DEVELOPMENT AND PROGRESSION OF OVARIAN CANCER: INSIGHTS FROM THE HEN MODEL OF THE DISEASE

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Ovarian cancer is the leading cause of reproductive cancer death in U.S. women. This high mortality rate is due, in part, to the lack of early detection methods and incomplete understanding of the origin of the disease. Animal models of ovarian cancer can shed light on the genetic and biological factors that influence tumor development and/or progression, as well as identify strategies for prevention, early detection and treatment. One animal model, the domestic hen, has a high spontaneous incidence of the disease that is age-dependent, similar to women. Although previous studies utilizing the hen as a model for ovarian cancer have characterized chicken ovarian tumors and tested putative prevention and treatment strategies of the disease, our understanding of the development and progression of ovarian cancer in the hen is still limited. Our objectives were 1) to further characterize chicken ovarian tumors through global gene expression analysis; 2) to test the effect of progestin and estrogen together, as commonly delivered in "the pill", as well as progestin and estrogen alone on ovarian cancer prevalence in the hen; and 3) to determine estrogen receptor subtype expression in chicken ovarian tumors. The second and third objectives were based on evidence in women that steroid hormones play a role in ovarian cancer, with estrogen associated with an increased risk and progesterone associated with a decreased risk of the disease. We have shown that administration of "the pill" is associated with a significant decrease in ovarian cancer prevalence, as well as egg production,

suggesting that ovulation is important for the initiation of ovarian cancer. Furthermore, we observed that chicken ovarian tumors over-express oviduct-related genes, even at early stages, providing evidence that tumors possibly arise from the epithelial cells of the oviduct. Finally, our results also support a role for steroid hormones, particularly estrogen, in mediating ovarian tumor progression. Collectively, our studies have provided information regarding the development and progression of ovarian cancer in the hen that may help unlock the mysteries of the disease in women.

BIOGRAPHICAL SKETCH

Lindsey Starr Treviño was born February 2, 1982 in San Antonio, TX. She graduated from the Science Academy of South Texas in Mercedes, TX in 2000 and enrolled in St. Mary's University in San Antonio, TX. At St. Mary's she was a fellow in the Minority Access to Research Careers (MARC) Undergraduate Student Training in Research (U*STAR) program sponsored by the National Institute of General Medical Sciences (NIGMS). Through this program, she performed summer research in the labs of Dr. Jamboor K. Vishwanatha at the University of Nebraska Medical Center in Omaha, Nebraska and Dr. O. David Sherwood at the University of Illinois at Urbana-Champaign in Urbana-Champaign, Illinois. Her experiences in these labs stimulated her interest in cancer and reproductive biology research. In 2004, she was the recipient of the university's Presidential Award, an honor bestowed on graduating students who exemplify the leadership ideals of scholarship and service and obtained her B.S. in chemistry with a minor in biology with honors. She was accepted into the graduate field of Molecular and Integrative Physiology at Cornell University in Ithaca, NY that same year, where she joined the lab of Dr. Patricia Johnson in the Department of Animal Science. She applied for and was awarded a pre-doctoral fellowship from the NIGMS to study the role of steroid hormones in ovarian cancer of the hen.

To Aunt Nelda, who had a heart as big as Texas

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TABLE OF CONTENTS

BIOGRAPHICAL SKETCH iii

DEDICATION iv

ACKNOWLEDGEMENTS v

LIST OF FIGURES viii

LIST OF TABLES ix

CHAPTER 1 1

LITERATURE REVIEW 1

Ovarian Cancer 1

Steroid Hormones and Ovarian Cancer 11

The Use of Oral Contraceptives for Chemoprevention of Ovarian Cancer 20

Hen as a Model for Ovarian Cancer 25

REFERENCES 33

CHAPTER 2 49

GENE EXPRESSION PROFILING REVEALS DIFFERENITIALLY EXPRESSED GENES IN OVARIAN CANCER OF THE HEN: SUPPORT FOR OVIDUCTAL ORIGIN? 49

Abstract 49

Introduction 50

Materials and Methods 51

Results 56

Discussion 65

Acknowledgements 72

REFERENCES 73

CHAPTER 3 78

ORAL CONTRACEPTIVES DECREASE THE PREVALENCE OF OVARIAN CANCER IN THE HEN 78
Abstract 78
Introduction 79
Materials and Methods 80
Results 83
Discussion 88
Acknowledgments 98
REFERENCES 99
CHAPTER 4 104
CHARACTERIZATION OF ESTROGEN RECEPTOR EXPRESSION IN AN ANIMAL MODEL OF OVARIAN CANCER 104
Abstract 104
Introduction 105
Materials and Methods 107
Results 111
Discussion 118
Acknowledgments 121
REFERENCES 122
CHAPTER 5 126
SUMMARY AND CONCLUSIONS 126
REFERENCES 133
APPENDICES 136

LIST OF FIGURES

Figure 1.1 Schematic diagram of a model of ovarian cancer development 12

Figure 2.1 Gene profile of 50 differentially expressed genes 58

Figure 2.2 Graphical representation of microarray results 59

Figure 2.3 Real-time quantitative PCR analysis 60

Figure 2.4 Representative images of oviduct as well as tumor 62

Figure 2.5 Real-time PCR analysis in microdissected tissue 63

Figure 2.6 Real-time PCR analysis of ESR1 mRNA expression 64

Figure 2.7 Immunohistochemistry with antibodies against ovalbumin 66

Figure 3.1 Effect of treatment on body weight 84

Figure 3.2 Representative H&E images of tumor subtypes 89

Figure 3.3 Effect of treatment on egg production 91

Figure 3.4 Effect of treatment on plasma estradiol levels 92

Figure 3.5 Effect of treatment on apoptosis in the OSE 93

Figure 4.1 Estrogen receptor mRNA expression in ovaries 112

Figure 4.2 ESR2 mRNA expression in tumor subtypes 113

Figure 4.3 There is abundant ESR1 protein expression 115

Figure 4.4 There is little to no expression of ESR2 116

Figure 4.5 Plasma estradiol level of normal hens 117

Figure 5.1 Model of ovarian tumor development and progression 127

LIST OF TABLES

Table 2.1 Stages of ovarian cancer in the hen 52

Table 2.2 Primers used for real-time quantitative PCR 55

Table 2.3 Summary of ovalbumin and PAX2 protein expression 67

Table 3.1 Number of hens that died before experiment termination 83

Table 3.2 Number of hens with ovarian cancer that involved metastases 85

Table 3.3 Stage of ovarian cancer for hens diagnosed with cancer 86

Table 3.4 Prevalence of ovarian cancer among treatments 87

Table 3.5 Histological subtypes of chicken ovarian tumors 90

Table 3.6 Number of hens with metastases based on tumor subtype 90

CHAPTER 1

LITERATURE REVIEW

Ovarian cancer

Ovarian cancer has a high mortality rate

Ovarian cancer is broadly defined as cancer that forms in tissues of the ovary. It has been estimated that, in 2010, approximately 22,000 women will be diagnosed with ovarian cancer and more than 13,800 women will die of the disease [1]. Ovarian cancer is the leading cause of reproductive cancer death in the U.S [2]. This high mortality rate can be attributed to the fact that greater than 80% of women are diagnosed once the cancer has metastasized and the 5-year survival rate is less than 30% [3]. The 5-year survival rate is similar for the approximately 7% of women with unknown stage at diagnosis [3]. In contrast, the 5-year survival rate is greater than 90% for the approximately 15% of women diagnosed with cancer localized to the ovary [3].

Detection strategies for ovarian cancer are ineffective

These statistics highlight the importance of early detection of ovarian cancer; however, efforts to identify a widely acceptable screening strategy have thus far failed. One obstacle to early detection is the vagueness of symptoms experienced by women with the disease. These symptoms include pressure or pain of the back or abdomen, fatigue, bloating, constipation and urinary problems. Although these symptoms are not specific to ovarian cancer, their severity and frequency may indicate the presence of benign and malignant ovarian masses [4]. Current methods of screening and detecting ovarian cancer include physical and pelvic exams, blood tests, ultrasound and biopsy. Physical and pelvic exams can determine whether there is abnormal accumulation of fluid in the abdomen (ascites) and the size and shape of the ovaries and surrounding organs, respectively. Transvaginal ultrasound (TVS) can also be used

to detect abnormalities in ovarian size and shape, but this technique suffers from poor sensitivity [5]. Blood tests can also be conducted and the most widely used serum marker is CA125. Elevated levels of CA125 are found in >80% of patients with ovarian cancer, but are also associated with benign gynecological and non-gynecological diseases [6]. CA125 has proven a useful marker to monitor disease status and is one of two serum biomarkers approved by the FDA for this purpose, along with human epididymis protein 4 (HE4) [7]. For cancer detection, levels of these markers can be determined alone, although sensitivity may be increased when multiple markers are assessed together [7], or in combination with TVS [5]. Based on the results of these tests, a doctor may recommend surgery to obtain a biopsy to confirm the diagnosis. Unfortunately, these detection methods are better suited for detecting late stage disease, once the tumor is large enough for physical detection (if confined to the ovary), has spread beyond the ovary, or is producing large enough quantities of serum biomarkers suitable for detection in the blood.

Ovarian tumor stage and grade determine course of treatment

Ovarian cancer is a surgically staged disease and tumors are staged according to the criteria set by the International Federation of Gynecology and Obstetrics (FIGO). These criteria are similar to the American Joint Committee on Cancer (AJCC)/TNM system that describes the extent of the primary tumor (T), absence or presence of metastasis to lymph nodes (N), and absence or presence of metastasis to distant sites (M) [2]. Stage I is the least advanced stage with the cancer still confined to the ovary (ovaries). In stage II cancer, the tumor is in one or both ovaries and has spread to other organs in the pelvis (i.e. uterus, fallopian tubes, etc.). A woman is diagnosed with stage III cancer if the cancer is in one or both ovaries and has spread beyond the pelvis to the lining of the abdomen and/or the lymph nodes. Stage IV is the most advanced stage of ovarian cancer where the cancer has spread to organs

outside of the peritoneal cavity, including the liver and the lungs. Ovarian tumors are also graded depending on tissue morphology and how likely it is that the cancer will spread. Grade 1 tumors are well-differentiated (look like normal ovarian tissue) and less likely to spread. Grade 2 tumors are not as well-differentiated compared to grade 1. Grade 3 tumors are poorly-differentiated (do not look like ovarian tissue) and are more likely to spread. Accurate staging and grading of ovarian tumors is important in order to determine the best course of treatment.

Current treatment options for women with ovarian cancer include surgery and chemotherapy. There are two main goals of surgery for ovarian cancer: staging (as mentioned previously) and debulking (the removal of as much tumor tissue as possible) [2]. Patients who have undergone successful debulking have a more favorable prognosis than those left with larger tumors after surgery [8]. Surgery is generally followed by chemotherapy (the use of chemicals to treat disease). This form of treatment is generally systemic and specifically targets cancer cells that are rapidly dividing. There are frequently side effects, however, since normal cells that have similar characteristics (i.e. epithelial lining of the stomach and hair follicles) may also be damaged resulting in nausea, vomiting, and loss of hair. Common chemotherapy regimens for ovarian cancer include combination therapy with a platinum compound, such as carboplatin or cisplatin, and a taxane, such as paclitaxel [8]. Although patients with advanced disease will initially respond to chemotherapeutic regimens, a large proportion (50%) will have recurrent disease and overall poor survival [8]. The initial treatment for women with stage I disease is surgery to remove the tumor. In these women, chemotherapy is recommended if the tumor is high grade [2]. For stage II , III and IV cancer, treatment starts with debulking surgery followed by chemotherapy and may include removal of the uterus, fallopian tubes, ovaries and omentum (stage III

and IV) as well as successive rounds of surgery and chemotherapy to improve quality of life (stage IV).

Ovarian cancer is a heterogenous disease comprised of different types of cancers

Ovarian cancer is a heterogenous disease that includes several types of cancer with different etiologies, pathologies and responses to treatment. There are three main types of ovarian tumors: epithelial, germ cell and sex cord stromal. Epithelial ovarian tumors are the most common type of ovarian cancer in women (90%) [9], and are thought to be derived from the cells that cover the outer surface of the ovary (ovarian surface epithelial cells; OSE). Germ cell tumors account for ~4% of ovarian cancer cases [9] and originate from the cells that produce the ova (eggs). Sex cord-stromal tumors account for about 6% of ovarian tumors [9] and are derived from the stromal cells in the connective tissue of the ovary and produce estrogen and testosterone. Since epithelial ovarian tumors are the most common, further discussion will focus on this type.

Epithelial ovarian tumors can be further divided into 4 subtypes: serous, endometrioid, mucinous and clear cell [10]. This classification is based on the predominant pattern of differentiation of tumor cells. The prevalence of these subtypes varies in the literature, but serous epithelial ovarian tumors are by far the most common. Serous tumors resemble fallopian tube epithelium and have a prevalence of 80-85% [11]. In general, serous tumors can display a broad spectrum of characteristics including glands, solid sheets of cells, or slit-like spaces [10]. Endometrioid ovarian tumors resemble endometrial glands and account for about 10% of epithelial ovarian tumors [11, 12]. These tumors are characterized by distinctive glands lined by columnar cells with large, atypical nuclei and basophilic cytoplasm [10]. Mucinous ovarian tumors can resemble either endocervical or intestinal epithelium and have a prevalence of about 3% [11, 12]. These tumors are composed

of irregular cysts and glands lined by atypical mucinous cells [10]. Clear cell ovarian tumors resemble uterine epithelial cells and accounts for approximately 5-13% of all ovarian tumors [11, 12]. These tumors are characterized by clear cells containing abundant cytoplasmic glycogen and peg-shaped (hobnail) cells [10]. Less common types of ovarian tumors include Brenner, undifferentiated, and mixed epithelial tumors.

Risk factors for ovarian cancer include family history, reproductive factors, and environmental factors

Epidemiological studies have identified risk factors associated with the development of epithelial ovarian cancer. The most significant risk factor is a family history of the disease, which accounts for approximately 5-10% of ovarian cancers [8]. Mutations of the breast cancer type 1 protein (BRCA1) and breast cancer type 2 protein (BRCA2), explain approximately 90% of hereditary ovarian cancer cases [13], with the lifetime risk for ovarian cancer being 30-40% and 27% for women carrying the BRCA1 or BRCA2 mutation, respectively [8]. Ovarian cancer is also strongly associated with hereditary nonpolyposis colorectal cancer (HNPCC) syndrome (also known as Lynch II syndrome), characterized by mutations in DNA mismatch repair genes, including MSH2, MLH1, PMS1, and PMS2 [14]. The risk for ovarian cancer for women with this syndrome is 10% [8].

Reproductive factors are also associated with risk of ovarian cancer with nulliparity, infertility, and late menopause increasing risk and multiparity and lactation decreasing risk [13]. Exogenous hormones contribute to the associated risk of ovarian cancer and the role of steroid hormones in ovarian cancer development and/or progression will be discussed later in further detail. Briefly, hormone replacement therapy (HRT) is associated with an increased risk and oral contraceptive use is associated with a decreased risk [13]. Gynecologic-related conditions such as

endometriosis and polycystic ovarian syndrome (PCOS) are associated with increased risk, while tubal ligation and hysterectomy are associated with decreased risk [13]. Environmental factors possibly associated with increased risk of the disease are use of talcum powder, cigarette smoking, and obesity, while consumption of fruits and vegetables, green and black tea, and dietary vitamin D are associated with decreased risk [13]. Future studies are needed to confirm the association between these environmental factors and risk of ovarian cancer.

The numerous hypotheses proposed to explain the development of ovarian cancer highlight the complexity of the disease

Based on the epidemiological evidence, several theories have been proposed to explain the development of ovarian cancer. These are the incessant ovulation, inflammation, gonadotropin, and hormonal hypotheses. The incessant ovulation hypothesis proposes that the repeated rupture and repair of the ovarian surface epithelium (OSE) during ovulation predisposes the OSE to genetic mutations that could lead to development of ovarian cancer [15]. Related to the incessant ovulation hypothesis is the inflammation hypothesis. The process of ovulation resembles an inflammatory reaction and it is thought that inflammatory factors produced may lead to genetic mutations in OSE that could result in ovarian cancer development [16]. The gonadotropin hypothesis postulates that elevated levels of gonadtotropins, related to the preovulatory surge and loss of negative feedback during menopause, play a role in the development and progression of ovarian cancer [17]. Finally, hormones are also thought to play a role in ovarian cancer development with androgens and estrogens promoting cancer formation, and progesterone inhibiting cancer formation [18]. The hormonal hypothesis will be discussed later in further detail. These hypotheses are not mutually exclusive and it is likely that a combination of factors determines development of ovarian cancer. For example, pregnancy is associated with a

decreased risk of ovarian cancer accompanied by a decrease in ovulatory events and exposure to gonadtotropins, as well as elevated progesterone levels. Thus, pregnancy supports the incessant ovulation, gonadotropin and hormonal hypotheses, highlighting the complexity of ovarian cancer development.

