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Estrogen Receptor Subtype Expression is Altered in the Hen Model of Ovarian Cancer

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Abstract

There is growing evidence that estrogens may promote tumor progression, including ovarian tumors. Estrogens exert their actions in tissues through two different receptor subtypes (ESR1 and ESR2). Studies have shown that hens develop ovarian cancer spontaneously, therefore providing a suitable animal model for the disease. Our aim was to determine the expression of mRNA and protein of the estrogen receptor subtypes in ovaries of normal hens and ovaries from hens with ovarian cancer. Ovarian tissue from normal hens and hens with ovarian cancer was collected for quantitative real-time PCR and immunofluorescence analysis. Quantitative real-time PCR results showed that the relative mRNA expression of ESR1 and the ratio of ESR1/ESR2 are significantly greater from hens with ovarian cancer when compared to normal ovarian tissue. Immunofluorescence analysis showed differential ESR1 and ESR2 protein expression in ovarian tissue sections from normal hens and hens with ovarian cancer, with results parallel to the mRNA data. There was no significant difference in plasma estradiol levels between normal hens and hens with ovarian cancer. These data suggest an increase in downstream estrogen-mediated actions in chicken ovarian tumors and, indeed, microarray analysis reveals a functionally significant estradiol-signaling pathway in chicken ovarian tumors. Interestingly, expression of a putative ovarian tumor suppressor, EPB41L3, is down regulated in this pathway. Taken together, these results suggest that, in the hen, ESR1 may be mediating a proliferative response in ovarian cancer cells. Although additional studies are required to define the role of ESR1 in tumor formation in the hen, these results support the utility of the hen for testing possible endocrine therapies.

Keywords: Ovarian cancer; Estrogen receptor; Hen; Animal model; Estrogen

Introduction

Ovarian cancer is the leading cause of death due to gynecologic cancer in the United States and the fifth leading cause of cancer death among US women. Currently, only 15% of cases are diagnosed at an early stage. If ovarian cancer is detected and treated early, the five-year survival rate is greater than 90% [1]. The fact that most ovarian cancers are diagnosed at later stages illustrates our lack of understanding of the etiology of the disease. Although the molecular mechanisms of ovarian cancer initiation remain unclear, there is growing evidence that estrogens may be involved in tumor progression. It was initially thought that ovarian cancer was not estrogen-dependent because only 5-18% of ovarian cancers have been shown to be sensitive to tamoxifen treatment [2,3]. Paradoxically, as many as 60% of malignant ovarian tumors are estrogen receptor (ER) positive [2-4]. Furthermore, estrogens directly promote proliferation in ER positive ovarian cancer cell lines [5-8]. Epidemiological evidence also suggests that estrogens may promote ovarian cancer in women on hormone replacement therapy, possibly due to persistently elevated estrogen levels [9]. These lines of evidence point to a possible role of estrogen in ovarian cancer in women.

Estrogen can exert effects on target tissues through interaction with estrogen receptors. There are two subtypes of the nuclear estrogen receptor (ESR1 and ESR2) encoded by separate genes that are expressed in a tissue-specific manner and thought to regulate differential gene functions. Some studies have shown that the loss of ESR2 and/or increased ESR1/ESR2 ratio may be a common step in estrogen-dependent ovarian tumor progression [4,10-13]. It is thought that ESR1 and ESR2 regulate opposing cellular functions, with ESR1 involved in cellular proliferation and ESR2 mediating growth inhibition. There may be a balance of factors that regulates cellular growth because normal rat and human ovarian cells express greater amounts of ESR2 and less ESR1 [14]. In tumor cells, however, this balance is disrupted and cells are allowed to proliferate abnormally [14]. The importance of this disruption is evident in a study that reported that ESR1, but not ESR2, mediates estrogen-driven growth of epithelial ovarian cancer [8]. More recently, ESR2 expression in ovarian tumors was associated with poor progression-free survival [15]. Combined, these studies lend support to the idea that the relative levels of ESR1 and ESR2 may be important regulators of ovarian cell growth. Similar to the balance between ESR1 and ESR2 thought to be important in cancer progression, it is also believed that oncogenic and tumor suppressor genes may contribute to tumor development. EPB41L3 has been proposed as an ovarian tumor suppressor whose expression is frequently lost in ovarian cancer cell lines compared to normal ovarian epithelial cell lines [16].

