GI. Cytochrome P450 CYP1B1 over-expression in primary and metastatic ovarian cancer. British Journal Of Cancer; 2001;85: 242-246.

[14] Downie D, McFadyen MC, Rooney PH, Cruickshank ME, Parkin DE, Miller ID, Telfer C, Melvin WT, Murray GI. Profiling cytochrome P450 expression in ovarian cancer: identification of prognostic markers. Clin Cancer Res; 2005;11: 7369-75.

[15] Cavalieri EL, Stack DE, Devanesan PD, Todorovic R, Dwivedy I, Higginbotham S, Johansson SL, Patil KD, Gross ML, Gooden JK, Ramanathan R, Cerny RL, Rogan EG. Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. Proc Natl Acad Sci U S A; 1997;94: 10937-42.

[16] Landen CN, Jr., Birrer MJ, Sood AK. Early events in the pathogenesis of epithelial ovarian cancer. J Clin Oncol; 2008;26: 995-1005.

[17] Fathalla MF. Incessant ovulation--a factor in ovarian neoplasia? Lancet; 1971;2: 163.

[18] Fleming JS, Beaugie CR, Haviv I, Chenevix-Trench G, Tan OL. Incessant ovulation, inflammation and epithelial ovarian carcinogenesis: revisiting old hypotheses. Mol Cell Endocrinol; 2006;247: 4-21.

[19] Fredrickson TN. Ovarian tumors of the hen. Environ Health Perspect; 1987;73: 35-51.

[20] Murdoch WJ. Metaplastic potential of p53 down-regulation in ovarian surface epithelial cells affected by ovulation. Cancer Lett; 2003;191: 75-81.

[21] Murdoch WJ, Martinchick JF. Oxidative damage to DNA of ovarian surface epithelial cells affected by ovulation: carcinogenic implication and chemoprevention. Exp Biol Med (Maywood); 2004;229: 546-52.

[22] Bancroft J, Stevens A. Theory and Practice of Histological Techniques. In. Third ed. Edinburgh London Melbourne and New York: Churchill Livingstone; 1990. p. P726.

[23] Lim H, Gupta RA, Ma WG, Paria BC, Moller DE, Morrow JD, DuBois RN, Trzaskos JM, Dey SK. Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARdelta. Genes Dev; 1999;13: 1561-74.

[24] Muskhelishvili L, Thompson PA, Kusewitt DF, Wang C, Kadlubar FF. In situ hybridization and immunohistochemical analysis of cytochrome P450 1B1 expression in human normal tissues. The Journal Of Histochemistry And Cytochemistry: Official Journal Of The Histochemistry Society; 2001;49: 229-236.

[25] Murdoch WJ, Van Kirk EA, Alexander BM. DNA damages in ovarian surface epithelial cells of ovulatory hens. Exp Biol Med (Maywood); 2005;230: 429-33.

[26] Murdoch WJ, Townsend RS, McDonnel AC. Ovulation-induced DNA damage in ovarian surface epithelial cells of ewes: prospective regulatory mechanisms of repair/survival and apoptosis. Biol Reprod; 2001;65: 1417-24.

[27] Bahr JM, Johnson PA. The female reproductive system. 4 ed: New York: Academic Press; 1991.

[28] Singh S, Chakravarti D, Edney JA, Hollins RR, Johnson PJ, West WW, Higginbotham SM, Cavalieri EL, Rogan EG. Relative imbalances in the expression of estrogen-metabolizing enzymes in the breast tissue of women with breast carcinoma. Oncol Rep; 2005;14: 1091-6.

[29] Wenham RM, Lancaster JM, Berchuck A. Molecular aspects of ovarian cancer. Best Pract Res Clin Obstet Gynaecol; 2002;16: 483-97.

[30] Urick ME, Johnson PA. Cyclooxygenase 1 and 2 mRNA and protein expression in the Gallus domesticus model of ovarian cancer. Gynecol Oncol; 2006;103: 673-8.

[31] Dale Buchanan Hales, Yan Zhuge, Jo Ann Jaen Lagman, Kristine Ansenberger, Cassandra Mahon, Animesh Barua, Judith Luborsky, Bahr. JM. CYCLOOXYGENASES EXPRESSION AND DISTRIBUTION IN THE NORMAL OVARY AND THEIR ROLE IN OVARIAN CANCER IN THE DOMESTIC HEN (GALLUS DOMESTICUS). Endocrine; 2008; in press.

[32] Stammer K, Edassery SL, Barua A, Bitterman P, Bahr JM, Hales DB, Luborsky JL. Selenium-Binding Protein 1 expression in ovaries and ovarian tumors in the laying hen, a spontaneous model of human ovarian cancer. Gynecol Oncol; 2008;109: 115-21.

[33] Barua A, Bradaric M, Edassary S, Rotmensch J, Bitterman P, Hales D, et al. Anti-tumor antibodies and ovarian cancer in women and hens. In: Special Ameriacan Association for Cancer Research Conference, Molecular Diagnostics in Cancer Theraperutic Development: Maximizing Opportunities for Individualized Treatment. Chicago, IL: Scholar-in-Training Award; 2006.

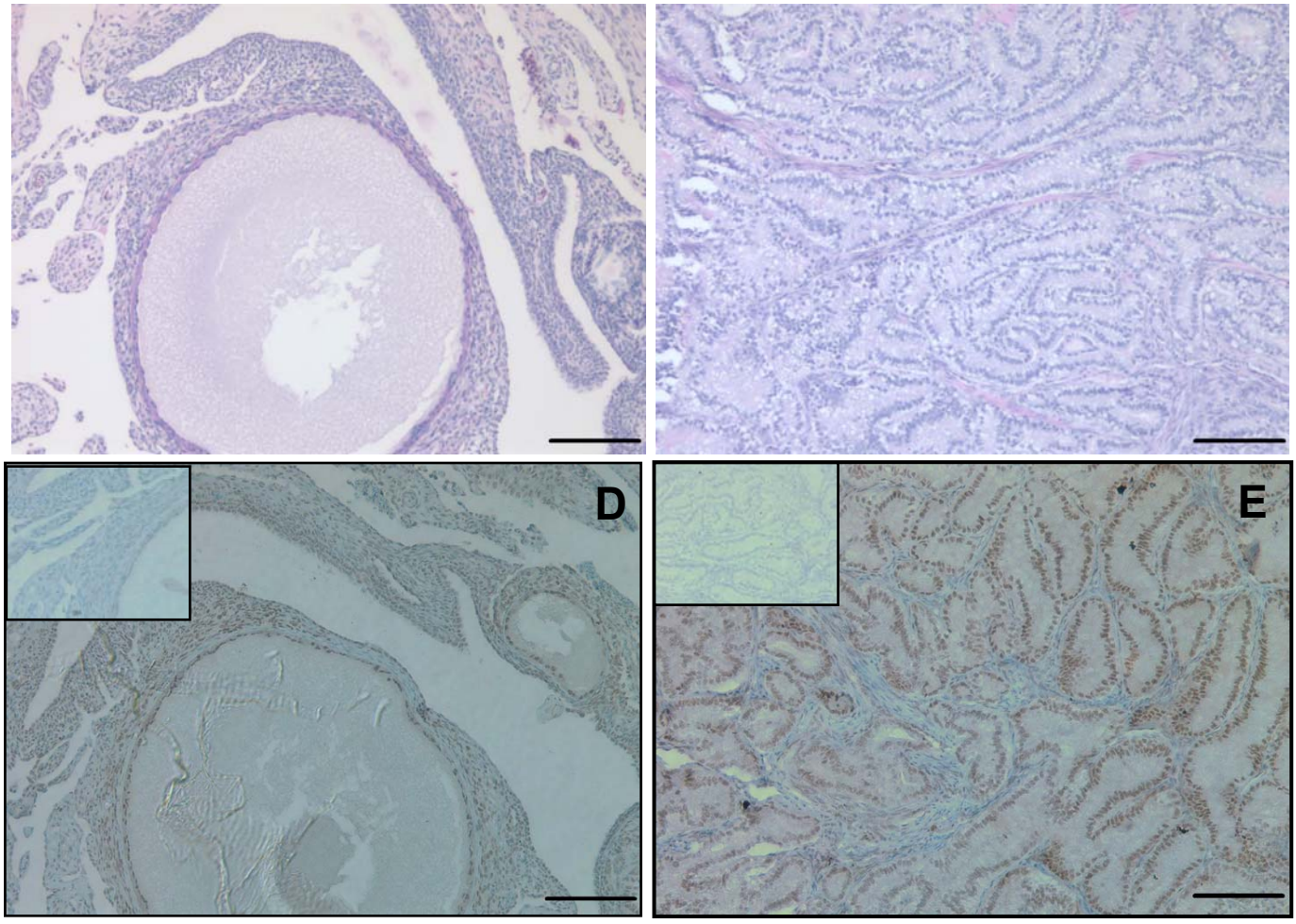


Figure 1. CYP1B1 H & E staining and immunohistochemistry: A) age-matched old normal ovary, H & E; B) cancer ovary, H & E; C) age-matched old normal ovary, IHC; D) cancer ovary, IHC. (insert: non-immune IgG, 20X). Calibration bar: 100 um

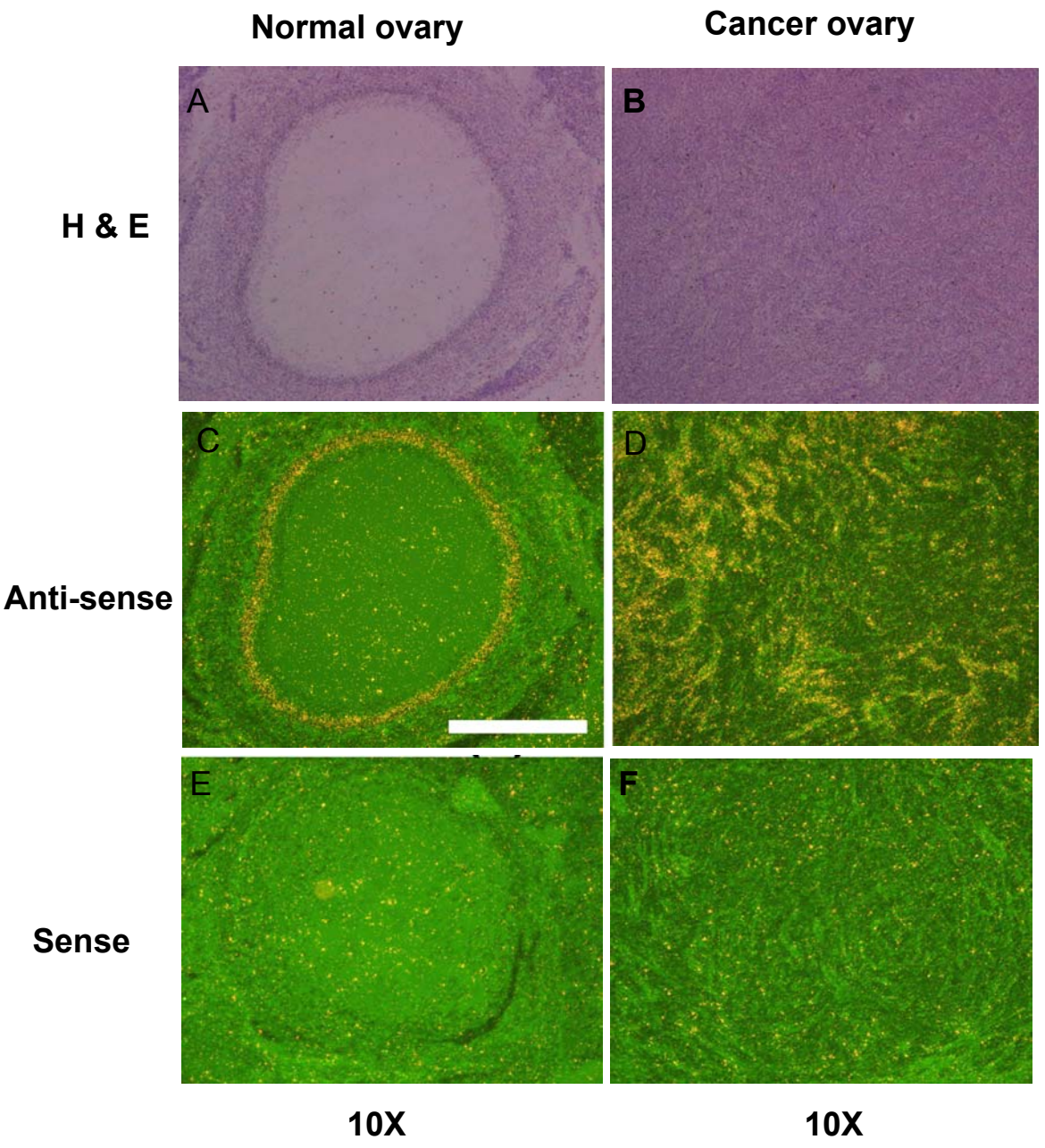


Figure 2. *In situ* hybridization of CYP1B1 in cancer and age-matched old normal ovaries. C & D from antisense probe; E & F from sense probes; A & B post-stained with Hemotoxylin and Eosin. Yellow spots are hybridization signals. Bar, 400 μm.

Figure 3. CYP1B1 mRNA expression in ovarian cancer (165 weeks, n=17), age-matched old normal hens (165 months, n=10) and young hens (40-50 weeks, n=6). Asterisks indicate a significant increase in ovaries with tumors as compared to age-matched old normal and young ovaries ($p<0.05$). Bars indicate standard error.

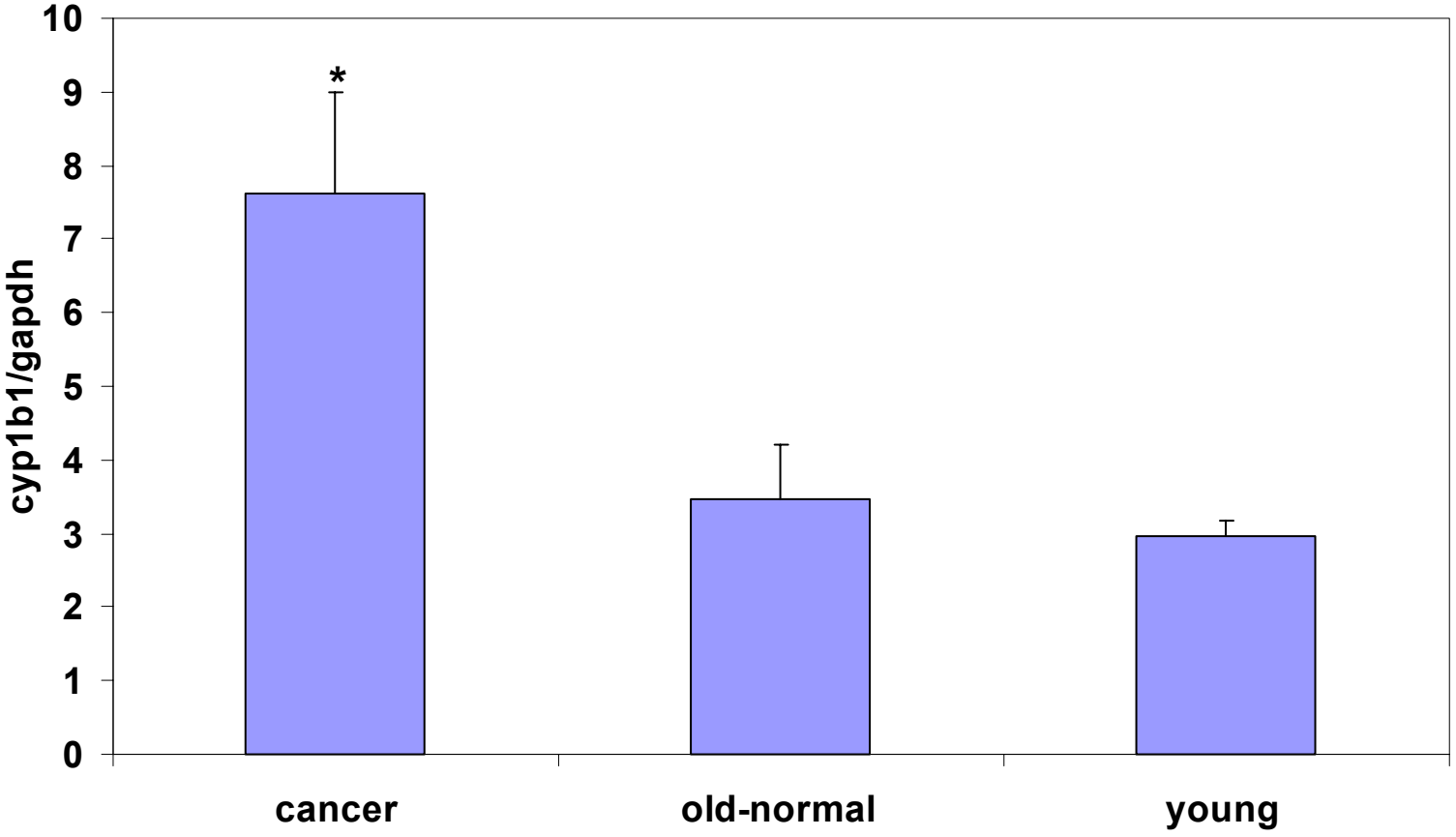
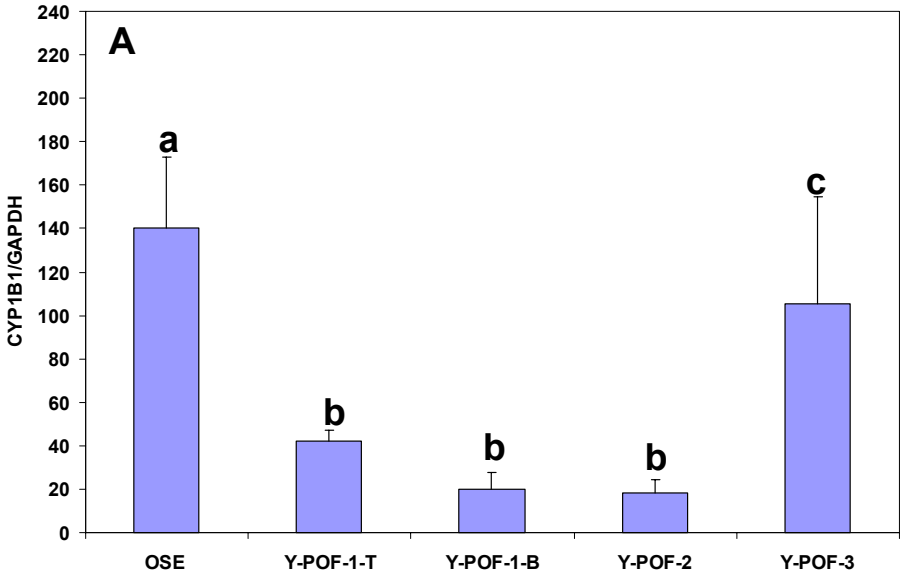
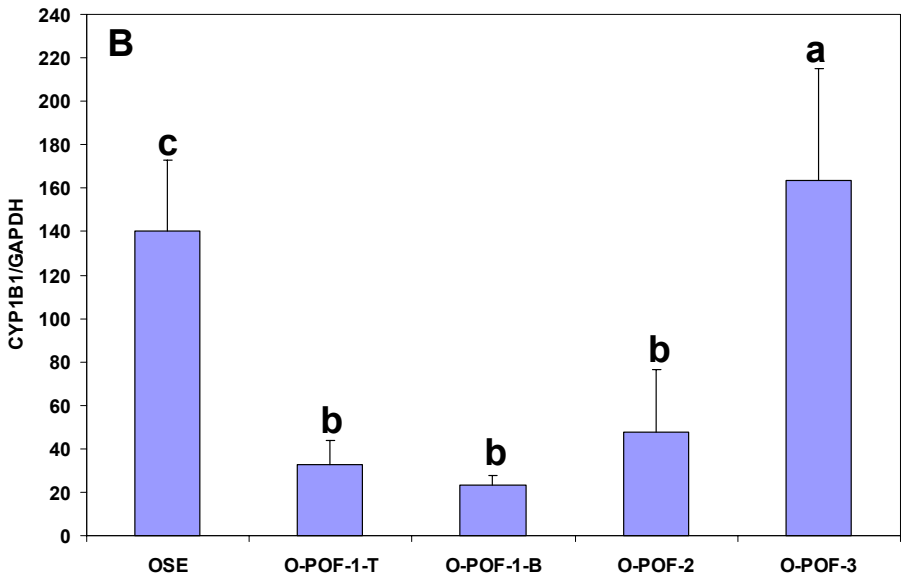


Figure 4. CYP1B1 mRNA expression in OSE (100 week old hen, n=6) as compared to (A): POF-1-top (n=7), POF-1-bottom (n=6), POF-2 (n=6), POF-3 (n=6) from young hen (40-50 weeks), and to (B): POF-1-top (n=7), POF-1-bottom (n=6), POF-2 (n=6) and POF-3 (n=6) from age-matched old normal hen (165 months). a vs. b, $P<0.01$; c vs. b, $P<0.05$. Bars indicate standard error





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Histopathology of ovarian tumors in laying hens, a preclinical model of human ovarian cancer

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Keywords:	ovarian cancer, preclinical model, laying hen, tumor histology

Histopathology of ovarian tumors in laying hens, a preclinical model of human ovarian cancer

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Running head: Histology of spontaneous ovarian tumors in hens in laying hen

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Abstract

The high mortality rate due to ovarian cancer is attributed to the lack of an effective early detection method. Due to the non-specificity of symptoms at early stage, most of the ovarian cancer cases are detected at late stages. This makes the access to women with early stage disease problematic and presents a barrier to development and validation of tests for detection of early stage of ovarian cancer in humans. Animal models are used to elucidate disease etiologies and pathogenesis that are difficult to study in humans. Laying hen is the only available animal that develops ovarian cancer spontaneously; however, detail information on ovarian tumor histology is not available. The goal of this study was to determine the histological features of malignant ovarian tumors in laying hens. A total of 155 young and old (1-5 years of age) laying hens (*Gallus domesticus*) were selected randomly and evaluated gross and microscopically for the presence of ovarian tumors. Histological classification of tumors with their stages and grades were performed with reference to those for humans. Similar to humans, all four types including serous, endometrioid, mucinous and clear cell or mixed carcinomas were observed in hen ovarian tumors. Some early neoplastic as well as putative ovarian lesions were also observed. Similarities in histology, metastasis and stages of hen ovarian cancer to those of humans demonstrate the feasibility of the hen model for additional delineation of the mechanism underlying ovarian carcinogenesis, preclinical testing of new agents for the prevention and therapy of this disease.

KEYWORDS: ovarian cancer; preclinical model; laying hen; tumor histology

Ovarian cancer (OVCA) is a fatal disease of women with the highest mortality rate of all gynecological malignancies. Approximately 70% of women with OVCA die of this disease [1, 2]. Survival is high in women who present with early stage disease [3, 4]. The lack of specific symptoms, the relative inaccessibility of the ovaries deep in the pelvis, and the absence of specific marker(s) represent barriers for early detection [5, 6]. In most cases, OVCA is diagnosed at a late stage [3]. Furthermore, our understanding of the early pathogenesis of OVCA has been hindered by the lack of sufficient number of patients with early stage disease [3, 4, 7]. Animal models are used to elucidate disease etiologies and pathogenesis that are difficult to study in humans. Animals that closely recapitulate human OVCA are crucial for understanding its pathogenesis and for testing new treatment strategies. Researchers have attempted for many years to develop a mammalian model of human OVCA using large domestic animals or rodents. Although large domestic mammals including bovine have similar reproductive traits and develop OVCA spontaneously similar to humans, the low incidence rate, multiple pregnancies, longer gestation and lactation period make them an inappropriate model for human OVCA. On the other hand, a number of rodent models, induced or genetically manipulated, have been developed and used successfully to elucidate some aspects of OVCA. However, the non-spontaneous nature of many of these models of OVCA limits their clinical relevance [8, 9]. Although several avian species have been reported to develop OVCA spontaneously, chickens (*Gallus domesticus*) are the most widely available avian species and develop spontaneous OVCA with a high incidence rate[10, 11]. Therefore, the laying hen is an appropriate animal model for the study of human OVCA.

Commercial egg laying hens (strains of Single Comb White Leghorn) attain sexual maturity (start laying eggs) at 20-22 weeks of age. They reach peak egg production at 30-32 weeks of age

[12]. Hens maintain a high laying rate (>90%) during the first year of lay (an average hen lays >280 eggs) and then egg production declines slowly indicating a decrease in ovarian function [12]. After two years, hens become economically unprofitable due to a reduction in their egg production rates and increased susceptibility to infections and other diseases. In the chicken, only the left ovary and oviduct become functional. A fully functional left ovary in young healthy laying hens of commercial strains contains 5 or 6 large preovulatory follicles arranged in a hierarchy based on their size (termed hierarchical follicles). The ovulatory cycle in hens ranges from 24-26 hours depending on the age of the hens (e.g., shorter in young laying hens and longer in older hens). A hen with normal reproductive function lays 5-6 or more eggs in a sequence (termed clutch size or sequence), followed by a pause of one day and then resumes laying. Clutch size decreases to <3eggs as the hen ages. Following ovulation of the largest follicle (F1), the second largest follicle becomes the largest, the third one becomes the fourth and so on and a small developing follicle is recruited from the pool into the hierarchy (Fig. 1A). The hen ovarian follicular pool contains many small developing yolky and white follicles. All preovulatory and small developing follicles protrude from the ovarian surface whereas primordial and primary follicles are embedded in the ovarian cortex. Similar to humans, both the follicular development and ovulatory cycles are under the control of pituitary gonadotrophins and ovarian steroids [12, 13]. Following ovulation, the egg passes through the oviduct and the remaining tissue of the ovulated follicle, now called postovulatory follicle, functions as an endocrine organ. Because the laying hen is an oviparous animal, the postovulatory follicle degrades within 3-4 days following ovulation. Therefore, similarities in some features of reproductive physiology between humans and hens, wide availability and easy accessibility make the hen an extraordinary animal to be explored as a model of human OVCA.

The incidence of spontaneous ovarian carcinoma and its epidemiology in laying hens was first described in two reports 20 years ago; however, those studies were performed from an agricultural interest rather than a biomedical perspective [10, 11]. Therefore, both reports lacked detailed information on tumor types and their stages, and as such are irrelevant to clinical applications. As egg production decreases and OVCA incidence increases with aging of hens, poultry farmers seldom maintain hens older than 2 years. Because older hens are not profitable, those reports were largely ignored by the poultry industry. Hence, studies on OVCA in hens were not pursued further by avian researchers. However, interest in laying hen OVCA is increasing as a few recent reports have shown it to be similar to human OVCA. Ovarian tumors in hens express several molecular markers including cytokeratin, Epidermal Growth Factor receptor (EGFR), Tag 72, Proliferating Cell Nuclear Antigen (PCNA), TGF-a and CA125 similar to humans [14, 15]. Moreover, treatment of hens with progesterone reduced the incidence of OVCA by 40% and progesterone has been suggested as a preventive agent for OVCA in hens [16]. This is similar to the epidemiological association of high progesterone states such as pregnancy and birth control pill use with reduced risk of OVCA in humans [17-19]. Also, DNA damage to ovarian surface epithelial cells as a result of frequent rupture due to ovulation in hens corresponds to the number of lifetime ovulations in humans [20]. Furthermore, similar to humans, hens of different genetic background (strains) have different rates of OVCA incidence [21]. However, this information will be of limited value in clinical settings as none of the reports described these OVCA-associated features in hens relative to tumor types and their stages. No detailed report on the tumor stages and histopathological features including tumor types in hens with OVCA and their similarities to human OVCA is available.

Tumor staging describes the extent and generally the severity of malignant tumors. Staging information helps in determining intervention strategies and their prognosis. Similar to staging, tumor histology plays an important role in the diagnosis and treatment of ovarian cancer in humans. The difference in the treatment and management of the different types of tumors underscores the importance of the correct histological diagnosis. Because laying hens are being considered as a feasible preclinical model for testing emerging chemotherapeutic agents, the precise identification of tumor types and their stages will be important for determining drug efficacy. In addition, most of the studies on hen OVCA were performed without distinguishing ovary from oviduct as the primary site. This further corroborates the urgency of a detailed study distinguishing the origin of hen OVCA, tumor types and their stages. All this information will be of enormous help to understand the etiology, pathophysiology, drug testing, and design treatment regimen of OVCA and will form the basis for clinical studies.

Laying hens may also offer an invaluable opportunity to explore the putative precursor lesions related to OVCA. The study of the precursor(s) of ovarian carcinoma in humans is complicated because the ovaries are not readily accessible for screening. Ovarian carcinomas are often large and present in advanced stage, obliterating or rendering unrecognizable any precursor that may have been present. Therefore, the goals of this exploratory study were to classify the histological types of malignant ovarian tumors and their stages in spontaneous OVCA in laying hens. Additionally, putative precursor lesions of OVCA in hens were also examined.

