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Overview

A Review of Principal Studies on the Development and Treatment of Epithelial Ovarian Cancer in the Laying Hen *Gallus gallus*

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Often referred to as the silent killer, ovarian cancer is the most lethal gynecologic malignancy. This disease rarely shows any physical symptoms until late stages and no known biomarkers are available for early detection. Because ovarian cancer is rarely detected early, the physiology behind the initiation, progression, treatment, and prevention of this disease remains largely unclear. Over the past 2 decades, the laying hen has emerged as a model that naturally develops epithelial ovarian cancer that is both pathologically and histologically similar to that of the human form of the disease. Different molecular signatures found in human ovarian cancer have also been identified in chicken ovarian cancer including increased CA125 and elevated E-cadherin expression, among others. Chemoprevention studies conducted in this model have shown that decreased ovulation and inflammation are associated with decreased incidence of ovarian cancer development. The purpose of this article is to review the major studies performed in laying hen model of ovarian cancer and discuss how these studies shape our current understanding of the pathophysiology, prevention, and treatment of epithelial ovarian cancer.

Abbreviations: AA, arachidonic acid; CTNNB1, beta-catenin; CA125, cancer antigen-125; COX, cyclooxygenase enzymes; DR6, death receptor 6; E-cad, E-cadherin; EC, endometrioid carcinoma; EOC, epithelial ovarian cancer; HGSOC, high-grade serous ovarian cancer; MPA, medroxyprogesterone acetate; OM3FA, omega 3 fatty acids; OSE, ovarian surface epithelia; PCOS, poly-cystic ovarian syndrome; PR, progesterone receptor; PCNA, proliferating cell nuclear antigen; PGE2, prostaglandin E_2; RO, Restricted Ovulators; SELENBP1, selenium-binding protein; VEGF, vascular endothelial growth factor

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Ovarian cancer is the leading cause of death among female gynecologic malignancies, with a 47% 5 y relative survival rate.[154] Early detection of the disease is necessary for decreasing the high mortality rate. However, early detection is difficult due to the lack of known specific biomarkers and clinically detectable symptoms until the tumor reaches at an advanced stage. The disease has multiple subtypes. Epithelial ovarian cancer (EOC) is the most common type of ovarian cancer, accounting for about 90% of all reported cases.[127,164] EOC is commonly subdivided into 5 histotypes: high-grade serous (HGSOC), low-grade serous, mucinous, endometroid (EC), and clear cell. The histotypes differ in terms of tumor cell morphology, severity, systemic effect, and response to treatment. Among the different subtypes, HGSOC accounts for about 70% of cases of EOC observed in women. HGSOC has a higher mitotic index and is a more aggressive form of cancer with a worse prognosis. HGSOC and low-grade serous histotypes exhibit distinctly different presentations of the disease[82,166] and demand different treatment modalities. EC (10% to 20%), mucinous (5% to 20%), and clear cell (3% to 10%) histotypes are less common forms of the disease. The subtypes of EOC also differ in terms of 5 y survival rates of patients; that is, HGSOC (20% to 35%), EC (40% to 63%), mucinous (40% to 69%), and clear cell (35% to 50%).[20,76,148]

Developing a representative animal model for EOC has been challenging due to the histologic and pathologic differences among different subtypes of EOC. While developing a reliable animal model is challenging due to the vast complexity and limited understanding of the origin of the disease, laying hens naturally develop EOC that is histopathologically very similar to the human form of the disease (Figure 1).[15] All the different human ovarian cancer histotypes have been observed in laying hen ovarian cancer (Figure 2). In addition, the presentation of the disease in chickens is remarkably similar to the human form of the disease, with early-stage ovarian cancer in laying hens having similar precursor lesions as occur in women.[15] The laying hen develops ovarian cancer spontaneously, allowing analysis of early events and investigation into the natural course of the disease, as tumors can be examined as they progress from normal to late-stage ovarian carcinoma. The gross appearance of these stages is shown in Figure 3.

Over the past 2 decades, the laying hen has emerged as a valuable experimental model for EOC, in addition to other in vivo models such as Patient-Derived Xenografts (PDX) and Genetically Engineered Mouse Models (GEMMs). Comparison of the hen model with other animal models has been reviewed elsewhere.[72]

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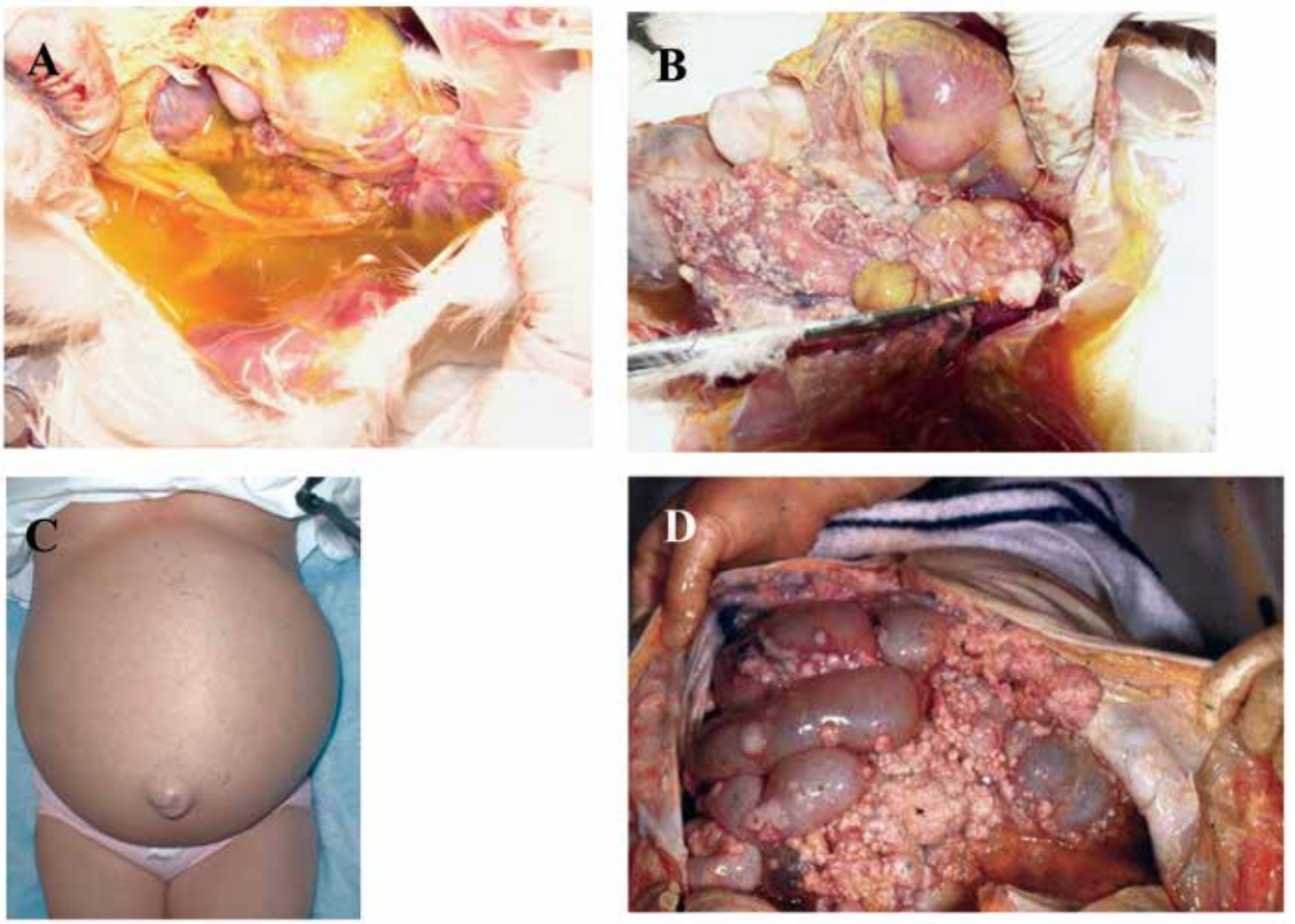