Alternative sites of origin of ovarian cancer, aside from the OSE, have been proposed

Historically, the OSE is believed to be the source of the majority of epithelial ovarian tumors in women. The OSE is the mesothelium that covers the ovary and consists of a single layer of flat-to-cuboidal epithelial cells which are separated by a basement membrane from an underlying stroma (tunica albuginea) that is comprised of collagenous connective tissue [19]. Normal OSE cells in the adult express both epithelial and mesenchymal markers and exhibit phenotypic plasticity, especially during post-ovulatory repair [20]. Studies have shown that the OSE shares some properties of relatively uncommitted pleuripotent cells, including the ability to proliferate, undergo morphological changes in response to environmental stimuli, and differentiate along several pathways [19]. These characteristics are believed to be responsible for the propensity of the OSE to undergo neoplastic transformation. Neoplastic transformation results in the acquisition of characteristics of Müllerian epithelial phenotypes, including characteristics of the fallopian tube, endometrium, endocervix, and vagina for the serous, endometrioid, mucinous and clear subtypes, respectively.

During ovulation, OSE cells proliferate and migrate to heal the ovulatory wound and may become trapped in the ovarian stroma where they form inclusion cysts (the preferred site of neoplastic progression). Inclusion cysts are not only related to ovulation, but may also form from invaginations of the OSE that become more common as the ovary ages [19] or due to inflammation and/or stromal-epithelial interactions [21]. Evidence that OSE and its cystic derivatives are sites of origin of

ovarian cancer rests on the fact that both OSE and inclusion cysts can differentiate into Müllerian epithelium and are sites of precancerous lesions and small carcinomas [21]. Studies have shown an increased incidence of ovarian cysts in the contralateral ovary from cancer patients [22], suggesting that these inclusion cysts represent early stages of neoplastic progression. It should be noted, however, that inclusion cyst formation can be increased in mice, with no observed progression to cancer [23, 24].

Some evidence has cast doubt on the idea that the OSE is the site of origin of all epithelial ovarian tumors. For one thing, ovarian epithelial tumors are similar to epithelial cells from other sites of the reproductive tract, not the ovary [25]. In fact, one study reported that the patterns of gene expression in different subtypes of ovarian cancer correlate with those in their normal Müllerian derived counterparts (i.e. fallopian tube, endometrium, etc.) and not OSE [26]. Furthermore, Cheng et al observed that the ovarian tumor subtypes expressed the same set of HOX genes as the epithelial cells of their normal Müllerian-derived counterparts [27]. HOX genes are highly specific for the different segments of the reproductive tract, supporting the idea of an alternative site of origin. Studies have also shown that paired box 2 (PAX2), a gene required for Müllerian duct development, is expressed in ovarian tumors, secondary Müllerian structures and normal oviduct, but not OSE [28, 29]. Second, ovarian tumors are thought to arise in cystic structures that have no counterpart in normal ovary [25]. In addition, primary peritoneal carcinomas, which are histologically and clinically identical to ovarian carcinomas, arise outside of the ovary, even in individuals in whom the ovaries have been removed [25]. Finally, the tissues which ovarian epithelial tumors resemble, share a common embryological origin that is unrelated to that of the ovary [25].

These inconsistencies suggest that alternative sites of origin for epithelial ovarian tumors exist. The secondary Müllerian structures have been proposed as one

alternative site [30]. Secondary Müllerian structures are normal structures outside of the uterus, cervix and fallopian tubes that are lined by Müllerian epithelium. These structures include paraovarian/paratubal cysts and rete ovarii, as well as structures associated with endosalpingiosis and endometriosis (conditions in which uterine tube-like epithelium is found outside of the uterine tube). In particular, Dubeau proposed that the rete ovarii may be a site of origin on the basis that ovarian-like tumors arise from this structure in humans, and that ovarian epithelial tumors in rodents, although rare, frequently arise in rete ovarii as well [25, 30]. Experimental evidence for this hypothesis is limited and further studies are needed to confirm this source. The fallopian tubes, another Müllerian site, have also been proposed as an alternative source of epithelial ovarian tumors. The fimbriated end of the fallopian tube is in close contact with the ovarian surface, especially during ovulation, possibly resulting in the incorporation of preneoplastic or neoplastic cells from the fallopian tube into the ovary. Evidence for this hypothesis comes from studies in women that are at high risk of ovarian cancer (BRCA+). One study has shown that the fallopian tubes from these women have a high incidence of dysplasia [31]. Another study uncovered a high incidence of serous tubal intraepithelial carcinomas (TICs) in the fallopian tubes of BRCA+ women, but no carcinomas in ovaries [32]. Also, approximately one half of ovarian serous carcinomas were found to be associated with TICs of the fimbria [33]. Furthermore, a putative precursor of serous carcinomas, termed the p53 signature, has been identified in the fimbria that shares characteristics with TICs [34]. Interestingly, p53 signatures are seen in the fallopian tubes of women that are BRCA+ and those with unknown BRCA status, but not inclusion cysts of the ovary [35], further supporting the idea that some epithelial ovarian cancers may arise in the fallopian tubes.

Ovarian tumors develop along two distinct pathways, possibly from different sites of origin

Recently, researchers have proposed that epithelial ovarian tumors develop along two main pathways designated type I and type II [36]. Type I tumors develop in a step-wise manner from well characterized precursors (i.e. adenofibromas, cystadenomas and borderline tumors), and tend to be low grade, exhibit slow growth and remain confined to the ovary while growing to a large size [36, 37]. Low-grade serous, endometrioid, mucinous, and clear cell tumors all fit into this category. Interestingly, these tumors typically acquire mutations in genes belonging to signaling pathways controlling cell growth and proliferation, including KRAS, BRAF, PTEN, β-catenin, and TGF-β RII [36]. Type I tumors are also associated with low cellular proliferation, a gradual increase in chromosomal instability and a 55% 5-year survival rate [36]. Type II tumors are thought to arise *de novo* (no identified precursors), and tend to be high grade and aggressive [36, 37]. This pathway includes high grade serous carcinoma and development of these tumors is associated with mutations in tumor suppressors that function in DNA damage signaling and repair including p53 [38], or BRCA1, BRCA2, MLH1 and MLH2 [39]. Furthermore, these tumors exhibit high cellular proliferation, high chromosomal instability, and a 30% 5-year survival rate [36]. It should be noted that both pathways share common characteristics including escape from the immune response, the invasion into the stroma, survival and attachment within the peritoneal cavity, as well as growth and angiogenesis [40]. Interestingly, Type I tumors constitute only 25% of ovarian tumors, while the vast majority (75%) of ovarian cancers are Type II [37]. This fact has a significant impact on clinical parameters such as early detection, diagnosis, and treatment [41].

A new model of ovarian cancer development has emerged that incorporates the alternative sites of origin discussed previously and Shih and Kurman's two pathways

of tumor development [42] (Figure 1.1). The first part of the model involves the OSE (Figure 1.1A). In this scenario, OSE (or in some cases fallopian tube epithelium, endometrium, and peritoneum) can become trapped in inclusion cysts and induced to undergo Müllerian metaplasia within the ovarian stroma. This process would give rise to endometrioid, mucinous, and serous borderline tumors in a step-wise progression [43] eventually resulting in the formation of type I tumors. The second part of the model involves the fallopian tube fimbria (Figure 1.1B). Accumulation of genotoxic stress and mutations in p53 could result in the formation of a preneoplastic lesion in which additional mutations could occur, leading to the formation of TIC. TICs can spread to adjacent pelvic structures or exfoliate into the peritoneal cavity, resulting in the formation of type II tumors. The lack of a precursor lesion in the ovary for type II tumors would be explained by this model. Future studies are needed to validate this model, but it is clear that the origin and pathogenesis of ovarian cancer have implications for the clinical management of the disease [43, 44].

Steroid hormones and ovarian cancer

Estrogen is associated with increased risk of ovarian cancer

As discussed, steroid hormones, especially estrogen and progesterone, are thought to play a role in ovarian cancer. Androgens have also been implicated in ovarian cancer progression, but the following discussion will focus on estrogen and progesterone. Numerous studies have shown that exogenous estrogen, in the form of hormone replacement therapy (HRT), is associated with an increased risk of ovarian cancer [13]. HRT is commonly prescribed to alleviate the symptoms experienced during menopause, and approximately 60 million prescriptions were written in the U.S. in 2003 [45]. Current formulations of HRT are estrogen only or estrogen plus progestin,

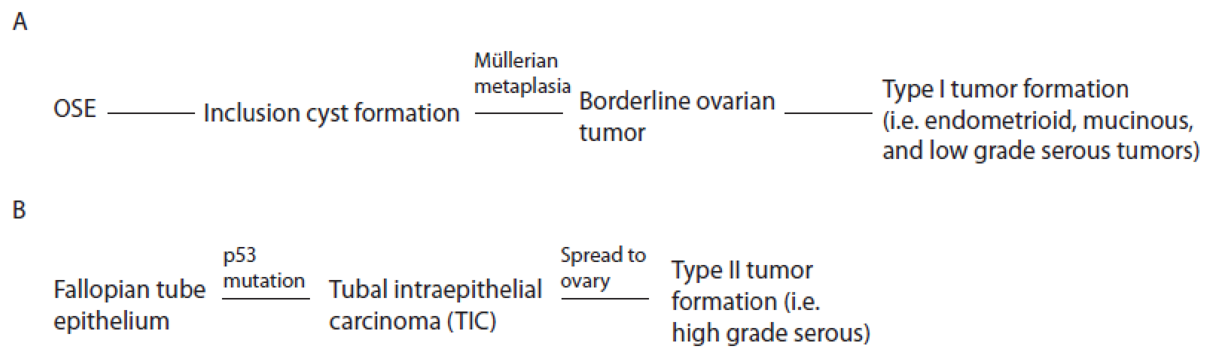

Figure 1.1 Schematic diagram of a model of ovarian cancer development that incorporates alternative sites of origin [42].

and it is believed that the risk of ovarian cancer is higher with estrogen only than with estrogen plus progestin [46-48]. This observation suggests that that addition of a progestin ameliorates the effect of estrogen and is consistent with the purported protective effect of progesterone in ovarian cancer. Analyses have also shown that the duration of treatment may be important for ovarian cancer risk, with longer duration associated with increased risk that wanes after cessation of use [46, 47]. It should be noted, however, that some studies found HRT was associated with an increased risk of ovarian cancer regardless of the duration of use [49], as well as the formulation, estrogen dose, progestin type, regimen and route of administration [49, 50].

Interestingly, studies have shown that the adverse effect of HRT use was stronger for endometrioid and clear cell tumors [18]. Endometrioid and clear cell tumor subtypes are associated with endometriosis, an estrogen-dependent condition, and it is thought that estrogen plays a role in the development of these subtypes [18]. There are some beneficial effects of HRT, including protection against osteoporosis, heart disease and colorectal cancer [51], but the clear association with ovarian cancer should be taken into consideration regarding the use of HRT in women. Obesity is another condition that is associated with increased ovarian cancer risk [13]. This may be due to an increase in unopposed estrogen through various mechanisms, including increased conversion of androgens to estrogen in adipose tissue, in obese women [52]. Obesity also affects the clinical outcome of ovarian cancer with obesity associated with a higher mortality rate [53]. Conversely, lactation and oral contraceptive use, which are both associated with decreased estrogen levels, decrease the risk of ovarian cancer [54].

Progesterone is associated with decreased risk of ovarian cancer

As mentioned previously, pregnancy and oral contraceptive use are associated with a decreased risk of ovarian cancer [13], and this may be due to levels of

progesterone and action of progestins, respectively. Circulating progesterone levels during pregnancy are increased 10-fold due to progesterone synthesis by the placenta [54]. Pregnancy is associated with a 40% decrease in risk of ovarian cancer and there is a 10-16% decrease in risk for each additional pregnancy [18]. Twin pregnancies are also associated with decreased risk of ovarian cancer, which is interesting because these women have higher gonadotropin levels and are more likely to double ovulate, factors that may put them at increased risk of the disease based on the incessant ovulation and gonadotropin hypotheses [54]. Twin pregnancies involve higher serum progesterone levels compared to single pregnancies [54], so this observed decreased risk suggests that progesterone is indeed protective. It should be noted that pregnancy also raises serum estrogen levels about 100-fold [54], seemingly contradicting the aforementioned role of estrogen in ovarian cancer development. After the first 4 to 5 weeks of pregnancy, estrogens are mainly synthesized in the trophoblasts and the ovarian contribution is minimal, indicating that the OSE would not be exposed to high local levels of estrogen, and hence would accumulate less DNA damage [18].

Oral contraceptives that contain progestin have also been shown to decrease the prevalence of ovarian cancer [13]. In fact, oral contraceptive use is associated with a 20% decrease in relative risk of ovarian cancer for every 5 years of use and longer duration of use further decreases the risk [55]. The effect is long-lasting with a reduction in risk for 30 years or more after use has ceased [55]. The effect of oral contraceptives on ovarian cancer prevalence has been attributed to the suppression of ovulation with these compounds (related to the incessant ovulation hypothesis); however, studies have shown that it may be the potency of the synthetic progestin itself that is responsible for the decrease in risk. One line of evidence to support this hypothesis is that a decrease in ovarian cancer risk is seen in women who take progestin-only types of oral contraceptives comparable to that seen with combined OC

[54]. Up to 40% of women using the progestin-only methods continue to ovulate [16], suggesting that the risk reduction cannot be explained by the reduction of ovulatory events alone. It should be noted that contraceptive progestins also exhibit variable androgenic properties and may lower androgen levels [54], providing another mechanism of action for the protective effect observed with respect to ovarian cancer. The use of oral contraceptives as chemopreventive agents will be discussed in the next section of the literature review.

Steroid hormones exert their effects in target tissues through steroid receptors

Effects of steroid hormones are mediated by interactions with their receptors in multiple target tissues throughout the body. Steroid receptors are part of the nuclear receptor superfamily, which also include thyroid receptor, vitamin D receptor, retinoic acid receptor and others [56]. In general, steroid receptors are modular proteins composed of distinct regions, including a transactivation region (responsible for transcriptional activation), a DNA-binding domain, and a ligand-binding domain, which determine function and specificity [57]. Steroids bind to their respective receptors, resulting in binding of the ligand/receptor complex to response elements within the promoter of target genes. Co-activators or co-repressors are then recruited to the promoter and gene transcription is either activated or repressed. This represents the classical or genomic mechanism of signaling, but genomic signaling can also occur in the absence of ligand where phosphorylation of the receptor by kinases occurs in the absence of ligand binding and in a response-element-independent manner where the ligand/receptor complex binds to alternative binding sites in promoters of target genes. Steroids can also signal through non-genomic mechanisms in which they bind to a receptor at the cell surface, triggering signal transduction pathways independent of gene transcription [58]. It is becoming increasingly clear that biological effects of

steroid hormone in target tissues are due to cross-talk between the genomic and non-genomic signaling pathways [58].

Thus far, two estrogen receptor subtypes, estrogen receptor 1 (ESR1) and estrogen receptor 2 (ESR2), have been identified. These subtypes are encoded by different genes, have distinct biological functions and exhibit differential localization and expression patterns. Knockout mice of the two subtypes exhibit different phenotypes, highlighting their distinct functions [59]. ESR1 and ESR2 exhibit 17%, 97% and 60% sequence homology in the N-terminal domain (transactivation region), DNA-binding domain, and ligand-binding domain, respectively [59]. Similar to estrogen, progesterone also signals through its receptor resulting in downstream biological effects in the target tissue. There are two isoforms of the progesterone receptor, PR-A and PR-B, which are the products of a single gene [60]. PR-A is a truncated form of PR-B, lacking amino acids 1-164 of the N-terminus of PR-B, due to differential splicing [60]. For the most part, PR-B acts as a transcriptional activator, while PR-A is transcriptionally inactive and acts a repressor of PR-B [60]. Selective ablation of PR-A and PR-B has confirmed that the tissue specific functions of the individual isoforms are distinct [60].