Materials and Methods

Animals: A total of 155 young (n=14, 1 to 1.5 years old with more than 5 eggs in a sequence) and old (n=141, 2 to 5 years old with 3-5 eggs in sequence) Single Comb White Leghorn laying hens (W/96 strain) were reared at the University of Illinois at Urbana-Champaign (UIUC) Poultry Research Farm. Hens were provided with commercial layer ration and water *ad libitum* and kept under 14h: 10h light and dark regimen. Egg production and mortality rates were recorded on a daily basis. All animal handling and husbandry practices were performed according to the Institutional Animal Care and Use Committee approved protocol.

Gross evaluation: Hens were examined for abnormal ovarian morphology upon euthanasia and following features were noted:

a) Primary ovarian carcinomas: To distinguish carcinomas of ovarian origin from that of oviductal origin, oviducts of hens were excised and the mucosal layer (inner layer) was examined for the presence of solid tissue masses or tumors. Large tumors and small nodules of solid masses of various sizes were seen in the oviducal mucosal layer in some hens (Fig. 3b and c) and these hens were excluded from the study. Metastatic carcinomas to the ovary were identified by the presence of intact large preovulatory hierarchical follicles. In hens with cancers metastasized to ovary, large preovulatory follicles were mostly unaffected indicating that the entire ovary was not involved by tumor (metastatic) and egg production in these hens was normal. These hens were also excluded from the study.

b) Tumor staging: Staging of the tumors was performed with reference to the International Federation of Gynecology and Obstetrics (FIGO, Rio de Janeiro 1988) [22] staging Systems for Ovarian Cancer in Humans (Table 1).

c) Non-tumor ovarian pathology: Non-tumor ovarian abnormalities were characterized by cysts, atretic large preovulatory follicles and regressed ovaries.

Human subjects: Ovarian tissues were obtained from patients undergoing prophylactic surgery for a family history of breast cancer or ovarian cancer without any gross ovarian abnormalities at Rush University Medical Center. All protocols were performed according to the IRB approved protocol and with the patient's written informed consent.

Microscopic Pathology

Tumor, non-tumor abnormal and normal ovaries of hens as well as ovarian tissues of women were collected and immediately fixed in 10% buffered formalin. Tissues were processed for routine histology. Sections of 5 μm thickness were cut and stained with hematoxylin and eosin and examined under light microscopy. Tumor types were classified according to the WHO criteria used for human OVCA.

Results

Ovaries of young and some of the old laying hens were more functional as determined by their egg laying rates. Some old hens had reduced ovarian function and laid fewer eggs irregularly whereas some other old hens ceased egg laying. Physical examination before euthanasia revealed that most of these hens had a distended abdomen which suggested the presence of ascites.

Gross evaluation

Non-tumor ovarian abnormalities: The ovaries of all young (n= 14) and old hens with a higher egg laying rate (n=89, with normal ovaries) had a set of 5 or more large preovulatory hierarchical

follicles without gross abnormality of any organ including the ovary and oviduct (Fig. 1A). Occasionally, ovulated ova were present in the oviducts. These hens showed no abnormality in the mucosal layers of the oviducts and therefore were considered healthy and normal. Non-tumor ovarian abnormalities in hens (n= 14) including regressed reproductive tract, atresia of large preovulatory hierarchical follicles and polycystic ovarian abnormalities were determined based on their gross appearance and not on histology. In hens with regressed reproductive tracts, both the ovary and oviduct were fully regressed though the ovaries in some hens contained a few small follicles (Fig. 1B). Although regression and rejuvenation of the reproductive tract are common physiological phenomena in hens, regression may also be a consequence of ovarian carcinoma *in situ*. In some hens, all of the large preovulatory follicles were hemorrhagic and atretic (Fig. 1C). Although atresia of stromal follicles is a natural event both in aves and mammals, atresia of large preovulatory hierarchical follicles is an abnormal condition in laying hens. Multiple cysts of various sizes were seen in the ovary of some laying hens (Fig.1D). Although the presence of one or two cysts in the normal ovaries are not rare but the presence of multiple cysts indicates ovarian abnormality.

Primary ovarian carcinomas and their staging in hens: Primary ovarian carcinomas were distinguished from that of secondary carcinoma to the ovary using the criteria described in the Methods section. Staging of ovarian carcinomas in hens was performed with reference to the FIGO system for human OVCA with emphasis on: location of tumors, presence or absence of metastasis and peritoneal ascites. Similar to humans, all four stages (stage I to stage IV) of OVCA were seen in hens (n=30). In hens with Stage I (n=5) OVCA, tumors were confined to the ovary, appeared firm and resembled cauliflower-like nodules with no, or minimal ascites (because the hen ovary is not covered by a capsule as in humans, ascites may leak to the

peritoneal cavity at Stage I) (Fig. 2A). In hens with Stage II (n=5) OVCA, tumors were metastasized to the oviduct with occasional seeding to the pelvic sidewall with moderate ascites (Fig. 2B). In hens with Stage III (n=13) OVCA, tumors were metastasized to both abdominal and peritoneal organs including small and large intestine, mesentery, undersurface of the diaphragm and surface of the liver with moderate to profuse ascites (Fig. 2C). At the time of necropsy, multiple hens had evidence of carcinomatosis and massive ascites consistent with Stage IV (n=7). Tumors at this stage were metastasized to most of the pelvic, abdominal and thoracic organs including liver, spleen and lung (Fig. 2D).

Metastatic tumor to the ovary (secondary ovarian cancer): In most of the cases, primary ovarian cancers in hens were associated with atresia of large preovulatory hierarchical follicles. In the present study 8 hens had secondary ovarian carcinoma. In hens with primary gastrointestinal (GI, 3 hens) cancer, only a portion of the ovary appeared solid while the large preovulatory follicles remained uninvolved and the hens were laying regularly (Fig. 3A). The GI tracts in these hens were hardened, coiled, and tumor seeding and masses were found both outside and inside the wall of the tract. In 3 hens, oviducal tumors had metastasized to the ovary and large preovulatory follicles had become atretic appearing as hemorrhagic spots. These tumors had solid masses both on the exterior wall (serous like) and in the mucosal layers of uterus and some seeding on the upper part of the reproductive tract (Fig. 3B). In addition, early oviductal tumors were identified in the excision of oviductal mucosa and they (in 2 hens) were incidental findings (Figure 3C).

Histology

Primary ovarian epithelial carcinomas: Epithelial ovarian carcinomas were classified based on the cellular subtypes and patterns of cellular differentiation with reference to OVCA tumor types

in humans. Four histological types of ovarian malignant tumors resembling those of human OVCA (n=26; 7 *serous,* 8 *endometrioid*; 9 *mucinous*; 2 *clear cell*) showing low (G1) or high (G2-3) morphology were observed. Well differentiated tumors with features similar to serous ovarian carcinomas in human had marked nuclear atypia and papillary structures. In most cases the architecture was characterized by labyrinth of slit like glands or lacelike papillary folding with large pleomorphic nuclei containing mitotic figures (Fig. 4A). Some of these tumors displayed papillae-like features with fibrovascular cores lined by atypical epithelial cells. Tumors resembling human endometrioid carcinomas were generally characterized by a complex glandular architecture, cribriform foci and nuclear polymorphism with a brisk mitotic rate. The glands contained a single layer of epithelial cells with mitosis, sharp luminal margins (Fig. 4B). A few cases showed glands lined by columnar epithelium with apparent cytoplasmic mucin compatible with mucinous differentiation. Features reminiscent of human ovarian mucinous carcinomas were observed in hens as crowded glands merged together without intervening stroma forming clusters surrounded by a fibromascular layer. The tumor contained columnar epithelium with intercalated ciliated goblet cells. The nuclei were separated from the basement membrane and had moved towards the apical surface with occasional stratification and luminal secretion (Fig. 4C). Several hen tumors displayed “clear cell like” features with solid, tubulopapillary or their mixture. The solid pattern was characterized by sheets of polyhedral cells with abundant clear cytoplasm separated by delicate fibromuscular septae or dense hyalinized fibrotic stroma. Large cells with clear cytoplasm and vesicular, pleomorphic nuclei with large prominent nucleoli were also observed (Fig. 4D). One hen showed a germ cell tumor.

Poorly differentiated ovarian carcinoma: In addition to low grade (well differentiated) carcinomas, high grade (moderate to poorly differentiated) ovarian tumors were also seen in hen

ovaries albeit with low frequency. Poorly differentiated carcinoma with serous-like features displayed extensive solid areas composed of fibromuscular sheets and an occasional slit containing cells with marked nuclear pleomorphism (Fig. 5A). A few tiny glands were present without any papillae. Poorly differentiated endometrioid like carcinomas were characterized by a solid growth pattern with complex glands and microglandular foci (Fig. 5B). Nuclear polymorphism, mitotic activity and necrosis were marked. Poorly differentiated "mucinous like" carcinomas were characterized by confluent microglandular architecture in a cribriform pattern with marked nuclear atypia and no intervening stroma (Fig. 5C). Several marked eosinophilic foci were also characteristic features of these tumors. In poorly differentiated cancers with "clear cell like" features, vacuolated cells containing pleomorphic nuclei and a brisk mitotic rate invaded the stroma and theca layer of stromal follicles (Fig. 5D). Deposition of eosinophilic hyalinized matrix in the stroma was also present.

Mixed epithelial carcinomas: Malignant mixed tumors (n=4) of two epithelial cell types were also identified. Although "serous" and endometrioid mixed ovarian carcinomas were present in a few hens, "mucinous" and endometrioid or "mucinous" and clear cell mixed carcinomas were more frequent (micrographs are not shown).

Early neoplastic progression: In some hens (n=9) with regressed ovaries, microscopic examination showed microscopic changes consistent with nascent neoplasia and malignant progression leading to tumor development (Fig. 6A-B). These microscopic carcinomas were unanticipated because there were no gross abnormalities. Focal lesions were formed in the stroma below the ovarian surface and appeared as a solid sheet of condensed granules with eosinophilic staining (Fig. 6B). Small cysts with or without developing outpouches and

developing glandular structures with a single layer of epithelial cells with pleomorphic nuclei similar to endometrioid tumors are seen inside the focal lesions in the ovarian stroma of some hens (Fig. 6C-F).

Putative precursor lesions: Microscopic evaluations of the ovaries of some hens (n=5) with non-tumor ovarian abnormalities (no grossly visible tumor) revealed a spectrum of histological abnormalities which are similar to those observed in the vicinity of malignant ovaries. These microscopic abnormalities were considered tumor associated putative precursor lesions and compared with human prophylactic ovarian samples.

a) Transformation of surface epithelial layer: Similar to the surface epithelial layer in the human ovary (Fig. 7A), the normal ovarian layer in hens also consists of a single layer of columnar epithelial cells. However, these columnar epithelial cells in hens with non-tumor ovarian abnormalities had a rounded phenotype with mitotic nucleus consistent with a potential to become malignant (Figure 7B).

b) Stromal invagination and surface epithelial dysplasia: Epithelial dysplasia was seen in the surface layer of stromal invaginations in human ovaries obtained from patients who had undergone prophylactic hysterectomy without any ovarian abnormality (Fig. 7C). Similarly, marked epithelial dysplasia was also seen in some ovaries of hens with non-tumor ovarian abnormalities (Fig. 7D).

c) Stromal inclusion cysts: Simple glands lined by a single layer of columnar epithelial cells were seen in the ovarian cortex of human prophylactic oophorectomy specimens (Fig. 7E). Similar inclusion cysts were also seen in the cortex beneath the ovarian surface in hens with non-

tumor ovarian abnormalities. However, the epithelial cells in the inclusion cysts in hens were rounded instead of columnar as in humans (Fig.7F).

Discussion

This report is the first detailed and comprehensive review of the histological types and characteristic features of OVCA staging in laying hens, a spontaneous model for human OVCA. Some of the putative preneoplastic ovarian lesions in laying hens were also demonstrated in this study. The findings of the present study show remarkable similarities between OVCA in human and hens regarding the histological types and stages of epithelial tumors of the ovary as well as their putative precursor lesions.

Dissimilarities in the histopathology of OVCA between rodents and humans limit the use of rodents as an animal model of human OVCA which is also the reason for exploring new animal models in which OVCA has a similar histopathology to those seen in humans. Similarities in the association of OVCA with circulating anti-tumor antibodies [23] and the similar expression of some OVCA markers between hens and humans [14, 15] has led us to study whether histological types of hen ovarian tumors resemble those of humans. Four histological types of hen OVCA including: serous, endometrioid, mucinous, clear cell and their differentiation (Grades 1, 2, 3) are somewhat similar to their human counterparts. Ovarian tumors of mixed histopathology (two histotypes in the same specimen) were also observed in some hens. Similarities in tumor histology will facilitate the use of laying hens to improve our understanding of tumor biology in humans, explore new drugs to develop treatment modalities or to improve existing ones. Moreover, the ovary is a complex organ and its tumor types are varied. Because ovarian tumors are relatively uncommon and include several types, they are difficult to diagnose

without proper experience. Thus the histologic diagnosis may therefore be compromised. Therefore, hen ovarian tumors, in addition to preclinical drug testing may also contribute to our comprehension of OVCA histopathology and diagnosis of OVCA as they are similar to humans.

Similar to histological types, tumor staging plays a key role in devising the treatment path and much is unknown about the specifics of effective drug therapy in relation to OVCA stages. In the current study, hen ovarian tumors were staged according to the FIGO classification for humans. Similar to humans, all four stages (stage I to stage IV) of tumor progression ranging from confinement in the ovary to distant metastases were observed in hens with OVCA. One of the most intriguing similarities between hen and humans is the association of advanced stage OVCA with profuse ascites. Because the laying hen has only one functional ovary, the staging criteria relative to the contra-lateral ovary in humans is not applicable in hens. Nonetheless, the laying hen can be utilized to determine the stage related efficacy and specificity of drugs with their prognostic value and can constitute the basis of clinical studies.

The discovery of microscopic malignant tumors in regressed ovaries (which functionally resemble the postmenopausal ovary in women) was of interest. These tumors were not anticipated and not uncovered until extensive microscopic examination of sections made from all areas of the ovary. This observation suggests that a perfunctory analysis of prophylactically removed ovaries could, in some cases, fail to detect small malignant tumors. If such were the case and these tumors had acquired early metastasized potential, cells that metastasized from them could explain the discovery of peritoneal carcinomatosis subsequent to prophylactic oophorectomy [24-26]. These abnormalities can be identified and treated in the early stages of carcinogenesis, in order to prevent the development of invasive cancer.

One of the challenges related to the early detection and prevention of ovarian cancer has been the uncertainty as to whether a premalignant or precursor lesion in the pathway to the development of clinical OVCA exists. In other organ systems, such lesions or well-defined series of morphologic changes are recognized to occur that are critical to the success of early detection programs (e.g., cervical intraepithelial neoplasia for carcinoma of uterine cervix, ductal carcinoma *in situ* for breast carcinoma and advanced adenomatous polyps in colorectal cancer) [27-29]. The candidates for the precursors of ovarian cancer include epithelial dysplasia of the surface epithelium or germinal inclusion cysts. Alternatively, carcinomas could also arise directly from the surface epithelium without an intermediate precursor lesion [25]. It is conceivable that all these mechanisms account for ovarian carcinomas. As reported in humans, a series of putative precursor lesions like surface epithelial transformation, inclusion cysts and epithelial dysplasia were seen in hens in the present study. The identification of a premalignant lesion may improve the effectiveness of early detection screening. Therefore, the laying hen may also provide a better understanding of the putative precursor lesions leading to OVCA.

The controversy regarding the existence of morphologic precursors may in a large part be due to the fact that ovarian cancer is most frequently diagnosed at a late stage. Hence, the opportunity to examine a large number of early stage ovarian cancers where it might be possible to see these changes repeatedly and document a consistent pattern of transition between benign and malignant ovarian surface epithelium is rare. This lack of information in turn has obviously constrained our understanding as to the most frequent sequence of morphologic changes that occur as clinical ovarian cancers develop. Moreover, the morphological precursors of clinical ovarian cancer in humans are not well established. Previous investigations with OVCA patients aimed at defining the types of lesions that lead to ovarian cancer have involved a variety of

approaches, including the examination of the contralateral ovaries in patients with unilateral ovarian cancer or ovaries that contain stage I tumors [30-32]. We believe this study complements previous investigations that some precursor lesions precede OVCA and cancers of other organs in humans as well as in hens. Through access to hens, their ovaries can be examined *in vivo* where there is a very high probability that malignant transformation will occur sometime during the animal's lifetime. For the first time, we developed *in vivo* imaging of hen ovaries by transvaginal grey scale and Doppler ultrasound which allows us to detect very early lesions based on their Doppler blood flow velocity indices [33]. Therefore, the laying hen also offers a unique opportunity for preclinical development of an effective early detection of OVCA by evaluating changes in the tissue morphology in association with changes in ovarian vascularity.

One of the few limitations of this study is that hens with a low egg laying rate were selected and thus this study did not represent a totally blinded study. From our previous experience, we know that hens with reduced (or ceased) egg production are more prone to develop primary OVCA than those of high laying rate. This study was not intended to report the incidence rate of spontaneous OVCA in hens but our goal was to define the histological types of ovarian tumors and their stages. Therefore, we decided to obtain as many hens with potential OVCA as possible. In addition, we could not clearly distinguish all the sub-stages within a stage in hens as can be done in humans. One of the reasons for this is that laying hens do not possess a right ovary and hence staging (sub-stages of Stage I and II) in relation to the status of the contra-lateral ovary is not possible. Moreover, the lymph nodes in chicken are not as well organized as in humans and hence the nodal involvement in hen OVCA metastasis was not confirmed. Nevertheless these limitations do not reduce the feasibility of this model because sub-stages within a stage do not constitute significant differences either in diagnosis or drug efficacy.

In conclusion, this study confirmed that ovarian cancer in hens occurs spontaneously and demonstrated that their histological types as well as stages are similar to humans. This study additionally showed that similar to humans, several precursor lesions also exist in hens. The similarity in histology, metastasis and stages of hen OVCA to those of humans demonstrates the feasibility of the hen model for additional delineation of the mechanism underlying ovarian carcinogenesis. The laying hen model could be used for preclinical testing of new agents for the prevention and therapy of this disease. Thus this study will contribute to the establishment of laying hen as the preclinical model of human ovarian cancer.

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References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007;**57:**43-66.
2. Goodman MT and Howe HL. Descriptive epidemiology of ovarian cancer in the United States, 1992-1997. *Cancer* 2003;**97(10 Suppl):**2615-30.
3. Goodman MT, Correa CN, Tung KH, Roffers SD, Cheng Wu X, Young JL, Jr., Wilkens LR, Carney ME, Howe HL. Stage at diagnosis of ovarian cancer in the United States, 1992-1997. *Cancer*, 2003;**97(10 Suppl):**2648-59.
4. Ries LA. Ovarian cancer. Survival and treatment differences by age. *Cancer*, 1993; **71(2 Suppl):**524-9.
5. Bast RC, Jr., Urban N, Shridhar V, Smith D, Zhang Z, Skates S, Lu K, Liu J, Fishman D, Mills G. Early detection of ovarian cancer: promise and reality. *Cancer Treat Res*, 2002;**107:**61-97.

6. Pepe MS, Etzioni R, Feng Z, Potter JD, Thompson ML, Thornquist M, Winget M, Yasui Y. Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst*, 2001;**93:**1054-61.
7. Holschneider CH and Berek JS. Ovarian cancer: epidemiology, biology, and prognostic factors. *Semin Surg Oncol*, 2000;**19:**3-10.
8. Stakleff KD and Von Gruenigen VE. Rodent models for ovarian cancer research. *Int J Gynecol Cancer*, 2003; **13:**405-12.
9. Vanderhyden BC, Shaw TJ, Ethier JF. Animal models of ovarian cancer. *Reprod Biol Endocrinol*, 2003;**1:**67.
10. Damjanov, I. Ovarian tumours in laboratory and domestic animals. *Curr Top Pathol*, 1989;**78:**1-10.
11. Fredrickson, TN. Ovarian tumors of the hen. *Environ Health Perspect*, 1987;**73:**35-51.
12. Bahr, JM and Palmar SS. The influence of aging on ovarian function. *CRC Critical Reviews in Poultry Biology*, 1989;**2:**103-10.
13. Robinson, FE and Etches RJ. Ovarian steroidogenesis during follicular maturation in the domestic fowl (Gallus domesticus). *Biol Reprod*, 1986;**35:**1096-105.
14. Rodriguez-Burford, C, Barnes MN, Berry W, Partridge EE, Grizzle WE. Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention. *Gynecol Oncol*, 2001;**81:**373-9.
15. Jackson, E, Anderson K, Ashwell C, Petitte J, Mozdziak PE. CA125 expression in spontaneous ovarian adenocarcinomas from laying hens. *Gynecol Oncol*, 2007;**104:**192-8.
16. Barnes, MN, Berry WD, Straughn JM, Kirby TO, Leath CA, Huh WK, Grizzle WE, Partridge EE. A pilot study of ovarian cancer chemoprevention using medroxyprogesterone acetate in an avian model of spontaneous ovarian carcinogenesis. *Gynecol Oncol*, 2002;**87:**57-63.
17. Ness, RB, Cramer DW, Goodman MT, Kjaer SK, Mallin K, Mosgaard BJ, Purdie DM, Risch HA, Vergona R, Wu AH. Infertility, fertility drugs, and ovarian cancer: a pooled analysis of case-control studies. *Am J Epidemiol*, 2002;**155:**217-24.
18. Runnebaum, IB and Stickeler E. Epidemiological and molecular aspects of ovarian cancer risk. *J Cancer Res Clin Oncol*, 2001;**127:**3-9.
19. Terry, KL, De Vivo I, Titus-Ernstoff L, Sluss PM, Cramer DW. Genetic variation in the progesterone receptor gene and ovarian cancer risk. *Am J Epidemiol*, 2005;**161:**442-51.
20. Murdoch, WJ, Van Kirk EA, Alexander BM. DNA damages in ovarian surface epithelial cells of ovulatory hens. *Exp Biol Med (Maywood)*, 2005;**230:**429-33.
21. Johnson, PA and Giles JR. Use of genetic strains of chickens in studies of ovarian cancer. *Poult Sci*, 2006;**85:**246-50.
22. Pecorelli S. FIGO Annual report on the results of treatment in gynecological cancer. *J. Epidemiol Biostat*, 1998;**3:**1-168.
23. Barua, A, Bradaric MJ, Edassery SL, Sharma S, Rothmensch J, Bitterman P, Hales DB, Bahr JM, Luborsky JL. Anti-tumor antibodies and ovarian cancer in women and hens. *American Association of Cancer Research: Molecular Diagnostics in Cancer Therapeutic Development, Maximizing Oppotunities for Individualized Treatment, Sept. 12-15, 2006, Chicago, IL:* ***Scholar-in-Training Award Winner****,* *(http://www.aacrmeetingabstracts.org/cgi/content/abstract/2006/2/A40)*, 2006. **A**: 40.

24. Tobacman, JK, Greene MH, Tucker MA, Costa J, Kase R, Fraumeni JF, Jr. Intra-abdominal carcinomatosis after prophylactic oophorectomy in ovarian-cancer-prone families. *Lancet*, 1982;2: 795-7.
25. Salazar, H, Godwin AK, Daly MB, Laub PB, Hogan WM, Rosenblum N, Boente MP, Lynch HT, Hamilton TC. Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. *J Natl Cancer Inst*, 1996;**88:**1810-20.
26. Chen, KT, Schooley JL, Flam MS. Peritoneal carcinomatosis after prophylactic oophorectomy in familial ovarian cancer syndrome. *Obstet Gynecol*, 1985;**66(3 Suppl):**93S-94S.
27. Fearon, ER and Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*, 1990;**61:**759-67.
28. Castilla, LH, Couch FJ, Erdos MR, Hoskins KF, Calzone K, Garber JE, Boyd J, Lubin MB, Deshano ML, Brody LC, et al. Mutations in the BRCA1 gene in families with early-onset breast and ovarian cancer. *Nat Genet*, 1994;**8:**387-91.
29. Wright TC, Kurman RJ, Ferenczy A. Precancerous lesion of the cervix. In: Kurman RJ ed *Blaustein's Pathology of the female genetal tract* 5th ed. NY: Springer, 2001:253-324.
30. Mittal, KR, Zeleniuch-Jacquotte A, Cooper JL, Demopoulos RI. Contralateral ovary in unilateral ovarian carcinoma: a search for preneoplastic lesions. *Int J Gynecol Pathol*, 1993;**12:**59-63.
31. Resta, L, Russo S, Colucci GA, Prat J. Morphologic precursors of ovarian epithelial tumors. *Obstet Gynecol*, 1993;**82:**181-6.
32. Deligdisch, L, Einstein AJ, Guera D, Gil J. Ovarian dysplasia in epithelial inclusion cysts. A morphometric approach using neural networks. *Cancer*, 1995;**76:**1027-34.
33. Barua, A, Abramowicz JS, Bahr JM, Bitterman P, Dirks A, Holub KA, Sheiner E, Bradaric MJ, Edassery SL, Luborsky JL. Detection of ovarian tumors in chicken by sonography: a step toward early diagnosis in humans? *J Ultrasound Med*, 2007;**26:**909-19.

Figure legends

Figure 1: Ovarian morphology in laying hens without obvious tumors. A) Ovary of a normally laying hen. The ovary contains a set of five large preovulatory hierarchical (see the text for terminology) follicles (F1-F5) and small developing follicles. B) Ovary of a hen that ceased laying. As compared to the normal ovary, no large preovulatory hierarchical follicles are seen although a few small follicles are present. The oviduct also regressed in size significantly. The regressed ovary and oviduct indicate that ovarian function is reduced and this hen is out of egg laying. C) Ovarian failure in a laying hen. Although the ovary contains large preovulatory hierarchical follicles, all of them are either hemorrhagic or atretic. The oviduct appears normal. D) Polycystic Ovary Syndrome in a laying hen. Although some preovulatory follicles are seen, multiple cysts of varying size with or without hemorrhagic spots are present.

Figure 2: Primary malignant ovarian tumors in hens. A) Stage I ovarian cancer. The tumor is limited to the ovary only with no large preovulatory hierarchical follicles. The solid tissue mass resembles a cauliflower without any noticeable ascites. B) Stage II primary ovarian cancer. The tumor is metastasized to the oviduct with a little ascites but other organs appear uninvolved (asterisk). C) Stage III primary ovarian cancer. The tumor is metastasized to abdominal organs including the gastrointestinal tract accompanied by profuse ascites (not seen in the figure). White and greenish tumor seeding is seen on the superficial layer of liver (arrows). D) Stage IV ovarian cancer. The tumor is metastasized to distant organs with profuse ascites. Multiple solid tumor masses are seen.

Figure 3: Tumor metastasized to the ovary in hens. A) Tumor of gastrointestinal tract (GI) metastasized to the ovary. Although tumor growth and seeding are seen in the intestine, all the

large preovulatory follicles remain uninvolved and no ascites is seen. B) A case of oviductal tumor metastasized to the ovary. A solid tumor mass is seen in the uterus with the malignant seeding on the upper part of the tract, intestine and the ovary with the large preovulatory follicle atretic with hemorrhagic spots. C) Early tubal tumor in the reproductive tract. Tumor nodules of various sizes are seen in the magnum (egg albumen secreting part) of the oviduct.

Figure 4: Histological types of well differentiated malignant ovarian tumors in hens. A) Ovarian "serous carcinoma" showing sheets of lacelike papillary folding and cells with large pleomorphic nuclei with mitotic bodies. B) Ovarian endometrioid carcinoma in hens with confluent back to back glands. Glands contain a single layer of epithelial cells with sharp luminal margin. C) Ovarian "mucinous carcinoma" with crowded glands in clusters without intervening stroma surrounded by a fibromuscular layer. The epithelium contains columnar and intercalated ciliated goblet cells. The nuclei are separated from the basement membrane and have moved towards the apical surface with occasional stratification and luminal secretion. D) Ovarian "clear cell carcinoma". The tumor contains a solid sheet of polyhedral cells with abundant clear cytoplasm and dense hyalinized fibrotic stroma. Large cells with clear cytoplasm and vesicular, pleomorphic nuclei with large prominent nucleoli are also observed. H &E, 40X.

Figure 5: Histological types of poorly differentiated ovarian epithelial carcinoma in hens. A) Poorly differentiated ovarian "serous carcinoma" showing solid areas composed of slit like sheets containing cells with high-grade nuclear atypia. A few tiny glands are also seen without any papillae. B) Poorly differentiated ovarian endometrioid carcinomas showing complex glandular and microglandular patterns. Nuclear polymorphism, mitotic activities and necrosis are marked. C) Poorly differentiated ovarian mucinous carcinomas showing confluent

microglandular architecture in a cribriform pattern with a high grade of nuclear atypia and no intervening stroma. Moderate to strong eosinophic reactions are also seen in the stroma. D) Poorly differentiated ovarian clear cell carcinoma showing vacuolated cells containing high grade nuclear atypia that invade the stroma and theca layer of stromal follicles. Deposition of eosinophilic hyalinized matrix in the stroma and necrotic bodies are also seen. H &E, 40X.