Figure 1. Gross pathologic presentation of chicken compared with human ovarian cancer. The remarkably similar presentation in hens (A,B) and women (C,D) at the gross anatomic level with profuse abdominal ascites and peritoneal dissemination of metastasis. A) Ascites in abdominal cavity chicken with advanced ovarian cancer (photo credit: DB Hales); (B) Chicken ovarian cancer with extensive peritoneal dissemination of metastasis (photo credit: DB Hales); (C) Distended abdomen from ascites fluid accumulation in woman with ovarian cancer (http://www.pathguy.com/bryanlee/ovca.html) (D) Human ovarian cancer with extensive peritoneal dissemination of metastasis (http://www.pathguy.com/bryanlee/ovca.html).

Modern-day laying hens, such as the white leghorn, have been selected from their ancestor red jungle fowl[57] for decreased broodiness and persistent ovulation, resulting in approximately one egg per day, if proper nutrition and light-dark cycles are maintained. Daily rupture and consequent repair of the ovarian surface epithelia (OSE) due to the persistent ovulation promotes potential error during rapid DNA replication. This increases the probability of oncogenic mutations, ultimately leading to neoplasia.[137] Inflammation resulting from continuous ovulation also promotes the natural development of EOC.[81] By the age of 2.5 to 3 y, laying hens have undergone a similar number of ovulations as a perimenopausal woman. The risk of ovarian cancer in white leghorn hens in this time (4%) is similar to the lifetime risk of ovarian cancer in women (0.35% to 8.8%).[125] By

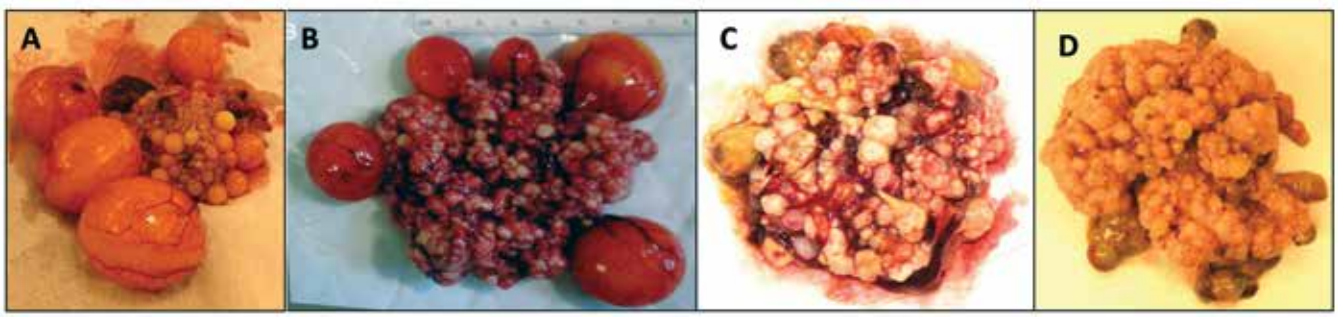

Figure 2. Gross anatomic appearance of different stages of ovarian cancer in the chicken The progression from the normal hen ovary to late-stage metastatic ovarian cancer. (A) Normal chicken ovary showing hierarchal clutch of developing follicles and postovulatory follicle; (B) Stage 1 ovarian cancer, confined to ovary with vascularized follicles; (C) Stage 2/3 ovarian cancer, metastasis locally to peritoneal cavity with ascites; (D) Stage 4 ovarian cancer, late stage with metastasis to lung and liver with extensive ascites (photo credits: DB Hales).