Hens have the same subtypes of ovarian cancer as seen in women, although the prevalence of the different subtypes varies between hens and women [17,18]. Estrogen receptors in the chicken are structurally and functionally similar to those in the human. According to UniGene, chicken ESR1 protein is ~79% similar to human ESR1, while chicken ESR2 protein is ~78% similar to human ESR2. Human ESR1 and chicken ESR1 share three regions of high homology that are most likely crucial for receptor function. These regions are the DNA-binding domain (100% homology), transcription activation domain (87% homology), and ligand-binding domain (94% homology) [19]. A conserved domain search through NCBI also identifies conserved domains of chicken

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ESR2 for ligand-binding and DNA-binding [20]. These conserved regions suggest that transcriptional action and regulation are similar between human and chicken estrogen receptor subtypes.

Our objective was to determine mRNA and protein expression of ESR1 and ESR2 in normal and cancerous ovarian tissue from hens. We also determined whether there were differences in plasma estradiol levels of normal hens and hens with ovarian cancer. Finally, we used pathway analysis of microarray data from hens with ovarian cancer to assess alterations of genes associated with the estradiol-signaling pathway. Collectively, the data suggest that ESR1 may be driving proliferation in hen ovarian tumors and support the use of the hen model to test endocrine therapies for the disease.

Materials and Methods

Tissue samples

Normal ovarian (n = 11) and ovarian tumor (n = 13) tissue samples from 2-5 year-old White leghorn hens were collected and stored in RNA later (Ambion, Inc., Austin, TX) for real-time PCR analysis or were fixed and paraffin-embedded and used for immunofluorescence. Normal oviduct tissue samples from a 3 year-old White leghorn hen were also collected and used as a positive control for immunofluorescence and Western blot analysis. We used the ovarian body (including stroma and follicles) as a source of RNA and protein in order to capture the estrogen receptor environment in the area of tumor development. Blood samples from a subset of the normal hens (n = 8) and hens with ovarian cancer (n = 11) were collected at the time of tissue collection for radioimmunoassay (RIA) analysis of plasma levels of estradiol. Ovarian tissue was collected from non-diseased 3-4 year-old White leghorn hens (n = 6) and frozen on dry ice for Western blot analysis. Diagnosis of ovarian cancer was confirmed by histological analysis of hematoxylin and eosin stained sections. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

Real-time quantitative PCR

RNA was extracted from hen ovarian tissue using the RNeasy Mini kit (Qiagen, Valencia, CA) and RNA integrity was verified using the 2100 Expert Bioanalyzer (Agilent Technologies, Santa Clara, CA). Extracted RNA was reverse-transcribed and the cDNA was used to determine mRNA expression levels of ESR1 and ESR2 relative to the internal control, ribosomal RNA (18S). Chicken specific Taqman primers and probes for ESR1 (GenBank accession #NM_205183) and ESR2 (GenBank accession #NM_204794) were designed using Primer Express software v2.0 from Applied Biosystems. The ESR1 primers defined a cDNA of 79 bp (forward: 5' CAGCATTCGTGAGAGGATGTCTA 3'; reverse: 5' ACAGTACCGGGTCTCCTTGGT 3'; probe: 6FAM TACCAATGAGAAAGGGAGCCTGTCCATG MGBNFQ) and the ESR2 primers defined a cDNA of 111 bp (forward: 5' GGAAATGCTATGAAGTGGGA ATG 3'; reverse: 5' TCTTGGTTTTGCCCATGCA 3'; probe 6FAM TGGGTATCGAAT CCTGCGCC MGBNFQ). Taqman primers and probes for 18S (Applied Biosystems, Foster City, CA) were used as the control and run for each sample. Control reactions containing no template and reactions lacking reverse transcriptase were also run. All sample reactions were run in duplicate using the ABI Prism 7000 Sequence Detection System. Mean values of estrogen receptor subtype mRNA were calculated relative to 18S reactions. The relative amount of mRNA was determined by the comparative threshold cycle (CT) method using Sequence Detection System Software (Applied Biosystems, Foster City, CA).