Figure 6: Early neoplastic progression leading to ovarian carcinoma in hen. A) Focal lesions are seen in the stroma below the ovarian surface and appear as a solid sheet of condensed granules with eosinophilic stain. B-D) Cysts developed from focal lesions are seen as dividing cells and out pouches. E) Multiple cystic division and formation of additional condensed eosinophilc glands. F) Cysts lined with a single layer of epithelial cells arising from focal lesions appear to be an early stage of ovarian endometrioid neoplasm with pleomorphic nuclei neoplasm. H &E, 40X.

Figure 7: Putative precursor lesions of ovarian carcinoma. A) Ovarian section of a normal human ovary showing the columnar surface epithelial layer. B) Section of hen ovary with non-tumor abnormalities showing transformation of the ovarian surface epithelial layer from single columnar epithelial cells to a rounded phenotype with mitotic nuclei consistent with a malignant potential. Inset showing magnified (120X) view of transformed and normal surface epithelial cells C) Section from a normal human ovary of a patient undergoing prophylactic surgery showing stromal invagination and surface epithelial dysplasia (arrow). D) Section of hen ovary with non-tumor abnormalities. Marked epithelial dysplasia is seen in the surface layer with stromal invaginations. E) Normal human ovary from a prophylactic oophorectomy patient showing stromal inclusion cysts. Simple glands lined by a single layer of columnar epithelial

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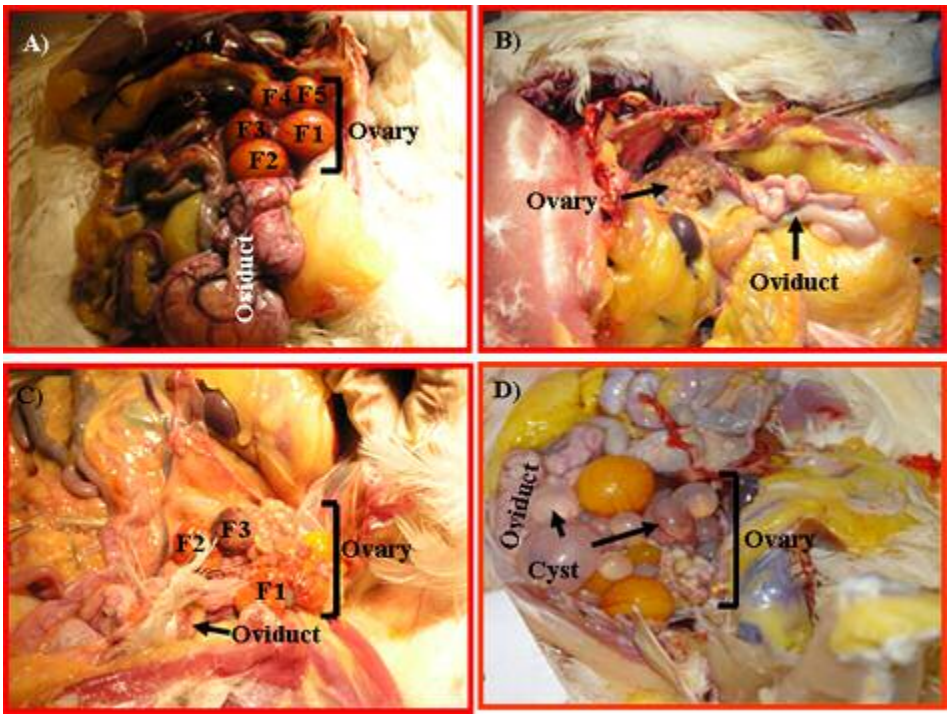

Figure 1: Ovarian morphology in laying hens without obvious tumors. A) Ovary of a normally laying hen. The ovary contains a set of five large preovulatory hierarchical (see the text for terminology) follicles (F1-F5) and small developing follicles. B) Ovary of a hen that ceased laying. As compared to the normal ovary, no large preovulatory hierarchical follicles are seen although a few small follicles are present. The oviduct also regressed in size significantly. The regressed ovary and oviduct indicate that ovarian function is reduced and this hen is out of egg laying. C) Ovarian failure in a laying hen. Although the ovary contains large preovulatory hierarchical follicles, all of them are either hemorrhagic or atretic. The oviduct appears normal. D) Polycystic Ovary Syndrome in a laying hen. Although some preovulatory follicles are seen, multiple cysts of varying size with or without hemorrhagic spots are present.

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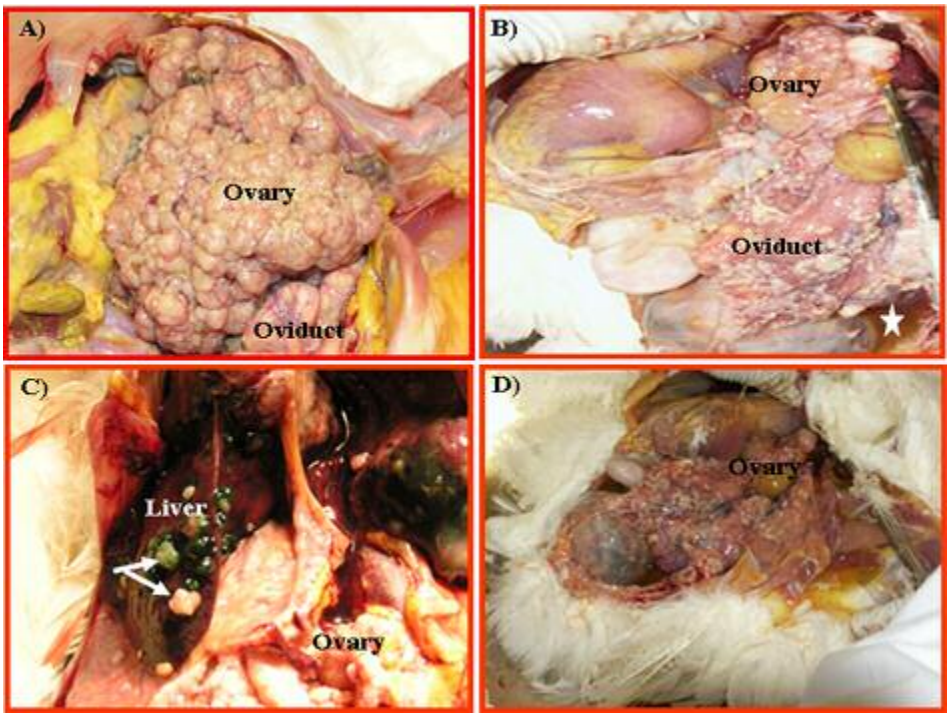

Figure 2: Primary malignant ovarian tumors in hens. A) Stage I ovarian cancer. The tumor is limited to the ovary only with no large preovulatory hierarchical follicles. The solid tissue mass resembles a cauliflower without any noticeable ascites. B) Stage II primary ovarian cancer. The tumor is metastasized to the oviduct with a little ascites but other organs appear uninvolved (asterisk). C) Stage III primary ovarian cancer. The tumor is metastasized to abdominal organs including the gastrointestinal tract accompanied by profuse ascites (not seen in the figure). White and greenish tumor seeding is seen on the superficial layer of liver (arrows). D) Stage IV ovarian cancer. The tumor is metastasized to distant organs with profuse ascites. Multiple solid tumor masses are seen.

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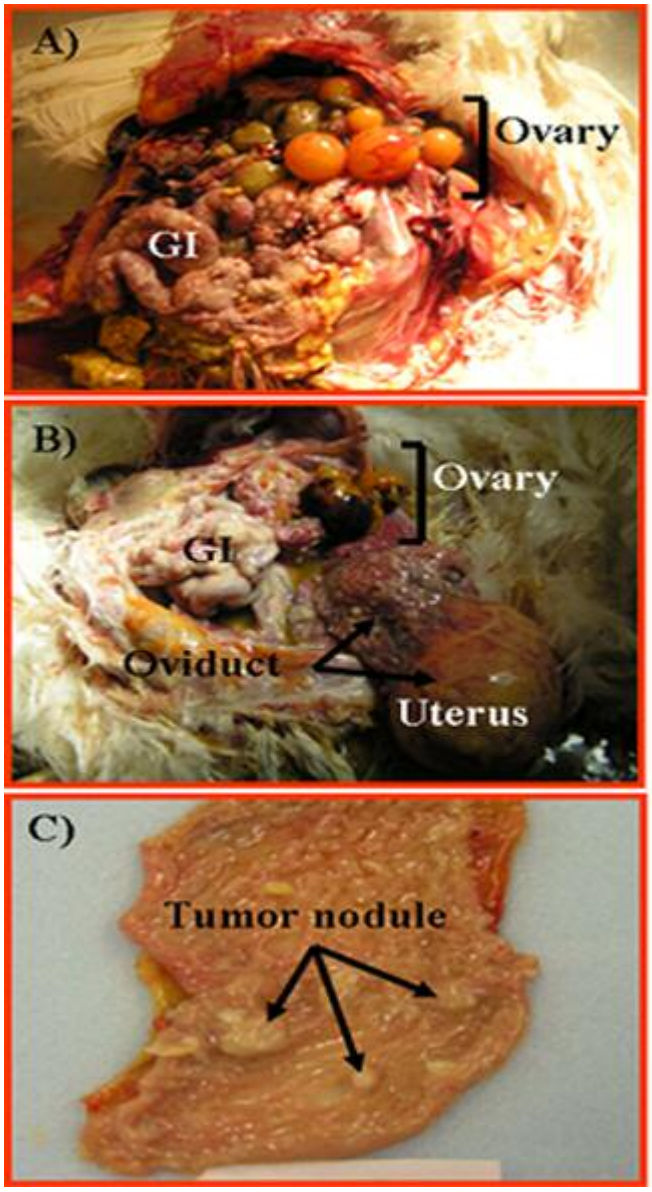

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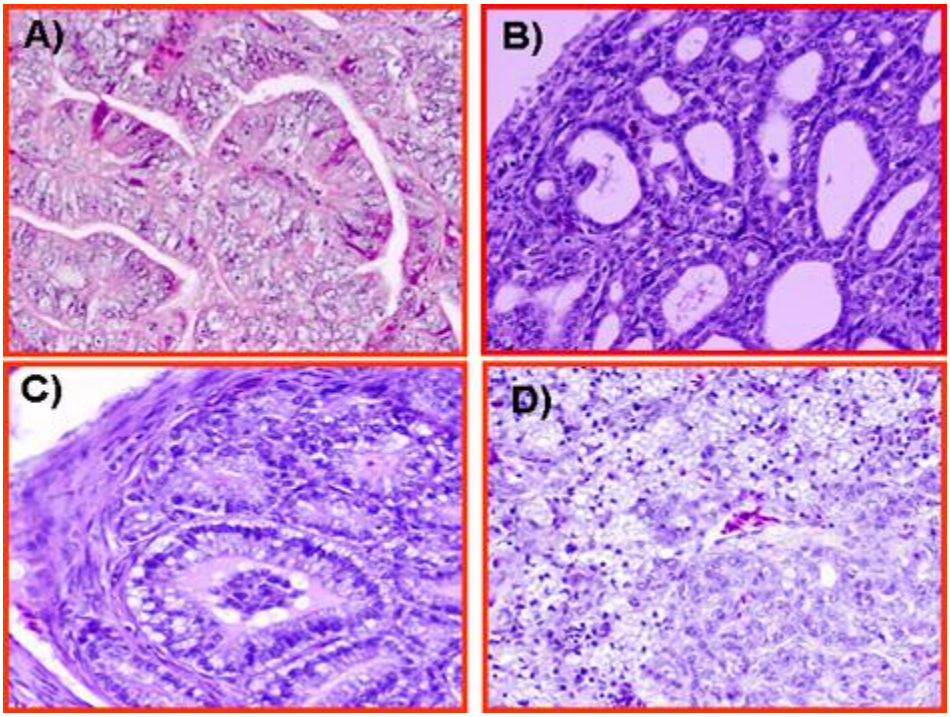

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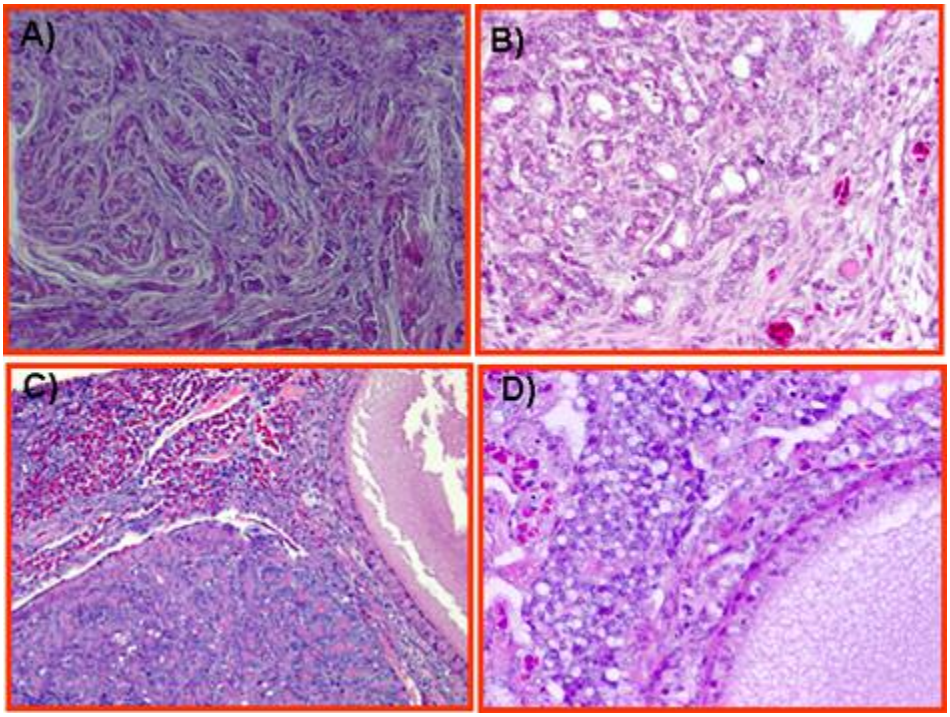

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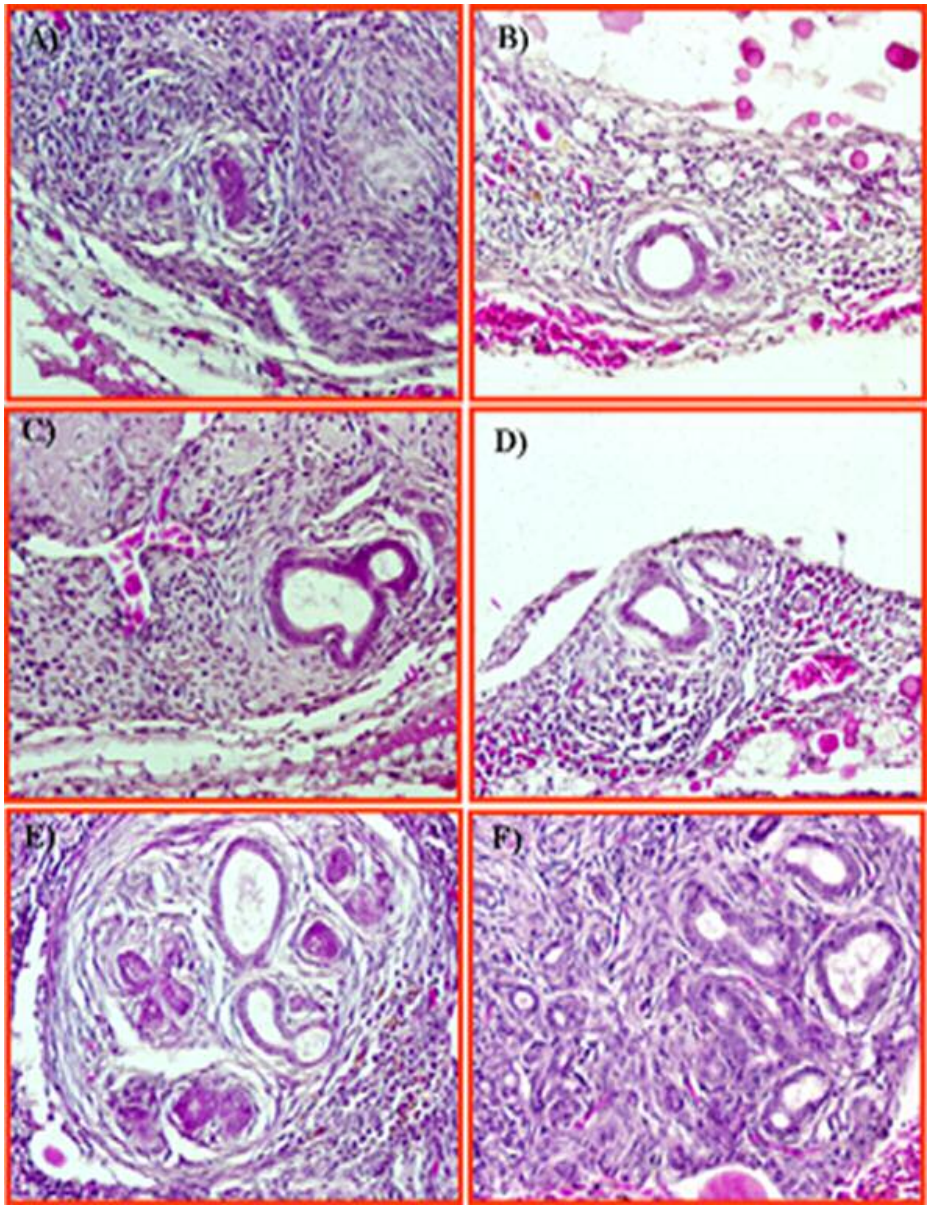

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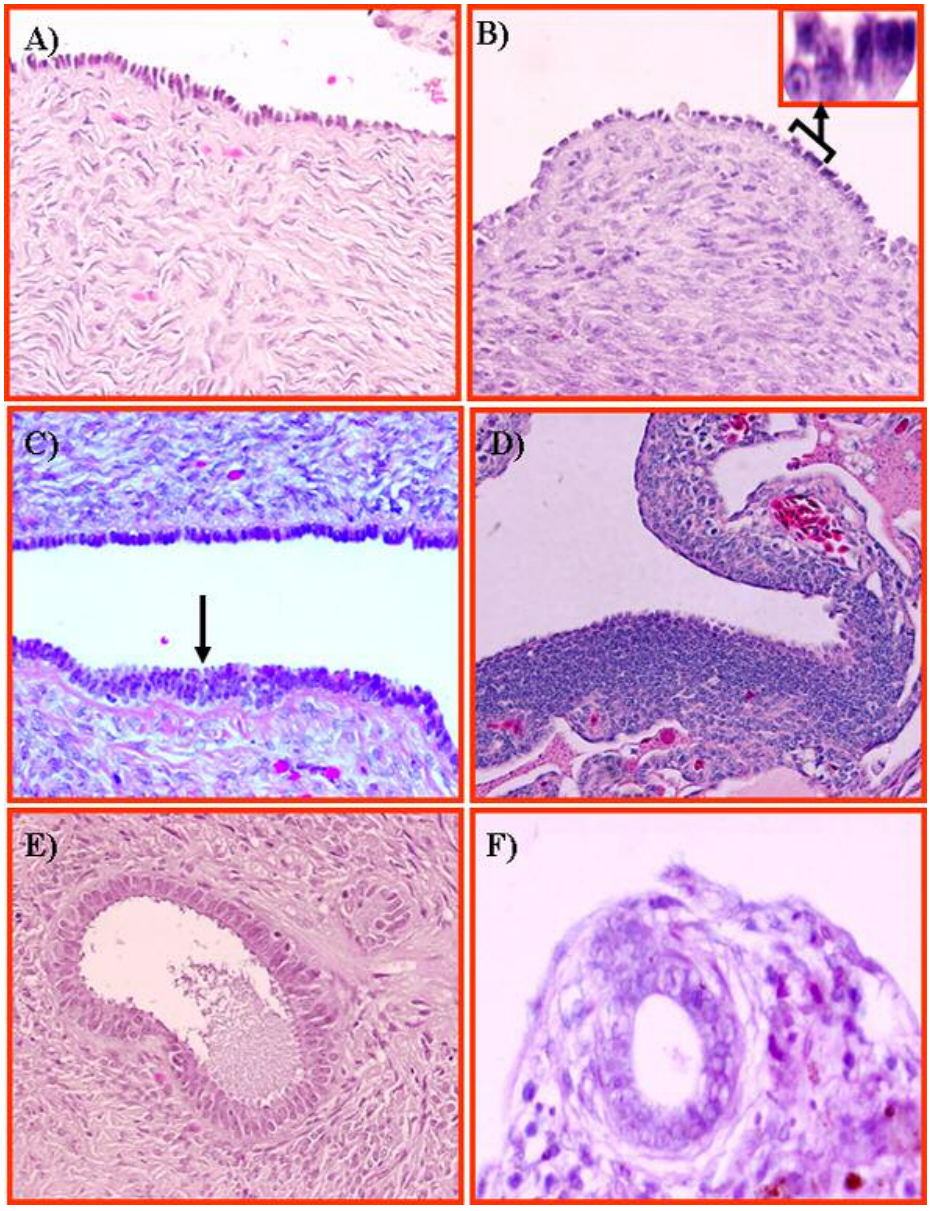

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inclusion cysts in the cortex beneath the ovarian surface epithelium. The epithelial cells in the inclusion cysts in hens are rounded instead of columnar as in humans. H &E, 40X.
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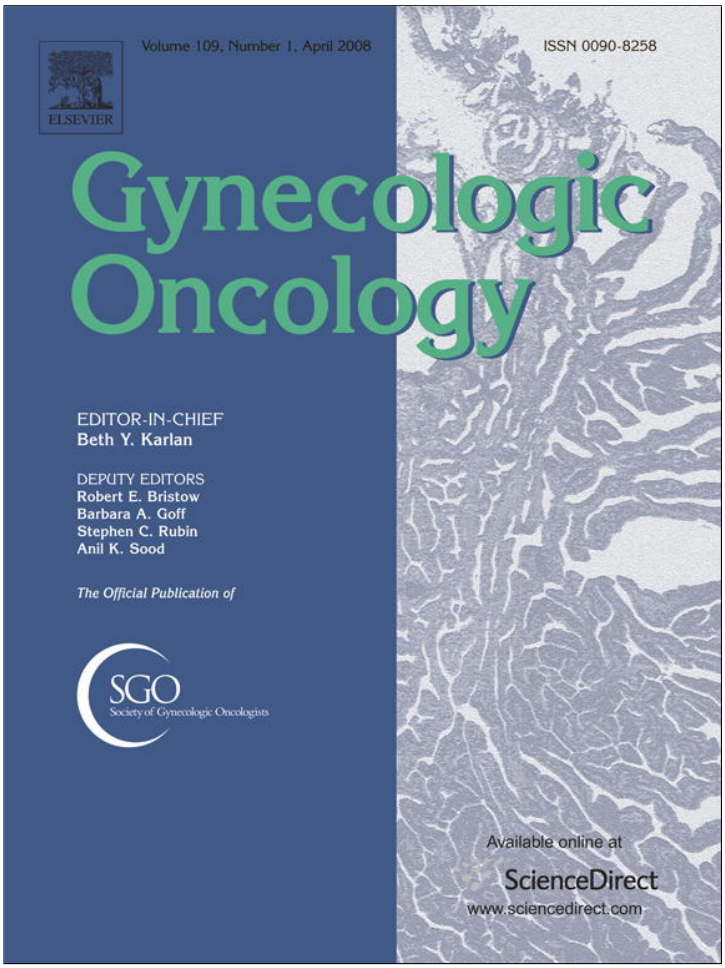
Table 1: Staging of ovarian cancer in laying hens

Stages	Characteristic features
Stage I	Growth limited to the ovary; very little or no ascites
Stage II	Tumor extended or metastasized to the oviduct, moderate ascites
Stage III	Tumor seeding metastasized to the pelvic organs; peritoneal and abdominal implants; gastrointestinal tract and superficial liver metastasis with profuse ascites.
Stage IV	Tumor metastasized to distant organs including liver parenchyma and lung; multiple solid tumors in mother organs with profuse ascites.



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Selenium-Binding Protein 1 expression in ovaries and ovarian tumors in the laying hen, a spontaneous model of human ovarian cancer

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Abstract

Objective. Reduced Selenium-Binding Protein 1 (SELENBP1) expression was recently shown in multiple cancers. There is little information on the expression and function of SELENBP1 in cancer progression. In order to develop a better understanding of the role of SELENBP1 in ovarian cancer, our objective was to determine if SELENBP1 is expressed in the normal ovaries and ovarian tumors in the egg-laying hen, a spontaneous model of human ovarian cancer.

Methods. SPB1 mRNA expression in normal ovary ($n=20$) and ovarian tumors ($n=23$) was evaluated by RT-PCR. Relative levels of mRNA were compared by quantitative RT-PCR (qRT-PCR) in selected samples. SELENBP1 protein expression was evaluated by 1D Western blot and immunohistochemistry with a commercial anti-human SELENBP1 antibody.

Results. SELENBP1 mRNA and protein was expressed in 100% of normal and ovarian tumors and qRT-PCR confirmed decreased mRNA expression in 80% of ovarian tumors. SELENBP1 was primarily localized in surface epithelial cells of normal ovaries. In ovaries containing early tumor lesions, SELENBP1 expression was reduced in the surface epithelium near the tumor and was expressed in tumor cells, while more distant regions with normal histology retained SELENBP1 expression in the surface epithelium.

Conclusions. We have shown for the first time that SELENBP1 is expressed in both normal ovaries and ovarian tumors in the hen and that SELENBP1 expression is altered in the vicinity of the tumor. Furthermore, SELENBP1 expression in normal ovarian surface epithelium and in ovarian tumors parallels that previously reported for ovarian cancer in women.

Keywords: Selenium-Binding Protein 1; Ovarian cancer; Chickens; Animal model

Introduction

Selenium is an essential micronutrient known for its role in the alleviation or prevention of inflammatory and autoimmune diseases, infertility, immunodeficiency diseases (HIV), thyroid function, cardiovascular disease and neurological diseases[1–5]. Because selenium inhibits cellular proliferation and promotes apoptosis of prostate, breast, endometrial, and lung cancer cells directly, it also plays a significant role in the reduction or prevention of multiple cancers [6–8]. The importance of the anti-cancer action of selenium has been well documented in epidemiological studies which have shown a correlation between high cancer rates and low dietary selenium intake [9]. Clinical trial results for the preventative role of selenium in prostate cancer are particularly strong [10–13].

Selenium uptake and distribution in mammals involves a variety of mechanisms and biochemical pathways [14]. In cancer progression, attention has primarily focused on glutathione

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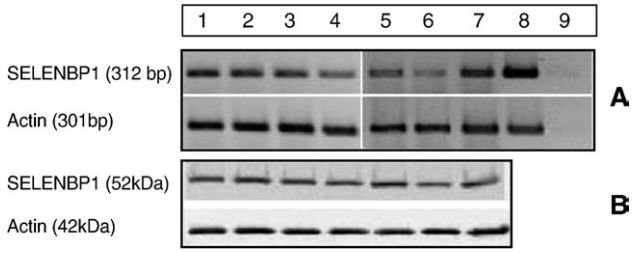

Fig. 1. Examples of SELENBP1 mRNA and protein expression in normal hen ovaries. A) SELENBP1 mRNA expression was identified in 100% of normal ovaries ($n=23$) by RT-PCR. B) SELENBP1 protein was identified in 100% of normal ovaries ($n=23$) by one-dimensional Western blot. β-Actin was used as an internal control for RT-PCR and one-dimensional Western blot. In the examples shown, lanes 1–7 represent the same tissues for SELENBP1 mRNA and protein. For mRNA, lane 8 is a positive control and lane 9 is a negative control. For protein detection, no stain occurred in the absence of primary antiserum (not shown).

peroxidase, a selenoprotein known to mediate oxidative stress in cancer cells [3,6]. There is relatively little information on Selenium-Binding Protein 1 (SELENBP1) which was originally identified from mouse liver [15] and subsequently cloned from human liver [16]. Interestingly, SELENBP1 may have an important role in cancer because significantly decreased SELENBP1 mRNA expression was observed in ovarian cancer [17] as well as colorectal [18], prostate [19], lung [20], gastrointestinal [21] and papillary thyroid cancer [22] by proteomic and differential array analysis. Decreased expression of SELENBP1 was associated with poorer survival of patients diagnosed with poorly differentiated lung tumors [20]. In addition, a paired comparison of colorectal adenoma and carcinoma tissues revealed decreased SELENBP1 levels in 87.5% of carcinomas versus 12.5% of adenomas, providing additional support for the potential role of SELENBP1 in malignant transformation and cancer progression [18]. This suggests that loss of SELENBP1 has a significant impact on the ability of selenium to control tumor cell growth. However, there is little information on expression and regulation of SELENBP1 during early tumor stages.