Aberrant steroid hormone/receptor signaling may play a role in ovarian cancer

Approximately 60% of human ovarian tumors express estrogen receptors [61, 62] and they are most highly expressed in serous and endometrioid subtypes [63]. Interestingly, ESR2 expression is significantly decreased in ovarian tumors compared to normal ovary [64, 65]. Decreased ESR2 is also evident in other cancers, including breast, colon and prostate, suggesting that loss of ESR2 may be a common step in estrogen dependent ovarian tumor progression [66]. On the other hand, ESR1 is the main form expressed in malignant ovarian tumors [65]. The ESR1/ESR2 ratio is also significantly increased in ovarian tumors [65] and ovarian cancer cell lines [67]. It is

thought that estrogen-driven growth in ovarian cancer cells is mediated by signaling through ESR1 [68], highlighting the importance of the increase in this ratio. The significance of ESR2 expression is also supported by a recent study that reported an association of ESR2 expression with stage I disease, as well as longer disease-free and overall survival [69]. Approximately 49% of malignant ovarian tumors express progesterone receptors [70], with higher expression in the endometrioid subtype [71]. Furthermore, one study showed that PR-B protein expression is up-regulated in malignant ovarian cancer cells versus normal ovarian epithelial cells [67]. Another study found a reduction in PR-A in ovarian cancer tissue compared to normal ovarian tissue, as well as an elevation of the PR-B/PR-A mRNA ratio in cancer cell lines treated with estrogen [53]. Further research is required to determine the significance of differential PR isoform expression in ovarian cancer tumorigenesis.

There is some evidence that defects in the progesterone receptor itself may be related to increased ovarian cancer risk [54], but subsequent studies have failed to confirm this link [53]. Numerous studies have shown that progesterone receptor expression is a favorable prognostic indicator for ovarian cancer [72-74] and is associated with disease-free survival [75, 76], overall survival [76-78] early stage disease [77], low ascites volume [74], and higher tumor differentiation [74]. Interestingly, one study found that low grade ovarian tumors expressed more estrogen and progesterone receptors, suggesting that these tumors may respond more readily to hormone therapy [79].

Steroid hormones mediate cellular pathways that affect tumor development and progression

Through interactions with their receptors, estrogen and progesterone affect multiple cellular pathways resulting in ovarian tumor development and progression. Ovarian tissue levels of estrogen are 100-fold higher than circulating levels [80]

suggesting that the OSE and its cystic derivatives are exposed to high levels of this steroid. Studies in breast cancer have shown that estrogen, particularly its oxidative metabolites, can be genotoxic and directly damage DNA [81]. Estrogens stimulate proliferation in normal OSE [82], which could contribute to mutagenesis of these cells as well. Estrogens have also been implicated in the progression of ovarian cancer by affecting cellular proliferation as well as invasion and metastasis [53, 71]. Angiogenesis is an important process required for tumor growth, and estrogen metabolites have been implicated in this process as well [71]. One *in vivo* study reported that treatment of mice with estradiol resulted in an earlier onset of ovarian tumors and decreased overall survival time [83], further supporting the importance of estrogen in the development and progression of ovarian cancer. As compared to estrogen, progesterone has been shown to inhibit ovarian cancer development and progression. Several *in vitro* studies have shown that progesterone inhibits cell growth and induces apoptosis in OSE and ovarian cancer cells [53]. These effects are observed at higher concentrations of progesterone, similar to what is experienced during pregnancy. One study in primates reported that levonorgesterol, a progestin, induced apoptosis in the OSE [84], which could be mediated by transforming growth factor β [85]. Progesterone has also been shown to induce differentiation-like characteristics in ovarian cancer cells and decrease ovarian cancer invasion by decreasing plasma membrane fluidity [71]. These studies support the hypothesis that progesterone is protective for ovarian cancer.

Steroid hormone/receptor signaling can be targeted for therapy

Clinical studies have been conducted to determine the efficacy of agents that target hormone signaling in ovarian tumors. In general, hormonal therapy is well-tolerated and relatively inexpensive, but has not been shown to be more effective than conventional chemotherapy as a first-line treatment [86]. Therefore, it is currently

used in women with refractory or recurrent disease that exhibits poor response to chemotherapy [86]. Selective estrogen receptor modulators (SERMs) and aromatase inhibitors have been tested to inhibit estrogen signaling. SERMs, like estrogen, can bind to estrogen receptors and act either as agonists or antagonists in a tissue-dependent manner. Tamoxifen, one example of a SERM, has been shown to have antagonistic properties in breast tissue and behaves as an agonist in the uterus and is currently used as a therapeutic and chemopreventive agent for breast cancer [87]. In ovarian cancer, it is thought to exert an antiestrogen role by competitively binding to the estrogen receptor, blocking estrogen's effects on transcription [71]. Studies have shown a 13-17% response rate in women with ovarian cancer previously treated with tamoxifen [86]. There is some indication that patients with ER-positive tumors may have a higher response rate to tamoxifen, but further studies are needed to clarify this [71]. Chemotherapy is the first line of treatment for ovarian cancer and there is currently little evidence to indicate that the addition of tamoxifen enhances its effects [71].

Aromatase inhibitors have also been tested as a therapeutic for ovarian cancer. Aromatase is the enzyme that is responsible for the conversion of androgens to estrogens, and contributes to the elevated circulating estrogens in post-menopausal and obese women [88]. Aromatase inhibitors act by inhibiting the production of estrogen, thereby decreasing the availability of estrogen for gene transcription, angiogenesis and DNA mutations [71]. Letrozole and anastrozole, aromatase inhibitors that are currently used to treat breast cancer, have been shown to have little to moderate activity in ovarian cancer [86]. Progesterone has been used to treat women with refractory or recurrent ovarian cancer with some success [71]. Although both estrogens and tamoxifen can induce progesterone receptor expression and may enhance treatment with progesterone, little to no response was seen with combination

hormonal therapy in recurrent or advanced ovarian cancer [71]. No response was observed in studies that utilized progestin in combination with chemotherapy, either [71]. One study tested the effects of a progestin as a front-line therapy in women with the endometrioid subtype of ovarian cancer [89]. The authors reported an overall response rate of 53.5%, with the highest incidence of tumor regression observed in tumors positive for both estrogen and progesterone receptors [89]. These results suggest that receptor status may be important in determining response to hormonal therapies.

The use of oral contraceptives for chemoprevention of ovarian cancer

Chemoprevention strategies for ovarian cancer are desirable

Ovarian cancer has a high mortality rate and there are no accepted screening strategies which have been shown to decrease morbidity and mortality from the disease. In fact, screening for ovarian cancer is not currently recommended for the general population, only for high risk individuals [90]. The high mortality of the disease and the difficulties with screening emphasize the need for prevention strategies. Studies have shown that prophylactic oophorectomy can significantly reduce the risk of ovarian cancer in women with a family history of the disease [91]. Women at high risk (BRCA+) are advised to undergo prophylactic oophorectomy at a young age (~35 years) and this procedure has some disadvantages in these patients, including the psychological impact of losing the ovaries, and early onset of menopausal symptoms [91]. Furthermore, women who undergo this procedure are still at risk for development of peritoneal carcinomas even in the absence of the ovaries [91]. It is clear that an alternative method of prevention, such as chemoprevention, is needed to protect women, especially those with a high risk, from developing ovarian cancer.

Possible chemopreventive agents for ovarian cancer include NSAIDs, retinoids and oral contraceptives

Chemoprevention is the use of natural, synthetic, or biologic chemical compounds to prevent, inhibit or reverse the progression of cancer [92]. A diverse array of compounds have been shown to prevent the initiation or progression of cancer, including vitamins, antioxidants, flavanoids, inhibitors of the cyclooxygenase pathway, steroid hormones, and retinoids [93]. These compounds can be divided into two major categories based on when they exert their preventive effects: 1) blocking agents, which inhibit tumor initiation by preventing carcinogens from reaching or reacting with target sites and 2) suppressing agents, which inhibit tumor progression [94]. Blocking agents can exert their effects by preventing carcinogen activation, enhancing detoxification of carcinogenic agents, or trapping cancer-producing compounds before they reach their targets [95]. Suppressing agents act by decreasing proliferation, increasing apoptosis and/or differentiation, and blocking oncogene activation [93, 95].

Studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs), retinoids, and oral contraceptives may be putative chemopreventive agents of ovarian cancer. NSAIDs, such as aspirin and ibuprofen, are drugs with analgesic, antipyretic, and anti-inflammatory effects. Most NSAIDs work as non-selective inhibitors of the prostaglandin-endoperoxide synthases (PTSGs) which catalyze the formation of prostaglandins from arachidonic acid. Prostaglandins can act as messengers in the inflammatory pathway, so blocking this conversion is beneficial for treating diseases associated with inflammation. NSAIDs have been shown to decrease the risk of ovarian cancer [96-99], especially in nulliparous women and those who have never used oral contraceptives [100]. It is thought that NSAIDs can exert their protective effects by inhibiting proliferation, angiogenesis, and oxidative DNA damage in cancer

cells, as well as by increasing apoptosis [101]. Long-term use of NSAIDs may, however, result in adverse gastrointestinal effects, especially in individuals with previous history of gastrointestinal ulcers, bleeding disorders, and allergic reactions [102]. Further clinical trials are needed to assess the efficacy of NSAIDs as chemopreventive agents.

Another group of putative chemopreventive agents, the retinoids, are natural and synthetic compounds that are related to vitamin A. They have been shown to play roles in cell differentiation and proliferation [103]. Fenretinide (4-HPR), a synthetic retinoid, has been shown to inhibit the growth of ovarian cancer cells and induce changes typical of apoptosis [91]. One study showed that administration of 4-HPR induced apoptosis in monkey ovaries [104], further supporting the use of retinoids as chemopreventive agents. In this study, 4-HPR was also tested in combination with oral contraceptives and this combination was shown to have a greater effect on markers associated with growth inhibition and apoptosis, than 4-HPR or oral contraceptives alone [104]. Side effects of retinoids are reversible and include skin dryness and irritation, although some retinoids have been identified as teratogens [105]. Prolonged use of 4-HPR, in particular, has minimal side effects, making it a good candidate as a form of chemoprevention [105].

Oral contraceptives are associated with a decreased risk of ovarian cancer, even in women at high risk for the disease (BRCA+) [106], supporting their use as chemopreventive agents for the disease. It has been estimated that greater than 50% of ovarian cancer cases in the United States can be prevented by the use of oral contraceptives for at least 4-5 years [91]. Oral contraceptives are commonly prescribed for the purpose of birth control and have been used by more than 300 million women [107]. There are two widely available forms of oral contraceptives: combination estrogen and progestin and progestin only. Combination oral

contraceptives are the most widely used oral contraceptives and contain low doses of estrogen (<35 ug) and progestin (0.1 to 1.5 mg) [108]. These types may or may not vary dosage throughout the regimen, but all are taken for 21 days followed by 7 days without medication [108]. They prevent pregnancy mainly by suppressing ovulation (estrogen) and through effects on cervical mucus and on the endometrium (progestin) [108]. The progestin-only oral contraceptive (the "mini-pill") contains a small dose of progestin (<0.5 mg norethindrone or norgestrol) and must be taken every day continuously [108]. Women who take the progestin-only oral contraceptives may still ovulate, but pregnancy is prevented through progestin's effects on cervical mucus, and on the endometrium [108]. Another progestin-only contraceptive, the injectible contraceptive, is also a popular method of contraception world-wide. Depo-medroxyprogesterone acetate (DMPA) is one such injectible contraceptive available in 2 formulations: 150 mg/ml for intramuscular injection (IM) and 104 mg/0.65ml for subcutaneous injection (SC) [109]. Both are given at 3-month intervals and prevent pregnancy by inhibiting gonadotropin secretion thereby inhibiting follicle development and ovulation [109]. Combined oral contraceptives decrease the risk of ovarian cancer by 20% for each 5 years of use and the longer the duration of use, the greater the reduction in risk [55]. This reduction persists for more than 30 years after use has ceased, but becomes attenuated over time [55]. A protective effect of progestin-only oral [110] and injectible [54] contraceptives has also been observed, but further studies are needed to confirm this effect.

The mechanism of oral contraceptive action is unclear

Little is known about the mechanism of the protective effect of oral contraceptives. It is thought that the protective effect might be related to the decrease in ovulatory cycles associated with oral contraceptive use. This is consistent with the incessant ovulation hypothesis that states that the OSE are susceptible to genetic

mutations during ovulatory rupture and repair [15]. In women who have used oral contraceptives for at least 5 years, however, there is a 50% decrease in ovarian cancer associated with an estimated 15% decrease in ovulatory cycles [91]. In addition, women who use progestin-only oral contraceptives can ovulate, but still have a decreased risk as discussed previously. These observations suggest that the protective effect of oral contraceptives may be due to a mechanism other than suppression of ovulation. DMPA has been shown to inhibit the gonadotropin peak [111] and inhibition of gonadotropin release from the pituitary may also play a role in the protective effect. This is consistent with the gonadotropin hypothesis that states that elevated gonadotropins play a role in the development and progression of ovarian cancer [17]. Finally, the hormonal components of oral contraceptives may induce apoptosis (progestin) and stimulate proliferation (estrogen) of susceptible OSE cells, consistent with the hormonal hypothesis [18]. The mitogenic role of estrogen has already been discussed in some detail with regard to hormone replacement therapy. There is some evidence for the protective effect of progestin from a study in monkeys [84]. In that study, progestin alone, and in combination with estrogen, increased apoptosis in the OSE, with maximal effect seen in the progestin alone group [84]. Another study in a mouse model, however, showed no effect of progestin, alone or in combination with estrogen, on apoptosis, although a reduction of tumor weight was observed in both treatment groups [112]. The latter study was not directly testing the chemopreventive effect of progestin, since tumors were already present, but lack of apoptosis seen with progestin administration should be noted. Further studies are needed to determine the mechanism of oral contraceptive action on ovarian cancer.

The side effects of oral contraceptive use, although minimal for most women, should be taken into consideration

One problem with the use of oral contraceptives as chemopreventive agents is their association with negative side effects. One major concern regarding the long-term oral contraceptive use is the reported negative effect on cardiovascular health. Studies have shown that these effects are minimal in most patients; however, hypertension, smoking, obesity and diabetes may increase these risks [113]. Oral contraceptive use has also been associated with increased risk of breast and cervical cancers, but studies have shown that these increases are small [113]. There are some non-contraceptive health benefits of oral contraceptive use, including prevention of endometriosis and endometrial cancer, as well as treatment of menorrhagia, ovulatory pain, and acne [114]. In general, the benefits seem to outweigh the risks, but these side effects should be taken into consideration when considering the use of oral contraceptives for chemoprevention.

Hen as a model for ovarian cancer

Few animal models for ovarian cancer, aside from hens, develop the disease spontaneously at a high frequency

Animal models of human ovarian cancer can shed light on the genetic and biological factors that influence tumor development and/or progression, as well as identify strategies for prevention, early detection and treatment. Current animal models of ovarian cancer involve spontaneous or induced carcinomas and human xenografts [115]. Each model has its strengths and weaknesses, but a spontaneous model is desirable because it would most accurately recapitulate disease initiation and progression in women. Most mammals, however, do not spontaneously develop ovarian carcinomas. This is most likely due to the fact that most sexually mature

female mammals are either pregnant, lactating, or seasonally anestrous and hence have reduced numbers of ovulations. Ovulation is associated with increased risk of ovarian cancer; therefore species that ovulate intermittently might have a decreased risk of the disease. There have been reports of spontaneous ovarian cancer in rodents [116, 117] and non-human primates [118], but these cases are rare.

In contrast, laying hens have a high spontaneous incidence of the disease that is age-dependent [119]. Numerous studies have characterized hen ovarian tumors and validated the hen as a model for human ovarian cancers. Chicken ovarian tumors histologically resemble human ovarian tumors. There are 4 subtypes of human ovarian adenocarcinomas including serous, endometrioid, mucinous and clear cell tumors and these subtypes are also observed in the hen [120-122]. Ovarian tumors of the hen express similar markers to tumors in women including progesterone receptor [123], prostaglandin synthases (PTGSs) [124, 125], CA-125 [126], vascular endothelial growth factor (VEGF) [127], cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B) [128], HER2/neu [129], and E-cadherin [130]. In addition, hen ovarian tumors exhibit mutations in the tumor suppressor p53, which frequently occur in human ovarian tumors as well [129]. Production of antitumor antibodies is associated with tumors in human cancers and this is also observed in hens with ovarian cancer [122]. These studies have provided evidence that hen ovarian tumors are similar to tumors in women and support the usefulness of the hen as a model of ovarian cancer.

Hens and humans exhibit several physiological differences limiting the use of the hen as a model for ovarian cancer

There are limitations to using the hen as a model for human ovarian cancer. First of all, there is a difference in physiology between mammals and chickens. In contrast to mammals, only the left ovary and oviduct develops in the hen. During

development of the reproductive tract, anti-Müllerian hormone (AMH) acts to regress the right ovary and oviduct and it is thought that ovarian estrogen protects the left side of the reproductive tract from the effects of AMH [131]. In women with unilateral ovarian cancer, the contralateral ovary is examined for evidence of precursor lesions (i.e. abnormal OSE morphology, cortical invaginations, and inclusion cysts), shedding light on early events in ovarian cancer progression. Involvement of the contralateral ovary (or lack thereof) is also used in staging ovarian cancer in women. Since the hen lacks a second ovary, the information provided by examining the contralateral ovary is not readily accessible. Hens and women also exhibit differences in reproductive cycles, including cycle length, steroid hormone responsible for the LH surge and formation of the corpus luteum. The menstrual cycle in women lasts an average of 28 days and the cycle in the hen is accelerated comparatively, lasting approximately 26 hours. In women, the LH surge is initiated by high levels of estradiol and occurs approximately 2 days before ovulation. The LH surge in the chicken is triggered by progesterone approximately 4-6 hours before ovulation. There is also no comparable luteal phase in chickens due to the lack of corpus luteum formation. Instead, the postovulatory follicle is resorbed through apoptosis. Finally, studies in the hen may be confounded by standard husbandry practices. Ovarian cancer prevalence increases in older hens and one study showed that mortality rates increased after handling older birds for cage cleaning [132]. The aforementioned limitations, including the lack of a contralateral ovary, difference in reproductive cycle and fragility of older hens do not exclude the hen as a suitable animal model for ovarian cancer, but should be taken into consideration when designing and conducting experiments.