SDS-PAGE and western blot

Frozen normal (n = 3) and tumor (n = 3) ovarian tissue samples were homogenized in lysis buffer (10 mM Tris pH 7.5, 1.0% Triton x-100, 150 mM NaCl, 1 mM EGTA, and protease inhibitors) and centrifuged. The supernatant was collected for SDS-PAGE and protein concentration was determined using the BCA protein assay (Pierce Biotechnologies, Rockford, IL). Approximately 25 ug of protein was added to sample buffer and samples were loaded onto 10% Tris-HCl gels (Pierce Biotechnologies, Rockford, IL) and run under denaturing conditions at 100V for 50 minutes. Twenty-five ug of protein from oviduct was processed similarly as a positive control. Although approximately equal amounts of protein were loaded, amounts were not corrected with a loading control as the results were used to assess appropriate molecular weight and the presence of a unique band. The separated proteins were transferred to a nitrocellulose membrane (Pierce Biotechnologies, Rockford, IL) and blocked for 30 minutes in 1X TBST + 5% non-fat milk. Membranes were incubated in rabbit polyclonal ESR1 antibody against the N-terminal epitope of human ESR1 (described in ref. [21]; kindly provided by Dr. W.L. Kraus of Cornell University) at a dilution of 1:10,000 for 2 h, followed by incubation in HRP-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) at a dilution of 1:10,000 for 1 h prior to detection of specific protein through enhanced chemiluminescence with LumiGLO (KPL, Gaithersburg, MD). Membranes were stripped (1M Tris-HCl pH 6.7, 10% SDS, β-mercaptoethanol, and water) and processed as described above to examine ESR2. The rabbit polyclonal ESR2 antibody was made against the N-terminal epitope of human ESR2 (kindly provided by Dr. W.L. Kraus of Cornell University) and used at a dilution of 1:10,000 for 2 h, followed by incubation in HRP-conjugated goat anti-rabbit IgG (KPL, Gaithersburg, MD) at a dilution of 1:10,000 for 1 h. This was repeated at total of 2 times with similar results.

Immunofluorescence

Paraffin sections were deparaffinized and rehydrated through a series of treatments with xylene and ethanol. Sections were boiled in citrate buffer for antigen retrieval and blocked in 10% goat serum in PBS for 30 minutes. Sections were then incubated with the polyclonal ESR1 antibody (described above) at a dilution of 1:100 or the polyclonal ESR2 antibody (described above) at a dilution of 1:50 for 1 h at 39˚C. Control slides were incubated with rabbit IgG at a dilution of 1:100 or 1:50, respectively. Incubation with primary antibody (or control IgG) was followed by incubation in AlexaFluor 488 goat anti-rabbit IgG secondary antibody (1 μg/mL) for 1 h at 39˚C. Slides were viewed using a Nikon eclipse E600 microscope with fluorescence capability and images were captured with a Spot RT Slider camera at the same exposure in each figure. This was repeated a total of 3 times with similar results.

Estradiol radioimmunoassay (RIA)

Plasma was assayed for estradiol using the Coat-A-Count estradiol RIA kit (DPC, Los Angeles, CA). All samples were assayed in duplicate. The intra-assay coefficient of variation was 3.9%.

Microarray: ingenuity pathway analysis

Microarray analysis was previously performed with ovarian tissue from normal hens that showed no gross or histological evidence of ovarian cancer (n = 3), or ovarian tissue from hens with tumors (n = 3) as described [22]. Briefly, total RNA was extracted from frozen tissue using TRIZOL (Invitrogen, Carlsbad, CA) and labeled antisense RNA (aRNA) targets were produced. The labeled aRNA was hybridized

to GeneChip® chicken genome arrays (Affymetrix, Santa Clara, CA) and scanned by the GeneChip scanner 3000-7 G. Analysis of the raw data were carried out at the DNA-microarray core facility at Cornell University and the data were deposited in the Gene Expression Omnibus (GEO) public repository (accession number GSE21706). To identify relevant pathways that were altered in hen ovarian cancer, the data was analyzed through the use of Qiagen's Ingenuity Pathway Analysis (IPA®, Qiagen, www.qiagen.com/ingenuity) software version 8.7.

Statistics

Data were graphed and analyzed by Student's t-test for statistical significance using GraphPad Prism 5.04. A p-value of <0.05 was considered significant.

Results

Real-time quantitative PCR

Results indicate that ESR1 mRNA expression is significantly increased in ovarian tumor samples as compared to normal ovarian samples (Figure 1A; $p < 0.01$). There is a trend for ESR2 mRNA to be decreased in ovarian tumor samples as compared to normal ovarian samples, however this difference was not significant (Figure 1B; $p = 0.07$). Figure 1C shows that the ratio of ESR1/ESR2 mRNA expression is significantly higher in ovarian tumor samples as compared to normal ovarian samples ($p < 0.05$).