In ovarian cancer, decreased SELENBP1 expression was observed in 87% of borderline and invasive tumors [17]. A greater decrease in SELENBP1 expression was associated with better survival unlike other cancers in which a greater decrease was associated with poorer survival. Thus, the role of decreased SELENBP1 in ovarian cancer prognosis is not clear. Ovarian cancer remains the most lethal gynecologic malignancy for women and it represents 2.5% total cancer deaths in the United States [23–26]. Identifying altered SELENBP1 expression may be significant for the treatment of ovarian cancer since selenium has been shown to have an important role in the reduction and prevention of other cancers.

Currently, diagnosis of ovarian cancer primarily occurs at advanced stages, making it difficult to study progressive events involved in ovarian cancer in women. Animal models have historically complemented the discovery of disease etiology and progression by making it possible to examine events that are difficult to study in humans [27–29]. The egg-laying hen is the only spontaneous model of ovarian cancer. Ovarian cancer in the hen has significant similarities to human ovarian cancer. The incidence of ovarian cancer in hens is strain and flock dependent and in general the incidence of ovarian cancer is high (up to 40% by age 6) which contributes to its utility as a model [30]. Also, the incidence of ovarian cancer in hens is age dependent [30], similar to ovarian cancer in women [25]. Hormone cycles, hormone regulation, and ovulation are also similar in women and hens. Hens ovulate about 250 eggs a year [31], which is the equivalent of 20 years of ovulation for a woman. An accepted epidemiologic risk factors for human ovarian cancer is the number of life-time ovulations, because reduced lifetime ovulation – due to pregnancy and birth control pills (elevated progesterone) – reduces ovarian cancer risk [32–35]. Likewise, inhibition of ovulation with progesterone partly reduces ovarian cancer incidence in hens [36].

The morphologic, histological and molecular features of hen ovarian tumors are remarkably similar to human ovarian cancer. Epithelial tumors represent 60% of human ovarian tumors, 90% of which are malignant [33,37]. In the hen, most tumors are malignant and have epithelial cell histology [30,38–40]. In addition, the common histological subtypes of tumors that are seen in humans, such as, serous, endometrioid, clear cell and mucinous, are represented in hen tumors [41]. Hen ovarian tumors express molecular markers that are expressed in human ovarian tumors [42] such as cytokeratin AE1/AE3, pan cytokeratin, TGF-a (growth factor), EGF-R (growth factor receptor) erbB-2 (proto-oncogene), Lewis Y, CEA and Tag 72 (oncofetal tumor markers), PCNA (proliferation marker), p27 (cell cycle inhibitor) [42] and the well known CA-125 [43]. In addition, we showed for the first time that hens with ovarian cancer have anti-tumor antibodies [44,45], similar to humans.

Ovarian adenocarcinomas that bear a striking histological resemblance to human ovarian cancer develop spontaneously in the egg-laying hen [29,30]. In addition, proteins commonly expressed in human ovarian cancer have been shown to occur in the laying hen using antibodies to human proteins [42]. In order to study the changes in SELENBP1 expression during tumor development, our objective was to determine if SELENBP1 is expressed in the normal ovary and ovarian tumors of the laying hen.

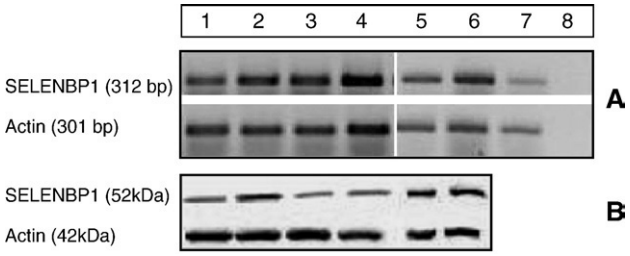

Fig. 2. Examples of SELENBP1 mRNA and protein expression in hen ovaries containing tumors. A) SELENBP1 mRNA expression was identified in 100% of ovaries containing tumors ($n=20$) by RT-PCR. B) SELENBP1 protein expression was identified in 100% of ovaries containing tumors ($n=20$) by one-dimensional Western blot. β-Actin was used as an internal control for RT-PCR and one-dimensional Western blot. In the examples shown, lanes 1–7 represent the same tissues for SELENBP1 mRNA and protein. The tumor types are, lane 1: endometrioid, lane 2: endometrioid, lane 3: endometrioid, lane 4: serous, lane 5: muicinous, lane 6: clear cell histology. For mRNA, lane 7 is a positive control and lane 8 is a negative control. For protein detection, no stain occurred in the absence of primary antiserum (not shown).

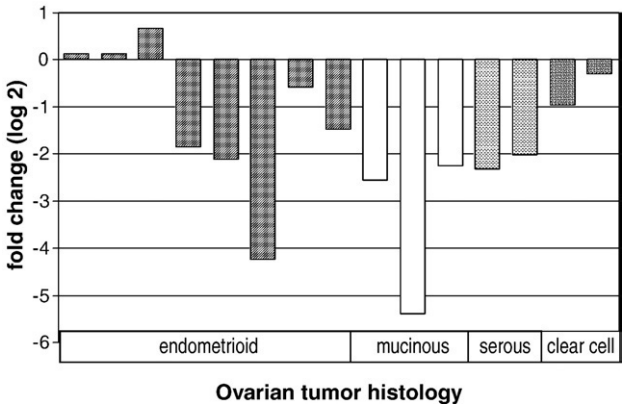

Fig. 3. Relative expression of SELENBP1 mRNA in ovarian tumors. The graph shows that SELENBP1 mRNA expression, evaluated by qRT-PCR, is decreased in 80% (12/15) of ovarian tumors compared to normal ovaries. Fold changes were calculated by converting the $\Delta\Delta$Ct exponential values to linear values using the formula $2^{\Delta\Delta Ct}$ as described in the Methods.

This would permit subsequent studies of the role of SELENBP1 in ovarian tumor progression and its regulation by selenium.

Materials and methods

Animals

Commercial strains of White Leghorn laying hens ($n=43$, 2.5 to 3 years old) were housed at the Poultry Research Farm of the University of Illinois at Urbana-Champaign and kept under a controlled light regimen (14 h light:10 h dark) with food and water provided *ad libitum*. Egg production and mortality records were maintained on a daily basis. Hens with normal ovarian morphology and histology had ≥ 5 eggs per clutch, while those with ovarian tumors had ≤ 2 eggs per clutch. Hens were euthanized according to an approved Institutional Animal Care and Use Committee (IACUC) protocol and the presence of tumors was detected by gross morphology and histology.

Tissue

Normal ovaries ($n=23$) and ovarian tumors ($n=20$) were collected at the time of euthanasia and portions snap frozen and stored at −80 °C for biochemical analysis or fixed for histological analysis. Frozen tissues were pulverized in a dry ice-acetone bath and homogenized with a Polytron (Brinkman Instruments, Westbury, NY) in ice-cold Tris–sucrose buffer (40 mM HCl, 5 mM $MgSO_4$, 0.25 M sucrose) containing 1 μl/ml protease inhibitor cocktail (Sigma, St. Louis, MO), pH 7.4. The homogenate was centrifuged (1000 ×*g*, 10 min) and the supernatant was collected. The protein content of the extract was measured with a Bradford protein assay kit (BioRad, Hercules, CA) with bovine serum albumin as a standard.

For RNA isolation, tissues were homogenized in Trizol reagent (1 ml Trizol/100 mg tissue, Invitrogen) and phase separation by adding 0.2 ml chloroform/ml of trizol used. The homogenate was centrifuged (10,000 ×*g*, 15 min, 4 °C) and the aqueous, RNA-containing phase was collected. RNA was precipitated from the aqueous phase with isopropanol(0.5 ml/1 ml Trizol; 10 min, 22 °C). Samples were centrifuged (10,000 ×*g*, 10 min, 4 °C) and the supernatant was removed. The remaining RNA pellet was washed with 75% ethanol (1 ml/1 ml Trizol), centrifuged (7500 ×*g*, 5 min, 4 °C) and allowed to air dry. The RNA pellet was dissolved in 250 μL of diethylpyrocarbonate (DEPC) treated water and stored at −80 °C. The RNA-containing solution was diluted (1:100) in HPLC grade water and concentrations as well as RNA quality were measured.

Western blot

Thirty micrograms of total protein lysate was loaded per lane onto a 10% Tris–HCL gradient gel (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved by one-dimensional SDS PAGE and transferred to a nitrocellulose membrane. Membranes were blocked (16 h, 4 °C) in Starting Block in Tris-Buffered Saline (TBS), Pierce Biotechnology, Rockford, IL) containing 0.05% Tween. Blots were washed with TBST (TBS containing 0.05% Tween-20). The membranes were incubated (1 h, 22 °C) in mouse anti-human SELENBP1 antibody (1: 1000, purified IgG clone 4D4, MBL, Nagoya, Japan) and washed in TBST prior to incubation (1 h, 22 °C) with horseradish peroxidase-conjugated secondary antibody (1:1000, goat anti-mouse IgG HRP, Pierce Biotechnology, Rockford, IL). Antibody reactions were visualized with an enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL) and images were acquired using a Chemidoc XRS (Bio-Rad Laboratories, Hercules, CA).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

First strand synthesis was completed according to the manufacturer's recommendations using the Superscript III first strand synthesis (Invitrogen, Carlsbad, CA). Oligoperfect Designer software (Invitrogen, www.invitrogen.com) was used to design SELENBP1 (BX935001.2) and actin (endogenous control, NM_205518.1) *Gallus gallus* primer sequences as follows: SELENBP1 Forward (5′-TGC TGC AGA AGG ATT TGT TG-3′) and Reverse (5′-CAC CAC AGT

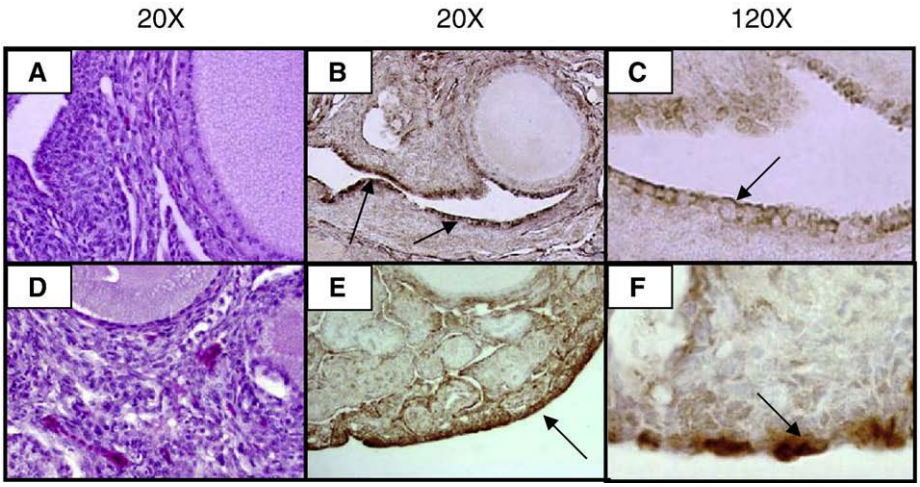

Fig. 4. Immunohistochemical localization of SELENBP1 in normal hen ovaries. Each row contains an H&E stained section (A and D) and a corresponding example of the SELENBP1 expression (B, C, E and F) at two magnifications (20× and 120×). SELENBP1 is localized primarily in surface epithelium (arrows) of normal ovaries. In cells (C) of the normal surface epithelium, SELENBP1 is frequently located adjacent to the plasma membrane and characteristically exhibits a ring-like staining pattern. Cells (F) with stained nuclei and cytoplasm are observed less often.

CAC AGG TCC AC-3′); Actin Forward (5′-TGC GTG ACA TCA AGG AGA AG-3′) and Reverse (5′-ATG CCA GGG TAC ATT GTG GT-3′).

The PCR reaction was performed according to the suggested protocol for taq DNA polymerase (Invitrogen, Carlsbad, CA). The PCR reaction included one denaturation step (94 °C, 3 min) and 35 cycles of amplification (94 °C, 30 s; 53 °C, 30 s; 72 °C, 60 s; 72 °C, 5 min). PCR products were visualized using agarose (2%) gel electrophoresis and ethidium bromide staining. SELENBP1 amplicon was observed at 312 base pairs.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR primers for SELENBP1 and β-actin were designed using Primer Express software (Applied BioSystems, Foster City, CA). SELENBP1 sequence (BX935001.2) was used to design the qRT-PCR primer as follows: SELENBP1 Forward (5′-GGA TGG CTC CTC CCT GAC A-3′) and Reverse (5′-TCG TCC AGC GAG ATG AGG AT-3′). β-actin (endogenous control) sequence (NM_205518.1) was used to design the qRT-PCR primer as follows: Forward (5′-GCC CTC TTC CAG CCA TCT TT) and Reverse (5′-TGG AGT TGA AGG TAG TTT CAT GGA T-3′).

Total RNA was isolated by methods described previously. 1.0 μg of total RNA was treated with DNase1 according to the manufacture's recommended protocol (DNase1, catalog number EN0521, Fermentas, Hanover, MD). cDNA synthesis and first strand synthesis were completed according to the manufacturer's recommendations using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA).

To perform a PCR assay, SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA), primers, template, and nuclease-free water (Applied Biosystems, Foster City, CA) were added to a reaction mixture (50 μl final volume) according to the manufacturer's instructions. Primers were added to a final concentration of 200 nM. From each 50 μl reaction mixture two 25 μl aliquots were drawn, each containing the cDNA template from 25 ng of total RNA, and placed into wells in a 96-well PCR reaction plate. For negative control reactions (minus template), nuclease-free water was substituted for solutions of template DNA. PCR assays were perfomed on an ABI 7500 System (Applied BioSystems, Foster City, CA).

Relative levels of SELENBP1 mRNA were calculated using the Relative Quantitation (RQ ΔΔCt) method. In order to use the RQ ΔΔCt method, both the target gene and endogenous control should have similar amplification efficiency and that was evaluated in a preliminary qRT-PCR run that produced similar slopes for SELENBP1 and β-actin (−3.07 and −3.08). The average Ct value for each

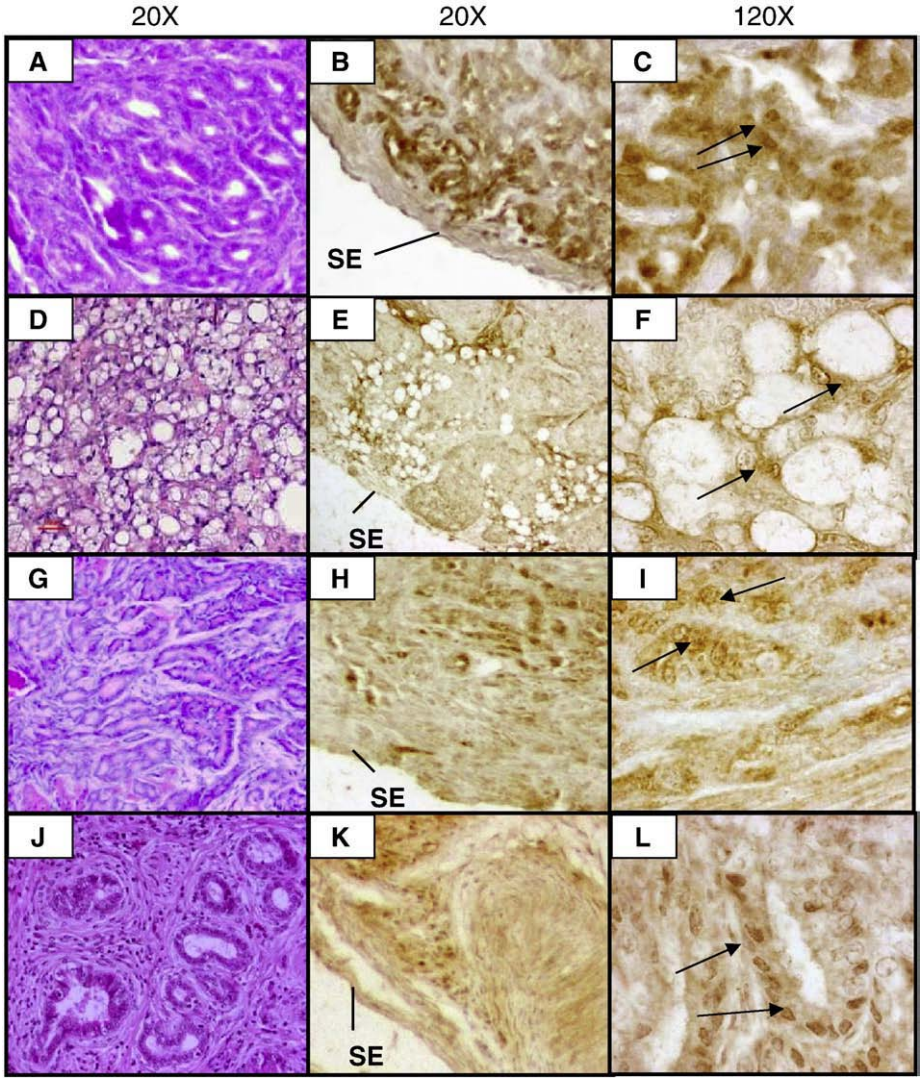

Fig. 5. Immunohistochemical localization of SELENBP1 in hen ovarian tumors. Each row contains an H&E stained section and an example of the same tumor type at two magnifications (20× and 120×). SELENBP1 expression is reduced in ovarian surface epithelium (SE) (middle column) and is localized in tumor cells (right column) in endometrioid (top row), clear cell (second row), serous (third row) and mucinous (bottom row) tumors. SELENBP1 expression is typically observed in the cytoplasm and nuclei of tumor cells (arrows).

SELENBP1 and β-actin sample was determined and the ΔCt for SELENBP1 in each sample was calculated by subtracting the average Ct value of β-actin from average Ct value of SELENBP1. The average ΔCt value was also calculated for normal ovary and used as a calibrator. ΔΔCt was determined by subtracting ΔCt for β-actin (calibrator) from the ΔCt for SELENBP1 (normalized target). The fold change for each tumor sample was calculated by converting the ΔΔCt exponential values to linear values using the formula $2^{-\Delta\Delta Ct}$.

Histology and Immunohistochemistry

Tissues were fixed in a10% buffered formalin, paraffin-embedded and 6μm tissue sections were cut, mounted on microscopic slides and incubated (2 h, 35 °C). Normal histology and ovarian tumor pathology were verified by H&E staining. In order to determine the location of SELENBP1 expression, selected normal ($n=2$) and tumor ($n=8$) tissues were further processed for immunohistochemistry. De-paraffinized sections were boiled (10 min) in an antigen unmasking solution (1:100, Vector Laboratories, Burlingame, CA) and incubated in 0.3% hydrogen peroxide–methanol (20 min, 22 °C) to block endogenous peroxidase activity. Sections were washed in phosphate buffered saline (PBS) and blocked with normal horse serum (30 min, 22 °C). Anti-human SELENBP1 monoclonal antibody (purified IgG Clone CD4, MBL, Nagoya, Japan) was diluted in PBS plus 1% BSA (1:250) and incubated with sections (1 h, 22 °C then 12 h, 4 °C). Control staining was performed by (1) omitting the primary antibody (replaced with PBS), or (2) by omitting the secondary antibody (replaced with PBS). Sections were washed in PBS and incubated (1 h, 22 °C) with a universal secondary antibody (pan-specific bioatinylated anti-immunoglobulin, Vector Laboratories, Burlingame, CA) followed by incubation (1 h, 22 °C) with Avidin-Biotin Complex reagent (Vector Laboratories, Burlingame, CA). Reaction product was produced with diaminobenzidine substrate (R.T.U. Vectastain Kit, Vector Laboratories, Burlingame, CA). Sections were counterstained lightly in Hematoxylin.

Results

Expression of SELENBP1 in normal ovary and ovarian tumors

SELENBP1 PCR amplicon was detected at the expected size of 312 bp in all normal ovaries ($n=23$) examined in the egg-laying hen (Fig. 1). Immunoreactive SELENBP1 protein was detected at 52 kDa in all normal ovaries by Western blot (Fig. 1). SELENBP1 amplicon and immunoreactive protein was expressed in all ovarian tumors ($n=20$) (Fig. 2), including endometrioid, serous, mucinous and clear cell ovarian tumors.

Because mRNA density varied and because previous findings suggested that SELENBP1 expression in ovarian tumors in women is altered, we examined SELENBP1 mRNA levels by quantitative RT-PCR. Compared to normal hen ovaries, SELENBP1 mRNA was down regulated in 80% (12/15) of hen ovarian tumors (Fig. 3).

Localization of SELENBP1 in normal ovary and ovarian tumors

In order to assess the location of SLENBP1 expression, SELENBP1 expression was detected by immunohistochemistry in selected normal ($n=2$) and tumor ($n=8$) ovaries. In normal ovaries, SELENBP1 was primarily expressed in ovarian surface epithelium (Fig. 4). In cells of the normal surface epithelium, SELENBP1 was frequently localized in the cytoplasm adjacent to the plasma membrane region and to a lesser extent throughout the cytoplasm and nuclei.

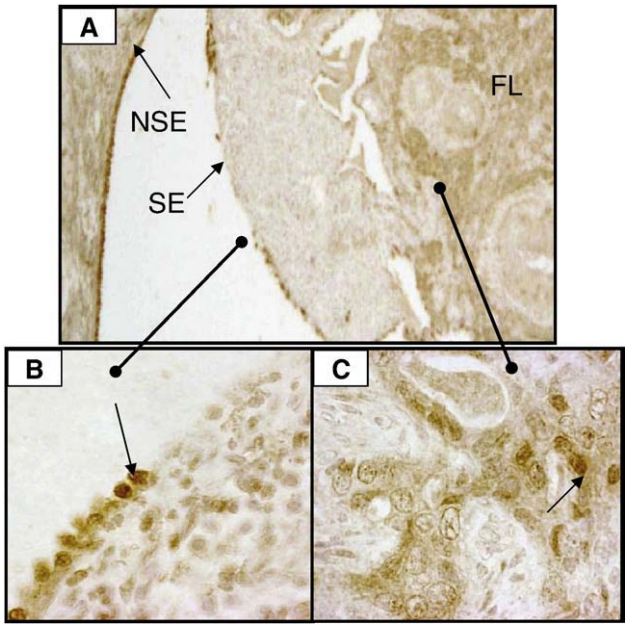

Fig. 6. Reduced SELENBP1 expression in the surface epithelium is associated with SELENBP1 expression in tumor cells of focal lesions. (A) Reduced expression of SELENBP1 in the surface epithelium (SE) of the ovary was seen primarily in the vicinity of focal lesions (FL) while other more distant areas retained SELENBP1 expression in the normal surface epithelium (NSE). (B) The area in (A) is enlarged to show the disrupted expression of SELENBP1 in the surface epithelium (arrow) near tumor cells. (C) The area in (A) is enlarged to show tumor cell staining in cytoplasm and nuclei (arrow). The intracellular distribution of SELENBP1 in tumor cells differs from cells (see Fig. 4) in normal surface epithelium.

Hen ovarian tumor histology was strikingly similar to the histology observed in ovarian tumors in women (Fig. 5) and represented endometrioid, serous, clear cell, and mucinous tumor cell types. Within the tumors studied, ($n=20$), 6 (30%) had endometrioid, 4 (25%) had mucinous, 3 (15%) had serous, and the remaining had a mixed histology with 1 (5%) serous/mucinous, 2 (10%) serous/endometrioid and 2 were undetermined (late stage with extensive metastasis). SELENBP1 was expressed in all and ovarian tumors and was down regulated in the surface epithelium of ovaries containing tumors. Moreover, in the vicinity of small focal lesions, decreased SELENBP1 expression at the nearby surface epithelium appeared to coincide with the presence of a tumor, while SELENBP1 was expressed in surface epithelium in more distant areas of the ovary (Fig. 6). In contrast to cells of the normal surface epithelium, SELENBP1 in tumor cells was frequently located throughout the cytoplasm and in nuclei.

Discussion

We demonstrated for the first time that SELENBP1 mRNA and protein is expressed in both normal ovary and ovarian tumors in the hen. In addition, SELENBP1 mRNA and protein was similar in size to human SELENBP1 mRNA and protein, consistent with the nucleic acid homology of 73% and the protein homology of 76%. Furthermore, the tissue distribution of SELENBP1 in hens parallels its expression in the human ovary

and ovarian tumors [17]. Likewise, hen SELENBP1 mRNA was decreased in 80% of ovarian tumors compared to the normal ovary. This is also consistent with the 87% decrease in SELENBP1 expression reported for human ovarian cancer [17].

The histology of ovarian tumors in the hen resembles the histology of human ovarian tumors. We observed expression of SELENBP1 in endometrioid, mucinous, serous, and clear cell tumors by immunohistochemistry, similar to that reported for human ovarian cancer [17]. However, the observations of human ovarian cancer by Huang *et al* [17] were based on late stage ovarian cancer in humans. The advantage of studies in the hen is that we routinely observed early stage focal lesions. At these early stages, large areas of the ovary retain normal morphology. Interestingly, reduced expression of SELENBP1 in the surface epithelium of the ovary was seen in the immediate vicinity of the focal lesions, while other more distant areas retained expression in the surface epithelium. The disappearance of SELENBP1 in the epithelial layer appeared to coincide with SELENBP1 expression the focal lesion. This is a novel result, which supports the concept that epithelial ovarian cancer arises from the surface epithelium. Furthermore, the intracellular distribution of SELENBP1 differed in tumor cells; in normal surface epithelium SELENBP1 is located in the sub-plasma membrane region of the cell while in tumor cells it appeared to be more diffusely distributed throughout the cytoplasm and the nucleus. One interpretation of this result is that the functional relationships of SELENBP1 are altered in transformed cells.

Because of its similarity to human ovarian cancer, researchers have sought to utilize the egg-laying hen to examine the expression of proteins thought to play a role in human ovarian cancer. For example, hen ovarian tumors express molecular markers that are expressed in human ovarian tumors [42] such as cytokeratin AE1/AE3, pan cytokeratin, TGF-a (growth factor), EGF-R (growth factor receptor) erbB-2 (proto-oncogene), Lewis Y, CEA and Tag 72 (oncofetal tumor markers), PCNA (proliferation marker), p27 (cell cycle inhibitor) [42], CA-125 [43] and COX-1 and COX-2 [46]. In turn, these findings have helped to validate the use of the laying hen as an emerging model of ovarian cancer. Likewise, the findings of this study of SELENBP1 support the use of the hen as a model of human ovarian cancer.

While there are significant changes in the expression of SELENBP1 in ovarian cancer in *both* humans and hens, the role of SELENBP1 in selenium utilization and ovarian cancer progression remains to be determined. The demonstration that changes in SELENBP1 expression in the hen are similar to those in humans will permit studies to examine the role of selenium and SELENBP1 in ovarian cancer progression.