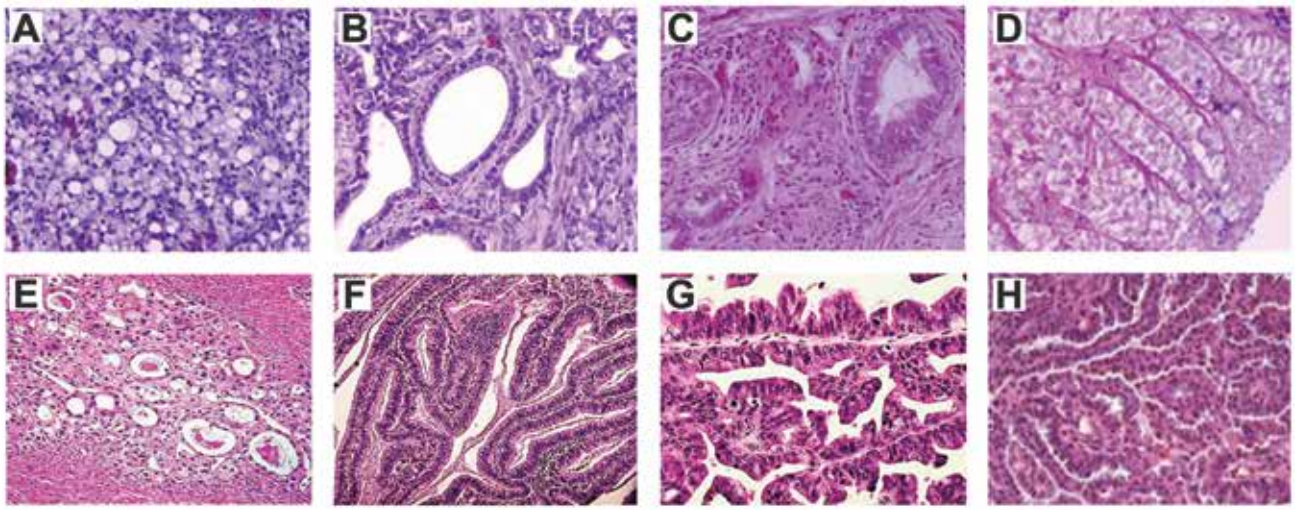

Figure 3. Histologic subtypes in chicken compared with human ovarian cancers. H and E staining of formalin fixed paraffin embedded tissues from hens with ovarian cancer (A through D) and women (E through G). (A) Chicken clear cell carcinoma; (B) Chicken endometrioid carcinoma; (C) Chicken mucinous adenocarcinoma; (D) Chicken serous papillary adenocarcinoma (photo credits: DB Hales). (E) Human clear cell carcinoma; (F) Human endometrioid carcinoma; (G) Human mucinous cystadenocarcinoma; (H) Human serous adenocarcinoma (https://www.womenshealthsection.com).

the age of 4 to 6 y, the risk of ovarian cancer in hens rises to 30% to 60%.[54] The incidence of ovarian carcinoma in the hens, however, depends on the age, genetic strain,[80] and the egg-laying frequency of the specific breed.[54] The common white leghorn hen has routinely been employed in chicken ovarian cancer studies. On average, hens are exposed to 17 h of light per day, with lights turned on at 0500 h and turned off at 2200 h. The laying hen model of EOC does present some considerable challenges. Despite its great utility for research, the model is still used mainly by agricultural poultry scientists and a small number of ovarian cancer researchers.

Comprehensive and proper vivarium support is required to conduct large-scale cancer prevention studies. Only a few facilities are available for biomedical chicken research, including University of Illinois Urbana-Champaign, Cornell University, Penn State University, NC State, Auburn University, and MS State University. Another difficulty is a lack of available antibodies specific for chicken antigens. Because of the structural dissimilarities between most human proteins and murine antigens to their chicken counterparts, cross-reactivity of available antibodies is also limited. The entire chicken genome was sequenced in 2004;[78] however, the chromosomal locus of many key genes, such as p53, are still unknown. Overall, humans and chickens share about 60% of genetic commonality, whereas humans and rats share about 88% of their genes. Specific pathway-mutated strains of chickens are not yet available, limiting the ability to study key pathways in carcinogenesis and prevention of cancer using this model. Although all 5 different subtypes of ovarian cancer are present in hens, their most predominant subtype is different from women. Close to 70% of women diagnosed with ovarian cancer have serous EOC, while the predominant subtype reported in hens is endometrioid.[15] However, these comparisons are complicated because observations of cancer in hens consist of both early and late stages of the disease, wherein women, most of the data is from late stage and aggressive ovarian carcinoma.

The spontaneous onset of ovarian cancer and the histologic and pathologic similarities to the human form of the disease make laying hens an excellent model for continued research on EOC. To date, a large number of studies have been performed on laying hens. Here we have divided the current studies into 2 groups— (A) studies that have described the molecular presentation of EOC to be similar to that in women; (Table 1) and (B) chemoprevention studies performed on large cohorts of laying hens (Table 2). The purpose of this review is to outline the major studies conducted using the laying hen model for EOC, and to describe how the research has advanced the field of ovarian cancer.

Etiology of the disease: current hypotheses

To understand, diagnose, treat, and prevent any malignancy requires the determination of the origin of the tumor. Summary of all the current hypotheses have been summarized in Table 3. Unlike many other epithelial carcinomas such as colon and cervical cancer, which have well-defined precursor lesions, the cell of origin for EOC is poorly understood. An emerging body of evidence suggests an important role for the fallopian tube in initiating serous ovarian cancer.[35,86] The proposed tubal hypothesis states that some ovarian cancers may arise from the fimbriae or the distal fallopian tube in women. Embryologically, OSE originates from the coelomic epithelium, whereas the fallopian tube, uterus and cervix originate from the paramesonephric (Müllerian) ducts. Neoplastic OSE is present in Müllerian-like tissues in which the epithelium is not of Müllerian origin, supporting this hypothesis. The tubal hypothesis classifies ovarian carcinoma in 2 groups, 'Type I' are low-grade cancers often lacking p53 mutations and originate from the ovaries; 'Type II' are aggressive high-grade carcinomas that arise from the fallopian tube with mutated p53 in over 90% of all cases reported.[27,88,142]

Among multiple contributing factors for carcinogenesis, chronic inflammation seems to have a significant role, giving rise to the inflammation hypothesis.[116,122] A prolonged and sustained inflammatory response is well established as a potent activator of tumor growth and invasion.[4,12,13] The ovulatory process involves the rupture of the OSE, which triggers a strong inflammatory reaction due to the wound healing response; cyclic wounding and healing exacerbate the inflammation, with infiltrating leukocytes, production of inflammatory cytokines, and a marked upregulation in major inflammatory signaling

Table 1. Studies investigating key molecular signatures in laying hen ovarian cancer