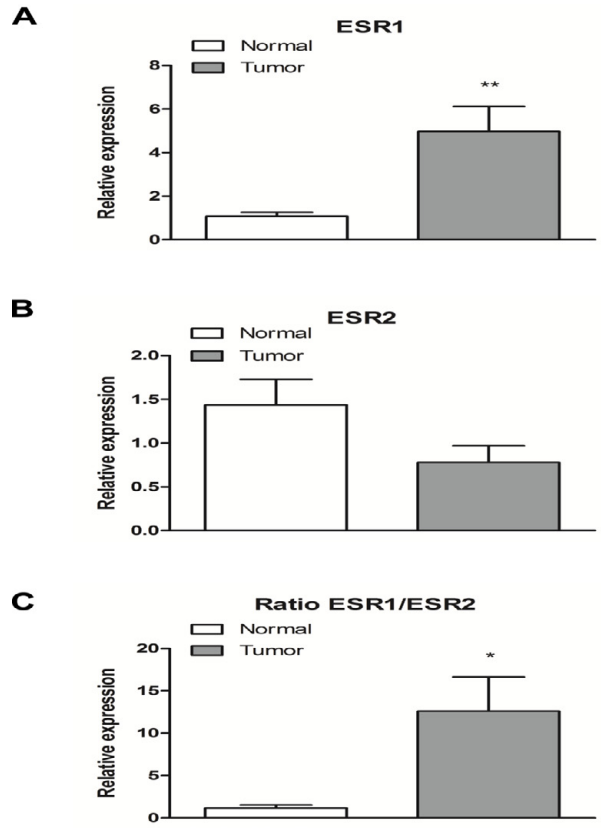

Figure 1: Estrogen receptor mRNA expression in ovaries with tumors (n = 13) and normal ovaries (n = 11). A. ESR1 mRNA expression. Asterisks indicate a significant increase in ovaries with tumors as compared to normal ovaries ($P < 0.01$). B. ESR2 mRNA expression. No significant difference was found ($P = 0.07$). C. Ratio of ESR1/ESR2 mRNA expression. Asterisk indicates a significant increase in ovaries with tumors as compared to normal ovaries ($P < 0.05$). Bars indicate mean ± standard error.

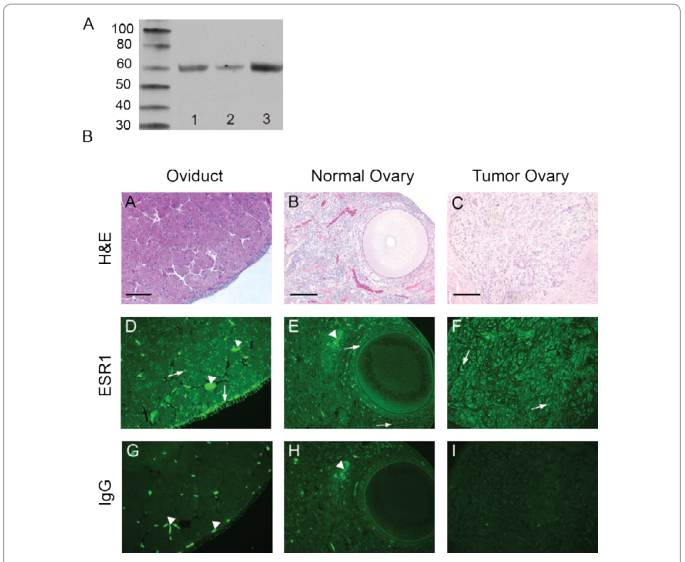

Figure 2: ESR1 protein expression in chicken ovarian and oviduct tissue. A. Western blot of chicken ovarian tissue from a normal hen (lane 1), ovarian tissue from a hen with ovarian cancer (lane 2) and oviduct (lane 3). B. Immunofluorescence with ESR1 antibody. H&E staining of oviduct tissue (A), normal ovarian tissue (B) and ovarian tissue from hen with cancer (C); ESR1 staining of oviduct tissue (D), normal ovarian tissue (E) and ovarian tissue from hen with cancer (F); Negative control (rabbit IgG) of oviduct tissue (G), normal ovarian tissue (H) and ovarian tissue from hen with cancer (I). Arrows indicate specific staining and arrowheads indicate autofluorescence. Scale bar = 100 µm.