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References

[1] Lanzillotti JS, Tang AM. Micronutrients and HIV disease: a review pre- and post-HAART. Nutr Clin Care 2005;8:16–23.
[2] Ryan-Harshman M, Aldoori W. The relevance of selenium to immunity, cancer, and infectious/inflammatory diseases. Can J Diet Pract Res 2005;66:98–102.
[3] Rayman MP. Selenium in cancer prevention: a review of the evidence and mechanism of action. Proc Nutr Soc 2005;64:527–42.
[4] Beckett GJ, Arthur JR. Selenium and endocrine systems. J Endocrinol 2005;184:455–65.
[5] Schweizer U, Schomburg L. New insights into the physiological actions of selenoproteins from genetically modified mice. IUBMB Life 2005;57:737–44.
[6] Letavayova L, Vlckova V, Brozmanova J. Selenium: from cancer prevention to DNA damage. Toxicology 2006;227:1–14.
[7] Dennert G, Horneber M. Selenium for alleviating the side effects of chemotherapy, radiotherapy and surgery in cancer patients. Cochrane Database Syst Rev 2006;3:CD005037.
[8] Aboul-Fadl T. Selenium derivatives as cancer preventive agents. Curr Med Chem Anti-canc Agents 2005;5:637–52.
[9] Klein EA. Selenium: epidemiology and basic science. J Urol 2004;171: S50–3 discussion S53.
[10] Klein EA. Selenium and vitamin E cancer prevention trial. Ann NY Acad Sci 2004;1031:234–41.
[11] Lee SO, Yeon Chun J, Nadiminty N, Trump DL, Ip C, Dong Y, et al. Monomethylated selenium inhibits growth of LNCaP human prostate cancer xenograft accompanied by a decrease in the expression of androgen receptor and prostate-specific antigen (PSA). Prostate 2006;66:1070–5.
[12] Dong Y, Zhang H, Gao AC, Marshall JR, Ip C. Androgen receptor signaling intensity is a key factor in determining the sensitivity of prostate cancer cells to selenium inhibition of growth and cancer-specific biomarkers. Mol Cancer Ther 2005;4:1047–55.
[13] Etminan M, FitzGerald JM, Gleave M, Chambers K. Intake of selenium in the prevention of prostate cancer: a systematic review and meta-analysis. Cancer Causes Control 2005;16:1125–31.
[14] Behne D, Kyriakopoulos A. Mammalian selenium-containing proteins. Annu Rev Nutr 2001;21:453–73.
[15] Bansal MP, Mukhopadhyay T, Scott J, Cook RG, Mukhopadhyay R, Medina D. DNA sequencing of a mouse liver protein that binds selenium: implications for selenium's mechanism of action in cancer prevention. Carcinogenesis 1990;11:2071–3.
[16] Chang PW, Tsui SK, Liew C, Lee CC, Waye MM, Fung KP. Isolation, characterization, and chromosomal mapping of a novel cDNA clone encoding human selenium binding protein. J Cell Biochem 1997;64:217–24.
[17] Huang KC, Park DC, Ng SK, Lee JY, Ni X, Ng WC, et al. Selenium binding protein 1 in ovarian cancer. Int J Cancer 2006;118:2433–40.
[18] Kim H, Kang HJ, You KT, Kim SH, Lee KY, Kim TI, et al. Suppression of human selenium-binding protein 1 is a late event in colorectal carcinogenesis and is associated with poor survival. Proteomics 2006;6:3466–76.
[19] Yang M, Sytkowski AJ. Differential expression and androgen regulation of the human selenium-binding protein gene hSP56 in prostate cancer cells. Cancer Res 1998;58:3150–3.
[20] Chen G, Wang H, Miller CT, Thomas DG, Gharib TG, Misek DE, et al. Reduced selenium-binding protein 1 expression is associated with poor outcome in lung adenocarcinomas. J Pathol 2004;202:321–9.
[21] He QY, Cheung YH, Leung SY, Yuen ST, Chu KM, Chiu JF. Diverse proteomic alterations in gastric adenocarcinoma. Proteomics 2004;4:3276–87.
[22] Brown LM, Helmke SM, Hunsucker SW, Netea-Maier RT, Chiang SA, Heinz DE, et al. Quantitative and qualitative differences in protein expression between papillary thyroid carcinoma and normal thyroid tissue. Mol Carcinog 2006;45:613–26.
[23] Goodman MT, Correa CN, Tung KH, Roffers SD, Cheng Wu X, Young Jr JL, et al. Stage at diagnosis of ovarian cancer in the United States, 1992–1997. Cancer 2003;97:2648–59.
[24] Goodman MT, Howe HL. Descriptive epidemiology of ovarian cancer in the United States, 1992–1997. Cancer 2003;97:2615–30.
[25] Goodman MT, Howe HL, Tung KH, Hotes J, Miller BA, Coughlin SS, et al. Incidence of ovarian cancer by race and ethnicity in the United States, 1992–1997. Cancer 2003;97:2676–85.
[26] Pecorelli S, Favalli G, Zigliani L, Odicino F. Cancer in women. Int J Gynaecol Obstet 2003;82:369–79.
[27] Rose NR. Avian models of autoimmune disease: lessons from the birds. Poult Sci 1994;73:984–90.

[28] Sundick RS, Bagchi N, Brown TR. The obese strain chicken as a model for human Hashimoto's thyroiditis. Exp Clin Endocrinol Diabetes 1996;104 (Suppl 3):4–6.
[29] Vanderhyden BC, Shaw TJ, Ethier JF. Animal models of ovarian cancer. Reprod Biol Endocrinol 2003;1:67.
[30] Fredrickson TN. Ovarian tumors of the hen. Environ Health Perspect 1987;73:35–51.
[31] Lewis PD, Long SE. Incidence of non-laying in domestic hens. Br Poult Sci 1992;33:289–95.
[32] Holschneider CH, Berek JS. Ovarian cancer: epidemiology, biology, and prognostic factors. Semin Surg Oncol 2000;19:3–10.
[33] Tung KH, Goodman MT, Wu AH, McDuffie K, Wilkens LR, Kolonel LN, et al. Reproductive factors and epithelial ovarian cancer risk by histologic type: a multiethnic case–control study. Am J Epidemiol 2003;158:629–38.
[34] Runnebaum IB, Stickeler E. Epidemiological and molecular aspects of ovarian cancer risk. J Cancer Res Clin Oncol 2001;127:73–9.
[35] Cramer DW, Hutchison GB, Welch WR, Scully RE, Ryan KJ. Determinants of ovarian cancer risk. I. Reproductive experiences and family history. J Natl Cancer Inst 1983;71:711–6.
[36] Barnes MN, Berry WD, Straughn JM, Kirby TO, Leath CA, Huh WK, et al. A pilot study of ovarian cancer chemoprevention using medroxyprogesterone acetate in an avian model of spontaneous ovarian carcinogenesis. Gynecol Oncol 2002;87:57–63.
[37] Chen VW, Ruiz B, Killeen JL, Cote TR, Wu XC, Correa CN. Pathology and classification of ovarian tumors. Cancer 2003;97:2631–42.
[38] Damjanov I. Ovarian tumours in laboratory and domestic animals. Curr Top Pathol 1989;78:1–10.
[39] Giles JR, Olson LM, Johnson PA. Characterization of ovarian surface epithelial cells from the hen: a unique model for ovarian cancer. Exp Biol Med (Maywood) 2006;231:1718–25.
[40] Giles JR, Shivaprasad HL, Johnson PA. Ovarian tumor expression of an oviductal protein in the hen: a model for human serous ovarian adenocarcinoma. Gynecol Oncol 2004;95:530–3.
[41] Barua A, Abramowicz J, Bahr J, Bitterman P, Dirks A, Holub K, et al. Detection of ovarian tumors in chicken by ultrasonography — a step towards early diagnosis in humans? J Ultrasound Med 2007;26:909–19.
[42] Rodriguez-Burford C, Barnes MN, Berry W, Partridge EE, Grizzle WE. Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention. Gynecol Oncol 2001;81:373–9.
[43] Jackson E, Anderson K, Ashwell C, Petitte J, Mozdziak PE. CA125 expression in spontaneous ovarian adenocarcinomas from laying hens. Gynecol Oncol 2006;104(1):192–8.
[44] Barua A, Bradaric M, Edassary S, Rotmensch J, Bitterman P, Hales D, et al. Anti-tumor antibodies and ovarian cancer in women and hens. Special American Association for Cancer Research Conference, Molecular Diagnostics in Cancer Therapeutic Development: Maximizing Opportunities for Individualized Treatment. Chicago, IL: Scholar-in-Training Award; 2006.
[45] Barua A, Edassery S, Bitterman P, Bahr J, Hales D, Luborsky J. Anti-tumor antibodies in serum of chickens with ovarian cancer. submitted for publication.
[46] Urick ME, Johnson PA. Cyclooxygenase 1 and 2 mRNA and protein expression in the *Gallus domesticus* model of ovarian cancer. Gynecol Oncol 2006;103:673–8.

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Prevalence of anti-tumor antibodies in laying hen model of human ovarian cancer

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Running head: Anti-tumor antibodies and ovarian cancer in laying hen

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Abstract

Anti-tumor antibodies are associated with tumors in human cancers. There is relatively little information on the timing and progression of antibody response to tumors. The objective of the study was to determine if spontaneous ovarian cancer in the egg-laying hen is associated with anti-tumor antibodies. Antibodies were detected by immunoassay and immunoblotting using proteins from normal ovary and ovarian tumors. Candidate antigens were identified by mass spectrometry of immunoreactive spots cut from two-dimensional gels and Western Blot. Anti-tumor (serum reacting against tumor ovarian extract) and anti-ovarian (serum reacting against normal ovarian extract) antibodies were significantly associated with ovarian cancer (67%, $P \leq 0.001$) compared to normal control hens. Hens with abnormal histology but no gross tumor had anti-tumor antibodies (63%, $P \leq 0.025$) but not anti-ovarian antibodies. There were common as well as different immunoreactions against normal ovary, and homologous and heterologous tumor proteins in two-dimensional Western blots. The candidate antigens included those commonly associated with human cancers and other diseases such as vimentin, apolipoprotein A1, Annexinn II, enolase, DJ-1 etc. The results suggest that anti-tumor antibodies are associated with ovarian cancer in hens, similar to human ovarian cancer. The egg-laying hen may be a model for understanding the anti-tumor humoral immune response, particularly at early tumor stages that are not readily accessible in human ovarian cancer.

KEYWORDS: ovarian cancer; chicken; anti-tumor antibodies; ovarian antigens

Despite several decades of research, there is no reliable screening or diagnostic test for ovarian cancer (OvCa). Consequently, OvCa tends to be diagnosed at later stages and is the most fatal gynecological cancer [1]. A pre-clinical animal model that recapitulates spontaneous OvCa, would supplement studies in humans, and improve understanding of disease progression and potentially facilitate the identification and validation of markers of early disease.

Cancers arise from the host's tissues and modifying the tissues as an altered "self" against which the immune system produces an immune response[2]. There is clear evidence that there are both humoral and cellular immune responses to tumors[3, 4]. Anti-tumor antibodies are associated with most cancers[5, 6] and antibodies to a number of immunoreactive intracellular and cell surface antigens have been reported for OvCa[7, 8]. Correlation between humoral and cellular immune responses against same tumor antigen has been reported[9]. We[10] and others[11, 12] have shown anti-tumor antibody responses to OvCa in humans. This suggests that immune responses against tumor antigens have potential as diagnostic markers for OvCa.

As shown in autoimmune diseases, antibodies are excellent disease markers because they are specific and biochemically stable[5]. The advantage of anti-tumor antibodies as early cancer markers is that they can detect antigens at low levels that might not be detectable by protein measurement. Published reports on individual antigens support the concept that only cancer patients tend to have anti-tumor antibodies to normally occurring antigens that are expressed in tumors[12-15]. Anti-tumor antibodies have been used to identify candidate cancer markers by screening tumor derived, cDNA expression libraries with patient sera[16, 17]. Analytic modeling showed the feasibility of using antigen specific antibodies to diagnose cancers in a cohort of patients with breast, lung, prostate, colorectal, gastric and hepatocellular cancers[6], although few

studies have validated the early detection of cancers with anti-tumor antibodies[18, 19]. Furthermore, there are few studies of antibodies associated with early stage OvCa[5]. Our overall objective is to demonstrate the utility of anti-tumor antibodies to detect early stage OvCa. Since there is a paucity of early stage patients and since following the disease trajectory in humans is extremely difficult, we examined animal models of OvCa.

Historically, animal models have facilitated understanding of disease-related events that are difficult to study in humans. Models are selected as much as possible because of their similarity to the human disorder. For OvCa, most of the common models are rodents in which ovarian tumors are artificially induced by transplant of human tissue, chemical or genetic manipulation[20]. Frequently, the tumor types and histopathology of ovarian carcinomas induced by chemical or genetic manipulation are not similar to spontaneous human OvCa [20, 21]. In contrast, the egg-laying hen (*Gallus domesticus*) is the only available animal known to develop OvCa spontaneously[22]. Ovarian carcinoma in hens has remarkable similarities with human OvCa based on incidence, histopathology and expression of molecular markers. Similar to humans[23], the incidence of OvCa in egg-laying hens increases with age and, depending on the flock, up to 40% of hens may have ovarian tumors by 6 years of age[22]. Although there is no comprehensive summary of the histopathology of malignant ovarian tumors in hens, the available published reports show that histological types of ovarian tumors in hens are similar to human tumors[24-26]. Furthermore, common human tumor associated molecular markers including cytokeratin, several growth factors and their receptors, carcinoembryonic antigen, proto-oncogene, onco-fetal tumor markers and PCNA (proliferation cell nuclear antigen)[27] are also expressed in hen ovarian tumor tissues. CA125, the only marker in routine diagnostic use to gauge tumor recurrence, was recently shown to be expressed in hen ovarian tumors by

immunohistochemistry[28]. Furthermore, COX-1 is elevated in hen ovarian tumors[29] consistent with the specific effect of COX-1 inhibitors on reduction of human ovarian tumors. We recently showed that ovarian tumors could be detected by ultrasound using the similar equipment used to evaluate human ovaries, thus making it possible to monitor the morphological and blood vessel changes associated with onset and progression of ovarian cancer in egg-laying hens. Therefore, given the similarities in hen and human ovarian cancer, we investigated the anti-tumor antibody response in hens. This represents the first investigation of an immune response to ovarian tumors in the hen. The specific goal of this study was to determine if ovarian tumors in hens were associated with circulating anti-tumor antibodies. The results would provide the basis for subsequent studies of the onset and progression of immune responses to ovarian tumors.

Materials and Methods

Animals

Commercial strains of Single Comb White Leghorn egg-laying hens (*Gallus domesticus*) 2.5-3 years old were kept in individual cages under a light regimen of 14h light:10h dark with *ad libitum* access to feed and water. Approximately 10-20% of laying hens at this age with low egg production (≤ 3eggs/clutch/hen) will have spontaneous ovarian tumors[24]. A commercial White Leghorn hen with normal ovarian function lays 5 or more eggs in a clutch. The ovary of young normally laying hens (11 months old, serum of these hens were used as negative control in immunoassay) contained 5 large yolk-filled preovulatory follicles, numerous developing small follicles and 2-3 degrading postovulatory follicular tissues indicating their frequent laying status. Hens lay an egg/day and a clutch is defined as the number of days of continuous egg laying by a

hen in a sequence. After laying eggs in a sequence, a hen pauses for one or two days and then resumes laying. A smaller clutch size indicates partially reduced ovarian function. All procedures including the care and management, handling as well as subsequent euthanasia of animals were performed according to the Institutional Animal Care and Use Committee approved guidelines. A work flow diagram showing tissue collection, processing and subsequent steps are summarized in Figure 1.

Serum and tissue

Blood was collected into a 10ml red top tube from wing veins, centrifuged (1,000 x g, 20 min) and sera stored at -80 ^{o}C. Hens were euthanized by carbon dioxide inhalation just after oviposition approximately 30 minutes before the expected time of ovulation[30]. Hens with apparent non-ovarian pathologies upon post-mortem gross examination were excluded from the study. In the chicken, only the left ovary is functional while the right is rudimentary. The gross morphology of ovaries was recorded and included number of viable or atretic large preovulatory follicles and cystic and solid masses. A portion of the ovary was frozen immediately and stored at -80 ^{o}C for later biochemical analysis. Another portion was processed for histological examination. In some cases, the ovary may be a secondary tumor site as tumors may metastasize to the ovary from the oviducts. Therefore, the oviducts of all hens with or without ovarian tumors were excised to confirm that the ovary was the primary site of malignancy. Hens with tumor seeding in the inner wall of the oviduct (oviductal mucosa) were excluded from the study. Ovaries were classified into normal (n=39, age-matched hens without any ovarian abnormality), ovaries with tumor (n=18) and ovaries with non-tumor pathology (n=8). Non-tumor pathology included atrophied ovaries with no preovulatory follicles, ovaries with atretic or fluid filled

cystic preovulatory follicles without any solid tissue masses. Although atresia of small developing follicles is the normal physiological phenomena, the developed larger preovulatory follicles usually do not undergo atresia in hens and such conditions are considered abnormal.

Histology

Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin. Sections (6µm thick) were cut from each block, stained with hematoxylin and eosin, examined by light microscopy and classified as normal, ovaries with tumor or ovaries with non-tumor pathology. Microscopically, ovarian malignancies were confirmed by the presence of focal lesions, glandular structures, cells with pleomorphic nucleus with mitotic bodies and hyperplastic surface or stromal hyperplasia. Non-tumor pathologies included increased atresia of developing stromal follicles, cystic structures, hyperplasia without any focal lesion or malignant cells with a pleomorphic nucleus or glandular structures. Tumors were classified based on histological types with reference to the FIGO classification for human. Staging of tumors were performed based on the gross morphology[24].

Preparation of ovarian homogenates for biochemical analysis

Frozen tissues were pulverized in a dry ice-acetone bath and homogenized with a Polytron (Brinkman Instruments, Westbury, NY) in ice cold Tris-sucrose buffer (40 mM Tris, 4mM $MgCl_2$, 0.25 M sucrose, 1µl/ml protease inhibitor cocktail (Sigma, St. Louis, MO), pH 7.4). The homogenate was centrifuged (1,000xg, 10 min) and the supernatant was collected. The protein content of the extract was measured (Bradford protein assay kit, BioRad, Hercules, CA) with

bovine serum albumin (BSA) as a standard (Sigma, St. Louis, MO). Extracts were stored at -80°C.

Immunoassay

Autoantibodies were detected by minor modification of a previously described tissue-based immunoassay[31]. Immunoassay plates (MaxiSorp, Nunc) were coated with the extracts of normal ovaries or malignant ovarian tumors diluted in a phosphate based buffer (0.2 µg protein/well). Sera were diluted (1:100) in Wash buffer (10 mM phosphate buffer, 30 mM NaCl, 1mM $MgCl_2$, pH 7.4; 0.05% Triton X-100) containing 1% BSA (Sigma, St. Louis, MO) and tested in duplicate. Sera were tested using 100, 200, 500, 1000, 1500 and 2000 dilutions. The 1:1000 dilution was found to be the minimum threshold for positive reactivity against ovarian antigens. The bound autoantibodies were detected with alkaline phosphatase conjugated affinity purified rabbit anti-chicken IgY (IgG) (H+L) (Jackson Immuno Research Laboratories, Inc., West Grove, PA) and washed to remove unbound conjugate. The bound alkaline phosphatase was reacted with p-nitrophenylphosphate substrate (Sigma, St. Louis, MO) and plates were read at 405 nm in a plate reader (Thermomax; Molecular Devices, Sunnyvale, CA).

Two-dimensional electrophoresis and Western Blots

Two-dimensional gel electrophoresis is a process of protein separation from a complex proteome homogenate based on their isoelectric point (is the pH at which a particular molecule or surface carries no net electric charge and will not migrate in an electric field) and mass. In the first dimension, proteins are separated by their isoelectric point and in the second dimension by their mass (MW) perpendicularly to the first dimension. Two dimensional gel electrophoresis was

performed as reported previously[32] with minor modification. Extracts (300µg protein/strip) were diluted in rehydration buffer (Bio-Rad ReadyPrep 2-D rehydration buffer) containing 1% (v/v) TBP reducing agent (Bio-Rad), 0.5% (v/v) carrier ampholyte (Bio-Lyte 3-10, Bio-Rad, Hercules, CA) and 1.2% (v/v) destreaking agent (DeStreak Reagent, Amersham Biosciences, Upsala, Sweden). Samples were incubated with immobilized pH gradient (IPG) strips (Bio-Rad, Hercules, CA) according to the manufacturer's instructions (16 h, 20°C). Following rehydration of the strip, proteins were separated by isoelectric focusing in a PROTEAN IEF apparatus (Bio-Rad, Hercules, CA), in three steps according to the manufacturer's instructions (step 1: 250V, 15 min; step 2: 8,000V, 2.5 h; step 3: 8000V, 35,000 V-hr). After focusing, strips were equilibrated in two steps (20 min each, 20°C) using equilibration buffer 1 (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol and 2.5% (w/v) DTT) and buffer 2 (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol and 2.5% (w/v) iodoacetamide). Ovarian proteins in IPG strips were resolved in discontinuous 10% Tris-HCl SDS-PAGE preparative well precast gel (BioRad, Hercules, CA) in the second dimension. Molecular size was determined by comparison to a chemiluminescent protein standard (MagicMarker Mix, broad range, Invitrogen, Carlsbad, CA). Proteins were transferred to a nitrocellulose membrane (0.45 µm; BioRad, Hercules, CA)[33]. and reactions of four representative sera (positive for antibodies in immunoassay) against normal and tumor ovarian proteins were assessed in immunoblotting as reported previously[10]. Blots were blocked overnight in Starting Blocker (Pierce, Rockford, IL) containing 0.05% Tween-20, and then incubated with sera (1:100, 2 h) and washed 3X with Tris-buffered saline containing 0.05% Tween 20. Blots were then incubated with peroxidase-conjugated $F(ab)_2$ fragment affinity purified rabbit anti-chicken IgY (IgG) (H+L) (Jackson Immuno Research Laboratories Inc., West Grove, PA) (1:10,000, 1 h, 20°C). Immunoreactions were visualized as a chemiluminescence

product (Super Dura West substrate, Pierce, Rockford, IL) and the image was captured using a Chemidoc XRS (BioRad, Hercules, CA).

Mass Spectrometry (MS) and protein identification

Sypro Ruby stained protein spots matched with two-dimensional Western blots were excised manually from gels and proteins were identified by LC-MS/MS. Proteins were digested with trypsin according to the manufacturer's protocol (Pierce, In-Gel Trypsin Digestion kit). Cystine disulfides were reduced to free cysteine and alkylated with iodoacetamide. Peptide digests were desalted on a Michrom (Auburn, CA) peptide CapTrap. The CapTrap was placed in-line as a pre-column, to a New Objective (www.newobjective.com) 10cm x 75 □m i.d. C18 ProteoPrep nano-HPLC column attached to a NewObjective nano-ESI source interfaced to ThermoFinnigan (San Jose, CA) LTQ ion trap mass spectrometer. Peptides were separated and eluted into the nano-ESI interface with a reverse-phase LC gradient of solvent A (0.1% formic acid, Fluka)/water (Riedel-deHaen) versus solvent B (0.1% formic acid/70% acetonitrile (Riedel-deHaen). A linear gradient of 5% to 100% solvent B, at a flow rate of 200 nanoL/min for 70 min was delivered by a Dionex (www.dionex.com) Ultimate 3000 nanoLC. Full scan MS spectra, m/z 440 -2000, high resolution zoom scan, and MS/MS spectra were obtained by a data dependent data acquisition "Triple Play" experiment. Instrument parameters include: ESI voltage 2.1kV, heated capillary 180°C; for MS/MS, isolation width 1.5 m/z, activation Q 0.25, activation time 30 msec, collision energy 35%, 2 x 100 msec microscans. The resultant MS/MS spectra were assessed for peptide sequence with SEQUEST. A peptide mass tolerance of 1.0 amu and fragment ion tolerance of 1.0 amu, strict trypsin cleavage at R & K, dynamic

differential modifications of 16 amu for M and 1 amu for N. Spectra were then searched against a complete set of Avian proteins in GenBank v.156.

Analysis

In immunoassay, samples were assessed against the same negative control sera from young laying hens (11 months old, laying rate of 5 or more eggs/clutch, n=5) in every assay. Values considered positive for antibody had an optical density greater than three standard deviations (P<.01) above the mean optical density value for negative control sera[31]. The proportion of sera positive for ovarian or tumor antibodies was determined for each group and results were compared using a Chi-square test with P<0.01 considered significant. In addition, the mean optical density values were compared using a two tailed Student's t test for unequal variance with P<0.05 considered significant. The correlation of optical density values for normal ovarian antigens and tumor antigens was compared using Spearman's rho two-tailed test, means were compared by ANOVA using Games-Howell GH method for unequal variances and unequal sample sizes with p<0.05 considered significant. Statistical analysis was performed with SPSS (Chicago, IL)

Results

Gross morphology and histology

Upon necropsy, 2 or 3 large yolk-filled preovulatory follicles, few developing small follicles and 1 or 2 degrading postovulatory follicles were observed consistent with reduced ovarian function (Fig. 1A). In hens with abnormal ovaries, the 2-3 large preovulatory follicles were either atretic or cystic (Fig. 1B). In hens with OvCa, ovaries contained solid nodular masses or firm white

cauliflower-like structures, with no yolky preovulatory follicles (Fig. 1C). In some hens, tumors were metastasized to peritoneal organs accompanied by profuse reddish brown ascites (Fig. 1D).

More developing follicles were seen in ovarian sections of hens with normal ovaries than in hens with tumors or abnormal ovaries. Frequent atresia of follicles containing fat globules with ruptured theca and granulosa layers and cysts were seen in the ovary of hens with non-tumor abnormalities. Although occasional stromal hyperplasia was seen, neither focal lesions nor cells with pleomorphic nuclei were seen in abnormal ovaries. Serous OvCa (n=5, 2 early and 3 late stages) was characterized by malignant cells with pleomorphic nuclei and mitotic figures. Endometrioid carcinoma (n=5, 2 early and 3 late stages) was identified by glandular structures resembling endometrial glands with a lining of columnar, cuboidal or oval epithelial cells. In mucinous carcinoma (n=4, 3 early and 1 late stages), glandular ciliated goblet cells with luminal secretion were present and clear cell carcinoma (n = 1, early stage) was identified by cells with clear cytoplasm (Fig. 2a-d). In addition, poorly differentiated or mixed ovarian carcinomas (n = 3, 2 early and 1 late stage) were observed occasionally in some hens.

Anti-tumor antibodies detected by immunoassay

The mean optical density values for both anti-tumor and anti-ovarian antibodies in the serum of hens with OvCa was significantly higher than in hens with a normal ovary or hens with abnormal ovarian pathologies ($P<0.05$) (Fig 3). Moreover, the optical density values for anti-tumor and anti-ovarian antibodies were significantly correlated except for hens with ovarian abnormalities [experimental controls (young hens), correlation co-efficient = 0.92, $P = 0.05$; normal (age matched old hens without any ovarian abnormality), correlation co-efficient = 0.90, $P = 0.01$; ovarian cancer, correlation co-efficient = 0.88, $P = 0.01$).

The proportion of sera positive for anti-tumor antibodies (67%, $P < 0.001$) or anti-ovarian antibodies (56%, $P < 0.01$) in hens with OvCa was significantly higher than hens with a normal ovary (15% and 21%, respectively). Anti-tumor antibodies (63%) but not anti-ovarian antibodies (38%) were more prevalent in hens with abnormal ovaries than in hens with normal ovary ($P < 0.03$). The proportion of serum antibodies in hens with OvCa using tumor or normal ovarian antigens did not differ significantly. Differences in the prevalence of anti-tumor antibodies by tumor type were not observed.

Anti-tumor antibody reactions with normal and tumor ovarian antigens in western blot

Two-dimensional electrophoresis and Western blots were performed using selected OvCa sera ($n = 4$, 1 each of serous, endometrioid, mucinous and clear cell carcinoma) with representative immunoreactions based on immunoassay and one-dimensional Western blot (data not shown). Owing to the heterogeneity in protein expression, serum from individual hens with OvCa was examined against homologous as well as heterologous tumor antigens (Fig. 4). Common immunoreactive spots of approximately 50 kDa were seen against normal or tumor ovarian antigens (Fig. 4b-d). Some spots at approximately 30-40 kDa range were different between normal and tumor ovarian antigens. In addition, among the tumors of different sources, spots of similar (dotted lines and circles) as well as different (triangular and square) molecular weights (20-40 kDa, Fig. 4) were seen. Two dimensional electrophoresis and western blot separates the individual immunoreactive antigens from the whole ovarian proteome but it does not identify the ovarian antigenic proteins. Therefore, mass spectrometry was used to identify these immunoreactive ovarian tumor antigens.

Identification of antigens

Proteins identified are summarized in Table 1 and an example of a representative 2-DE gel with hen ovarian tumor ovarian proteins indicating their approximate location are shown in Figure 5. Among identified proteins, number of low molecular wt (20-50 kDa) proteins was more than high molecular wt (higher than 50kDa) proteins. Heat shock cognate and chaperonin GroEL were the two identified proteins from spots at 60-80 kDa. Proteins identified on the 50-60 kDa range include vimentin and ATP synthase subunit β. Enolase and protein disulfide isomerase A6 precursor were the two proteins identified at the range of 40-50 kDa. Proteins identified in the low molecular wt range (20-40 kDa) include apolipoprotein A1, Annexin A2/Annexin II, UDP-glucose-4-epimerase, ester hydrolase, peroxiredoxin (PRX), Rho GDP-dissociation inhibitor-1, D4-GDP-dissociation inhibitor, Protein DJ-1 (Fig. 5).

Discussion

The results of this study, for the first time, demonstrated an anti-tumor antibody response in the egg-laying hen, a spontaneous animal model of OvCa. The prevalence of antibodies associated with OvCa in hens is similar for ovarian antigens from both normal and tumor ovary. This suggests the reactive antigens are not unique to the tumors, similar to anti-tumor antibodies in human OvCa[10]. The ovarian tumors have the same epithelial cell derived histology as human tumors. Antibodies were associated with all histological types including endometrioid, mucinous, clear cell and mixed types of OvCa. Furthermore, anti-tumor antibodies were detected in hens irrespective of OvCa stage suggesting the possibility that the antibody response occurs early in tumor progression.

The antibody response to tumors is a characteristic feature of cancer including OvCa [3, 10, 34]. The frequency of anti-tumor antibodies to individual antigens in patients with OvCa ranges from 5- 67%[35]. Previously, we reported that 69% of patients with OvCa had anti-tumor antibodies that reacted to antigens in ovarian tumor homogenates [10]. In the present study, a similar prevalence of anti-tumor antibodies was observed in hens with OvCa. Immunoreactive antigens detected in 2D-WB showed some commonality in their expression in both normal ovaries and ovarian tumors with a predominant reaction at 50 kDa. However, there were obvious differences in immunoreactions between antigens from normal and tumor ovaries as well as between homologous and heterologous (different) sources. Therefore, as reported in humans with OvCa, immunoreactive ovarian antigens are also heterologus in hens OvCa[10]. Thus, in both hen and human OvCa, there is a humoral immune response against ovarian tumor antigens detected by serum anti-tumor antibodies.