Author	Year	Significance	Key molecular targets	Citation
Haritani and colleagues.	1984	Investigating ovarian tumors for key gene signatures	Ovalbumin	71
Rodriguez-Burford and colleagues.	2001	Investigating expressions of clinically important prognostic markers in cancerous hens	CA125, cytokeratin AE1/AE3, pan cytokeratin, Lewis Y, CEA, Tag 72, PCNA, EGFR, erbB-2, p27, TGF{α}, Ki-67, MUC1, and MUC2	135
Giles and colleagues.	2004, 2006	Investigating ovarian tumors for key gene signatures	Ovalbumin, PR, PCNA, Vimentin	62, 63
Jackson and colleagues.	2007	CA125 expression in hen ovarian tumors	CA125	79
Stammer and colleagues.	2008	SELENBP1 downregulation in hen ovarian tumors	SELENBP1	149
Hales and colleagues.	2008	Cyclooxygenase expressions in hen ovarian tumors	COX1, COX2, PGE2	67
Urick and colleagues.	2008-2009	VEGF expression in cultured ascites cells from hen ovarian tumors	VEGF	160, 161
Ansenberger and colleagues.	2009	Elevation of E-cadherin in hen ovarian tumors	E-cad	6
Hakim and colleagues.	2009	Investigating oncogenic mutations in hen ovarian tumors	p53, K-ras, H-ras	66
Zhuge and colleagues.	2009	CYP1B1 levels in chicken ovarian tumors	CYP1B1	175
Seo and colleagues.	2010	Upregulation of Claudin-10 in hen ovarian tumors	Claudin-10	145
Trevino and colleagues.	2010	Investigating ovarian tumors for key gene signatures	Ovalbumin, Pax2, SerpinB3, OVM, LTF, RD	157
Choi and colleagues.	2011	Upregulation of MMP-3 in hen ovarian tumor stroma	MMP-3	28
Barua and colleagues.	2012	Upregulation of DR6 in hen ovarian tumors	DR6	16
Lee and colleagues.	2012-2014	Upregulation of DNA methylation in hen ovarian tumors	DNMT1, DNMT3A, DNMT3B, SPP1, SERPINB11, SERPINB13	94, 101, 103, 104
Lim and colleagues.	2013-2014	Key genes upregulated in endometrioid hen tumors	AvBD-11, CTNNB1, Wnt4	102, 11, 100
Bradaric and colleagues.	2013	Investigating immune cells in hen ovarian tumors		23
Ma and colleagues.	2014	Identifying unique proteins from proteomic profiling	F2 thrombin, ITIH2	106
Hales and colleagues.	2014	Key genes upregulated in hen ovarian tumors	PAX2, MSX2, FOXA2, EN1	68
Parada and colleagues,	2017	Unique ganglioside expressed in hen ovarian tumors	NeuGcGM3	124

pathways. The group of activated inflammatory molecules during ovulation include IL8, CCL2 and CCL5/RANTES and is similar to those activated during EOC.[55] OSE is a continuum of the peritoneal lining, unlike most other organs in the peritoneal cavity.[7] Therefore, the OSE is also exposed to any environmental or xenobiotic stress present in the peritoneum, which are likely to be extremely inflammatory in nature.

The gonadotropin hypothesis[33] of ovarian cancer arose from early observations of an increased incidence of ovarian cancer in rodents after transplanting their ovaries to their spleen. This tumorigenesis was attributed to elevated pituitary gonadotropin levels caused by disruption of negative feedback of estrogen to the pituitary gland.[167] Ovarian tumors did not form if one ovary was left intact while the other was transplanted to the spleen.[19] Exposure to carcinogens or X-irradiation that causes loss of functional oocytes and ovarian failure also induced ovarian cancer in animals.[107,114] The mouse strain ((C57BL/6J x C3H/HeJ)F1 WxWv), which is congenitally deficient in or has few functional oocytes, also rapidly develops ovarian cancer.[114,152] These models all have increased gonadotropin release due to disruption of the hypothalamus-pituitary-ovarian negative feedback loop. Tumor development could be reduced or prevented by suppressing gonadotropin in these animals.[21,105] Ectopic expression of hormone receptors (that is, receptors for GnRH-I/II,[29,48] activin,[110,150] inhibin,[56] estrogen,[74,92,128] progesterone[3,92,95] and androgen,[26,69,92]) has also been reported in EOC. The gonadotropin hypothesis proposes that the surge of gonadotropins due to lack of gonadal negative feedback in menopause and/or premature ovarian failure contributes to the development and progression of EOC.

The incessant ovulation hypothesis,[51] which arose in 1971, is based on an analysis of epidemiologic data from patients and animals and postulates that continuous ovulation causes continuous damage to the OSE.[51] (Table 3) This damage triggers rapid wound healing and the generation of an immense inflammatory burden on the ovaries, increasing the likelihood of oncogenic mutations and carcinogenesis. While repairing the ovulation wound, the OSE often forms an indentation from retraction of corpus albicans or disintegration of a cystic follicle.[129] Such indentations lead to the deposition of the surface epithelium into the cavity of a corpus luteum, resulting in the formation of inclusion cysts that remain in the ovarian stroma. Presence of inclusion cysts in the contralateral ovary in women with ovarian cancer has provided strong evidence that cysts could play a major role in the development of ovarian cancer.[111] Ovulation frequency is reportedly higher in the right ovary than in the left ovary[126] and studies have also reported a higher propensity for the right ovary to develop ovarian cancer.[34] Ovulation-inducing agents (clomiphene and gonadotropins) are suggested risk factors for developing ovarian cancer.[5,91,132,138,163] In contrast, reducing the total number of ovulations has reduced the risk of getting ovarian cancer. Oral contraceptive users have about a 30% lower risk of getting EOC than do nonusers due to decreased ovulatory events.[22,113,158]

Table 2. Ovarian cancer prevention studies using laying hen model

Author	Year	Significance	Citation
Barnes and colleagues.	2002	Medroxyprogesterone study	14
Johnson and colleagues.	2006	Different genetic strain of laying hens (C strain and K strain)	80
Urick and colleagues.	2009	Dietary aspirin in laying hens	161
Giles and colleagues.	2010	Restricted Ovulator strain	61
Carver and colleagues.	2011	Calorie-restricted hens	25
Eilati and colleagues.	2012-2013	Dietary flaxseed in laying hens	43, 44, 45
Trevino and colleagues.	2012	Oral contraceptives in laying hens	156
Rodriguez and colleagues.	2013	Calorie-restricted hens with or without Vitamin D and progestin	136
Mocka and colleagues.	2017	p53 stabilizer CP-31398 in laying hens	112

Also, each additional pregnancy after the first reduces the risk of getting EOC by 10% to 16%.[70,134] Thus, the incessant ovulation hypothesis encompasses other pre/coexisting hypothesis for onset of ovarian cancer, such as the gonadotropin hypothesis[32,133] and the inflammation hypothesis.[4,12,13,49] However, some evidence contradicts the hypothesis that gradually accumulating postovulatory, benign inclusion cysts leads to the onset of a malignant carcinoma.[119,131,139] Although patients suffering from Poly-Cystic Ovarian Syndrome (PCOS) have a high number of inclusion cysts in their ovaries,[131] the high number of cysts is not positively correlated with increased ovulation; in fact, PCOS patients are predominately hypo-ovulating and are subfertile.