Studies in the hen have provided support for the "incessant ovulation" hypothesis, as well as a role for inflammation in ovarian cancer

Despite the limitations discussed, studies in the hen have provided insight into the factors that may play a role in the development of ovarian cancer, including ovulation, inflammation and hormones. As mentioned previously, most mammalian species do not spontaneously develop ovarian cancer and this may be related to the fact that these species do not ovulate with as much frequency as the human or hen. The hen ovulates approximately every 26 hours and it has been suggested that once a hen has reached her third year of egg-laying, she has ovulated approximately as many times as a peri-menopausal woman (~500 times) [130]. This observation of high ovulatory frequency in the hen and increased incidence of ovarian cancer supports the "incessant ovulation" hypothesis previously discussed. In a previous study, our lab reported an association between the number of ovulatory events and the risk of ovarian cancer. Giles et al discovered a decreased incidence of ovarian cancer in restricted ovulator (RO) hens compared to wild-type (WT) hens [133]. RO hens have a mutation that affects their ability to incorporate yolk into growing follicles and consequently ovulate fewer times than their WT counterparts [134]. It should be noted that although this association has been observed, studies have reported no significant difference in egg production in hens that develop ovarian cancer versus those that do not [119, 133], suggesting that other factors contribute to development of ovarian cancer in the hen.

Related to the "incessant ovulation" hypothesis is the idea that inflammation is linked to ovarian cancer development. Ovulation has been described as an inflammatory process [135] and it is thought that inflammatory factors produced during ovulation may induce DNA damage in the ovarian surface epithelium (OSE) at the site of ovulation [136]. Our lab has previously isolated and characterized chicken

OSE and shown that these cells express markers similar to human OSE [137]. Another study has shown that DNA damage accumulates in OSE associated with pre- and postovulatory follicles in the laying hen [136], supporting the inflammation hypothesis. Prostaglandins are thought to play a role in the inflammatory process and prostaglandin synthase 1 (PTGS1; one of the enzymes responsible for prostaglandin synthesis) is overexpressed in chicken ovarian tumors compared to normal ovaries [124, 125] and in the post-ovulatory follicle in normal hen ovaries [124]. Interestingly, non-steroidal anti-inflammatory drugs (NSAIDs) that target prostaglandin synthase 1 have been shown to inhibit proliferation of chicken ascites cells *in vitro* [127] and inhibit progression of chicken ovarian cancer *in vivo* [139]. Another study reported decreased severity of ovarian cancer in hens fed flaxseed, a source of omega-3 fatty acid which has been shown to reduce inflammation [140]. These studies suggest that the inflammatory process associated with ovulation may play a role in the progression of ovarian cancer in the hen.

More research is needed to elucidate the role of steroid hormones in ovarian cancer of the hen

Few studies have directly examined the role of either gonadotropins or steroid hormones in hen ovarian cancer. As mentioned previously, gonadotropins, estrogens and androgens are thought to promote ovarian tumor progression, while progesterone is protective. Interestingly, the RO hens previously discussed have a hormone profile associated with increased risk of ovarian cancer including elevated levels of LH, FSH, and estrogen [133, 141] and decreased levels of progesterone [141]. These hens, however, have a decreased prevalence of ovarian cancer [133]. Fredrickson observed that plasma levels of steroid hormones were not associated with disease progression, except for elevated levels of plasma estrogen in hens with granulosa cell tumors [119]. These studies would suggest that hormone levels may not be a contributing factor to

tumor progression in the hen; however, the effects of hormones were not measured directly in these studies. One study directly tested the effect of progestin on ovarian cancer prevalence in the hen. Hens treated with the progestin, medroxyprogesterone acetate (MPA), exhibited a decreased risk of ovarian cancer [142]. This effect could be due to the progestin itself, or the fact that progestin administration also resulted in a decline in egg production, thus further supporting the "incessant ovulation" hypothesis. The information that we have regarding the role of hormones in ovarian cancer in the hen is thus far incomplete and further study is needed in this area.

Studies in the hen can provide information about prevention, early detection, and development of ovarian cancer

The hen has also proven to be a useful pre-clinical model to study prevention and early detection strategies. Chicken ovarian tumors have been shown to express antigens that are useful surrogate biomarkers in chemoprevention trials, including proliferating cell nuclear antigen (PCNA), p27 and transforming growth factor-α (TGF-α) [143]. As mentioned previously, several studies have been conducted testing the preventative agents, progestin [142], aspirin [139], and flaxseed [140]. A significant reduction in either prevalence or stage was seen in all three studies demonstrating the merit of prevention studies in the hen. The hen has also proven to be useful to study methods of early detection of ovarian cancer. Studies have shown that the use of sonography aids in the accurate detection of ovarian tumors in the hen [120] and may help monitor early changes in the ovary associated with neoplastic progression, including tumor-associated neoangiogensis [144].

The hen animal model should also be used to identify early markers of the disease and to investigate the origin of ovarian cancer, since these areas of research will have a major impact on how women are diagnosed and treated. Several immunohistochemical-based studies have been conducted to determine the origin of

ovarian cancer in the hen. Haritani et al used an antibody against the oviductal protein ovalbumin to determine where tumors in the hen arise [145]. They found that all ovarian tumors examined expressed ovalbumin and concluded that most of the tumors in hens are of oviductal origin. Our lab also determined ovalbumin expression in ovarian tumors, and divided the tumors into two groups: those with or without oviductal involvement [146]. Similar to Haritani et al, all ovarian tumors expressed ovalbumin, even in the absence of oviductal involvement. It is clear that ovalbumin cannot be used to distinguish between ovarian tumors that originate in the ovary or the oviduct, but another marker that may be useful in this regard has been identified. Hakim et al reported that the majority of ovarian carcinomas, but few oviductal carcinomas exhibited significant HER2/neu expression [129]. HER2/neu expression may prove useful in distinguishing between these two sites of origin if it is shown that metastases from the oviduct to the ovary are also negative for HER2/neu. Future studies regarding origin of ovarian cancer should take advantage of the hen model.

Previous studies in the hen have characterized chicken ovarian tumors, yielded information about the development of ovarian cancer, as well as tested putative prevention and treatment strategies of the disease. There are, however, major questions that remain unanswered. For one thing, chicken ovarian tumors have yet to be fully characterized. Most studies to date have focused on the expression and function of individual genes in chicken ovarian tumors, but no global gene expression profiling studies have been performed. Gene expression profiling has yielded valuable prognostic and classification information for human ovarian tumors and can do the same for chicken ovarian tumors. In order to further characterize chicken ovarian tumors, we performed microarray analysis to identify differentially expressed genes in chicken ovarian tumors versus normal ovary using microarray technology. We also examined gene expression in early stage tumors, to obtain information about early

neoplastic events. Second, it is known that steroid hormones play a role in ovarian cancer in women, but their roles in ovarian cancer of the hen has yet to be elucidated. In particular, oral contraceptive use is associated with a decreased risk of ovarian cancer. We performed an *in vivo* study to determine the effects of the hormonal components of oral contraceptives, alone and in combination, on ovarian cancer prevalence in the hen and to investigate the mechanism of oral contraceptive action. Finally, estrogen/estrogen receptor signaling has been implicated in human ovarian cancer, but the estrogen receptor status of chicken ovarian tumors is unknown. We determined the mRNA and protein expression of estrogen receptor subtypes in ovaries from hens with cancer versus in ovaries from normal hens. We hypothesized that these studies would yield information about the development and progression of ovarian cancer in the hen, as well as in women.

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CHAPTER 2

GENE EXPRESSION PROFILING REVEALS DIFFERENTIALLY EXPRESSED GENES IN OVARIAN CANCER OF THE HEN: SUPPORT FOR OVIDUCTAL ORIGIN?

Abstract

Ovarian cancer has a high mortality rate due, in part, to the lack of early detection and incomplete understanding of the origin of the disease. The hen is the only spontaneous model of ovarian cancer, and can therefore aid in the identification and testing of early detection strategies and therapeutics. Our aim was to combine the use of the hen animal model and microarray technology to identify differentially expressed genes in ovarian tissue from normal hens compared to hens with ovarian cancer. We found that the transcripts up-regulated in chicken ovarian tumors were enriched for oviduct-related genes. Quantitative real-time PCR and immunohistochemistry confirmed expression of oviduct-related genes in normal oviduct and in ovaries from hens with early- and late-stage ovarian tumors, but not in normal ovarian surface epithelium (OSE). In addition, one of the oviduct-related genes identified in our analysis, paired box 2 (PAX2) has been implicated in human ovarian cancer and may serve as a marker of the disease. Furthermore, estrogen receptor 1 (ESR1) mRNA is over-expressed in early stage tumors, suggesting that expression of the oviduct-related genes may be regulated by estrogen. We have also identified oviduct-related genes that encode secreted proteins that could represent putative serum biomarkers. The expression of oviduct-related genes in early stage tumors is similar to what is seen in human ovarian cancer, with tumors resembling

normal Müllerian epithelium. These data suggest that chicken ovarian tumors may arise from alternative sites, including the oviduct.

Introduction

Ovarian cancer is the fifth leading cause of cancer death in U.S. women and the leading cause of death from gynecologic cancer [1]. In 2009, the American Cancer Society estimated that greater than 14,000 women died from the disease. The high mortality rate is due, in part, to limited early detection strategies and incomplete understanding of the origin of the disease.

Gene expression profiling studies using DNA-microarray technology can advance the understanding of the etiology and progression of ovarian cancer. The use of such technology has made it possible to analyze the expression of genes in small samples of tumor tissue on a global level. Thus far, gene expression profiling has provided prognostic and classification information, as well as predictions of responsiveness to chemotherapeutics [2]. Further insights gained from these types of studies could eventually enhance the clinical management of the disease by providing potential markers for early diagnosis as well as novel therapeutic strategies.

The hen is the only spontaneous animal model of ovarian cancer and can therefore aid in the identification and testing of early detection strategies and therapeutics. Hens have a high incidence of ovarian cancer and similar to women, this incidence increases with age [3]. Several studies have reported that ovarian tumors of the hen express antigens that are frequently expressed in human ovarian tumors including cytokeratin, EGFR, HER-2/neu, COX-1, VEGF, and E-cadherin [4-9]. Barua et al found that chicken tumors resemble all 4 phenotypes found in women, with serous and endometrioid tumors present at about equal frequencies [10]. A recent

study also showed that approximately half of ovarian tumors in the hen exhibit mutations in p53 similar to ovarian adenocarcinomas in women [9].

These studies and others have demonstrated the usefulness of the hen as a model to study the etiology and progression of ovarian cancer. However, no studies to date have examined global gene expression in ovarian cancer of the hen. To this end, we combined the use of the hen animal model with microarray technology to identify differentially expressed genes in ovarian tissue from normal hens compared to hens with ovarian cancer. One advantage of this approach is that differentially expressed genes identified can be evaluated in early stage tumors, providing information about changes that occur during early neoplastic events. We also evaluated expression of particular genes at different stages to assess changes associated with tumor progression.

Materials and Methods

Animals and tissue collection

Single-comb White Leghorn hens were individually caged with access to food and water *ad libitum*. Hens were maintained on a 15h light and 9h dark schedule. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

Normal ovarian tissue samples and ovarian tumor tissue samples from 2.5 to 4.5 year old hens were collected and either frozen in liquid nitrogen or placed in RNAlater [11]. Additional samples were placed in cassettes in formalin for paraffin embedding for immunohistochemical analysis. Diagnosis of hens was made by gross observation and confirmed by analysis of hematoxylin and eosin stained paraffin embedded sections. Ovarian tumors were staged as previously described [11] (Table 2.1).

Microarray analysis

Hens used for microarray analysis were between 2.5 and 3.5 years of age. Normal hens (n=3) showed no gross or histopathological evidence of ovarian cancer, while cancer specimens (n=3) were stage 2 or 3 (Table 2.1). Total RNA was extracted

Table 2.1 Stages of ovarian cancer in the hen [11].

Stage	Description
1	Tumor restricted to ovary and only detectable by histology
2	Tumor restricted to ovary and observable at necropsy
3	Ovarian tumor with abdominal seeding
4	Ovarian tumor with abdominal seeding and ascites

from the frozen normal ovarian tissue and ovarian tumor tissue using TRIZOL (Invitrogen, Carlsbad, CA) and RNA integrity was verified using the 2100 Expert Bioanalyzer (Agilent Technologies, Santa Clara, CA). Linear amplification and labeling was carried out to produce an antisense RNA (aRNA) target. The labeled aRNA was hybridized to GeneChip® chicken genome arrays (Affymetrix, Santa Clara, CA) and scanned by the GeneChip scanner 3000-7G. Analysis of the raw data (including the .CEL files) was carried out at the DNA microarray core facility (Cornell University, Ithaca, NY) using the Affymetrix GCOS software. A set of 273 differentially expressed genes was selected based on a t-test P value less than 0.01 and fold change greater than 1.5. These data are included as Appendix 1 and have been deposited in the Gene Expression Omnibus (GEO) public repository (accession

number GSE21706). Functional annotation analysis was subsequently carried out using DAVID Bioinformatics Resources 2008 [12, 13].

Real-time quantitative PCR

Total RNA was extracted from normal hen ovarian tissue (n=8), ovarian tissue from hens with stage 1 (n=3), stage 2 (n=2), stage 3 (n=6) and stage 4 (n=7) tumors using the RNeasy Mini kit (Qiagen, Valencia, CA). Extracted RNA was reverse-transcribed into cDNA and the cDNA was used for quantitative PCR with SYBR Green to determine mRNA expression levels of several genes selected for further study based on the functional annotation analysis of microarray data (DAVID). These were the oviduct-related genes: *Serpinb14*, more commonly known as ovalbumin (*Oval*), paired box 2 (*Pax2*), ovomucoid (*Ovm*), *Serpinb14b*, more commonly known as serpin peptidase inhibitor clade B member 3 (*Serpinb3*), transferrrin, also known as lactotransferrin (*Ltf*), and *LOC396449* which encodes riboflavin binding protein (RD). Chicken specific primers (designed to span introns) were designed using OligoPerfect^{TM} Designer (Invitrogen, Carlsbad, CA) or Primer Express software v2.0 (Applied Biosystems, Foster City, CA). The oligonucleotide sequences of primers are listed in Table 2. Control reagent was Quantum RNA Universal 18S primers (Ambion, Austin, TX). Control reactions containing no template and reactions lacking reverse transcriptase were also run. Total volume of reactions was 25 uL with a final concentration of 1X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.3 nM of primer pairs for the oviduct-related genes, and 0.1 nM of primer pairs for 18S. All sample reactions were run in duplicate using the ABI Prism 7000 Sequence Detection System. The relative amount of mRNA in a particular sample was determined by the comparative threshold cycle (C_t) method using Sequence Detection System software (Applied Biosystems, Foster City, CA).

Laser capture microdissection

Hens with early stage (stage 1, as defined in Table 2.1) ovarian cancer were identified by analyzing paraffin embedded sections that were stained with hematoxylin and eosin. Ovarian samples from these hens had also been embedded in Histo Prep (Fisher Scientific, Pittsburgh, PA) and snap frozen in cryomolds. Frozen sections of ovary from these hens (n=5) as well as sections of ovary and oviduct from normal hens (n=5) were cut, placed on PEN-membrane slides (Leica Microsystems, Wetzler, Germany), stained briefly with hematoxylin, and processed in a solution of 70% ethanol. Slides were allowed to dry before laser capture of tissue. Normal oviduct, ovarian epithelial cells (OSE), tumor and adjacent stromal tissue were captured using a laser capture microdissecting scope (Leica ASLMD, Leica Microsystems, Wetzler, Germany). Total RNA was extracted using the RNeasy Plus Micro kit (Qiagen, Valencia, CA) and was reverse transcribed into cDNA. As described previously, the cDNA was used for quantitative PCR with SYBR Green to determine mRNA expression levels of the oviduct-related genes *Oval*, *Pax2*, *Ovm*, *Serpinb3*, *Ltf*, and *Rd*.