Western blot and immunofluorescence

Figure 2A is a representative Western blot of ESR1 protein expression. A primary band appears at the approximate expected size (~67 kDa) in ovarian tissue from a normal hen (lane 1) and a hen with ovarian cancer (lane 2), as well as the positive control (oviduct; lane 3). Figure 2B illustrates representative immunofluorescence results using the ESR1 antibody and the IgG control. Panels A, B, and C are hematoxylin and eosin (H&E) stained sections of oviduct tissue, normal ovarian tissue and tumor ovarian tissue, respectively. As expected, there is widespread ESR1 expression in the oviduct tissue in the epithelial cells and in the cells of the tubular glands (Panel D arrows). There is also ESR1 protein expression in the stroma and theca of normal ovarian tissue (Panel E arrows). ESR1 protein expression is intense and widespread in ovarian tissue from hens with ovarian cancer, especially in the nests of tumor cells (Panel F arrows). Arrowheads in panels D-I indicate autofluorescence. There is no specific staining evident in IgG control sections (Panels G, H, I), although there is some autofluorescence associated with red blood cells (arrowheads).

Figure 3A shows a representative blot of ESR2 protein expression. There is a single band close to the expected size (~55 kDa) in ovarian tissue from a normal hen (lane 1) and a hen with ovarian cancer (lane 2), and in the positive control (lane 3). Figure 3B illustrates representative immunofluorescence results using the ESR2 antibody and the IgG control. Panels A, B, and C are H&E stained sections of oviduct tissue, normal ovarian tissue, and tumor ovarian tissue, respectively. There is weak ESR2 protein expression in the oviduct tissue restricted to the epithelial cells (Panel D arrows). ESR2 protein expression is seen in the stroma and granulosa cells of normal ovarian tissue (Panel E arrows) while limited ESR2 protein expression is seen in the stroma or glandular areas in the ovary of the hens with cancer (Panel F). Arrowheads in panels D-I indicate autofluorescence. Once again, there is no specific staining evident in IgG control sections (Panels G, H, I), although some autofluorescence of red blood cells is seen (arrowheads).

Estradiol radioimmunoassay (RIA)

Figure 4 shows that plasma estradiol levels are not significantly different between normal hens and hens with ovarian cancer ($p = 0.5$).

IPA analysis

Figure 5 shows the pathway with beta-estradiol as the signaling node identified by IPA analysis in chicken ovarian tumors. These genes include several that are upregulated (signified by green symbols) and several that are downregulated (signified by red symbols) in chicken ovarian tumors. We performed a literature search for the genes in the beta-estradiol signaling pathway to identify genes that may also play a role in human ovarian cancer. Interestingly, expression of the putative tumor suppressor EPB41L3 is significantly decreased in samples from hens (Figure 6; $p < 0.01$) with ovarian cancer compared to samples from normal ovary as measured by microarray.

Discussion

Our study is the first to show mRNA and protein expression of ER subtypes in ovarian cancer of the hen. We find that the ESR1 mRNA level and the ESR1/ESR2 mRNA ratio are significantly increased in ovarian tissue from hens with ovarian cancer as compared to tissue from normal hens. This is consistent with a number of studies in women [10-13]. We also observed a non-significant decrease in ESR2 mRNA expression in tumors, suggesting that a possible role of ESR2 in ovarian cancer cannot be ruled out. In fact, a growth inhibitory role for ESR2 in ovarian cancer has been proposed [23-25]. Interestingly, studies have reported that ESR2 might modulate the transcriptional activity of ESR1 [26,27], suggesting that the relative expression levels of these subtypes may determine cellular responses to agonists and antagonists. For example, one study showed that selective ER mediators might alter the response to hormonal treatment in women with ovarian cancer [28]. The fact that we see a trend of decreased ESR2 mRNA lends support to the idea that the decrease/loss of ESR2 might be important in ovarian