The specific etiology of ovarian cancer is still not resolved, perhaps predominantly because ovarian cancer is rarely detected in early stages, and therefore, the early molecular events that promoting the neoplastic changes are unknown.

Investigating key molecular signatures in laying hen ovarian cancer: similarities between the chicken model and human EOC

Detection of ovarian cancer is a challenge, primarily because of the lack of sensitive predictive biomarkers. Cancer antigen-125 (CA125)/mucin-16 (MUC-16) has been one of the earliest developed biomarkers for EOC.[17] While detection of the gradually elevating serum level of CA125 glycoprotein has long been used as a diagnostic marker for ovarian cancer,[83] CA125 levels cannot be used to distinguish ovarian cancer from other cancers. A previous study[18] reported that approximately 29% of patients with nongynecological cancer presented with elevated serum CA125. In laying hens, 2 studies have investigated CA125 expression; one study found that about 90% of ovarian tumors in laying hens express CA125,[79] whereas the other did not detect CA125 in chicken ovarian tumors, using the same CA125 antibody.[135] The first study[79] used a high-temperature antigen retrieval method, whereas the second,[135] which failed to detect CA125, used a low-temperature antigen retrieval method for CA125 immunostaining. This difference might be the key reason for these contradicting results. The second study also investigated expression of several other prognostic markers that were routinely used for clinical evaluation of ovarian cancer in women; of these, they found expression of cytokeratin AE1/AE3, pan cytokeratin, Lewis Y, CEA, Tag 72, PCNA, EGFR, erbB-2, p27 and TGF α to be positive in chicken ovarian tumors, while Ki-67, Muc1 and Muc2 were not detected in the hens.[135] More recently, one study[159] reported have shown Ki-67 positive staining and have expanded the number of known angiogenic and proliferation markers with chicken-cross reactive antibodies.[159] A separate report[106] conducted a shotgun proteomic analysis using combinational peptide ligand libraries (CPLL)-LC-MSMS workflow of chicken blood proteins and identified 264 unique proteins. From the unique proteins identified, 10 potential biomarkers were selected through semiquantitative spectral counting analysis; of these, interalpha inhibitor heavy chain (ITIH2) and F2 thrombin were found to be elevated in hens with cancer, as compared with normal hens. The human homolog of F2 thrombin, prothrombin fragment F2[58] and another human heavy chain of ITIH4[174] are both reported to be elevated in women with ovarian cancer. These studies allowed the identification of similar key biomarkers of EOC in laying hens and women.

One of the unique features in ovarian cancer is an upregulation of a transmembrane cell-cell adhesion glycoprotein, E-cadherin (E-cad), which is expressed by the normal oviductal and endometrial surface epithelia but is not found in the ovarian stroma.[7,60] During its early neoplastic changes, the OSE undergoes a conformational change from cuboidal to columnar

Table 3. Key hypotheses describing the origin of ovarian cancer

Hypotheses	Main features	Key citations
Tubal hypothesis	OSE is not embryologically derived from Mullerian ducts, however ovarian neoplasms present Mullerian features. This suggests that OC originates from the epithelium of the fallopian tube where p53 signatures have been observed and the disease later migrates onto the ovarian surface.	35, 27, 86, 89, 142
Inflammation hypothesis	Prolonged and sustained inflammation in the ovary gives rise to neoplastic changes. Ovulation is an inflammatory process. The OSE is also exposed to the peritoneal cavity and therefore is exposed to environmental and xenobiotic stress.	4, 13, 55
Gonadotropin hypothesis	Factors that induce gonadotropin release from the pituitary (such as loss of negative feedback by estrogens to the pituitary) induce ovarian carcinogenesis.	19, 21, 105, 107, 118, 167
Incessant Ovulation hypothesis	Continuous ovulation and consequential rapid wound healing of the OSE results in an immense inflammatory burden on the ovaries and can trigger oncogenic changes. Ovarian cancer incidence can also be positively correlated with the number of ovulations. This hypothesis combines elements of both the inflammation and gonadotropin hypotheses.	34, 51, 52, 111, 126

epithelial cells, which also occurs in prostate cancer. During these neoplastic changes, E-cad is reported to be gradually elevated in women.[8,144] Another report found that E-cad is significantly upregulated in ovarian cancer in laying hens.[6] In addition, E-cad is expressed in the granulosa cell layer around the follicles in normal ovary, around the inclusion cysts in the early neoplastic ovaries, and throughout the cancerous epithelium in late-stage ovarian tumors.[6] They found that the metastasized secondary tumors in the peritoneal cavity of hens form similar glandular structures with high E-cad expression.[6] Recently, an ectopic miR-200 expression in OSE cells in 3D culture was reported to stabilize the formation of inclusion cysts with a subsequent increase in E-cad expression.[30] Several gene expression studies have also reported upregulation of E-cad in chicken ovarian tumors.[64,153,157] One report assessed gene expression and performed a bioinformatic analysis of chicken ovarian tumors and reported that key genes upregulated in chicken ovarian cancer included PAX2, MSX2, FOXA2 and EN1.[68] All of these upregulated genes are involved in controlling branching morphogenesis during gland development, and parallel upregulation of E-cad and miR-200 family members.[68] At about the same time, another publication reported that the junction adherence molecule Claudin-10 is elevated in chicken ovarian cancer as also occurs in women.[145]