Expression levels of estrogen receptor 1 (estrogen receptor alpha; *Esr1*) and estrogen receptor 2 (estrogen receptor beta; *Esr2*) were also determined in these samples. Briefly, chicken specific TaqMan primers and probes were designed using Primer Express software v2.0 (Applied Biosystems, Foster City, CA). The sequences of primers and probes can be seen in Table 2.2. TaqMan primers and probes for 18S (Applied Biosystems, Foster City, CA) were used as control. Total volume of reactions was 25 uL with a final concentration of 1X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 nM of estrogen receptor primer pairs (*Esr1* and *Esr2*), 50 nM of 18S primer pair, and each respective TaqMan probe (250 nM of estrogen receptor probes (*Esr1* and *Esr2*) and 200 nM of 18S probe). All

sample reactions were run in duplicate and the relative amount of RNA in a particular sample was determined as described above.

Table 2.2 Primers used for real-time quantitative PCR analysis.

Gene	Gene accession no.	Forward primer	Reverse primer	Probe
OVAL	NM_205152	cacaagcaatgcctttcaga	gacttcatcaggcaacagca	N/A
OVM	NM_001112662	tcgtgctgttctctttcgtg	taggcacacagcaagcaatc	N/A
SERPINB3	NM_001031001	ggggcaagaggtaacactga	gtagcatttggcctggtgat	N/A
LTF	NM_205304	ctacagtgccatccagagca	tgatgcagtcctttgtctg	N/A
RD	NM_205463	gcctgcaaagatgattccat	gccaccatgtccttccttgtt	N/A
PAX2	NM_204793	cgagtttttgagcgtccttc	aaggtggtgcttgccatatc	N/A
ESR1	NM_205183	cagcattcgtgagaggatgtcta	acagtaccgggtctccttggt	taccaatgagaaagggagcctgtccatg
ESR2	NM_204794	ggaaatgctatgaagtgggaatg	acagtaccgggtctccttggt	tgggtatcgaatcctgcgcc

Immunohistochemistry

Previously collected ovarian samples (n=6 normal; n=14 tumors of various stages) had been fixed in formalin, embedded in paraffin and cut at 4µm. 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 mouse anti-chicken ovalbumin (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:500 and rabbit anti-human PAX2 (Abcam, Cambridge, MA) at a dilution of 1:50 overnight at 4°. Control slides were incubated without primary antibody. This was followed by incubation in AlexaFluor 594 goat anti-mouse IgG secondary antibody (0.24 ug/ml) for ovalbumin and AlexaFluor 488 goat anti-rabbit IgG (0.24 ug/ml) for PAX2 for 1h at 39°. Normal ovarian samples (n=6) and ovarian samples from hens with stage 1

(n=3), stage 2 (n=2), stage 3 (n=4) and stage 4 (n=5) cancer were incubated with both primary antisera. Slides were viewed using a Nikon eclipse E600 and pictures were taken with a Spot RT Slider camera.

Statistics

Relative expression values from real-time quantitative PCR analysis were log transformed and analyzed for significance using proc GLM. Means were compared using Duncan's multiple range tests. Relative expression of estrogen receptor in microdissected tumor tissue compared to the adjacent stroma was analyzed using proc TTEST. All tests were carried out using SAS version 9.2 with a significance level of $p<0.05$.

Results

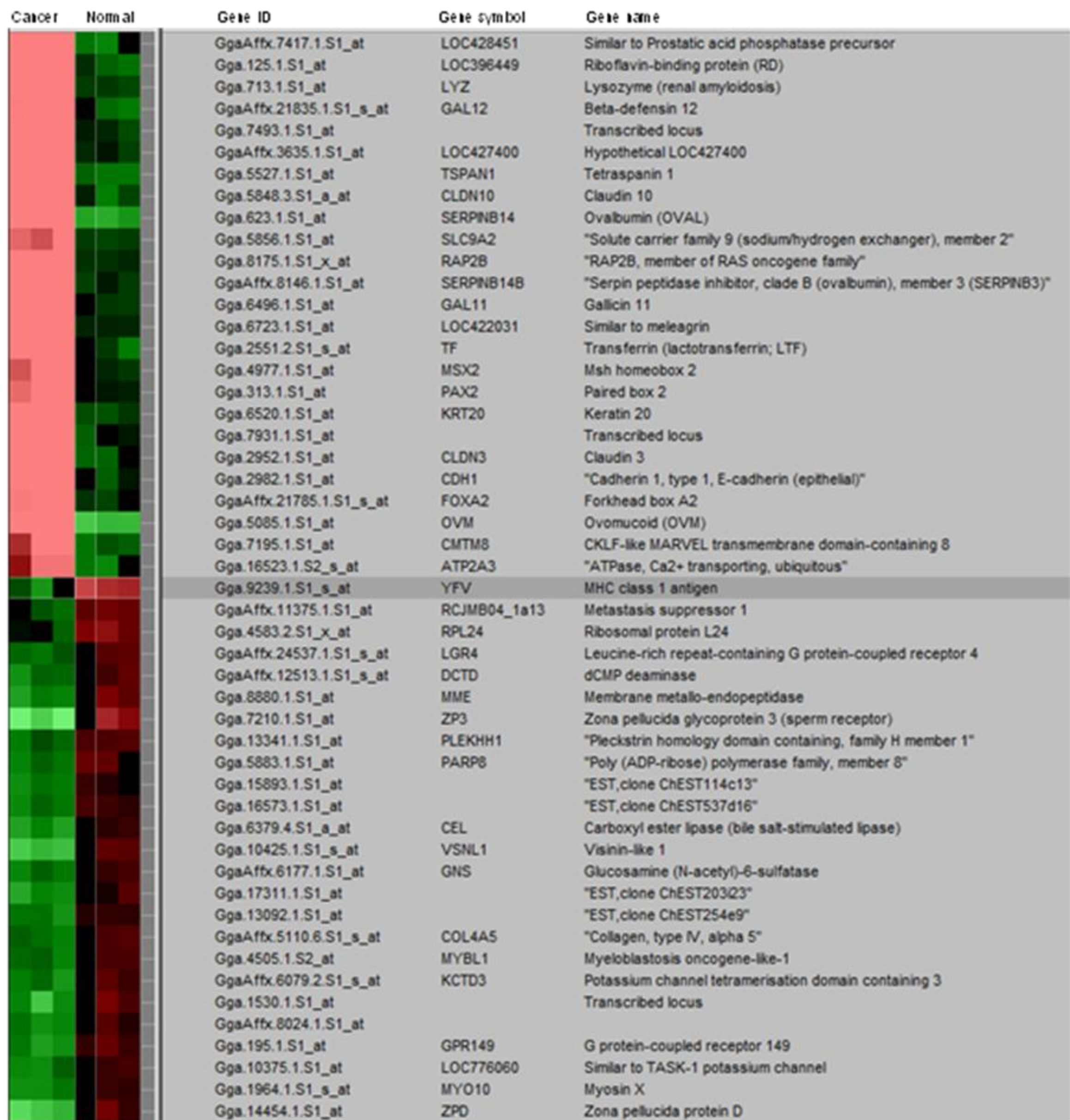
Up-regulated transcripts in ovarian tumors are enriched for oviduct-related genes

Out of >32,000 transcripts represented on the chicken GeneChip, 273 were differentially expressed between normal ovarian tissue and ovarian tissue from hens with cancer. Of these, 153 transcripts were up-regulated and 120 were down-regulated in tumor tissue. A full list of these genes is provided in Appendix 1 and can be accessed through GEO (accession number GSE21706). Figure 1 is a heat map of the top 25 up-regulated and down-regulated genes. Functional annotation analysis (DAVID) revealed that the up-regulated transcripts are enriched for genes that are normally expressed in the oviduct. Several of these genes are associated with the annotation keywords allergen and egg white, including *Oval*, *Ovm*, *Ltf*, *Rd*, and *Serpinb3*. A literature search revealed several more oviduct-related genes identified in our microarray analysis (including meleagrin, lysozyme, gallicin 11 and beta-defensin 12) and also confirmed that these genes encode proteins that are either expressed in the chicken oviduct [14], or secreted by oviductal cells to form membranes of the

developing egg [15, 16]. *Pax2* is expressed in the female reproductive tract, including the oviduct, and is required for Müllerian duct development [17]. In total, 10 out of the top 25 up-regulated genes (40%) are oviduct-related. These oviduct-related genes are consistently up-regulated in all three ovarian tumors as compared to normal ovary (Figure 2.2).

Real-time quantitative PCR confirms increased expression of a subset of oviduct-related genes in ovarian cancer of the hen

Quantitative PCR was performed for a subset (selection was made based on our ability to design intron-spanning primers) of the 10 oviduct-related genes: *Oval*, *Pax2*, *Ovm*, *Serpinb3*, *Ltf*, and *Rd*. All of these genes exhibited significantly higher ($p<0.01$) expression in tumor tissue (particularly later stages) as compared to normal tissue validating the microarray results (Figure 2.3). Moreover, there was a stage-dependent increase in expression of these genes in the tumors. In all cases, expression was significantly greater in advanced tumors (stages 3 and 4) as compared to the earliest tumors (stage 1) or normal ovary (Figure 2.3). Furthermore, several of the genes exhibited significantly higher expression in stage 2 tumors compared to normal ovary, including *Oval*, *Pax2*, *Ovm*, and *Ltf* (Figure 2.3). The limited expression of these genes in stage 1 tumors could be due to the fact that samples from these tumors are composed primarily of normal tissue with only scattered areas of neoplasia.



Cancer	Normal	Gene ID	Gene symbol	Gene name
		GgaAffx.7417.1.S1_at	LOC428451	Similar to Prostatic acid phosphatase precursor
		Gga.125.1.S1_at	LOC396449	Riboflavin-binding protein (RD)
		Gga.713.1.S1_at	LYZ	Lysozyme (renal amyloidosis)
		GgaAffx.21835.1.S1_s_at	GAL12	Beta-defensin 12
		Gga.7493.1.S1_at		Transcribed locus
		GgaAffx.3635.1.S1_at	LOC427400	Hypothetical LOC427400
		Gga.5527.1.S1_at	TSPAN1	Tetraspanin 1
		Gga.5848.3.S1_a_at	CLDN10	Claudin 10
		Gga.623.1.S1_at	SERPINB14	Ovalbumin (OVAL)
		Gga.5856.1.S1_at	SLC9A2	"Solute carrier family 9 (sodium/hydrogen exchanger), member 2"
		Gga.8175.1.S1_x_at	RAP2B	"RAP2B, member of RAS oncogene family"
		GgaAffx.8146.1.S1_at	SERPINB14B	"Serpin peptidase inhibitor, clade B (ovalbumin), member 3 (SERPINB3)"
		Gga.6496.1.S1_at	GAL11	Gallicin 11
		Gga.6723.1.S1_at	LOC422031	Similar to meleagrin
		Gga.2551.2.S1_s_at	TF	Transferrin (lactotransferrin; LTF)
		Gga.4977.1.S1_at	MSX2	Msh homeobox 2
		Gga.313.1.S1_at	PAX2	Paired box 2
		Gga.6520.1.S1_at	KRT20	Keratin 20
		Gga.7931.1.S1_at		Transcribed locus
		Gga.2952.1.S1_at	CLDN3	Claudin 3
		Gga.2982.1.S1_at	CDH1	"Cadherin 1, type 1, E-cadherin (epithelial)"
		GgaAffx.21785.1.S1_s_at	FOXA2	Forkhead box A2
		Gga.5085.1.S1_at	OVM	Ovomucoid (OVM)
		Gga.7195.1.S1_at	CMTM8	CKLF-like MARVEL transmembrane domain-containing 8
		Gga.16523.1.S2_s_at	ATP2A3	"ATPase, Ca2+ transporting, ubiquitous"
		Gga.9239.1.S1_s_at	YFV	MHC class 1 antigen
		GgaAffx.11375.1.S1_at	RCJMB04_1a13	Metastasis suppressor 1
		Gga.4583.2.S1_x_at	RPL24	Ribosomal protein L24
		GgaAffx.24537.1.S1_s_at	LGR4	Leucine-rich repeat-containing G protein-coupled receptor 4
		GgaAffx.12513.1.S1_s_at	DCTD	dCMP deaminase
		Gga.8880.1.S1_at	MME	Membrane metallo-endopeptidase
		Gga.7210.1.S1_at	ZP3	Zona pellucida glycoprotein 3 (sperm receptor)
		Gga.13341.1.S1_at	PLEKHH1	"Pleckstrin homology domain containing, family H member 1"
		Gga.5883.1.S1_at	PARP8	"Poly (ADP-ribose) polymerase family, member 8"
		Gga.15893.1.S1_at		"EST,clone ChEST114c13"
		Gga.16573.1.S1_at		"EST,clone ChEST537d16"
		Gga.6379.4.S1_a_at	CEL	Carboxyl ester lipase (bile salt-stimulated lipase)
		Gga.10425.1.S1_s_at	VSNL1	Visinin-like 1
		GgaAffx.6177.1.S1_at	GNS	Glucosamine (N-acetyl)-6-sulfatase
		Gga.17311.1.S1_at		"EST,clone ChEST203l23"
		Gga.13092.1.S1_at		"EST,clone ChEST254e9"
		GgaAffx.5110.6.S1_s_at	COL4A5	"Collagen, type IV, alpha 5"
		Gga.4505.1.S2_at	MYBL1	Myeloblastosis oncogene-like-1
		GgaAffx.6079.2.S1_s_at	KCTD3	Potassium channel tetramerisation domain containing 3
		Gga.1530.1.S1_at		Transcribed locus
		GgaAffx.8024.1.S1_at		
		Gga.195.1.S1_at	GPR149	G protein-coupled receptor 149
		Gga.10375.1.S1_at	LOC776060	Similar to TASK-1 potassium channel
		Gga.1964.1.S1_s_at	MYO10	Myosin X
		Gga.14454.1.S1_at	ZPD	Zona pellucida protein D

Figure 2.1 Gene profile of 50 differentially expressed genes in chicken ovarian tumors. The top 25 up- and down-regulated genes are depicted. Genes were identified by Affymetrix ID (Gene ID), gene symbol and gene name. Each column represents a single sample. The shades of red indicate induced genes and the shades of green indicate the repressed genes. Colored pixels indicate the magnitude of gene response.

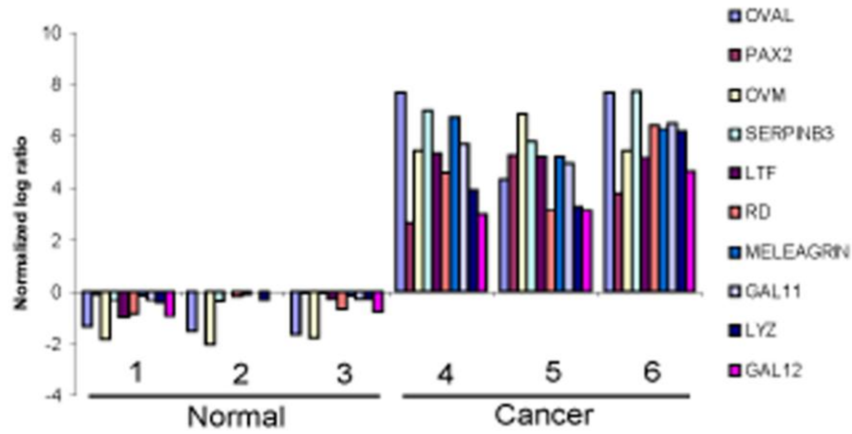


Figure 2.2 Graphical representation of microarray results for the 10 oviduct-related genes in the top 25 up-regulated genes in ovarian tissue from three individual normal hens (1-3) compared to tissue from three individual hens with cancer (4-6).