This is the first report on the immunoreactive ovarian antigens detected by serum anti-tumor antibodies. Fourteen ovarian proteins were identified as the best match in this study as possible antigens for anti-tumor antibodies. Some of the identified proteins are structural or regulatory proteins where as others are members of enzyme families. None of these proteins is known to be unique to OvCa either in hens or humans. No published information on the ovarian antigens associated with OvCa in hens is available. Among these proteins, increased expression of vimentin was reported in the pulmonary sarcoma in hens[36]. Interestingly, some of the identified proteins were suggested to be expressed in the several cancers and autoimmune diseases in humans and other animals. Apolipoprotein A1 is a by-product of host's response to tumor and down regulated in OvCa in humans[37]. Annexin II and DJ-1 were reported to be involved in the tumor invasion and metastasis of breast, prostate GI and lung cancers [38-40]. Vimentin and

enolase are the two antigens reported in several autoimmune diseases including animal model of lupus, inflammatory diseases, endometriosis and tumors of several organs[41-44].

One of the limitations of the present study is that the immunoassay using ovarian antigens could not differentiate hens with non-tumor ovarian abnormalities and those with ovarian tumors with regard to the presence of ovarian autoantibodies. Abnormal ovaries may be an early event in the onset of malignancy. Hens with abnormal ovaries including, those neither with developed preovulatory follicles, follicular atrophy nor with gross tumors also showed the presence of anti-ovarian antibodies as compared to their old normal counterpart. It is possible that these hens might have developed ovarian autoimmunity. Previous study reported that circulatory ovarian autoantibodies were associated with reduced ovarian function and decreased egg production in older hens[45]. Therefore, it might be possible that ovarian autoimmunity may precede ovarian cancer. In contrast, it might also be possible that some of these hens were at the initial stage of molecular transformation of ovary that could not be perceived by light microscope. Another drawback of the present study is the differences in immunoreactions between hens that may limit the utility of normal ovarian and tumor antigen detection in routine and/or screening tests. However, the screening test could be based on more than one antigenic protein. In addition to the usefulness as detection tools, these antigens might be useful as markers for monitoring patient's response to treatment modalities.

The principle aim of our study is to understand the etiology and the early events in the progression of OvCa so as to develop an early detection tool for the disease. Over the years chickens have been used extensively as an established or emerging model in biomedical research and contributed immensely to the understanding of immune system as well as many other human diseases including genetic disorders, drug testing, immune suppression, multiple sclerosis and

autoimmune diseases. Moreover, chickens are the only animal model in which a vaccine has been developed against cancer (Marek's disease)[46]. Demonstration of anti-ovarian tumor immune response in hens similar to humans will be of immense significance to the understanding of OvCa in humans including diagnostic and therapeutic interventions. Numerous studies have attempted to evaluate the clinical value of circulating anti-tumor antibodies in the context of neoplastic process and the results were confusing. Following the detection of anti-tumor antibodies in circulation, hen can be followed for ovarian changes leading to OvCa by *in vivo* imaging. Recently, we have developed, for the first time, the modalities of *in vivo* imaging of ovarian tumors by transvaginal Doppler ultrasonography with using probes used for humans[47]. The results of this study will reinforce the suitability of laying hen as a preclinical animal model of OvCa.

In conclusion, the result of the present study showed that majority of the serum from hens with OvCa contained anti-ovarian and anti-tumor antibodies. Thus, antibodies are a potential diagnostic marker for OvCa and laying hens can be used to understand the etiology of OvCa in humans. The current study confirms the existence of spontaneous OvCa in laying hens with similar metastasis to abdominal tissues and profuse ascites as in humans. All major types of epithelial ovarian tumors including serous, endometrioid, mucinous, clear cell and mixed types were observed in hens and are similar to humans in histological features.

Acknowledgements

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References

1. Jemal A, Siegel R, Ward E *et al*. Cancer statistics, 2007. *CA Cancer J Clin* 2007;**57(1):** 43-66.
2. Turk MJ, Wolchok JD, Guevara-Patino JA *et al*. Multiple pathways to tumor immunity and concomitant autoimmunity. *Immunol Rev* 2002;**188:** 122-35.
3. Preuss KD, Zwick C, Bormann C *et al*. Analysis of the B-cell repertoire against antigens expressed by human neoplasms. *Immunol Rev* 2002;**188:** 43-50.
4. Guevara-Patino JA, Turk MJ, Wolchok JD, Houghton AN. Immunity to cancer through immune recognition of altered self: studies with melanoma. *Adv Cancer Res* 2003;**90:** 157-77.
5. Luborsky JL, Barua A, Shatavi SV *et al*. Anti-tumor antibodies in ovarian cancer. *Am J Reprod Immunol* 2005;**54(2):** 55-62.
6. Koziol JA, Zhang JY, Casiano CA et al. Recursive partitioning as an approach to selection of immune markers for tumor diagnosis. *Clin Cancer Res* 2003;**9(14):** 5120-6.
7. Hanash S. Harnessing immunity for cancer marker discovery. *Nat Biotechnol* 2003;**21(1):** 37-8.
8. Imafuku Y, Omenn GS, Hanash S. Proteomics approaches to identify tumor antigen directed autoantibodies as cancer biomarkers. *Dis Markers* 2004;**20(3):** 149-53.
9. Gnjatic S, Atanackovic D, Jager E *et al*. Survey of naturally occurring CD4+ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. *Proc Natl Acad Sci U S A* 2003;**100(15):** 8862-7.
10. Barua A, Bradaric MJ, Kebede T *et al*. Anti-tumor and anti-ovarian autoantibodies in women with ovarian cancer. *Am J Reprod Immunol* 2007;**57(4):** 243-9.
11. Taylor DD, Gercel-Taylor C. Tumor-reactive immunoglobulins in ovarian cancer: diagnostic and therapeutic significance? (review). *Oncol Rep* 1998;**5(6):** 1519-24.
12. Stockert E, Jager E, Chen YT *et al*. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J Exp Med* 1998;**187(8):** 1349-54.
13. Sugita Y, Wada H, Fujita S *et al*. NY-ESO-1 expression and immunogenicity in malignant and benign breast tumors. *Cancer Res* 2004;**64(6):** 2199-204.
14. Jager E, Stockert E, Zidianakis Z *et al*. Humoral immune responses of cancer patients against "Cancer-Testis" antigen NY-ESO-1: correlation with clinical events. *Int J Cancer* 1999;**84(5):** 506-10.
15. Scanlan MJ, Gout I, Gordon CM *et al*. Humoral immunity to human breast cancer: antigen definition and quantitative analysis of mRNA expression. *Cancer Immun* 2001; **1:** 4.
16. Chen YT, Scanlan MJ, Sahin U *et al*. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci U S A* 1997;**94(5):** 1914-8.
17. Li G, Miles A, Line A, Rees RC. Identification of tumour antigens by serological analysis of cDNA expression cloning. *Cancer Immunol Immunother* 2004;**53(3):** 139-43.
18. Li Y, Karjalainen A, Koskinen H *et al*. p53 autoantibodies predict subsequent development of cancer. *Int J Cancer* 2005;**114(1):** 157-60.

19. Suzuki H, Graziano DF, McKolanis J, Finn OJ. T cell-dependent antibody responses against aberrantly expressed cyclin B1 protein in patients with cancer and premalignant disease. *Clin Cancer Res* 2005;**11(4):** 1521-6.
20. Vanderhyden BC, Shaw TJ, Ethier JF. Animal models of ovarian cancer. *Reprod Biol Endocrinol* 2003;**1:** 67.
21. Stakleff KD, Von Gruenigen VE. Rodent models for ovarian cancer research. *Int J Gynecol Cancer* 2003;**13(4):** 405-12.
22. Johnson PA, Giles JR. Use of genetic strains of chickens in studies of ovarian cancer. *Poult Sci* 2006;**85(2):** 246-50.
23. Goodman MT, Howe HL, Tung KH *et al*. Incidence of ovarian cancer by race and ethnicity in the United States, 1992-1997. *Cancer* 2003;**97(10 Suppl):** 2676-85.
24. Fredrickson TN. Ovarian tumors of the hen. *Environ Health Perspect* 1987;**73:** 35-51.
25. Giles JR, Shivaprasad HL, Johnson PA. Ovarian tumor expression of an oviductal protein in the hen: a model for human serous ovarian adenocarcinoma. *Gynecol Oncol* 2004;**95(3):** 530-3.
26. Damjanov I. Ovarian tumours in laboratory and domestic animals. *Curr Top Pathol* 1989;**78:** 1-10.
27. Rodriguez-Burford C, Barnes MN, Berry W *et al*. Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention. *Gynecol Oncol* 2001; **81(3):** 373-9.
28. Jackson E, Anderson K, Ashwell C *et al*. CA125 expression in spontaneous ovarian adenocarcinomas from laying hens. *Gynecol Oncol* 2007; **104(1):** 192-8.
29. Urick ME, Johnson PA. Cyclooxygenase 1 and 2 mRNA and protein expression in the Gallus domesticus model of ovarian cancer. *Gynecol Oncol* 2006; **103(2):** 673-8.
30. Warren D, Scott, HM. Time factor in poultry production. *Poultry Science* 1935; **14:** 195-207.
31. Luborsky JL, Visintin I, Boyers S, *et al*. Ovarian antibodies detected by immobilized antigen immunoassay in patients with premature ovarian failure. *J Clin Endocrinol Metab* 1990;**70(1):** 69-75.
32. Le Naour F, Brichory F, Misek DE, *et al*. A distinct repertoire of autoantibodies in hepatocellular carcinoma identified by proteomic analysis. *Mol Cell Proteomics 2002*; *1(3):* 197-203.
33. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979;**76(9):** 4350-4.
34. Dong H, Strome SE, Salomao DR, *et al.* Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002;**8(8):** 793-800.
35. Naora H, Montz FJ, Chai CY, Roden RB. Aberrant expression of homeobox gene HOXA7 is associated with mullerian-like differentiation of epithelial ovarian tumors and the generation of a specific autologous antibody response. *Proc Natl Acad Sci U S A* 2001;**98(26):** 15209-14.
36. Hafner S, Goodwin MA, Smith EJ *et al*. Pulmonary sarcomas in a young chicken. *Avian Dis* 1998;*42(4):* 824-8.
37. Zhang Z, Bast RC, Jr., Yu Y *et al.* Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. *Cancer Res* 2004;**64(16):** 5882-90.

38. Sharma MR, Koltowski L, Ownbey RT *et al.* Angiogenesis-associated protein annexin II in breast cancer: selective expression in invasive breast cancer and contribution to tumor invasion and progression. *Exp Mol Pathol* 2006;**81(2):** 146-56.
39. Singh P. Role of Annexin-II in GI cancers: interaction with gastrins/progastrins. *Cancer Lett* 2007;**252(1):** 19-35.
40. Clements CM, McNally RS, Conti BJ, *et al.* DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2. *Proc Natl Acad Sci U S A* 2006;**103(41):** 15091-6.
41. Vossenaar ER, Despres N, Lapointe E, *et al*. Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin. *Arthritis Res Ther* 2004;**6(2):** R142-50.
42. Thebault S, Gilbert D, Hubert M *et al*. Orderly pattern of development of the autoantibody response in (New Zealand White x BXSB)F1 lupus mice: characterization of target antigens and antigen spreading by two-dimensional gel electrophoresis and mass spectrometry. *J Immunol* 2002;**169(7):** 4046-53.
43. Gitlits VM, Toh BH, Sentry JW. Disease association, origin, and clinical relevance of autoantibodies to the glycolytic enzyme enolase. *J Investig Med* 2001;**49(2):** 138-45.
44. He P, Naka T, Serada S, *et al.* Proteomics-based identification of alpha-enolase as a tumor antigen in non-small lung cancer. *Cancer Sci* 2007;**98(8):** 1234-40.
45. Barua A, Yoshimura Y. Ovarian autoimmunity in relation to egg production in laying hens. *Reproduction* 2001;**121(1):** 117-22.
46. Millers SH. Increased Marek's Condemnations….New Strains or Old Problems? *Broiler Industry* 1996;**(May):** 26-31.
47. Barua A, Abramowicz JS, Bahr JM, *et al.* Detection of ovarian tumors in chicken by sonography: a step toward early diagnosis in humans? *J Ultrasound Med* 2007;**26(7):** 909-19.

Figure Legends

Figure 1. Workflow schematic diagram showing the methods used for the separation and identification of immunoreactive ovarian antigens from laying hens with or without ovarian tumors. Please refer to the materials and methods for detailed description.

Figure 2. Gross morphology of ovaries of White Leghorn egg-laying hens (2.5-3.0 years old) with or without tumor. A) normal ovary showing 3 large preovulatory follicles (F_3-F_1). Following ovulation of the largest one (F_1), F_2 becomes F_1; F_3 becomes F_2 and one from the stock of growing follicles will be recruited to the set of largest preovulatory follicles. In between the preovulatory follicles, is a degrading postovulatory follicle, B) abnormal ovary. All the large preovulatory follicles are atretic and shrunken with yellow or black brown color (arrows). However, small growing follicles are present, C) early stage ovarian cancer. The tumor is solid with a cauliflower-like appearance (circle), well formed nodules and is limited to the ovary without ascites or preovulatory follicles, D) late stage ovarian cancer with extensive metastasis to the abdominal cavity and extra-ovarian solid masses accompanied by profuse ascites (*).

Figure 3. Histology of ovarian carcinoma in egg-laying hens. A) Clear cell carcinoma showing solid sheets of polyhedral cells with abundant clear cytoplasm with dense hyalinized fibrotic stroma. B) Well developed endometrioid carcinoma with confluent pattern of back to back glands. C) Mucinous carcinoma showing glandular epithelium containing ciliated goblet cells and mucin-like secretory products in the lumen of the gland. D) Serous carcinoma. Solid pattern of sheets beneath the surface epithelium containing moderate to large-sized cells with scanty cytoplasm and pleomorphic nuclei separated by septal tissue. Hematoxylin and eosin stain, 40X.

Figure 4. Anti-tumor antibodies in sera of hens with or without ovarian cancer determined by immunoassay. Optical density values are displayed as a box-and-whiskers plot with median, range (whiskers), 25th to 75th percentile (box) and outliers (circles). Antibodies were detected in two assays using antigens either from normal ovary or from ovarian tumors. Sera (n = 26; age range 2.5 to 3.0 years) from hens with ovarian cancer (n = 18), abnormal (non-tumor ovarian pathology, n = 8) were compared with normal hens (healthy hens of the same age, n = 39) and experimental controls (young laying hens). The mean optical density of ovarian cancer sera was significantly higher than those of controls.

Figure 5. An example of the immunoreactivity of a serum from a hen with ovarian cancer against normal ovarian antigens and autologous or heterologous tumor antigens in two-dimensional Western blot (2D-WB). A) control 2D-WB with ovarian tumor antigen but omitting serum shows the lack of background reaction except for interaction of the second antibody with IgY. Ovarian cancer sera reacted against B) normal ovarian antigens, C) homologous ovarian tumor antigens and D) heterologous ovarian tumor antigens. Anti-tumor antibodies reacted with both normal and ovarian tumor antigens, consistent with immunoassay results (see Fig. 3). Although variations in the immunoreactivities among different sources of antigens are seen, common immunoreactions at approximately 50 kDa are also observed. Sera reacted more with homologous tumor antigens than heterologous tumor antigens. Spots of similar (dotted lines and

circles) as well as different (triangular and square) molecular weights (20-40 kDa) were seen in heterologus tumors.

Figure 6. Representative two-dimensional gel of hen ovarian tumor protein (corresponding to Western blot immunostained with serum from same hen) stained with Sypro Ruby. The tryptic peptides from proteins in spots corresponding to Western blot reactions with ovarian cancer sera were analyzed by LC-MS/MS (see the methods for detail).

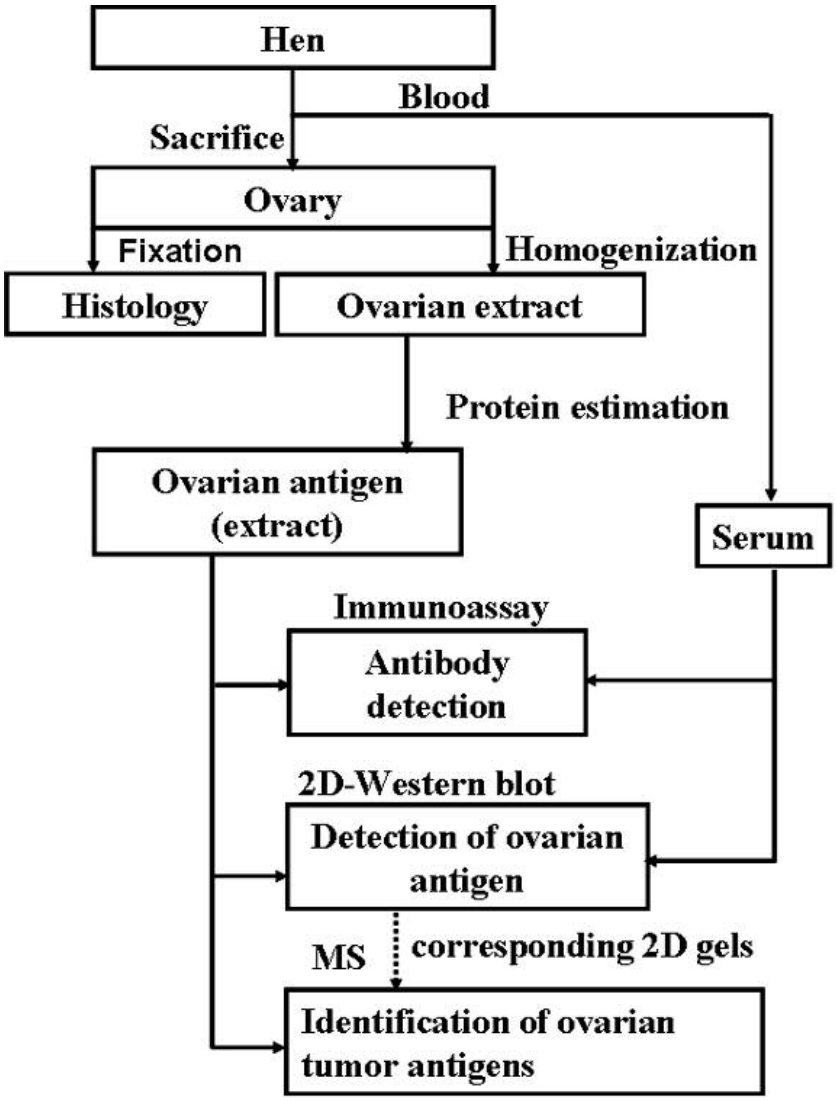

Figure 1.

Figure 1. Workflow schematic diagram showing the methods used for the separation and identification of immunoreactive ovarian antigens from laying hens with or without ovarian tumors. Please refer to the materials and methods for detailed description.
50x70mm (300 x 300 DPI)

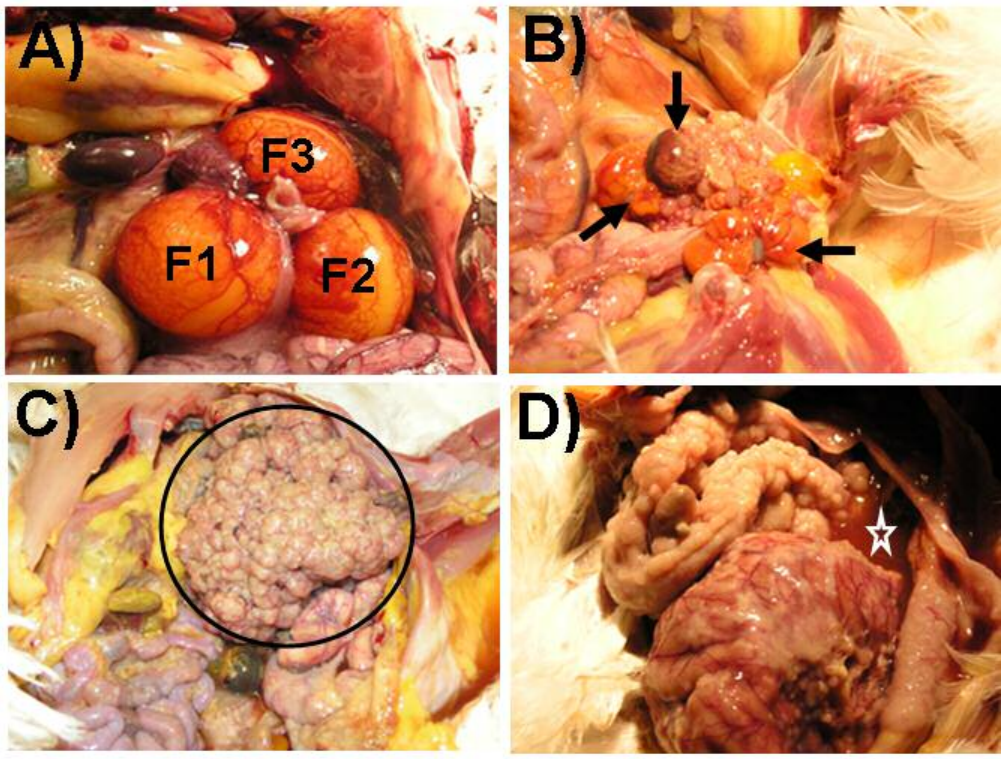

Figure 2

Figure 2. Gross morphology of ovaries of White Leghorn egg-laying hens (2.5-3.0 years old) with or without tumor. A) normal ovary showing 3 large preovulatory follicles (F3-F1). Following ovulation of the largest one (F1), F2 becomes F1; F3 becomes F2 and one from the stock of growing follicles will be recruited to the set of largest preovulatory follicles. In between the preovulatory follicles, is a degrading postovulatory follicle, B) abnormal ovary. All the large preovulatory follicles are atretic and shrunken with yellow or black brown color (arrows). However, small growing follicles are present, C) early stage ovarian cancer. The tumor is solid with a cauliflower-like appearance (circle), well formed nodules and is limited to the ovary without ascites or preovulatory follicles, D) late stage ovarian cancer with extensive metastasis to the abdominal cavity and extra-ovarian solid masses accompanied by profuse ascites (*).
176x165mm (96 x 96 DPI)

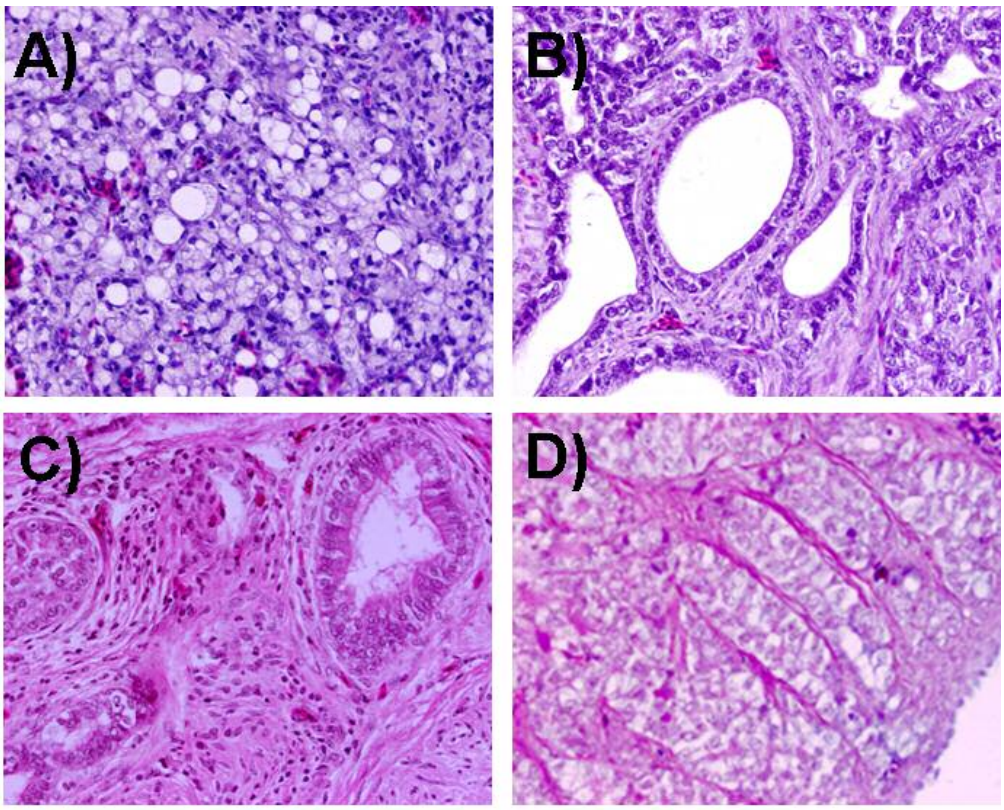

Figure 3

Figure 3. Histology of ovarian carcinoma in egg-laying hens. A) Clear cell carcinoma showing solid sheets of polyhedral cells with abundant clear cytoplasm with dense hyalinized fibrotic stroma. B) Well developed endometrioid carcinoma with confluent pattern of back to back glands. C) Mucinous carcinoma showing glandular epithelium containing ciliated goblet cells and mucin-like secretory products in the lumen of the gland. D) Serous carcinoma. Solid pattern of sheets beneath the surface epithelium containing moderate to large-sized cells with scanty cytoplasm and pleomorphic nuclei separated by septal tissue. Hematoxylin and eosin stain, 40X.
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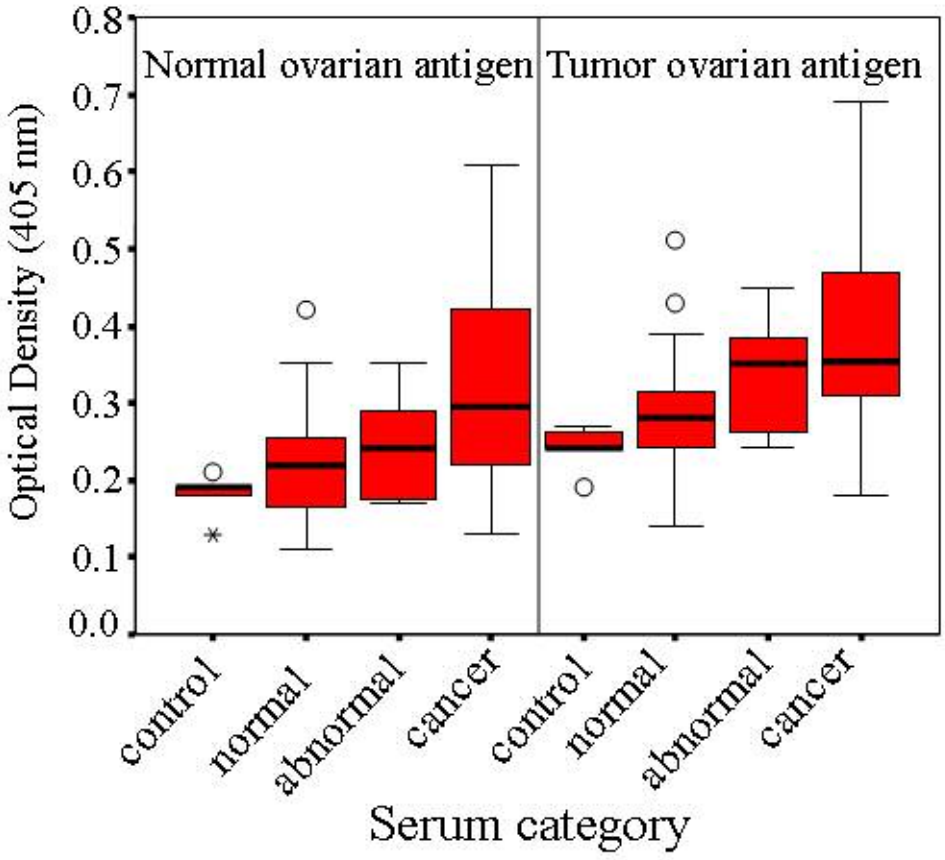

Figure 4

Figure 4. Anti-tumor antibodies in sera of hens with or without ovarian cancer determined by immunoassay. Optical density values are displayed as a box-and-whiskers plot with median, range (whiskers), 25th to 75th percentile (box) and outliers (circles). Antibodies were detected in two assays using antigens either from normal ovary or from ovarian tumors. Sera (n = 26; age range 2.5 to 3.0 years) from hens with ovarian cancer (n = 18), abnormal (non-tumor ovarian pathology, n = 8) were compared with normal hens (healthy hens of the same age, n = 39) and experimental controls (young laying hens). The mean optical density of ovarian cancer sera was significantly higher than those of controls.