One of the most intensively studied tumor suppressor genes is TP53. This gene codes for the p53 protein and is often referred to as the 'guardian of the genome'. Over 50% of all human cancers have a mutant TP53 gene.[121] In ovarian cancer, TP53 is mutated in about 95% of HGSOC.[24] This exclusive feature of HGSOC is widely accepted for prognostic distinction of this subtype.[9,141] One report[66] analyzed the TP53 mutation in chickens in 2 flocks from a previously-reported calorie-restricted chicken study.[25] This TP53 study[66] performed gene expression analysis from 172 4 y old white leghorn hens, grouped as calorie-restricted (n = 102) (flock A) and normal diet (n = 70) (flock B). Flock A birds had a significantly lower number of ovulations and a lower incidence of ovarian cancer. Gene expression analysis revealed that 48% of the chicken ovarian tumors had a mutated p53 gene, 14% in flock A and 96% in flock B. This was a striking resemblance to the human form of ovarian cancer in which p53 mutations correlate with the number of lifetime ovulations in women.[140,168] This study[66] also reported that most of the p53 mutations were found in the proline-rich and DNA-binding domains (82 of 90 mutations), similar to previous reports in women.[66] The type of mutation also differed between the 2 flocks of hens. All flock A mutations (14) were found within the DNA-binding domain and only 0.7% (1 out of 14) were a missense mutation, whereas 93% of flock B mutations (71 out of 76) were missense mutations, and all mutations were found within the proline-rich domain. Most of the flock B mutations (76%) involved a change in an aliphatic amino acid at position 62 (Ala) or 72 (Thr) into a proline. However, the effect of these mutations on p53 function is not known. This study[66] also found very few K-ras mutations (1.2%) and no H-ras mutations in these birds. Ras mutations in women are also extremely rare in aggressive ovarian carcinomas.[36,108,118,171] Positive Her-2/neu staining was reported in 53% of hen ovarian adenocarcinomas, which was similar to that reported in women.[117] Together, these findings reveal a similar oncogenic mutational landscape between the laying hens and human disease.

Selenium-binding protein (SELENBP1) downregulation has been observed in women with ovarian cancer.[77] Because selenium is an essential micronutrient involved in reducing cell proliferation and promoting apoptosis in tumor cells, a marked decrease in SELENBP1 has been positively correlated with cancer progression in women.[96] A previous study found that SELENBP1 is downregulated in all histotypes of ovarian cancer in laying hens.[149] In normal, noncancerous chicken ovaries, SELENBP1 is strongly expressed at or near the surface epithelium. This expression was significantly lower in tumors and early neoplastic lesions, indicating another significant similarity between the laying hen and human ovarian cancer.[96]

Several other studies have supported the use of the chicken model to study ovarian cancer. One article[29] reported an upregulation of matrix metalloproteinase 3 in the chicken ovarian tumor stroma, as had earlier been reported in human ovarian cancer.[115] Another group[16] reported elevated expression of Death receptor 6 (DR6), a receptor that mediates suppression of antitumor activities, in ovarian tumors in hens. DR6 expression found in serum and microvessels was low in normal hens, and gradually increased with ovarian cancer progression into the late stages of the disease. DR6 expression was also reported to be elevated in women with ovarian cancer.[143,146] Other articles identified that an avian homolog of the β-defensin (AvBD-11),[102] β-catenin (CTNNB1)[11] and Wnt4[100] were expressed abundantly in the glandular epithelium of the endometrioid type of ovarian cancer in hens. In women with ovarian cancer, human β-defensin (hBD) has been reported to influence vasculogenesis under the influence of VEGF-A.[31] Human EOC has been reported to express mutated CTNNB1[89,109] and Wnt4 has also been positively associated with a higher risk of ovarian cancer in women[173] A separate report found an increase in de novo DNA methylation in chicken ovarian tumors and an upregulation of DNMT1, DNMT3A and DNMT3B.[94] Other studies reported that a secreted phosphoprotein (SPP1)[101] and 2 serine proteinase inhibitors, SERPINB11[104] and SERPINB3[103] are significantly upregulated in ovarian cancer. All of these factors (that is, DNMT1, DNMT3A, DNMT3B,[1,2] SPP1,[172] SERPINB11 and SERPINB13)[10] were also found to be elevated in women with ovarian cancer. Another article[64] investigated differential gene expression patterns in localized and metastasized hen ovarian tumors and normal ovarian epithelium samples. A class comparison analysis with the human ovarian cancer microarray GEO database (GSE6008) revealed that the altered gene expression pattern in laying hen EOC is very similar (approximately 78%) to that in women.[64]

Studies involving immune system activation to target and kill cancer cells have been revolutionized since the discovery of immune checkpoint inhibitors. T-cells express immune checkpoint receptors such as CTLA-4 and PD-1 on their surface; these receptors send an "off' signal upon binding with their ligands, rendering the T-cell inactive. CTLA-4 binds with the B7 ligand on antigen-presenting cells with a higher affinity than CD28, impeding the co-stimulating signal for T-cell activation. PD-1 binds with PD-L1 ligand, which is expressed by many tumor cells and M2-like macrophages. Inhibiting the checkpoint inhibitors prevents the inactivation of T-cells, allowing them to attack the tumor cells more effectively. However, despite initial success in melanoma and lung cancer, many solid tumors have shown formidable resistance against the immune checkpoint inhibition therapy. The tumor microenvironment substantially aids this resistance, leading to an increasing interest in investigating how the tumor and its microenvironment harness host anti-tumor immunity. The immune system in chickens and humans are reported to be very similar,[39,50] although chickens lack IgE and IgD immunoglobins,[130] and the MHC regions of chickens are simpler and more compact than those of humans.[84,85] A report conducted to investigate the association of immune cells with

ovarian cancer showed that the immune cell content and locations in early to late ovarian cancer is similar in laying hens and women.[23] This work also provided evidence that CD4+ helper T-cells were less prevalent than CD8+ cytotoxic T-lymphocytes and B cells in both normal and cancerous ovaries. B cells were found in the stroma and were not associated with the follicles. These findings were similar to the immune landscape seen in women with ovarian tumors.[155] Another study reported that chicken ovarian tumors uniquely express a NeuGcGM3 ganglioside that is not expressed in normal ovarian tissue.[124] NeuGcGM3 ganglioside has also been reported to be highly expressed in women with breast and ovarian cancer.[90,120]