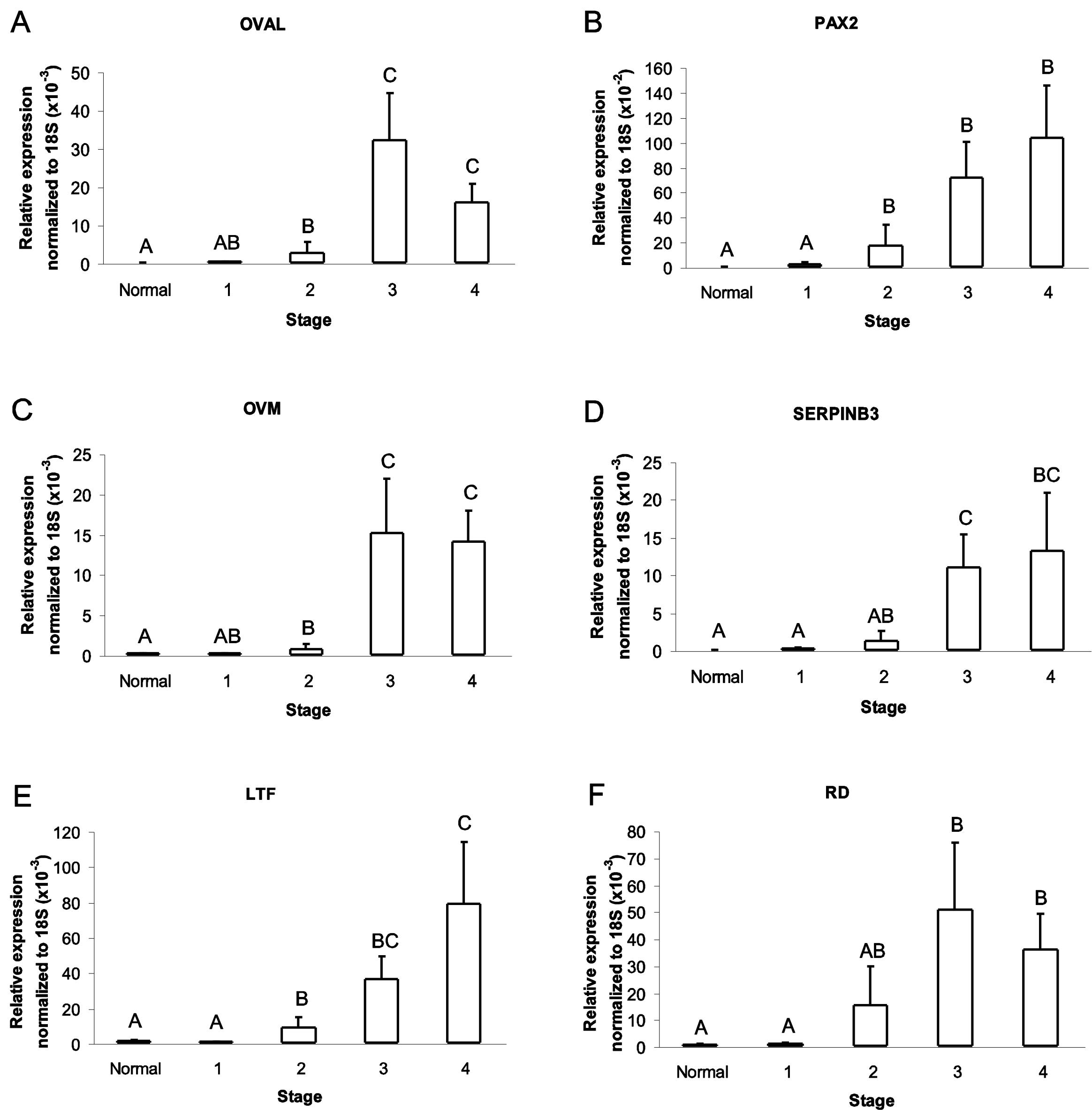


Figure 2.3 Real-time quantitative PCR analysis of OVAL (A), PAX2 (B), OVM (C), SERPINB3 (D), LTF (E), and RD (F) mRNA expression in normal ovary (n=8), as well as stage 1 (n=3), stage 2 (n=2), stage 3 (n=6) and stage 4 (n=7) ovarian cancer. Means were compared using Duncan's multiple range tests. Different letters indicate significant differences ($p<0.01$). Means that share a letter are not significantly different. Bars indicate standard error.

Expression of oviduct-related genes in early stage ovarian cancer compared to normal oviduct and OSE

In order to investigate expression of these genes in early stage ovarian tumors, as well as in oviduct and OSE, we performed laser capture microdissection. Oviduct and OSE from normal hens, as well as from neoplastic areas and adjacent stromal tissue from stage 1 ovarian cancers were microdissected from frozen ovarian sections. Figure 2.4 shows representative images of the microdissected oviduct, OSE, cancer and stromal tissue. Quantitative real-time PCR was performed for the oviduct-related genes: *Oval*, *Pax2*, *Ovm*, *Serpin3*, *Ltf*, and *Rd* as above. These oviduct-related genes are generally expressed in normal oviduct and early stage ovarian tumors, but not normal OSE (Figure 2.5). Specifically, expression of *Pax2* ($p<0.001$; Figure 2.5B), *Serpinb3* ($p<0.05$; Figure 2.5D), *Ltf* ($p<0.01$; Figure 2.5E) and *Rd* ($p<0.05$; Figure 2.5F) mRNA are significantly greater in early tumors compared to adjacent stroma and/or normal OSE. There is also a trend for higher *Oval* and *Ovm* mRNA expression in early stage tumors compared to the surrounding stroma and normal OSE (Figure 2.5A, 2.5C).

Expression of estrogen receptors in early stage ovarian cancer

We also determined expression of *Esr1* and *Esr2* in the microdissected neoplastic areas and adjacent stromal tissue. Relative expression of *Esr1* mRNA was significantly higher in microdissected tumor tissue compared to adjacent stroma ($p<0.05$; Figure 2.6). *Esr2* mRNA was not detectable in microdissected tumor or the adjacent stroma (data not shown).

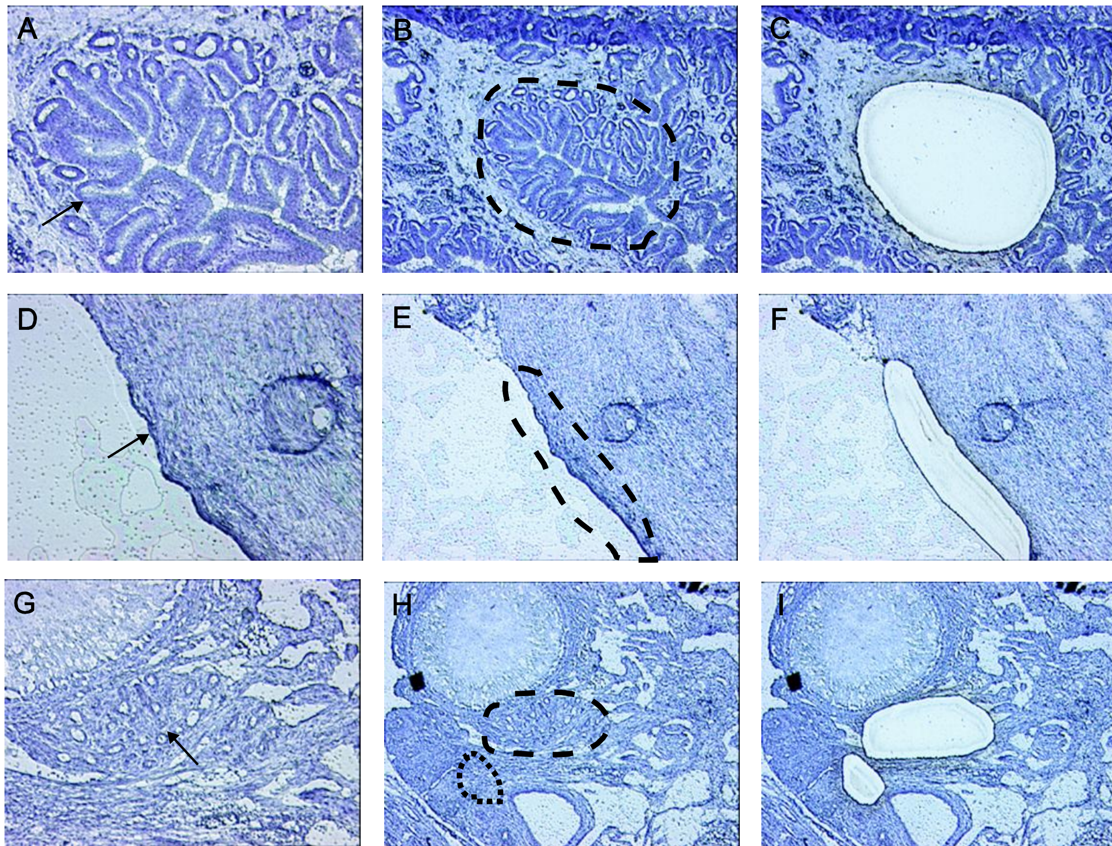


Figure 2.4 Representative images of oviduct (A-C), OSE (D-F), as well as tumor and adjacent stromal tissue (G-I) before and after microdissection. A) Frozen section of normal oviduct with tubular glands (arrow; 20X). B) Image of oviduct section before microdissection. Dashed line indicates area of tubular glands to be captured (10X). C) Image of oviduct section after microdissection (10X). D) Frozen section of normal ovary with OSE (arrow; 20X). E) Image of ovary section before microdissection. Dashed line indicates area of OSE to be captured (10X). F) Image of ovary section after microdissection (10X). G) Frozen section of ovary from hen with early stage cancer. Tumor exhibits gland-like structures (arrow; 20X). H) Image of ovary section before microdissection. Dashed line indicates area of neoplastic cells to be captured while dotted line indicates area of apparently normal adjacent stroma to be captured (10X). I) Image of ovary section after microdissection (10X).

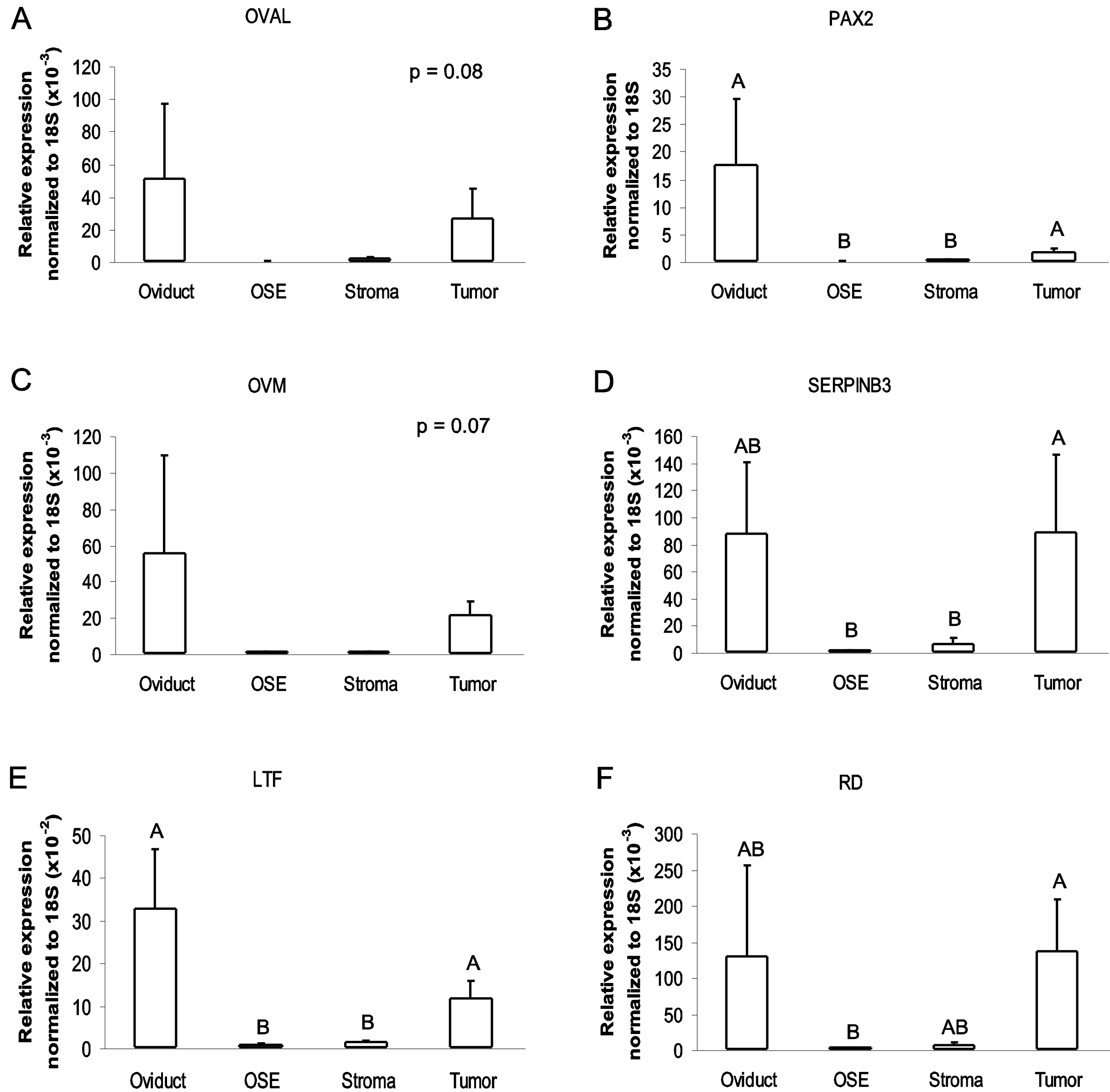


Figure 2.5 Real-time PCR analysis of OVAL (A), PAX2 (B), OVM (C), SERPINB3 (D), LTF (E), and RD (F) mRNA expression in microdissected oviduct (n=5), OSE (n=5), tumor (n=5) and adjacent stroma (n=5). Means were compared using Duncan's multiple range tests. Different letters indicate significant differences ($p<0.05$). Means that share a letter are not significantly different. Bars indicate standard error.

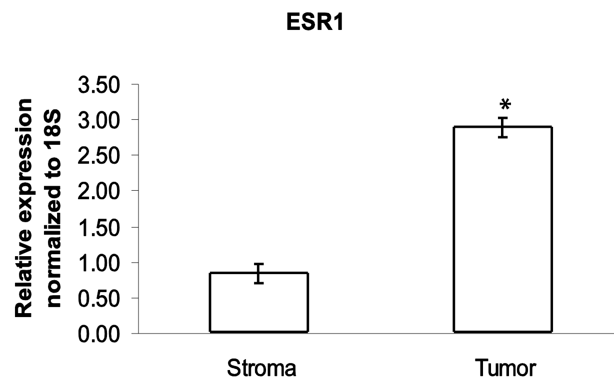


Figure 2.6 Real-time PCR analysis of ESR1 mRNA expression in microdissected tumor (n=5) and adjacent stroma (n=5). Asterisk indicates significant difference ($p<0.05$). Bars indicate standard error.

Chicken ovarian tumors express PAX2 protein

Figure 2.7 illustrates that ovalbumin and PAX2 protein were expressed in early (stage 1) and late stage (stage 3) ovarian cancers of the hen. Ovalbumin and PAX2 protein expression were assessed in normal ovarian structures, including OSE, in normal ovaries and ovaries from hens with cancer. In addition, expression of ovalbumin and PAX2 was also determined in neoplastic areas in ovaries from hens with cancer.

As expected, no ovalbumin expression was detected in normal ovary (Figure 2.7A), but ovalbumin was expressed in an early-stage tumor while the surrounding stroma was negative (stage 1; Figure 2.7C). As previously reported, ovalbumin expression was seen in a late-stage ovarian tumor (stage 3; Figure 2.7E). There was little to no expression of PAX2 in the normal ovary (Figure 2.7B). There was nuclear PAX2 expression localized to neoplastic cells in an early stage tumor (Figure 2.7D, arrow) and nuclear expression in a late stage tumor (Figure 2.7F). Control sections incubated without primary antibody were negative (insets Figures 2.7A-F). Table 2.3 summarizes the results obtained for all of the ovarian tissue sections examined. All of the ovarian cancers expressed ovalbumin regardless of stage. Similar to ovalbumin, PAX2 was expressed in ovarian tumors of all stages, but fewer stage 4 tumors were positive.