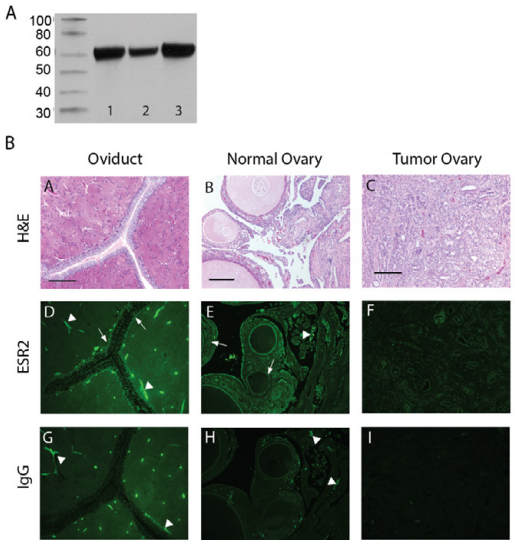

Figure 3: ESR2 protein expression in chicken ovarian and oviduct tissue. A. Western blot of chicken ovarian tissue from a normal hen (lane 1), ovarian tissue from a hen with ovarian cancer (lane 2) and oviduct (lane 3). B. Immunofluorescence with ESR2 antibody. H&E staining of oviduct tissue (A), normal ovarian tissue (B) and ovarian tissue from hen with cancer (C); ESR2 staining of oviduct tissue (D), normal ovarian tissue (E) and ovarian tissue from hens with cancer (F); Negative control (rabbit IgG) of oviduct tissue (G), normal ovarian tissue (H) and ovarian tissue from hen with cancer (I). Arrows indicate specific staining and arrowheads indicate autofluorescence. Scale bar = 100 µm.

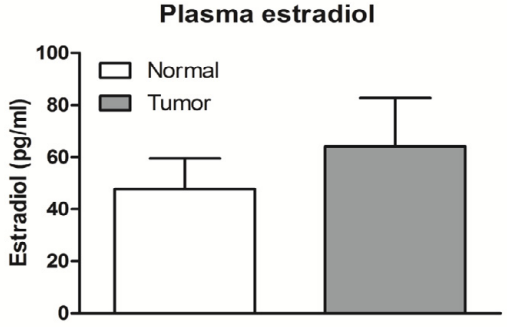

Figure 4: Plasma estradiol level of normal hens (n = 8) and hens with ovarian cancer (n = 11). No significant difference was found ($P = 0.5$). Bars indicate mean ± standard error.

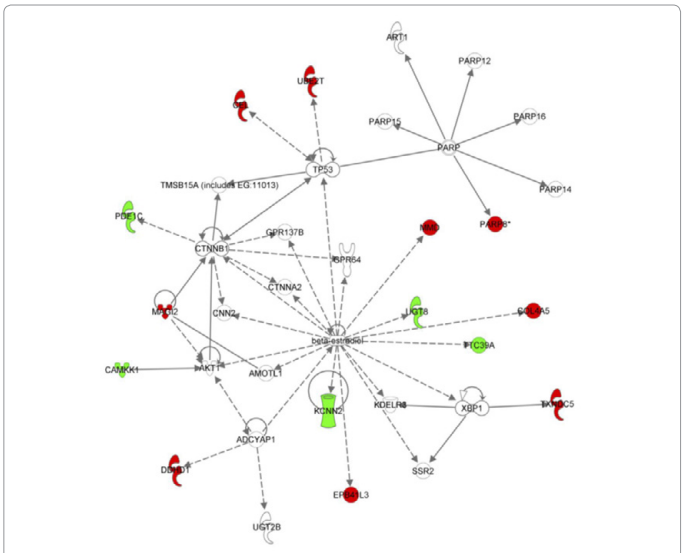

Figure 5: Beta-estradiol pathway identified from Ingenuity Pathway analysis of genes differentially regulated in chicken ovarian tumors compared to normal ovary. Microarray analysis of tissue from normal hens (n = 3) and tissue from hens with ovarian cancer (n = 3) was conducted in a previous study as described in Treviño et al., 2012 [17]. Pathway analysis was conducted using IPA version 8.7. Upregulated genes are illustrated with green symbols and down-regulated genes are illustrated with red symbols.