Other studies in laying hens have further supported the oviductal origin theory of ovarian cancer. One of the earlier studies that suggested an oviductal origin of ovarian cancer used tumor tissues that were collected from 12 hens with ovarian adenocarcinoma, immunostained with ovalbumin, and all found to have positive staining,[71] Another group analyzed 16 hen ovarian adenocarcinomas with or without oviductal involvement and 9 normal ovarian tissues for expression of ovalbumin, proliferating cell nuclear antigen (PCNA) and progesterone receptor (PR).[63] All ovarian cancer samples were positive for ovalbumin and PCNA, yet ovalbumin was absent in normal OSE. Progesterone receptor was present in 9 of 14 ovarian tumors. A follow up study reported that ovarian tumors strongly express cytokeratin and PCNA and weakly express vimentin in the gland-like regions of the tumors; normal OSE expressed cytokeratin, PCNA and PR.[62] Another group performed a microarray-based gene expression study in laying hens and found by functional annotation analysis that the top 25 genes altered in the ovarian tumors are related to the oviduct.[157] Of them, OVAL (Ovalbumin/SerpinB14), Paired-box 2 (Pax2), SerpinB3 (important in promoting EMT), OVM (Ovomucoid/SPIK7), LTF (lactotransferrin), and RD (riboflavin binding protein) were expressed in the hen in both early and late-stage ovarian cancer, but not in normal OSE. Another important observation was that all these altered genes, like several other oviduct-related genes, are driven by estradiol.[157] The expression of these oviductal genes is also involved in human ovarian carcinoma. One of these genes, Pax8, is a well-established marker for ovarian tumors in women and promotes tumor cell growth and differentiation (chickens do not have a Pax8 gene).[97,99] The laying hen studies have not helped to resolve the controversy surrounding the ovarian cancer cell of origin; however, the presence of the same controversy in the laying hens further endorses the model as a reliable equivalent to the human form of ovarian cancer.

Cancer prevention studies using the laying hen

The first large cohort study that connected the number of ovulations with the onset of ovarian cancer in laying hens monitored egg production in 3 different flocks of 466 white-leghorn hens.[54] The flocks were grouped in 2 y, 3 y and 4 y or older birds, and the incidence of EOC in those birds were 9%, 19% and 39%, respectively. The study also monitored incidences of oviductal adenocarcinoma, granulosa cell tumors, Sertoli cell tumors and other tumor types but only EOC was positively correlated with age across the flocks. The observations of the study and the incessant ovulation hypothesis proposed by others, led to a focus on the impact of ovulation and inflammation in ovarian cancer incidence.[52]

Prostaglandins are bioactive lipids that are produced from arachidonic acid (AA) through the action of the cyclooxygenase (COX) enzymes. Humans have 2 isoforms of COX: COX1, which is constitutively expressed in most cells and tissues, and COX2, which is inducible by various inflammatory stimuli. Upregulation of COX2 has been reported in many cancers.[40,147] COX1 is expressed in the laying hen by the OSE, granulosa cell layer, cortical interstitium, and postovulatory follicles in normal ovaries, while spreading largely to the tumor stroma with ovarian cancer.[67] However, expression of COX2 in the hen ovary increases with age yet is not affected by the onset of cancer.[67] To investigate the effect of inhibition of the COX1 and COX2 enzymes on ovarian cancer cells, cultured ascitic cells were collected from cancerous chickens and treated with aspirin, sc-560 (a specific COX1 inhibitor) or ns-398 (a specific COX2 inhibitor).[160] The data showed an increase in vascular endothelial growth factor (VEGF) expression in the ascitic cells, theca cells in normal ovaries, and the glandular areas of the tumor. VEGF levels in the peritoneal ascites was higher than in tumors. Aspirin and sc-560 significantly decreased the cell growth and VEGF production but ns-398 did not in the OVCAR-3 ovarian cancer cell line. These findings supported the previous observations that, unlike most carcinomas, COX1 is upregulated[37,38,65,87,93,98,162] and COX2 is downregulated or remains unaltered in ovarian cancer.[169] The proliferation of the ascites cells and subsequent VEGF production are dependent on COX1 but not on COX2. In a study of the effects of dietary aspirin in laying hens,[161] hens from 3 different age groups were fed either a control diet or a diet supplemented with 0.1% aspirin for one year. Dietary aspirin did not decrease the incidence but decreased the stage or severity of ovarian cancer. That study further found that dietary aspirin significantly decreased the liver prostaglandin E_2 (PGE2) levels in the birds, which indicated an inhibition of the systemic activity of the COX enzymes. An increase in PGE2, and other prostanoids (TxB_2) has also been reported in women with ovarian cancer.[73,170]

Eicosanoids such as PGE2, produced from AA (omega-6 fatty acids) are proinflammatory. In contrast, eicosanoids derived from omega 3 fatty acids (OM3FA) have antiinflammatory properties. Dietary ingestion of OM3FA is well known to increase their incorporation to cell membranes and therefore affect the inflammatory response. A cancer-preventative study in laying hens evaluated dietary supplementation of flaxseed, the richest plant source of OM3FA (mostly α-linolenic acid).[59] Feeding a 10% flaxseed supplemented diet to 2.5 y old hens for one year was associated with a decrease in severity of ovarian cancer.[44] The concentration of PGE_2 and COX2 expressions in the ovaries were both significantly lower with flaxseed diet, whereas the concentration of OM3FA in yolks more than doubled. This study[44] also reported that long-term consumption (4 y) of 10% flaxseed decreases both the severity and incidence of ovarian cancer. Long-term flaxseed consumption significantly diminishes PGE_2 and COX2 levels in chicken ovaries, protecting them from ovarian cancer.[43,45] Another study showed that a flaxseed supplemented diet decreased PGE_2 concentration and COX2 expression in the ovaries in a dose-dependent manner, accompanied by dose-dependent increases in the OM3FA/OM6FA ratio.[41] Dietary supplementation with fish oil (rich in OM3FA, mostly eicosapentanoic acid) has been shown to downregulate expression of COX1, COX2, and PGE_2 levels in chicken ovaries,[46] suggesting that antiinflammatory actions of the omega-3 fatty acids are pivotal in targeting the prostaglandin biosynthesis pathway and thereby hindering the onset of ovarian cancer. Dietary flaxseed has also been reported to alter the estrogen metabolism pathways, inducing the CYP1A1 pathway and enhancing systemic production of the 2-methoxyestradiol metabolite while suppressing the CYP1B1 and CYP3A4 pathways.[41,42] In addition, CYP1B1 levels in chicken ovarian tumors

are significantly higher than in the age-matched normal chicken ovaries,[175] which also supports an alteration in the estrogen metabolism pathway in chicken ovarian cancer. The flaxseed diet also activated the MAPK pathways (p38 MAPK and Erk-1/2) in the ovaries, which may be protective against ovarian carcinogenesis.[42,123]