Discussion

To our knowledge, we are the first to report analysis of gene expression of ovarian cancer in the hen on a global level. Through the current approach, we have identified genes that are differentially expressed in ovarian tissue from normal hens compared to tissue from hens with ovarian cancer. Furthermore, functional annotation analysis revealed that 10 of the top 25 up-regulated genes (40%) in chicken ovarian

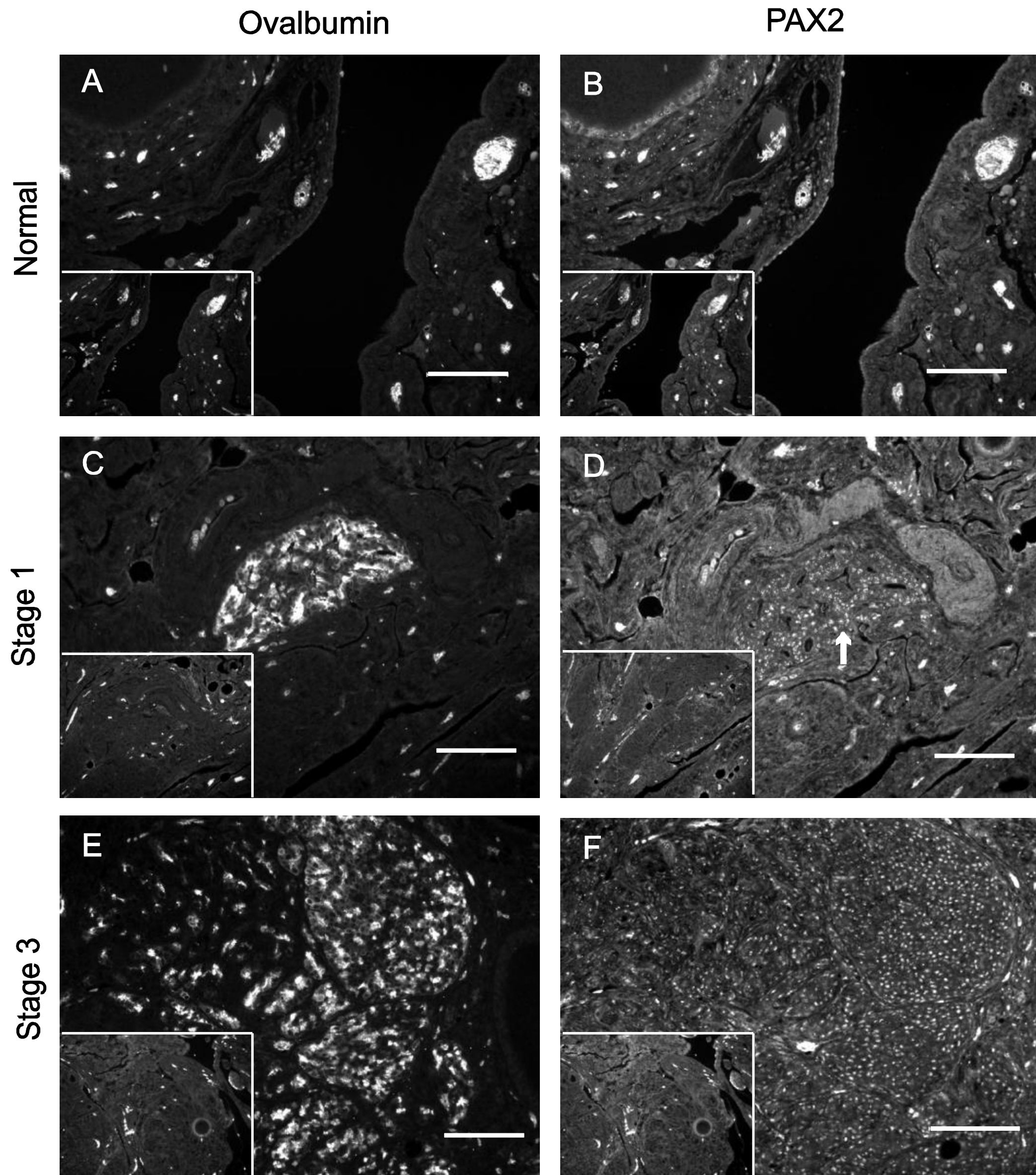


Figure 2.7 Immunohistochemistry with antibodies against ovalbumin and PAX2. Ovalbumin protein expression in normal ovary (A), early-stage ovarian cancer (stage 1; C), and late-stage ovarian cancer (stage 3; E). PAX2 protein expression in normal ovary (B), early-stage ovarian cancer (stage 1; D; arrow indicates area of neoplastic cells), and late-stage ovarian cancer (stage 3; F). Control sections were incubated without primary antibody (insets A-F). Scale bar = 100 um.

Table 2.3 Summary of ovalbumin and PAX2 protein expression in normal ovarian tissue (n=6), and ovarian tissue from hens with stage 1 (n=3), stage 2 (n=2), stage 3 (n=4), and stage 4 (n=5) ovarian cancer.

Type	Ovalbumin+ (%)	PAX2+ (%)
Normal	0/6 (0)	0/6 (0)
Stage 1	3/3 (100)	2/3 (67)
Stage 2	2/2 (100)	2/2 (100)
Stage 3	4/4 (100)	3/4 (75)
Stage 4	5/5 (100)	1/5 (20)

tumors are oviduct-related. We have shown that these genes are expressed in early and late stage chicken ovarian tumors, as well as in the oviduct, but not OSE (Figures 2.3 and 2.5), suggesting that ovarian tumors of the hen may originate from sites other than the OSE. Alternatively, expression of oviduct-related genes in chicken, as well as human, ovarian tumors might suggest secondary Müllerian origin of these tumors [reviewed in 18 and 19], or aberrant differentiation of OSE [reviewed in 20], as has been proposed in women. Despite the fact that ovarian cancer is a heterogeneous disease comprised of a variety of subtypes, the OSE has traditionally been viewed as the common origin of ovarian tumors. However, recent studies have suggested that a subset of ovarian tumors might arise from sites other than the OSE, including the fallopian tubes [21]. For one thing, BRCA+ women who have a high risk of ovarian cancer also have a high incidence of epithelial dysplasia [22] and serous tubal intraepithelial carcinomas [23] in the fallopian tubes. In addition women with serous ovarian carcinomas (regardless of BRCA status) exhibit involvement of the fallopian tubes as well as tubal intraepithelial carcinomas [24]. These results suggest that high

grade serous ovarian tumors may be of tubal origin. These tumors are characterized by p53 mutations and over-expression of the *HER2/neu* oncogene [25]. Interestingly, ovarian adenocarcinomas in the hen also exhibit mutations in p53 and over-expression of *HER2/neu* [9], and therefore resemble high grade human ovarian cancers.

Further evidence of a possible alternative site of origin of ovarian tumors comes from expression studies of paired box 2 (PAX2) in human ovarian tumors. Studies have shown that PAX2 mRNA and protein are expressed in the human reproductive tract and ovarian tumors [26, 27]. These studies also reported that OSE does not express PAX2 [26, 27], however, one study did find expression of PAX2 in normal OSE [28]. PAX2 expression has been reported in both low grade and high grade ovarian tumors [26], although Tung et al. found that few high grade ovarian tumors express PAX2 [27]. Although we did not evaluate PAX2 expression based on tumor grade, we did evaluate PAX2 expression in chicken ovarian tumors of all stages and found that fewer late stage tumors express PAX2 protein (Table 2.3). This result is in contrast to the PAX2 mRNA expression data (Figure 2.3), suggesting that translation of PAX2 protein is altered in late stage tumors and that PAX2 may play a role in early tumor development. Additionally, we have shown that PAX2 mRNA and protein are expressed in chicken ovarian tumors, but no *Pax2* mRNA was detected in OSE (Figure 2.5), although a few scattered positive cells were observed in the OSE by immunohistochemistry (Figure 2.7). These results are generally supportive of an oviductal site of origin, but could indicate a role for de-differentiation of the OSE. We have also observed PAX2 mRNA and protein (Figure 2.5 and Appendix 2) expression in chicken oviduct, confirming that it is an oviduct-related protein. It is possible that PAX2 may be playing a role in the progression of ovarian cancer in the hen, since it has been shown to be required for human ovarian cancer cell survival [29]. Future studies are needed to elucidate the role of PAX2 in ovarian cancer of the hen. It

should be noted that another PAX family member, PAX8, is similar to PAX2 in amino acid sequence and has also been implicated in the development of ovarian cancer [30]. PAX8 has yet to be identified in chicken, so it is not known how similar these sequences would be in the hen. We performed a BLAST search with the peptide sequence of the immunogen of our antibody and identified PAX2, but not PAX8, in the human. The BLAST search also identified PAX2 in other species, including the chicken. Therefore, assuming that chicken PAX8 exists and has a similar sequence to chicken PAX2 (as seen in humans), it is likely that our antibody is specific for PAX2.

Interestingly, a number of oviduct-related genes identified in our analysis are estrogen-regulated. It has long been known that estrogen is responsible for the differentiation of oviductal glands that synthesize the major egg-white proteins in the chicken oviduct [reviewed in 31]. Estrogen has been shown to have a direct effect on the synthesis of ovalbumin, lactotransferrin, ovomucoid and lysozyme in the chicken oviduct [32]. In humans, PAX2 expression has also been shown to be activated by estrogen in endometrial cancer cells [33, 34]. Estrogen is thought to promote ovarian tumor progression by regulating cellular proliferation, motility and invasion and these effects are thought to be mediated by ESR1 [35]. We have shown that *Esr1* mRNA expression is significantly higher in microdissected tumor tissue as compared to adjacent stroma (Figure 2.6), suggesting that expression of the oviduct-related genes in these tumors may be regulated by estrogen.

As mentioned previously, some of the oviduct-related genes identified in our analysis encode proteins that are secreted by oviductal cells to form the membranes of the developing egg [15, 16]. The identification of up-regulated genes that encode secreted proteins in ovarian cancer provides the possibility of utilizing serum levels of these proteins as biomarkers for the malignancy. Several current and potential serum biomarkers of ovarian cancer in humans have been shown to be normally expressed in

the oviduct including CA-125 [36], human epididymis protein 4 (HE4) [37] and oviductal glycoprotein 1 (OVGP1) [38, 39]. A number of the oviduct-related genes identified in chicken ovarian tumors encode proteins that are secreted and may represent serum biomarker candidates. In particular, the genes encoding PAX2, SERPINB3, LTF and RD are expressed in early stage chicken ovarian tumors (Figure 2.5), suggesting these proteins may serve as putative early markers of the disease.

Our results support previous studies in the hen which reported increased expression of ovalbumin and E-cadherin in chicken ovarian tumors [40, 8]. Ovalbumin and E-cadherin are in the list of the top 25 up-regulated genes in ovarian tumor tissue compared to normal ovarian tissue with expression levels increased >200-fold and 40-fold, respectively (Appendix 1). Our results also support previous studies in women which reported over-expression of E-cadherin [41] and claudin 3 [42] in human ovarian tumors. As mentioned previously, E-cadherin has been shown to be up-regulated in chicken ovarian tumors compared to normal ovarian tissue. Claudin 3 is also over-expressed in chicken ovarian tumors. Our microarray analysis revealed that it is in the top 25 up-regulated genes in chicken ovarian tumor tissue compared to normal ovarian tissue with expression levels increased >40-fold (Appendix 1). In the current study, we focused on the genes up-regulated in ovarian tumors of the hen and have yet to study the down-regulated genes in depth. It is possible, however, that putative tumor suppressors have been uncovered in our results and further studies are warranted.

Studies in women have identified common genetic alterations in ovarian cancer, including alterations of oncogenes such as *HER2/neu*, K-ras, PIK3CA, and c-myc, as well as of tumor suppressors including p53 and BRCA1/2 [41]. Some of these alterations include overexpression (i.e. HER2/neu) and, surprisingly, we did not identify these genes in our microarray analysis. In order to further investigate the

function of the genes identified, we performed a preliminary pathway analysis using the Ingenuity Pathway Analysis (IPA) program. The top 5 network pathways identified by IPA are provided in Appendices 3A-E. Interestingly, the oncogenes PI3K (Appendix 3B) and myc (Appendix 3C) and the tumor suppressor p53 (Appendix 3E) are represented in the pathways created. These data suggest that although these genes were not identified in our microarray analysis, they may possibly play a role in ovarian tumorigenesis in the hen. Furthermore, we have also identified VEGF signaling in this analysis (Appendix 3A) which supports a previous study in the hen [7]. Our pathway analysis also supports our current data which suggest a role for estrogen/ estrogen receptor signaling, with beta estradiol signaling represented in Appendix 3E. Further analysis is needed to fully comprehend the complex signaling pathways involved in ovarian tumor development and/or progression in the hen.

Our analysis has revealed up-regulation of oviduct-related genes in chicken ovarian tumors, particularly at early stages, compared to normal ovarian tissue. These results provide evidence that chicken ovarian tumors resemble normal Müllerian epithelium, similar to tumors in women and suggest a possible alternative site of origin of these tumors. Although we have not disproven the possibility of secondary Müllerian structures or the OSE as sites of origin, we hope that our data can stimulate additional work in this area. We have also shown that the oviduct-related genes may also be regulated by estrogen, highlighting the importance of estrogen signaling in these tumors. In addition, a number of these genes are secreted and may prove to be possible serum biomarkers. Importantly, our studies further validate the use of the hen as a model for human ovarian cancer.

Acknowledgements

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CHAPTER 3

ORAL CONTRACEPTIVES DECREASE THE PREVALENCE OF OVARIAN CANCER IN THE HEN

Abstract

Ovarian cancer is the leading cause of reproductive cancer death in U.S. women. This high mortality rate is due to the lack of early detection methods and ineffectiveness of therapy for advanced disease. Until more effective screening methods and therapies are developed, chemoprevention strategies are warranted. The hen has a high spontaneous prevalence of ovarian cancer and is suitable for studying ovarian cancer chemoprevention. Our objective was to determine the effect of progestin alone, estrogen alone, or progestin and estrogen in combination (as found in OCs) on ovarian cancer prevalence in the hen. We found that treatment with progestin alone and in combination with estrogen decreased the prevalence of ovarian cancer. A significant risk reduction of 91% was observed in the group treated with progestin alone (risk ratio 0.0909: 95% confidence interval 0.0117-0.704) and 81% was observed in the group treated with progestin plus estrogen (risk ratio 0.1916: 95% confidence interval 0.043-0.864). Ovulatory events were also significantly reduced in these treatment groups compared to control. We found no effect of progestin, either alone or in combination with estrogen, on apoptosis in the ovary, indicating that this is not the mechanism responsible for the protective effect of progestin in the hen. Our results suggest that ovulation is related to the risk of ovarian cancer in hens and that other factors, such as hormones, more than likely modify this risk. Our study supports the use of oral contraceptives to prevent ovarian cancer and further validates the hen as a suitable animal model to test chemoprevention strategies.

Introduction

Ovarian cancer is the leading cause of reproductive cancer death in U.S. women. This high mortality rate can be attributed to the fact that greater than 80% of women are diagnosed at late stages of the disease once tumors have metastasized. The 5-year survival rate is less than 30% at later stages, although this survival rate is greater than 90% for the ~15% of women diagnosed at earlier stages of the disease when the tumor is still confined to the ovary [1]. These data support the need for the development of early detection strategies of the disease. Unfortunately, efforts to identify a widely acceptable screening strategy have thus far failed. Chemopreventive agents, such as oral contraceptives, may decrease the number of deaths due to ovarian cancer.

Epidemiologic studies have consistently shown that ovarian cancer risk is decreased in women who use oral contraceptives [2]. In fact, a recent study showed that oral contraceptive use is associated with a 20% decrease in relative risk of ovarian cancer for every 5 years of use and longer duration of use further decreases the risk [3]. Additionally, the risk is reduced for 30 years or more after use has ceased [3]. Several mechanisms have been proposed to explain how oral contraceptives decrease the risk of ovarian cancer, including inhibition of ovulation, induction of apoptosis, and inhibition of proliferation [4]. Animal models can be used to test the efficacy and mechanism of action of chemopreventive agents.

The hen is a suitable animal model to test chemoprevention strategies because the hen spontaneously develops ovarian cancer which resembles the disease in women. Hens have a high incidence of ovarian cancer, and similar to women, this incidence increases with age [5]. The utility of the hen as a preclinical model of ovarian cancer has been tested in a number of studies. One study reported that chicken ovarian tumors express antigens that are frequently expressed in human

ovarian cancer as well as those that are useful as surrogate biomarker endpoints in chemoprevention trials [6]. Pilot studies have been conducted in the hen with putative chemopreventive agents including aspirin [7] and flaxseed [8]. An earlier study also tested the effect of medroxyprogesterone acetate (Depo-Provera), commonly used in progestin-only formulations of contraceptives, on ovarian cancer prevalence [9]. Hens treated with Depo-Provera exhibited a 15% reduction of risk of ovarian cancer compared to control hens [9].

We have previously shown that steroid hormone, particularly estrogen, signaling may play a role in ovarian tumorigenesis (Chapter 2). Oral contraceptives are comprised of steroid hormones and are associated with decreased risk of ovarian cancer. Therefore, our objective was to test the effect of progestin and estrogen together, as commonly delivered in “the pill”, as well as progestin and estrogen alone on ovarian cancer incidence in the hen. We also investigated the effect of these treatments on apoptosis in normal hen ovaries.

Materials and Methods

Animals

Single-comb White leghorn hens were individually caged with access to food and water *ad libitum*. Hens were maintained on a 15h light and 9h dark schedule. A total of 231 approximately one year-old hens were allocated to 4 treatment groups: control, progestin alone, estrogen alone, and progestin and estrogen combined. Hens were treated as described below. Egg production was monitored daily as a marker of ovulation and hens were weighed monthly. Necropsies were performed on hens that died before experiment termination (n=71) as well as those that were euthanized by CO2 asphyxiation at the end of the experiment (n=160). All animal procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

Treatments

Hens in the progestin alone and the combination treatment groups were injected with 50 mg of medroxyprogesterone acetate (MPA; Spectrum Chemical, Gardena, CA) dissolved in 1 ml of sesame oil. Initially, the hens were injected with 100 mg as previously described [8] resulting in an almost complete cessation of egg production; however, due to adverse effects of this dose, the dose was cut in half. Compared to the 100 mg dose, 50 mg resulted in fewer deaths and still exhibited an effect on egg production (although to a lesser extent). Hens in the estrogen alone and the combination treatment groups were implanted with 25 mg estradiol implants (Compudose 200; Elanco Animal Health, Indianapolis, IN) previously reported to be bioactive in the hen [10-13]. Control hens were injected with 1 mL of the sesame oil vehicle and implanted with an empty silastic tube. Injections were administered into the breast muscle (i.m.) every 3-4 weeks. Hens were administered a local anesthetic (bupivacaine; 5 mg/ml) between the wings where implants were inserted through an incision made with a scalpel. The incision was closed using tissue adhesive (VetBond). Control implants were inserted similarly. Treatments were administered for 16 months similar to the previous study where MPA was administered to hens [9].