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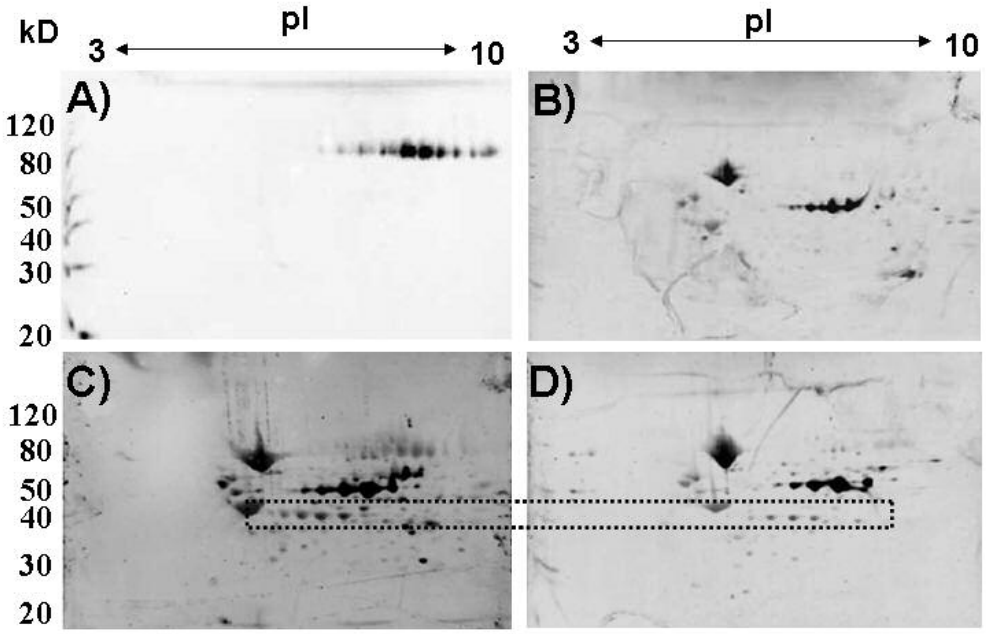

Figure 5

Figure 5. An example of the immunoreactivity of a serum from a hen with ovarian cancer against normal ovarian antigens and autologous or heterologous tumor antigens in two-dimensional Western blot (2D-WB). A) control 2D-WB with ovarian tumor antigen but omitting serum shows the lack of background reaction except for interaction of the second antibody with IgY. Ovarian cancer sera reacted against B) normal ovarian antigens, C) homologous ovarian tumor antigens and D) heterologous ovarian tumor antigens. Anti-tumor antibodies reacted with both normal and ovarian tumor antigens, consistent with immunoassay results (see Fig. 3). Although variations in the immunoreactivities among different sources of antigens are seen, common immunoreactions at approximately 50 kDa are also observed. Sera reacted more with homologous tumor antigens than heterologous tumor antigens. Spots of similar (dotted lines and

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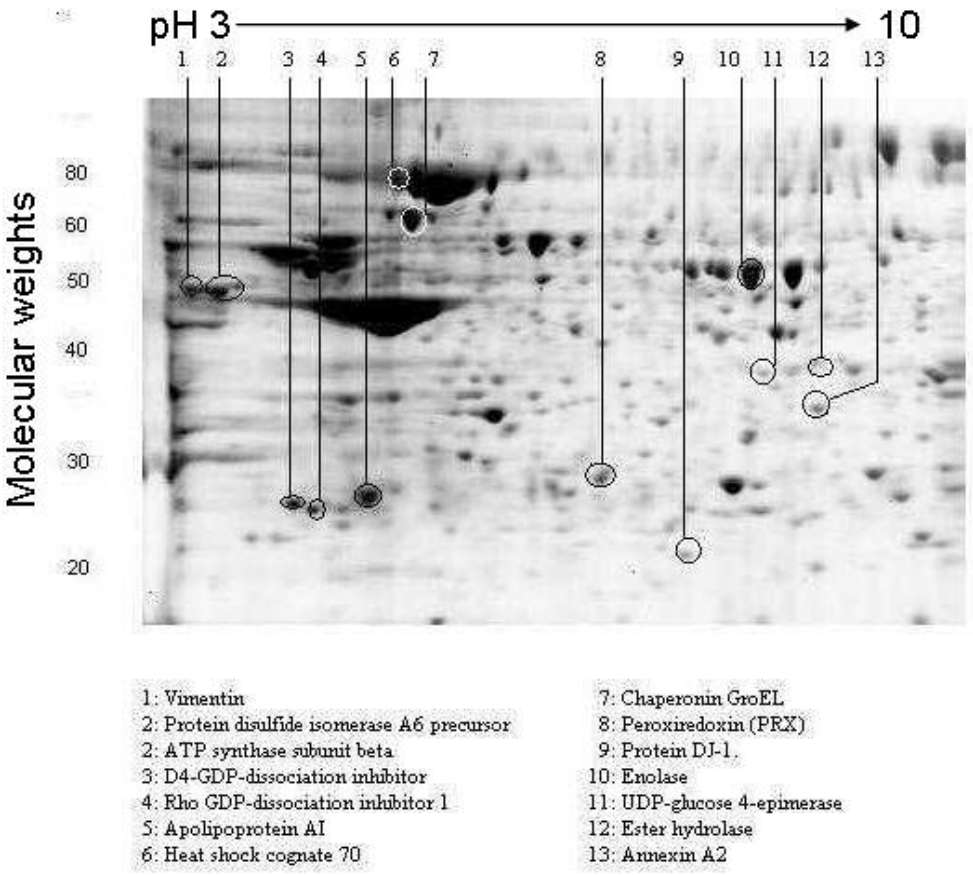

Figure 6

Figure 6. Representative two-dimensional gel of hen ovarian tumor protein (corresponding to Western blot immunostained with serum from same hen) stained with Sypro Ruby. The tryptic peptides from proteins in spots corresponding to Western blot reactions with ovarian cancer sera were analyzed by LC-MS/MS (see the methods for detail).

142x161mm (96 x 96 DPI)

Table 1. Immunoreactive ovarian antigens identified by mass spectrometry.

Acc number	Title	Mass (kDa)	pI	sequest score	flicka hit	coverage
NP_990856.1	apolipoprotein AI (put.); putative	30.7	5.6	19	15	51
XP_429266.1	heat shock cognate 70	71	5.6	6.2	5	15
CAG31521	Chaperonin GroEL (HSP60 family)	60	5.7	94	26	54
NP_990451.1	enolase	47.3	5.9	60.6	20	38.5
NP_990682.1	annexin A2; annexin II	38.6	6.9	2.6	5	20
XP_417833.1	UDP-glucose 4-epimerase	38.25	5.58	5.72	3	13.5
XP_417195.1	Predicted similar to Ester hydrolase	34.6	5.9	22.7	6	26.6
CAG32139.1	Peroxiredoxin (PRX) family, 1-cys PRX subfamily	24.5	5.8	41.5	11	44
BAB79527.1	Protein DJ-1.	19.9	6.8	12.5	7	51.3
hm11604	~human Rho GDP-dissociation inhibitor 1 (Rho GDI 1)	20		18.7	11	44
XP_416182.1	PREDICTED: ~ D4-GDP-dissociation inhibitor	22.8	4.9	9.6	5	49
CAG31468.1	ATP synthase subunit beta.	56.6	5.6	56.2	24	65.9
XP_418622.1	~ vimentin	53.1	5	30	26	57.6
XP_419952.1	PREDICTED: ~ Protein disulfide isomerase A6 precursor	48.8	5.1	12.5	10	29

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ORIGINAL PAPER

Cyclooxygenases expression and distribution in the normal ovary and their role in ovarian cancer in the domestic hen (*Gallus domesticus*)

Dale Buchanan Hales · Yan Zhuge · Jo Ann Jaen Lagman · Kristine Ansenberger · Cassandra Mahon · Animesh Barua · Judith Luborsky · Janice M. Bahr

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Abstract Cyclooxygenase (COX) (*PTGS*) is the rate-limiting enzyme in the biosynthesis of prostaglandins. Two COX isoforms have been identified, COX-1 and COX-2, which show distinct cell-specific expression and regulation. Ovarian cancer is the most lethal gynecological malignancy and the disease is poorly understood due to the lack of suitable animal models. The laying hen spontaneously develops epithelial ovarian cancer with few or no symptoms until the cancer has progresses to a late stage, similar to the human disease. The purpose of this study was to examine the relative expression and distribution of COX-1 and COX-2 in the ovaries of normal hens and in hens with ovarian cancer. The results demonstrate that COX-1 was localized to the granulosa cell layer and cortical interstitium, ovarian surface epithelium (OSE) and postovulatory follicle (POF) of the normal ovary. In ovarian cancer, COX-1 mRNA was significantly increased and COX-1 protein was broadly distributed throughout the tumor stroma. COX-2 protein was localized to the granulosa cell layer in the follicle and the ovarian stroma. COX-2 mRNA expression did not change as a function of age or in ovarian cancer. There was a significantly higher expression of COX-1 mRNA in the first POF (POF-1) compared to POF-2 and POF-3. COX-2 mRNA expression was not significantly different among POFs. There was no difference in COX-1 or COX-2 mRNA in the OSE isolated from individual follicles in the follicular hierarchy. The results confirm previous findings of the high expression of COX-1 in ovarian tumors further supporting the laying hen as a model for ovarian cancer, and demonstrate for the first time the high expression of COX-1 in POF-1 which is the source of prostaglandins needed for oviposition.

Keywords Ovarian cancer · Laying hen · Cyclooxygenase · Ovary · Oviposition

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Introduction

Ovarian cancer is the most lethal gynecological malignancy and the fifth leading cause of cancer death among women [1]. Ovarian cancer is usually detected only at a late stage with poor prognosis when significant peritoneal metastases and accompanying ascites have already developed. Research into ovarian cancer has been hampered by the lack of a suitable animal model for spontaneous ovarian cancer. In vivo animal models of ovarian cancer provide the opportunity to study each step of carcinogenesis from initiation through progression to late-stage metastatic disease. With the exception of the laying hen, no other accessible animal model spontaneously develops epithelial ovarian cancer similar in presentation and progression to the human disease [2–4].

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The OSE, a single layer of flat to cuboidal columnar epithelium consists of specialized mesothelial (celomic) cells that covers the entire ovarian surface [5]. The OSE is a unique epithelium that participates actively in the ovulatory process. Ninety percent of human ovarian carcinomas arise from the OSE [5]. The aging hen also develops OSE cancer spontaneously. The disease in hens appears to closely resemble the human disease, which in humans is correlated with ovulation frequency [6]. Whereas there are important rodent models for epithelial ovarian cancer, none of these ovarian carcinomas are linked to ovulation [7–13]. One of the most prevalent theories about the etiology of ovarian cancer is the "incessant ovulation hypothesis" first proposed by Fathalla [14]. He hypothesized that continuous ovulation, with successive rounds of surface rupture and OSE cell mitosis to repair the wound, renders the cells susceptible to malignant transformation. The observation that intensive egg-laying domestic hens frequently develop ovarian peritoneal carcinoma supports this hypothesis. Hens spontaneously develop significant numbers of ovarian adenocarcinomas similar in histological appearance and behavior to the common human ovarian epithelial carcinomas [6].

Cyclooxygenase (COX) (*PTGS*) is the rate-limiting enzyme in the biosynthesis of prostaglandins. COX catalyzes the conversion of arachidonic acid into prostaglandin H2, the precursor for all prostaglandins, prostacyclins and thromboxanes. Two COX isoforms have been identified, COX-1 and COX-2, which are encoded by different genes, *PTGS1* and *PTGS2* [15]. COX-1 and COX-2 have similar structural and kinetic properties but show distinct cell-specific expression and regulation. COX-1 is expressed constitutively in most cells, whereas the inducible COX-2 form is usually only expressed in response to various inflammatory stimuli [16]. COX-2 overexpression is observed in neurodegenerative diseases, acute and chronic inflammation, and a wide variety of epithelial cancers [17]. COX-2 expression contributes to tumor cell proliferation and survival in the majority of carcinomas [18, 19]. In contrast to most malignancies, however, COX-2 expression is down-regulated in ovarian cancer [20]. Instead, COX-1 expression has been shown to be elevated in ovarian cancer [8, 21–26]. Whereas considerable research has focused on the role of COX-2 in ovulation and normal ovarian function, much less is known about COX-1. The objective of this paper is to examine the distribution and relative expression of COX-1 and COX-2 in the ovary of normal hens and in hens with ovarian cancer, to gain insight into the origins and causes of ovarian cancer. The results of this study confirm and extend the prior observation that COX-1 expression is elevated in ovarian tumors of the hen [23] and further support the use of the hen model for ovarian cancer.

Results

Anatomy and pathology of the hen's ovary

A normal hen ovary consists of a hierarchy of 4–5 preovulatory follicles with F1 being the most mature and the next to ovulate. The most recent postovulatory follicle (POF-1) is shown immediately below F2 and consists of granulosa cells, a theca cell layer, and OSE (Fig. 1a). An ovarian carcinoma from a tumor confined exclusively to the ovary with no metastases or significant abdominal ascites fluid present on necropsy is shown in Fig. 1b. Hematoxylin and eosin (H&E) staining of a normal, healthy ovary shows a cortical follicle containing an oocyte surrounded by prominent granulosa and interstitial cell layers (Fig. 1c). H&E staining of a poorly differentiated ovarian tumor shows endometrioid-like glands (Fig. 1d). Gomori trichrome, which stains collagen green, connective tissue/cytoplasm red, and nuclei dark red, staining of normal ovary (Fig. 1e) and ovarian carcinoma (Fig. 1f) reveals how disorganized and poorly differentiated the carcinoma is compared to the normal ovary. All sections shown in Fig. 1 are from age matched old (165 weeks) hens.

COX-1 is upregulated in ovarian cancer and the postovulatory follicle

In the hen ovary (Fig. 2a), COX-1 is localized to the granulosa cells surrounding the oocyte. In the normal ovary, COX-1 is also present in the OSE and cortical stroma adjacent to the follicle (Fig 2a). In ovarian tumors from the hen (Fig. 2b), there is extensive COX-1 staining throughout the ovarian tumor. There is substantial COX-1 staining in POF-1 (Fig. 2c). COX-1 appears to be localized to the nuclei and peri-nuclear envelope.

COX-2 is widely distributed in the hen ovary and postovulatory follicle, but not increased in ovarian cancer

In the normal hen ovary, COX-2 is expressed in the granulosa cells of the follicle; it is also highly expressed in the interstitial tissue of the normal ovary (Fig. 3a). In ovarian tumors from the hen, (Fig. 3b), COX-2 expression is no longer associated with follicular structures, but distributed into discrete foci in the stroma of the ovarian tumor. COX-2 is widely distributed throughout POF-1 (Fig. 3c).

Quantification of COX-1 and COX-2 mRNA in normal ovaries from young and old chickens, compared to ovarian cancer

There was a significant ($P < 0.05$) increase in COX-1 mRNA levels in ovarian cancer compared to normal ovarian tissue

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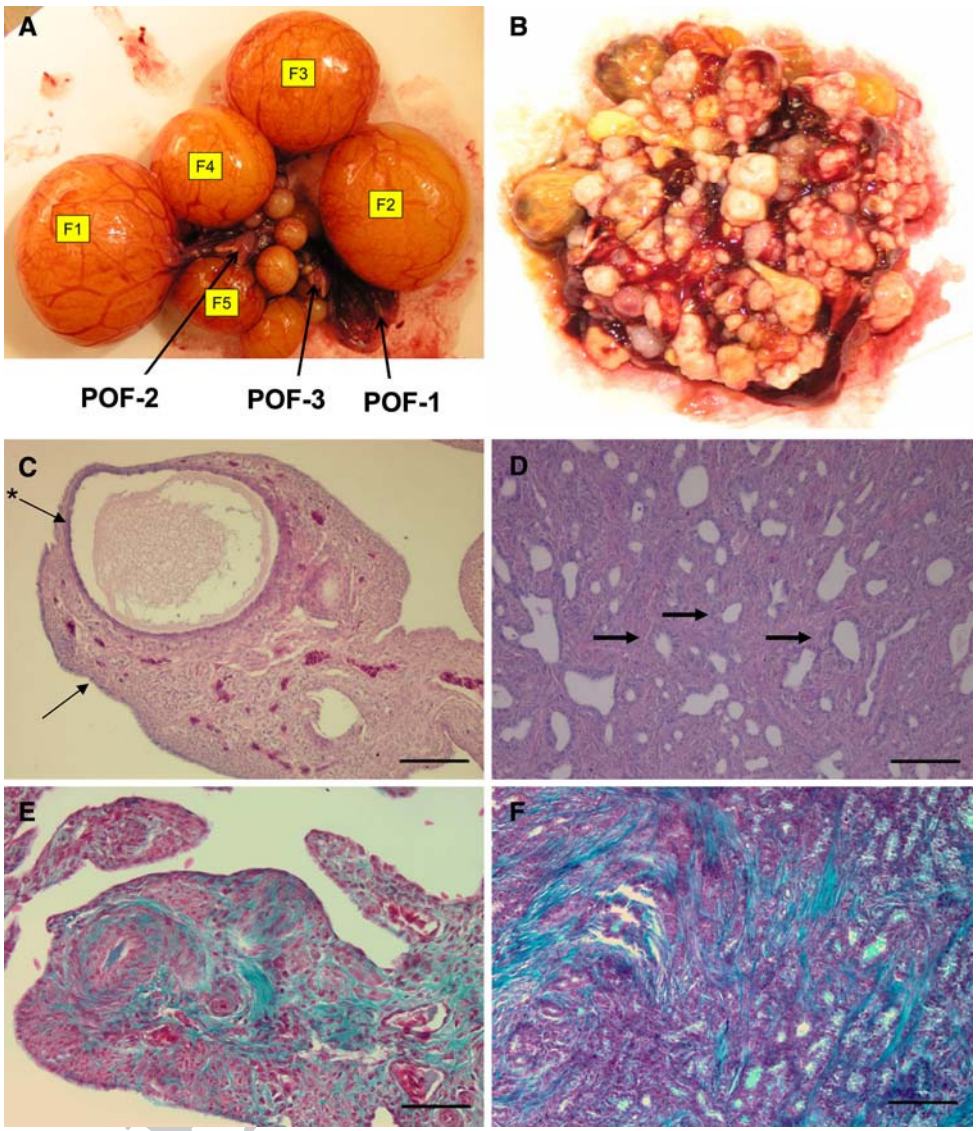

Fig. 1 Anatomy and pathology of the hen ovary: (**a**) Gross anatomy of normal ovary showing follicular hierarchy (F1–F5); arrows point to postovulatory follicles (POF-1–3). (**b**) Ovarian tumor from hen with cancer confined to the ovary. (**c**) H&E stain of normal ovary, showing small developing follicle, (arrow points to OSE, *arrow points to granulosa cell layer). (**d**) H&E stain of ovary with cancer (arrows points to endometrioid-like gland), (**e**) Gomori trichrome stain of normal ovary; (**f**) Gomori trichrome stain of ovarian tumor. Calibration bar, 100 μm

from young and age-matched old hens (Fig. 4A). In contrast, there was no significant change in COX-2 mRNA in normal ovarian tissue from old-normal hens, compared to young hens or in ovarian cancer compared to age-matched normal hens (Fig. 4B).

Quantification of COX-1 and COX-2 mRNA in postovulatory follicles from normal ovaries

COX-1 mRNA levels were significantly higher ($P < 0.01$) in POF-1 compared to POF-2 and POF-3 from young hens (Fig. 5A). There was no difference in COX-1 expression in POF-1 top versus POF-1 bottom (Fig. 5A).

COX-2 mRNA was quantified in POFs from normal ovarian tissue as described for COX-1 above (Fig. 5B). There was no significant change for COX-2 mRNA expression across the POFs. Also similar to COX-1, there was no difference in COX-2 mRNA levels in POF-1 top and bottom (Fig. 5B).

Quantification of COX-1 and COX-2 mRNA in OSE isolated from individual follicles and POFs from normal ovaries immediately after oviposition

COX-1 mRNA isolated from normal ovarian OSE from individual follicles (F3, F2, and F1) and from individual POFs (POF-1, POF-2, and POF-3), was quantified (Fig. 6A and B). There was no difference in COX-1 mRNA expression in OSE isolated from the three most mature preovulatory follicles (F1, F2, and F3, Fig. 6A). Strikingly, COX-1 mRNA expression was significantly higher ($P < 0.01$) in POF-1 compared to POF-2 and POF-3 (Fig. 6B). COX-1 mRNA levels in POF-2 and POF-3 were comparable to the level observed in the OSE of the preovulatory follicles (Fig 6A).

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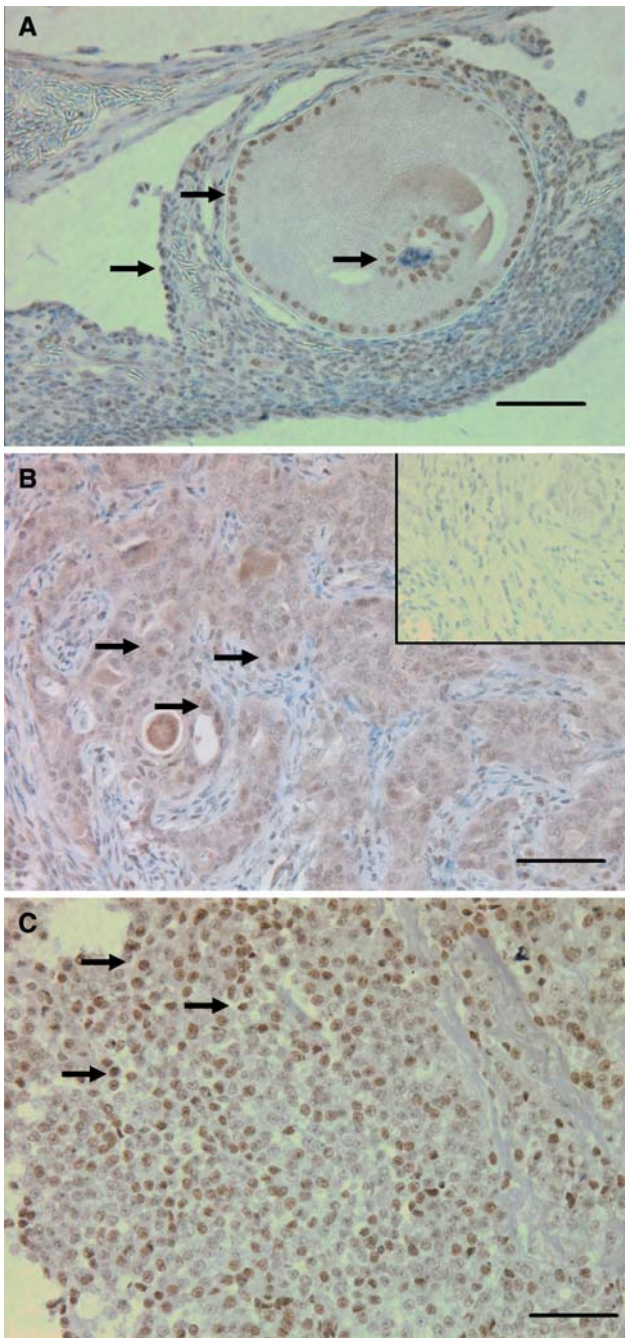

Fig. 2 COX-1 immunohistochemistry: (**a**) Normal ovary (arrows point to COX-1 positive nuclei); (**b**) Ovarian tumor (arrows point to COX-1 positive nuclei), *inset*: non-immune IgG. (**c**) POF-1 from normal ovary, COX-1 expression is seen throughout the ovarian tumor whereas in the normal ovary COX-1 is confined to the granulosa cells and adjacent stroma. COX-1 is highly expressed in POF-1 (arrows point to COX-1 positive nuclei). Calibration bar, 50 μm

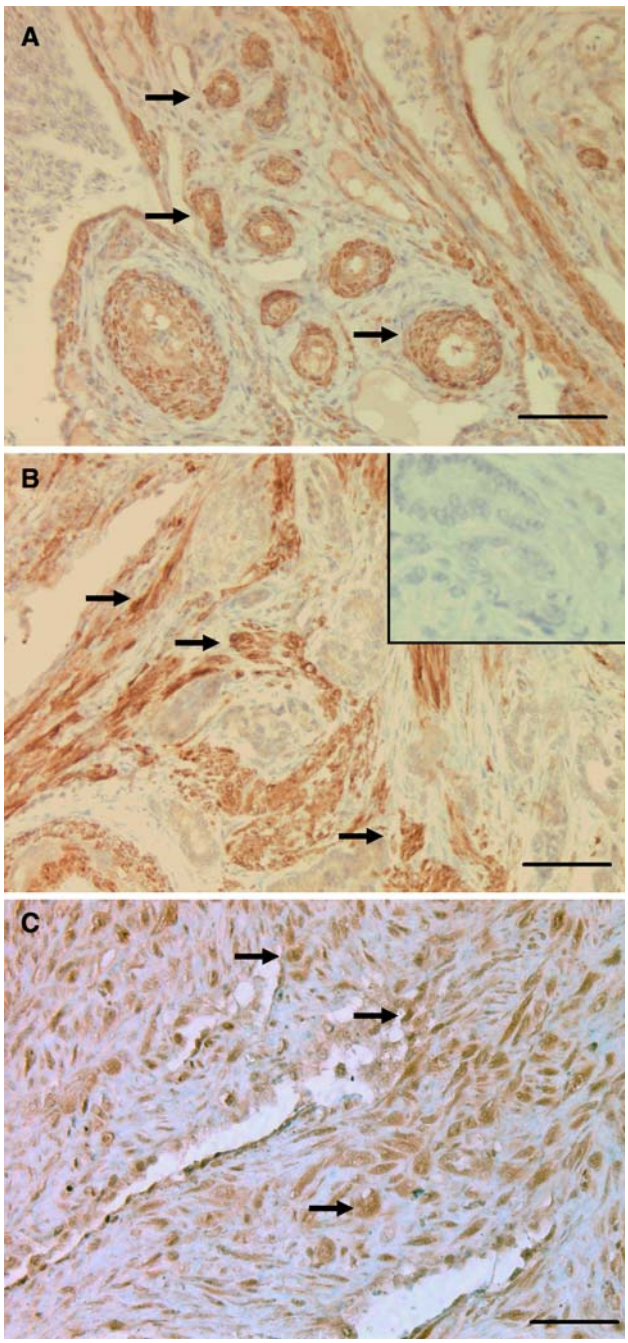

Fig. 3 COX-2 immunohistochemistry: (**a**) Normal ovary; (**b**) Ovarian tumor, (*inset*: non-immune IgG); (**c**) POF-1 from normal ovary. COX-2 expression is not increased in the ovarian tumor and is widely distributed in POF-1. Arrows point to COX-2 positive staining. Calibration bar, 50 μm

COX-2 mRNA was quantified as described for COX-1. There was no significant difference in COX-2 mRNA levels in F1 OSE compared to F2 OSE or F3 OSE (Fig. 6C); however, COX-2 mRNA levels were significantly higher ($P < 0.01$) in POF-2, than in POF-1 or in POF-3 (Fig. 6D).