Ovarian cancer was analyzed[59] in a unique, previously developed hyperlipidemic strain of white leghorns referred to as "Restricted Ovulators" (RO).[75] The RO strain has a mutated VLDL receptor; VLDL protein is necessary for oocytic uptake of major yolk precursor macromolecules such as VLDL and vitellogenin-2. Because of this mutation, oocytes fail to mature and lack a typical follicular hierarchy.[47] This failure results in massive lipid accumulation in blood plasma, making the hens hyperlipidemic, hypoprogesteronemic and hyperestrogenemic. The study monitored egg-laying and ovarian cancer incidence in 31 RO and 33 WT hens (around 4 y of age at euthanasia) over a period of 972 d and found that only 3% of the RO hens developed ovarian cancer as compared with about 27% of the WT hens.[61] The RO birds laid significantly fewer eggs than did wildtype birds. Another report made similar observations.[54] Another observation from this study[54] was that the RO birds had a significantly higher plasma estradiol concentration.

A study of 800 white leghorn hens used caloric restriction to suppress ovulation.[25] In this 2 y study, a normal caloric diet formulated for daily egg-production was compared with a diet designed to cause a 55% decrease in dietary energy consumption. Calorie-restricted birds had significantly lower body weight, produced about 64% fewer eggs and developed 23% less cancer of the reproductive tract. In chickens on the normal diet, 26% had ovarian cancer, as compared with only 6% of the calorie-restricted birds. These data support the hypothesis that reduction in ovulation reduces the incidence of carcinogenesis.

A subsequent study divided around 2,400 birds into 6 groups: regular diet, calorie-restricted diet, calorie-restricted supplemented with vitamin D_3, the progestin levonorgestrel, progestin provera, or a combination of levonorgestrel and vitamin D_3.[136] Ovulation rate did not change in the calorie-restricted groups, except that the levonorgestrel treated birds had fewer ovulatory events than did the other birds. Overall, progestin-treated birds had fewer reproductive tract cancers, including ovarian cancers, than did the other groups, whereas vitamin D_3 did not affect cancer incidence. However, the study concluded that suppressing ovulation (with progestins) reduces the incidence of ovarian cancer.[136] These findings were consistent with previous data from a study that administered medroxyprogesterone acetate (MPA; 100 mg dose), a progestin-only contraceptive, to 3 y old hens in 3 intervals over 16 mo.[14] The data showed a 15% reduction in all reproductive tract adenocarcinoma in the treated group as compared with the control. Egg-laying frequency was also reduced after MPA administration.

Suppressing ovulation by use of contraceptives and subsequent prevention of ovarian carcinogenesis was studied using oral contraceptives in laying hens.[156] In this study, 231 one-y-old white leghorns were divided into control and 3 treatment groups; progestin (MPA) alone, estradiol (compudose implant) alone, and a combination of both progestin and estradiol. Hens were euthanized after 16 mo and the recorded incidence of ovarian cancer in the 4 groups were as follows: control (19%), estradiol only (19%), progestin only (4%) and combination of progestin and estradiol (2%). Thus, in combination with estradiol, the efficacy of progestins improved, resulting in a further decrease the incidence of ovarian cancer and egg-laying frequency. Estradiol alone did not alter either egg-laying frequency or ovarian cancer onset. However, estradiol alone also did not increase ovarian cancer incidence. Another study that monitored ovarian cancer onset in 2 strains of domestic hens derived from a similar genetic background found that one strain (C strain) had a higher incidence of ovarian neoplasm than the other (K strain).[80] C strain birds had a higher plasma estradiol levels and a lower α-inhibin level (both in plasma and the granulosa cell layer of the ovaries) than did the K strain. RO birds also had higher plasma estradiol levels and a lower rate of ovarian cancer onset. These combined observations suggest that, unlike breast cancer, increased exposure to estradiol probably does not have a role in inducing ovarian cancer.

Another dietary chemoprevention study in the laying hens was performed[112] with a p53 stabilizing compound CP-31398.[53,165] CP-31398 is a styryl quinazoline compound[151] that is suggested to act in a chaperone-like manner, binding to newly synthesized p53 protein and maintaining its proper folding and wild-type conformation.[165] In this study, CP-31398 was fed to approximately 1.5 y-old hens for 94 wk in low (100 ppm), moderate (200 ppm) and high (300 ppm) doses.[112] Dietary CP-31398 in moderate and high doses significantly lowered onset of ovarian cancer (approximately 77% lower) as compared with the low-dose and control group. The moderate and high dose birds also had significantly lower feed consumption, body weight and number of eggs laid than did the low dose-fed and control group hens.

The ability to perform such chemopreventive studies on a model that is spontaneous and histopathologically similar to women is probably the greatest virtue of the laying hen model in ovarian cancer research. In addition, the possibility of performing long-term longitudinal analysis for potential biomarkers and studying key oncogenic molecular changes during early stages of carcinogenesis also makes it an extremely advantageous research model.

Perspectives

The advantages of the spontaneous development of EOC in hens likely transcend the challenges of using the laying hen model. The model is ideal for studying the progression of the disease. Similar to women, EOC in laying hens is an age-related disease, which contributes to the advantages of this model. Most of the large cohort studies in hens were able to describe early tumors that could only be identified histologically because the ovary was functional and appeared to be normal on gross inspection. These early neoplasms provide critical information that is likely relevant to elucidating the onset and early development of ovarian tumors in women. Studying the early events in carcinogenesis is an excellent way to identify biomarkers for early detection of the disease. Other indisputable benefits of using the laying hen as a model for EOC is the short generation time and the ability to perform large scale screening for chemoprevention trials. The laying hen provides the ovarian cancer research community with the critical resource of a natural experimental model, and with the current integrated multi-omics technologies, the laying hen remains as the most promising model system to harness key epidemiologic and molecular signatures to fight EOC.

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