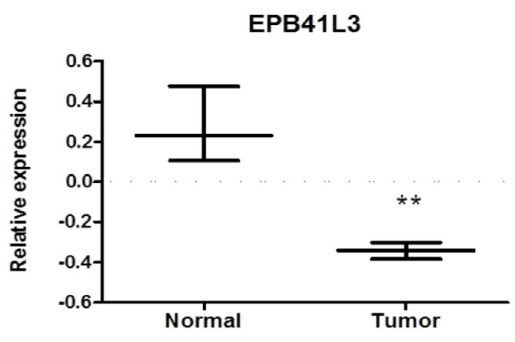

Figure 6: EPB41L3 gene expression is decreased in ovarian tumors compared to normal ovary in hens. Microarray analysis reveals that EPB41L3 expression is significantly decreased in ovarian tissue from hens with tumors compared to normal ovarian tissue. Statistical significance was determined by performing an unpaired t-test using GraphPad Prism. ¨$p < 0.01$.

carcinogenesis. Previous work has demonstrated that flaxseed decreases the severity and prevalence of ovarian cancer in hens [29]. Additionally, it was recently reported that ovarian ESR1 mRNA was decreased in a dose-related manner by treatment of hens with flaxseed [30] suggesting that at least part of the protective effect of flaxseed may be mediated through a decrease of estrogen action mediated through ESR1.

Western blot analysis indicates that our ER antibodies each detect one main band at the approximate expected size in hen ovarian lysates. Moreover, these heterologous antibodies stain oviduct samples differentially in accordance with the known distribution of ER in avian oviduct [31]. Our immunofluorescence analysis illustrates that ESR1 and ESR2 protein expression is primarily nuclear, although some cytoplasmic expression is also seen, more commonly in tumor samples (data not shown). ESR1 protein expression was widespread throughout the glandular areas comprised of tumor cells in the ovaries from hens with cancer. In women, estrogen receptor status is thought to be a predictor of response to hormonal therapy, although this is still under debate (reviewed in ref. [32]). In one study, women with estrogen receptor positive tumors seemed to respond better to treatment with the anti-estrogen tamoxifen (reviewed in ref. [32]). The detection of estrogen receptor protein (particularly ESR1) in ovarian tissue from hens with cancer suggests that the hen can be used to test endocrine therapeutics that may be appropriate for ovarian cancer in women.

We have previously reported that estrogen-stimulated genes are enriched in early- and late-stage chicken ovarian tumors [22], suggesting the importance of estrogen receptor signaling in the hen model of the disease. Interestingly, these up-regulated estrogen-stimulated genes are also oviduct-related genes [22] and these results support the emerging hypothesis that at least a subset of ovarian tumors in women may originate from the fallopian tube (reviewed in ref. [33]). EPB41L3 is another gene that was identified as differentially regulated in chicken ovarian tumors compared to normal ovary [22]. Specifically, EBP41L3 gene expression is down regulated in ovarian cancer tissue from hens. Interestingly, EPB41L3 has been shown to be a functional tumor suppressor in a variety of cancers, including prostate cancer [34], breast cancer [35,36], cervical cancer [37] and epithelial ovarian cancers [16]. As such, down regulation of its expression may play a role in the development/progression of ovarian cancer in hens and women. The presence of EBP41L3 in the beta-estradiol pathway may be suggestive of regulation by ER signaling. Further studies are needed to elucidate the mechanism(s) regulating EPB41L3 gene expression.

Numerous studies have been undertaken to determine whether estrogen receptor expression levels correlate with prognosis in women. To date, results have been contradictory with some studies finding a correlation between estrogen receptor level and patient prognosis and others not finding a correlation between the two (reviewed in [38]). These contradictory results may be attributed to the method of estrogen receptor measurement, as well as differences in ovarian cancer subtype, stage, and tumor composition. For example, estrogen receptor expression has been reported to be diagnostic when conventional analysis is adjusted to amount of tumor present [38]. Furthermore, ER expression has been reported to be higher in serous and endometrioid carcinomas [39-41] versus other subtypes. A recent study reported higher levels of estrogen receptor in low-grade serous carcinoma [42] versus high-grade serous carcinoma. As mentioned previously, ESR2 expression in ovarian tumors was associated with poor progression-free survival [15]. These results suggest that ovarian cancer subtypes and grades exhibit differences in receptor expression levels that may be exploited when developing therapeutic strategies. It is still not known whether signaling through ESR1 is involved in initiation of ovarian cancer or progression of the disease. We have previously shown that ESR1 mRNA expression is increased in lesions from hens with early-stage disease, suggesting that ESR1 signaling may indeed be important for initiation of the disease [22]. Future studies, therefore, may be particularly important in revealing potential causative factors in the initiation of ovarian cancer. The similarity of the spontaneous hen model to human ovarian cancer with respect to ER suggests that this animal model may be useful for testing endocrine therapeutics that could be helpful in combating this disease in women.

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