Discussion

Cyclooxygenase enzymes (*PTGS 1* and *2*) are essential for normal physiological processes yet their aberrant expression is a critical factor in a host of pathologies. COX enzymes are required for ovarian function and many female reproductive

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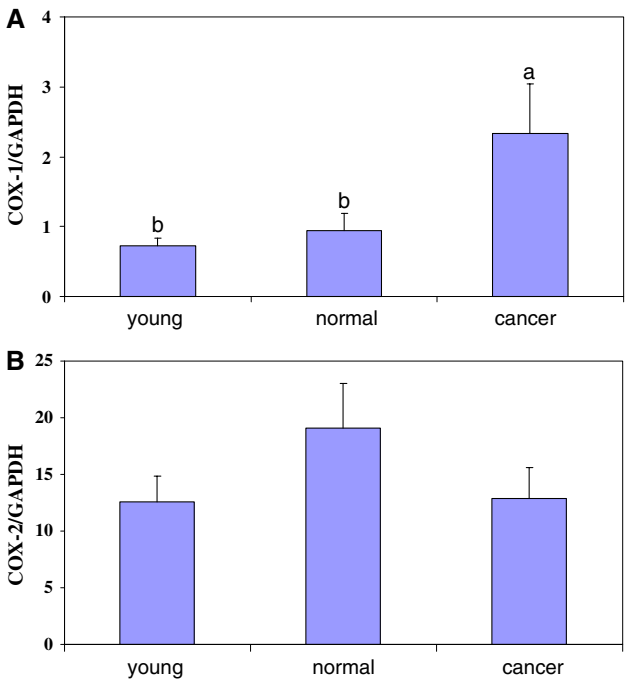

Fig. 4 Comparison of COX-1 (**A**) and COX-2 (**B**) mRNA in young chickens (40–50 weeks, $n = 6$), age-matched normal chickens (165 months, $n = 12$) and chickens with ovarian cancer (165 weeks, $n = 19$), quantified by real-time PCR. COX-1 and COX-2 mRNA by copy number, normalized to GAPDH. a versus b ($P < 0.05$) (±SEM)

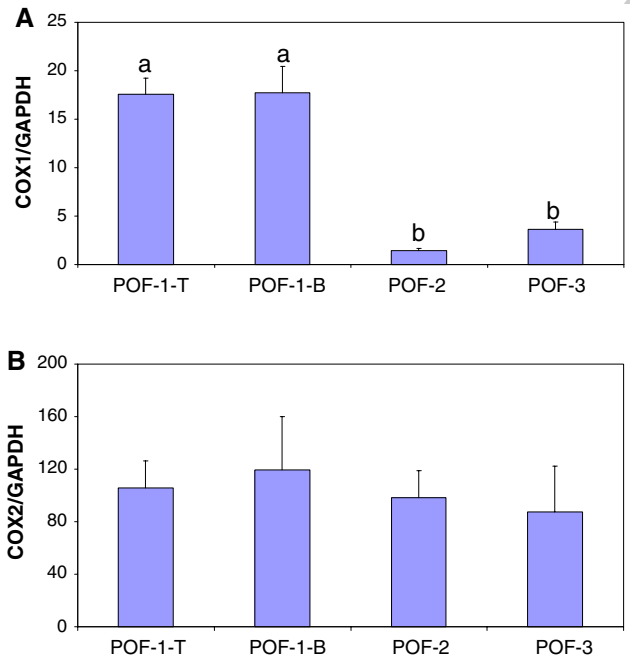

Fig. 5 Comparison of COX-1 (**A**) and COX-2 (**B**) mRNA in POFs, quantified by real-time PCR. POF-1 was removed and dissected into top (POF-1-T) and bottom (POF-1-B) portions. RNA was extracted from POF-1-T ($n = 7$) and POF-1-B ($n = 6$), POF-2 ($n = 6$) and POF-3 ($n = 6$) and quantified. COX-1 and COX-2 mRNA by copy number, normalized to GAPDH. a versus b, $P < 0.01$ (±SEM)

processes, but overexpression of COX is associated with significant pathology. Increased expression of COX-2 is associated with many epithelial carcinomas; however, COX-1 but not COX-2 overexpression has been shown to be associated with ovarian cancer [8, 21–26]. The laying hen provides an excellent model for studying normal ovarian functions, in particular ovulation [27, 28]. The hen also provides an important model for studying ovarian epithelial carcinoma [2, 3, 23, 29–32]. In the study reported here, the data demonstrate the relative expression and distribution of COX-1 and COX-2 in the normal ovary compared to alterations in expression observed in ovarian cancer. The data show for the first time that COX-1 expression is markedly increased in the postovulatory follicle (POF) of the normal ovary confirm the previous finding that COX-1 is elevated in ovarian carcinoma [23].

Histology of the normal hen's ovary compared to ovarian cancer

The normal anatomy of the hen's ovary (Fig. 1) features the follicular hierarchy, and the postovulatory follicular hierarchy. After the most mature follicle is ovulated and the OSE ruptures, POF-1 is formed. POFs consist of granulosa cells, interstitial theca cells, and OSE cells. The POF has been shown to be a site of oxidative DNA damage [33–35]. Common epithelial ovarian cancer is related to the successive rounds of ovulation and subsequent wound healing process characterized by OSE mitosis. The integrity of the DNA of these surface cells surrounding the ovarian rupture site is compromised during ovulation, and rapid growth of cells with mutated DNA may result in the initiation of carcinogenesis [36]. The role of inflammation and oxidative damage to the pathogenesis of cancer has gained much attention [37, 38]. Our results which demonstrate the high expression of COX-1 in the first postovulatory follicle (POF-1) may contribute to the pro-carcinogenic microenvironment where the malignant transformation of the OSE originates. In the mammalian ovary the remnant of the ruptured follicle forms the corpus luteum whose endocrine functions are important for preparation of the endometrium, implantation if fertilization takes place, and maintenance of the early embryo. In the hen, the POF persists for 2 or 3 days until it degenerates and becomes assimilated into the ovarian stroma, possibly forming clefts and inclusion cysts. Similar to its mammalian counterpart, the POF is an important endocrine tissue in the hen ovary. The POF produces prostaglandins for shell egg formation and oviposition. The original observation by Rothchild and Fraps in 1944, that removal of the POF resulted in a delay of oviposition indicated that the POF may play an important endocrine role in oviposition [39]. Subsequently, it was shown that prostaglandins peak

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at about the time of oviposition, and that inhibition of prostaglandin production with indomethacin perturbs egg laying, demonstrating the role that prostaglandins play in oviposition (for review, see [40]). Our data show, for the first time, that COX-1 as well as COX-2 mRNA and protein are expressed in the POF, and are the likely source of prostaglandins that control oviposition.

In contrast to the normal ovary shown in Fig. 1a, an ovarian tumor is shown in Fig. 1b. The tumor was confined to the ovary with no metastases or significant abdominal ascites fluid present on necropsy. Though the carcinoma had enveloped the entire ovary, it represents an early stage cancer as it had not progressed, and spread beyond the ovary.

Localization and quantification of COX-1 and COX-2 in the hen ovary

COX-1 is constitutively expressed in the mammalian ovary and confined to interstitial thecal cells and the corpus luteum [41]. In the hen ovary, as shown in Fig. 2a, COX-1 is localized to the granulosa cell layers, and confined to the OSE and cortical stroma adjacent to the follicle. In contrast to other cancers in which COX-2 is highly expressed, in ovarian cancer, COX-1 expression is markedly increased (Fig, 2b), as has been shown previously in the hen, human and in rodent models of ovarian cancer [8, 23, 24]. COX-1 has also been shown to be increased in feline oral squamous cell carcinomas, the only non-gynecological carcinoma reported to have increased COX-1 instead of COX-2 [42]. In the mammalian ovary, the ruptured follicle differentiates into the corpus luteum. There are no corpora lutea in the chicken ovary, but the POF is an important endocrine tissue, the site of prostaglandin production. As shown in Fig. 2c, POF-1 displays strong COX-1 staining, suggesting that COX-1 is important for the production of prostaglandins in POF-1, long known to be essential for oviposition [39].

In the mammalian ovary, COX-2 is localized to the granulosa cells and induced by LH at the time of the pre-ovulatory gonadotropin surge [43]. In the hen ovary, COX-2 is also expressed in the granulosa cells, as shown in Fig. 3a, but distinct from the reported distribution in the mammalian ovary, COX-2 is also highly expressed in the interstitial tissue of the ovary. Urick and Johnson have also reported that COX-2 is widely distributed in the hen ovary [23]. As shown in Fig. 3b, COX-2 expression in carcinoma becomes more localized to discrete foci, trapped within the glandular-like structures of the ovarian tumor.

Quantification of COX-1 and COX-2 mRNA levels in OSE and POF from normal ovaries

COX-1 and COX-2 mRNA expression in the OSE and POF was quantified by real-time PCR. It is important to note that OSE and POFs can only be collected from normal, non-cancerous ovaries due to the highly convoluted surface and afollicular presentation of ovarian cancer. POF-1 was dissected into "top" and "bottom" pieces. The POF was divided for COX analysis because it is likely that the outermost region of the POF was subjected to more oxidative damage, as has been reported by Murdoch and coworkers [33, 34], which presumably may influence COX expression. However, there was no difference in COX-1 or COX-2 mRNA expression observed between the top and bottom portions of POF-1. There was a significantly higher expression of COX-1 mRNA in POF-1, but this increase in

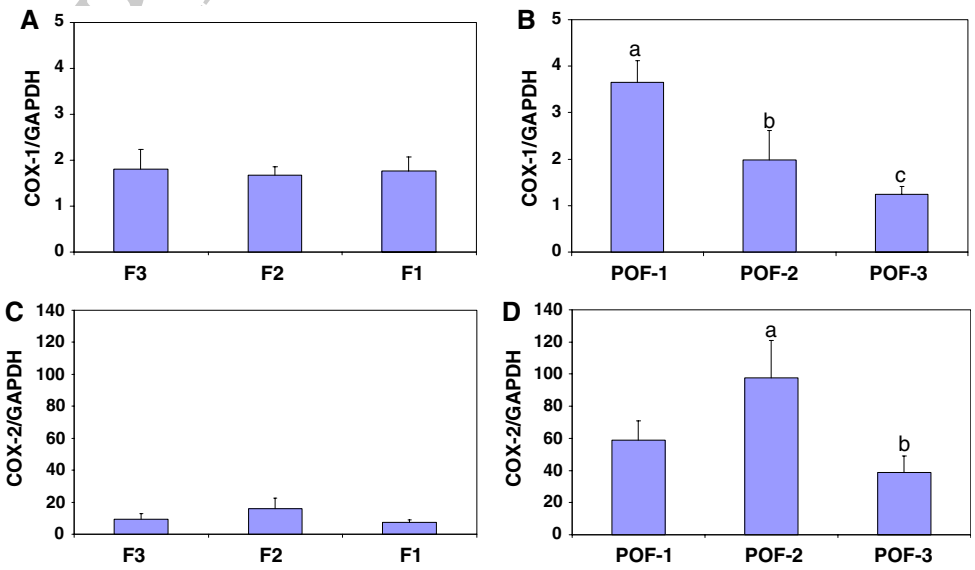

Fig. 6 Comparison of COX-1 mRNA in OSE isolated and analyzed from individual follicles F1 ($n = 8$), F2 ($n = 8$), F3 ($n = 8$) (**A**) and POF-1 ($n = 7$), POF-2 ($n = 6$) and POF-3 ($n = 6$) (**B**); COX-2 mRNA expression in OSE isolated from individual follicles (**C**) and POF-1–3 (**D**). Follicles and POFs were collected immediately after oviposition, RNA was extracted and quantified by real-time PCR. COX-1 and COX-2 mRNA by copy number, normalized to GAPDH. a versus b, $P < 0.05$; a versus c, $P < 0.01$ (±SEM)

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expression was transient in the normal ovary because in both POF-2 and POF-3, COX-1 mRNA expression was much lower (Fig. 5a).

The immunohistochemical staining suggests that COX-1 and COX-2 protein levels are comparable; however, COX-2 has ~20–30 times higher expression in terms of copy number of mRNA transcripts when normalized to GAPDH. It has been reported that COX-2 protein $t_{½}$ is approximately 20 to 50 times shorter than that of COX-1 protein $t_{½}$ in vitro [44]. This finding would indicate that even though COX-2 mRNA may be expressed at a higher level, the significant difference in protein stability would result in the protein levels of COX-1 and COX-2 in situ being comparable. Assessment of relative amounts of each protein by immunohistochemistry suggests their levels are comparable, thus COX-1 is likely to make a significant contribution to prostaglandin synthesis. The relative catalytic activities of COX-1 and COX-2 have been measured in vitro, and found to be very similar for conversion of arachidonic acid to PGH2. Thus, it is likely that the contribution of each enzyme to prostaglandin production in the hen ovary is comparable [45].

The results of this study have demonstrated that ovarian cancer in the hen shares many key features with human ovarian carcinoma, including elevated COX-1 expression thus further supporting the laying hen as an important model for the human disease. Our results show, for the first time that COX-1 mRNA is highly expressed in the POF of day one, indicating that COX-1 expression is important for the normal physiological functions of the ovary. These findings may provide the basis for clinical trials utilizing COX-1 specific inhibitors or dietary interventions targeting prostaglandin biosynthesis for the treatment and prevention of ovarian cancer.

Materials and methods

Materials

Bouin's fixative and neutral buffered formalin (NBF) were obtained from Sigma-Aldrich (St Louis, MO, USA); oligonucleotide primers were obtained from Sigma-Genosys, Sigma-Aldrich (St. Louis, MO, USA); antigen unmasking solution, Avidin/Biotin Blocking kit, Vectastain Elite Rabbit IgG kit, were obtained from Vector Laboratories (Burlingame, CA, USA); anti-human COX-1, and anti-human COX-2 antibodies were obtained from Cayman Chemical (Ann Arbor, MI, USA); High Capacity cDNA Archive Kit, *RNAlater* and SYBR® Green were obtained from Applied Biosystems (Foster City, CA, USA). Reverse Transcription System, Wizard Plus Miniprep DNA purification system, and RQ1 RNase-free DNase were obtained from Promega Corporation (Madison, WI, USA); TOPO TA Cloning kit, Trizol, and Quant-iT kit were obtained from Invitrogen (Carlsbad, CA, USA). Taq DNA Polymerase was obtained from Qiagen (Valencia, CA, USA). SuperFrost Plus microscope slides, Gil's hematoxylin, Histomount, diaminobenzadine (DAB), and all other reagents were from the Fisher Scientific (Itasca, IL, USA), or were the highest grade commercially available.

Animals and tissue collection

Single-comb White Leghorn hens, 40–50 weeks, $n = 20$ (young) and 165 weeks, $n = 65$ (old), were used for the study. 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. For hens in which tissue was collected relative to the time of ovulation, oviposition was monitored at 1 h intervals between 08:00 and 12:00 h, otherwise, oviposition was monitored daily. Animal management and procedures were reviewed and approved by the Division of Animal Research of the University of Illinois at Urbana-Champaign and the Animal Care Committee, University of Illinois at Chicago. Hens were sacrificed by CO_2 asphyxiation followed by cervical dislocation.

Normal and cancerous ovaries were removed from hens immediately after sacrifice and dissected. Large and small yellow follicles were removed prior to dissection. In normal ovaries, the three largest preovulatory follicles (F1, F2, and F3) and the three postovulatory follicles (POF-1, POF-2, and POF-3) were removed and the superficial epithelium (OSE) was collected from F1 to F3. OSE was collected from the largest preovulatory follicles after they were removed from the ovary by scraping with a cell scraper (Biologix Research Corp, Lenexa, KS, USA). Where indicated in the results, the POF-1 was dissected into "top" and "bottom" pieces. The top piece is the outermost region of the ruptured follicle, and the bottom piece is adjacent to, and the site of attachment to the ovarian cortex. The cancerous ovary lacks discernable or separable preovulatory and postovulatory follicles. Each normal or cancerous ovarian specimen was divided into four portions. The first portion was frozen in liquid nitrogen and later stored at −80°C; the second portion, together with POF-1–3 and superficial epithelium from F1 to F3 were put into RNA*later* solution and stored at 4°C before processing; the third and fourth portions were used for histological and immunohistochemical analysis and fixed in NBF and Bouin's fixative solution.

Histology and immunohistochemistry

Ovary tissues fixed in NBF or Bouin's fixative were processed and paraffin embedded. Tissues fixed in Bouin's were used for basic histology. Tissues fixed in NBF were

used for immunohistochemistry. Five micrometer sections were cut and mounted on SuperFrost Plus microscope slides. Slides were deparaffinized and rehydrated through xylene and graded ethanol solutions. Hematoxylin and eosin and Gomori Trichrome staining were performed as described [46, 47].

Immunohistochemistry was performed by using the Vectastain Elite ABC kit. Antigen retrieval was done using Antigen Unmasking Solution and pressure cooked at 20 psi for 5 min in a Decloaking Chamber electric pressure cooker (Biocare Medical, Walnut Creek, CA, USA). Slides were cooled and quenched in 0.3% H_2O_2 in methanol for 15 min. Slides were blocked with normal serum and incubated with primary antibody overnight at 4°C. Anti-human COX-1 (1:1000), and anti-human COX-2 (1:50) antibodies have previously been shown to be specific for the chicken by Urick and Johnson [23]. Non-immune IgG was used for negative control. After rinsing in Tris-buffered saline (TBS), sections were incubated with biotinylated secondary antibody and avidin–biotin complex. Specific binding was visualized using DAB in the presence of H_2O_2 and sections were counterstained with Gils hematoxylin, mounted with Histomount, examined on a Nikon ECLIPSE E400 microscope and were documented using SPOT Advanced version 4.0.1 software (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

RNA extraction and analysis

Preovulatory and small yellow follicles and POFs were removed from ovaries of normal hens prior to homogenization and RNA extraction. Total RNA was extracted from ovary, OSE, POF, and ovarian tumors using Trizol and was quantified by determination of absorbance at A_{260}. All RNA samples used in this study had a 260:280 ratio between 1.9 and 2.05. RNA samples were then treated with RQ1 RNase-free DNase prior to reverse transcription reaction. Synthesis of cDNA was performed using the high capacity cDNA archive kit and cDNA was quantified by Quant-iT fluorescent reagent. Equal amounts from all samples were subjected to real-time PCR.

Quantitative real-time PCR (qRT-PCR)

Chicken-specific primers were designed to recognize target genes using Primer Express (ABI). The primer pairs were designed so that at least one spanned an intron. Primer sequences for COX-1 (Prostaglandin G/H synthase 1, *PTGS1*, XM_425326): forward: 5′ TCAGGTGGTTCTGG GACATCA 3′; reverse: 5′ TGTAGCCGTACTGGGAG TTGAA 3′; for COX-2 (Prostaglandin G/H synthase 2, *PTGS2*, XM_422297): forward: 5′ CTGCTCCCTCCCAT GTCAGA 3′; reverse: 5′ CACGTGAAGAATTCCGGT GTT 3′; for internal control gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*, NM_204305): forward: 5′ GATGGGTGTCAACCATGAGAAA 3′; reverse: 5′ CAATGCCAAAGTTGTCATGGA 3′. Plasmid standards for each target of interest and internal control GAPDH were used for quantification. To clone plasmid standards, total RNA was extracted from chicken ovarian tissue, pooled, and reversed transcribed into cDNA with the Reverse Transcription System kit. Target gene fragments were amplified by *Taq* DNA Polymerase and cloned using TOPO TA Cloning Kit. Plasmid DNA was prepared with the Wizard Plus Miniprep DNA purification system. The identity of purified cDNA was verified by DNA sequencing. cDNA plasmid concentrations were measured by spectrophotometer and the corresponding copy numbers were calculated based on the formula that 1 µg of 1000 bp of DNA = 9.1×10^{11} molecules. qRT-PCR was conducted by amplifying cDNA with SYBR® Green (Applied Biosystems) on ABI 7900HT using a 384 well plate format and analyzed with AB1 Prism software. Control reactions lacking template were run for each gene. Reactions were 10 µl in total volume and 200 nM of each primer. 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, 40 cycles for 95°C 15 S, 60°C 1 min.

Statistical analysis

Statistical analysis was performed with GraphPad InStat by using One-way ANOVA and Student's Newman Keuls post-hoc comparison.

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References

1. UCSW Group, *United States Cancer Statistics: 2002 Incidence and Mortality*. (US Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute, 2005)
2. T.N. Fredrickson, Ovarian tumors of the hen. Environ. Health Perspect. **73**, 35–51 (1987)
3. C. Rodriguez-Burford, M.N. Barnes, W. Berry, E.E. Partridge, W.E. Grizzle, Immunohistochemical expression of molecular markers in an avian model: a potential model for preclinical evaluation of agents for ovarian cancer chemoprevention. Gynecol. Oncol. **81**, 373–379 (2001)
4. J.R. Giles, H.L. Shivaprasad, P.A. Johnson, Ovarian tumor expression of an oviductal protein in the hen: a model for human serous ovarian adenocarcinoma. Gynecol. Oncol. **95**, 530–533 (2004)

5. N. Auersperg, A.S. Wong, K.C. Choi, S.K. Kang, P.C. Leung, Ovarian surface epithelium: biology, endocrinology, and pathology. Endocr. Rev. **22**, 255–288 (2001)
6. C.H. Lingeman, Etiology of cancer of the human ovary: a review. J. Natl. Cancer Inst. **53**, 1603–1618 (1974)
7. S. Mabuchi, D.A. Altomare, D.C. Connolly, A. Klein-Szanto, S. Litwin, M.K. Hoelzle, H.H. Hensley, T.C. Hamilton, J.R. Testa, RAD001 (Everolimus) delays tumor onset and progression in a transgenic mouse model of ovarian cancer. Cancer Res. **67**, 2408–2413 (2007)
8. T. Daikoku, S. Tranguch, I.N. Trofimova, D.M. Dinulescu, T. Jacks, A.Y. Nikitin, D.C. Connolly, S.K. Dey, Cyclooxygenase-1 is overexpressed in multiple genetically engineered mouse models of epithelial ovarian cancer. Cancer Res. **66**, 2527–2531 (2006)
9. D.M. Dinulescu, T.A. Ince, B.J. Quade, S.A. Shafer, D. Crowley, T. Jacks, Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. Nat. Med. **11**, 63–70 (2005)
10. S.K. Bristol-Gould, C.G. Hutten, C. Sturgis, S.M. Kilen, K.E. Mayo, T.K. Woodruff, The development of a mouse model of ovarian endosalpingiosis. Endocrinology **146**, 5228–5236 (2005)
11. D.C. Connolly, R. Bao, A.Y. Nikitin, K.C. Stephens, T.W. Poole, X. Hua, S.S. Harris, B.C. Vanderhyden, T.C. Hamilton, Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. Cancer Res. **63**, 1389–1397 (2003)
12. A. Flesken-Nikitin, K.C. Choi, J.P. Eng, E.N. Shmidt, A.Y. Nikitin, Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. Cancer Res. **63**, 3459–3463 (2003)
13. R. Wu, N. Hendrix-Lucas, R. Kuick, Y. Zhai, D.R. Schwartz, A. Akyol, S. Hanash, D.E. Misek, H. Katabuchi, B.O. Williams, E.R. Fearon, K.R. Cho, Mouse model of human ovarian endometrioid adenocarcinoma based on somatic defects in the Wnt/beta-catenin and PI3K/Pten signaling pathways. Cancer Cell **11**, 321–333 (2007)
14. M.F. Fathalla, Incessant ovulation—a factor in ovarian neoplasia? Lancet **2**, 163 (1971)
15. D.A. Kujubu, B.S. Fletcher, B.C. Varnum, R.W. Lim, H.R. Herschman, TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J. Biol. Chem. **266**, 12866–12872 (1991)
16. C.S. Williams, M. Mann, R.N. DuBois, The role of cyclooxygenases in inflammation, cancer, and development. Oncogene **18**, 7908–7916 (1999)
17. H.R. Herschman, J.J. Talley, R. DuBois, Cyclooxygenase 2 (COX-2) as a target for therapy and noninvasive imaging. Mol. Imaging Biol. **5**, 286–303 (2003)
18. K. Subbaramaiah, A.J. Dannenberg, Cyclooxygenase 2: a molecular target for cancer prevention and treatment. Trends Pharmacol. Sci. **24**, 96–102 (2003)
19. S.M. Prescott, F.A. Fitzpatrick, Cyclooxygenase-2 and carcinogenesis. Biochim. Biophys. Acta **1470**, M69–78 (2000)
20. W.L. Yang, I.H. Roland, A.K. Godwin, X.X. Xu, Loss of TNF-alpha-regulated COX-2 expression in ovarian cancer cells. Oncogene **24**, 7991–8002 (2005)
21. R.A. Gupta, L.V. Tejada, B.J. Tong, S.K. Das, J.D. Morrow, S.K. Dey, R.N. DuBois, Cyclooxygenase-1 is overexpressed and promotes angiogenic growth factor production in ovarian cancer. Cancer Res. **63**, 906–911 (2003)
22. T. Daikoku, D. Wang, S. Tranguch, J.D. Morrow, S. Orsulic, R.N. DuBois, S.K. Dey, Cyclooxygenase-1 is a potential target for prevention and treatment of ovarian epithelial cancer. Cancer Res. **65**, 3735–3744 (2005)
23. M.E. Urick, P.A. Johnson, Cyclooxygenase 1 and 2 mRNA and protein expression in the Gallus domesticus model of ovarian cancer. Gynecol. Oncol. **103**, 673–678 (2006)
24. S. Li, K. Miner, R. Fannin, J. Carl Barrett, B.J. Davis, Cyclooxygenase-1 and 2 in normal and malignant human ovarian epithelium. Gynecol. Oncol. **92**, 622–627 (2004)
25. G. Lee, H.T. Ng, Clinical evaluations of a new ovarian cancer marker, COX-1. Intl. J. Gynaecol. Obstetr. Official Organ Intl. Feder. Gynaecol. Obstetr. **49**(Suppl), 27–32 (1995)
26. Y. Kino, F. Kojima, K. Kiguchi, R. Igarashi, B. Ishizuka, S. Kawai, Prostaglandin E2 production in ovarian cancer cell lines is regulated by cyclooxygenase-1, not cyclooxygenase-2. Prostaglandins, Leukotrienes, Essential Fatty Acids **73**, 103–111 (2005)
27. J. Bahr, The avian ovary: model for endocrine studies. J. Exp. Zool. **4**(Suppl), 192–194 (1990)
28. J.M. Bahr, A.L. Johnson, Regulation of the follicular hierarchy and ovulation. J. Exp. Zool. **232**, 495–500 (1984)
29. P.A. Johnson, J.R. Giles, Use of genetic strains of chickens in studies of ovarian cancer. Poult. Sci. **85**, 246–250 (2006)
30. J.R. Giles, L.M. Olson, P.A. Johnson, Characterization of ovarian surface epithelial cells from the hen: a unique model for ovarian cancer. Exp. Biol. Med. **231**, 1718–1725 (2006)
31. B.C. Vanderhyden, T.J. Shaw, J.F. Ethier, Animal models of ovarian cancer. Reprod. Biol. Endocrinol. **67**(1), 1–11 (2003)
32. M.N. Barnes, W.D. Berry, J.M. Straughn, T.O. Kirby, C.A. Leath, W.K. Huh, W.E. Grizzle, E.E. Partridge, A pilot study of ovarian cancer chemoprevention using medroxyprogesterone acetate in an avian model of spontaneous ovarian carcinogenesis. Gynecol. Oncol. **87**, 57–63 (2002)
33. W.J. Murdoch, J.F. Martinchick, Oxidative damage to DNA of ovarian surface epithelial cells affected by ovulation: carcinogenic implication and chemoprevention. Exp. Biol. Med. (Maywood) **229**, 546–552 (2004)
34. W.J. Murdoch, E.A. Van Kirk, B.M. Alexander, DNA damages in ovarian surface epithelial cells of ovulatory hens. Exp. Biol. Med. (Maywood) **230**, 429–433 (2005)
35. W.J. Murdoch, R.S. Townsend, A.C. McDonnel, Ovulation-induced DNA damage in ovarian surface epithelial cells of ewes: prospective regulatory mechanisms of repair/survival and apoptosis. Biol. Reprod. **65**, 1417–1424 (2001)
36. W.J. Murdoch, A.C. McDonnel, Roles of the ovarian surface epithelium in ovulation and carcinogenesis. Reproduction **123**, 743–750 (2001)
37. T.D. Oberley, Oxidative damage and cancer. Am. J. Pathol. **160**, 403–408 (2001)
38. L.M. Coussens, Z. Werb, Inflammation and cancer. Nature **420**, 860–867 (2002)
39. I. Rothchild, R.M. Fraps, On the function of the ruptured ovarian follicle of the domestic fowl. Proc Soc Exp Biol Med Soc Exp Biol Med N.Y **56**, 79–82 (1944)
40. J. Bahr, P. Johnson, Reproduction in poulty, in *Reproduction in Domestic Animals*, ed. P. Cupps (Academic Press, New York, 1991), pp. 555–575
41. W.Y. Wong, J.S. Richards, Evidence for two antigenically distinct molecular weight variants of prostaglandin H synthase in the rat ovary. Mol. Endocrinol. **5**, 1269–1279 (1991)
42. A. Hayes, T. Scase, J. Miller, S. Murphy, A. Sparkes, V. Adams, COX-1 and COX-2 expression in feline oral squamous cell carcinoma. J. Comparat. Pathol. **135**, 93–99 (2006)
43. J.S. Richards, D.L. Russell, S. Ochsner, L.L. Espey, Ovulation: new dimensions and new regulators of the inflammatory-like response. Annu. Rev. Physiol. **64**, 69–92 (2002)
44. U.R. Mbonye, M. Wada, C.J. Rieke, H.-Y. Tang, D.L. DeWitt, W.L. Smith, The 19-amino acid cassette of cyclooxygenase-2

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Article No. : **9080** ☐ LE ☐ TYPESET
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mediates entry of the protein into the endoplasmic reticulum-associated degradation system, 2006, pp. 35770–35778
45. M. Wada, C.J. Delong, Y.H. Hong, C.J. Rieke, I. Song, R.S. Sidhu, C. Yuan, M. Warnock, A.H. Schmaier, C. Yokoyama, E.M. Smyth, S.J. Wilson, G.A. Fitzgerald, R.M. Garavito, D.X. Sui, J.W. Regan, W.L. Smith, Enzymes and receptors of prostaglandin pathways with arachidonic acid- vs. eicosapentaenoic acid-derived substrates and products. J. Biol. Chem. (2007)
46. G. Gomori, A rapid one-step trichrome stain. Am. J. Clin. Pathol. **20**, 661–664 (1950)
47. D.C. Skeehan, B.B. Hrapchak, *Theory and practice of histotechnology, theory and practice of histotechnology* (Mosby, St. Louis, 1973), pp. 111–112

Journal : **Large 12020** Dispatch : **15-5-2008** Pages : **10**
Article No. : **9080** ☐ LE ☐ TYPESET
MS Code : **9080** ☑ CP ☑ DISK