Tissue collection

At necropsy, samples of ovary were placed in cassettes in formalin for paraffin embedding and hens were examined for presence of tumors within the peritoneal cavity (i.e. ovary, oviduct, and intestine) as well as for accumulation of ascites fluid. The paraffin embedded sections were cut and stained with hematoxylin and eosin (H&E). Diagnosis of hens was made by gross observation and confirmed by analysis of the H&E stained sections by two independent observers, one of whom is an avian

pathologist (ELB). Tumors identified in hens were subsequently staged as previously described [7].

Estradiol radioimmunoassay (RIA)

Blood samples were collected from the wing vein of a subset of hens 30, 60, 120, 180, and 360 days after the first implantation of estradiol or control implants. Plasma isolated from the blood samples was assayed for estradiol using the Coat-A-Count estradiol RIA kit (Siemens, Los Angeles, CA). All samples were assayed in duplicate. The average intra-assay coefficient of variation was 22%.

TUNEL assay

DNA fragmentation in apoptotic cells on normal ovarian tissue sections from a subset of each treatment group (n=6 each) was assessed by using the ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Billerica, MA) according to the manufacturer's instructions. Slides were scanned at 40X using the Aperio Scanscope (Aperio Inc., Vista, CA). Twenty random images of ovarian surface epithelium (OSE) and thirty random images of the ovarian body for each hen were obtained using the ImageScope Viewer program (Aperio Inc., Vista, CA). Positive staining of apoptotic nuclei was quantified in the images using the color range function of Adobe Photoshop. The intensity of positive staining was then graded on a scale from 0 to 3 (0=no staining; 1=low, 2=medium and 3=high intensity staining).

Statistical analysis

All tests were carried out using SAS version 9.2 with a significance level of $p<0.05$. The effect of treatment on ovarian cancer incidence was analyzed using proc GENMOD as recommended by staff at the Cornell Statistical Consulting Unit. Egg

production and plasma estradiol level among treatments were analyzed using proc MIXED. The effect of treatment on apoptosis in the OSE and the ovarian body was analyzed using proc GLM.

Results

Health parameters

A total of 71 out of 231 (31%) birds died before experiment termination (Table 3.1). A larger proportion of hens treated with MPA, either alone (37%) or in combination with estrogen (34%), died compared to the control (22%) or estrogen alone (30%) groups, however, we found no statistically significant effect of treatment on mortality. The higher mortality in the hens treated with MPA may be due to the fact that a higher dose of MPA (100 mg) was utilized at the beginning of the experiment. This dose was used in the Barnes et al study (2002) that also reported a higher mortality rate for hens treated with MPA (32%) compared to the control treatment [9]. The mechanism of this effect is unclear; however, MPA treatment can result in depression, polyuria, weight gain, liver damage and diabetes in birds [14]. We also observed no significant effect of treatment on body weight (Figure 3.1).

Table 3.1 Number of hens that died before experiment termination.

Treatment	Total hens	Mortality (%)
Control	59	13 (22)
MPA+E2	56	19 (34)
MPA	59	22 (37)
E2	57	17 (30)

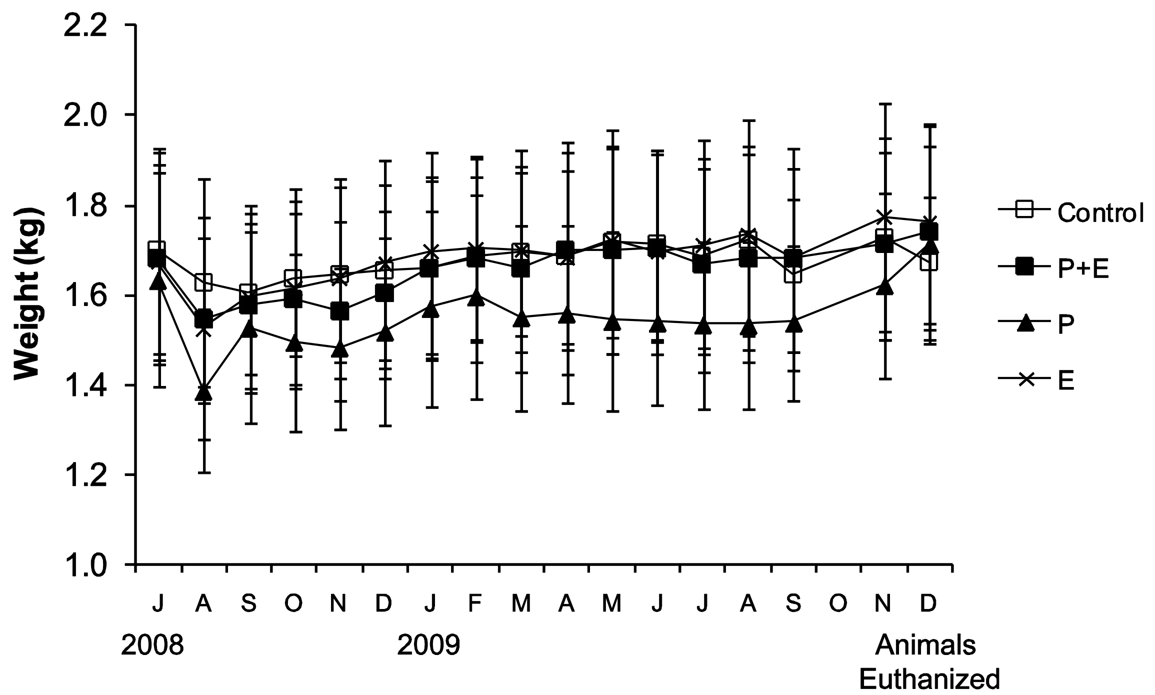

Figure 3.1 Effect of treatment on body weight (n=231). Hens were weighed approximately every 4 weeks. There was no effect of treatment on body weight. Bars indicate standard deviation.

Gross examination of chicken ovaries

Tumors in hens were identified grossly by the presence of firm, nodular outgrowths on the ovary, often accompanied by ascites and implants on the serosa of tissues within the abdominal cavity as described previously [5, 15-17]. Table 3.2 summarizes the data obtained at necropsy for the hens diagnosed with cancer. Seventeen and 19 ovarian tumors out of the 25 diagnosed also involved the oviduct and the intestines, respectively. There was a significant association of treatment with the presence of tumors in the intestine ($p<0.02$), but no association of treatment with oviductal tumors or ascites (Table 3.2). We also found no significant effect of treatment on stage of ovarian cancer (Table 3.3), and the majority of the tumors were late stage (metastases present outside of the ovary, with or without ascites). Hens were diagnosed at necropsy and confirmed by histology. Three tumors (all in the control group) were identified as early stage after examination of the H&E stained section. At necropsy, these ovaries had no visible signs of cancer, and two out of the three were regressed (no large or small yellow follicles present).

Table 3.2 Number of hens with ovarian cancer that involved the oviduct, intestines and ascites accumulation.

Treatment	Oviduct (%)	Intestine (%)	Ascites (%)
Control	6 (55)	6 (55)	3 (27)
MPA+E2	2 (100)	2 (100)[a]	2 (100)
MPA	1 (100)	0 (0)	0 (0)
E2	8 (73)	11 (100)[a]	6 (55)

[a] There is a significant increase in the number of hens with tumors involving the intestine compared to control ($p<0.02$)

Table 3.3 Stage of ovarian cancer [7] for hens diagnosed with the disease.

Treatment	Stage 1 (%)	Stage 2 (%)	Stage 3 (%)	Stage 4 (%)
Control	3 (30)	0 (0)	4 (40)	3 (30)
MPA+E2	0 (0)	0 (0)	0 (0)	2 (100)
MPA	0 (0)	0 (0)	1 (100)	0 (0)
E2	0 (0)	0 (0)	5 (45)	6 (55)

Cancer prevalence

We observed a significant effect of treatment on the prevalence of ovarian cancer ($p<0.01$; Table 3.4). There was a 19% prevalence of ovarian cancer in the control group. This is similar to the prevalence that has previously been reported (10-23%) in hens at this age [7, 18]. Treatment with MPA alone ($p<0.005$) and in combination with estrogen ($p<0.01$) significantly decreased prevalence of ovarian cancer compared to the control group. Administration of estrogen alone had no significant effect on cancer prevalence compared to the control treatment.

Histological classification of ovarian tumors

Microscopically, we identified three separate morphologies of tumors: endometrioid, serous, and "oviduct." Figure 3.2 illustrates examples of all three types. Endometrioid tumors resemble those found in women, and are generally solid with a dense stromal component. They can exhibit glands (Figure 3.2A), as well as squamous differentiation. Serous tumors also resemble those found in women and have very little stroma associated with the tumor cells (as opposed to endometrioid

tumors). These tumors can have papillary projections (Figure 3.2B). The third type, "oviduct", does not have a tumor counterpart in women. These tumors are associated with the production of secretory granules (Figure 3.2C) and resemble the oviduct in the chicken. They may represent a subtype of the other two main types, but this is not clear at this time. We found that hens treated with MPA, either alone or in combination with estrogen, did not develop the "oviduct" type of tumors (Table 3.5). In addition, there are a significantly greater number of hens with tumors that involve the intestines with serous type tumors compared to those with endometrioid type tumors (Table 3.6; $p<0.05$).

Egg production

Administration of MPA alone or in combination with estrogen significantly decreased egg production compared to the control treatment ($p<0.01$; Figure 3.3). There was no significant effect on egg production for the hens treated with estrogen alone compared to the control treatment.

Table 3.4 Prevalence of ovarian cancer among treatments.

Treatment	Total hens	Hens diagnosed with cancer (%)
Control	59	11 (19)
MPA+E2	56	2 (4)[a]
MPA	59	1 (2)[b]
E2	57	11 (19)

[a] Decreased prevalence compared to control ($p<0.01$)
[b] Decreased prevalence compared to control ($p<0.005$)

Plasma estradiol levels

Hens treated with estrogen alone ($p<0.01$) or in combination with MPA ($p<0.05$) had significantly higher plasma estradiol levels compared to the control group (Figure 3.4). As expected, treatment with MPA alone had no effect on plasma estradiol levels compared to the control treatment.

TUNEL assay

There was no significant effect of treatment on apoptosis in either the OSE (Figure 3.5A) or the ovarian body (Figure 3.5B) in normal (unaffected by ovarian cancer) hens.

Discussion

Our results suggest that ovulation is related to the incidence of ovarian cancer. We have shown that administration of progestin, either alone or in combination with estrogen, decreases ovarian cancer prevalence in the hen (Table 3.4). In fact, treatment with progestin alone, or in combination with estrogen, significantly reduced the risk by 91% and 81%, respectively. We also observed a significant reduction in ovulatory events (egg production) in hens treated with progestin alone or in combination with estrogen (Figure 3.3). These results extend a previous study in the hen. Barnes et al reported a 15% reduction in ovarian cancer risk in hens treated with MPA [9] and a significant decrease in egg production immediately after the progestin injection. This reduction of egg production lasted for approximately 4 weeks. It is possible that the marginal reduction in risk in the Barnes et al study (2002) was due to the fact that ovulation resumed for a significant portion of the experiment period. In contrast, we observed a consistent reduction in egg production throughout the treatment period and a greater reduction in risk.

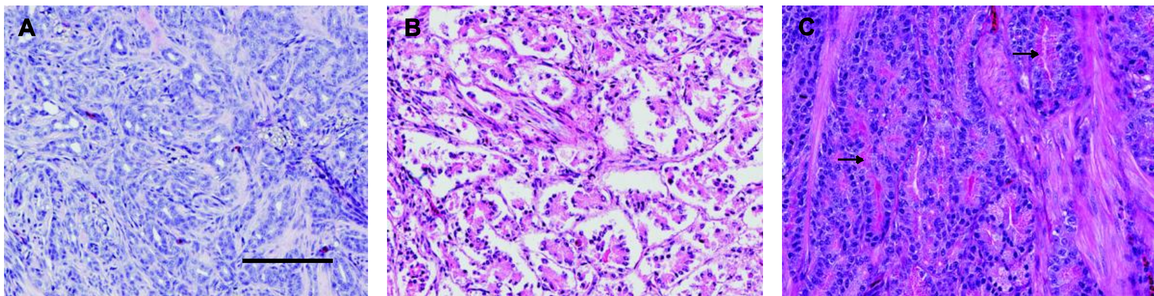

Figure 3.2 Representative H&E images of endometrioid (A), serous (B) and “oviduct” (C) types of chicken ovarian tumors. Note the secretory granules in the “oviduct” type tumors (C; arrows). Scale bar = 100µm.

Table 3.5 Histological subtypes of chicken ovarian tumors among treatments.

Treatment	Endometrioid (%)	Serous (%)	“Oviduct” (%)
Control	5 (45)	1 (9)	5 (45)
MPA+E2	1(50)	1 (50)	0 (0)
MPA	1 (100)	0 (0)	0 (0)
E2	3 (27)	4 (36)	4 (36)

Table 3.6 Number of hens with ovarian cancer that involved the oviduct, intestines and ascites accumulation based on tumor subtype.

Type	Oviduct (%)	Intestine (%)	Ascites (%)
Endometrioid	6 (60)	5 (50)	4 (40)
Serous	4 (67)	6 (100)[a]	1 (11)
Oviduct	7 (78)	8 (89)	5 (56)

[a] There is a significant increase in the number of hens with tumors that involve the intestines in the serous subtype compared to the endometrioid subtype ($p<0.05$)

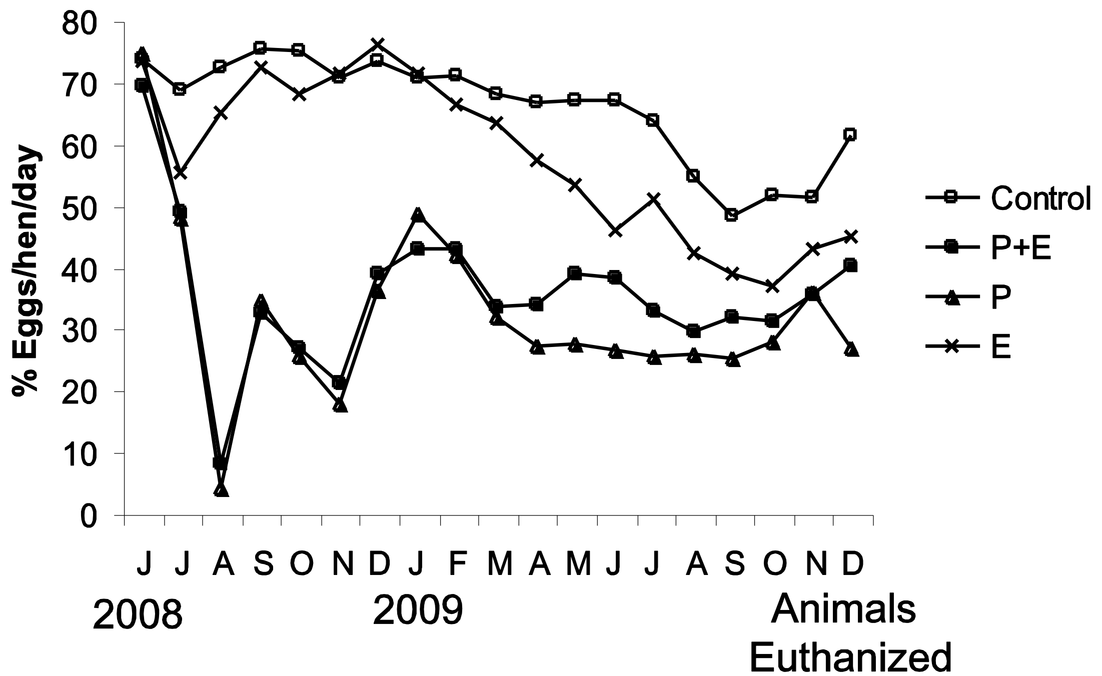

Figure 3.3 Effect of treatment on egg production (n=231). MPA+estrogen (P+E) or MPA (P) significantly decreased egg production compared to control ($p<0.01$). There was no significant effect on egg production for the hens treated with estrogen (E) compared to control.

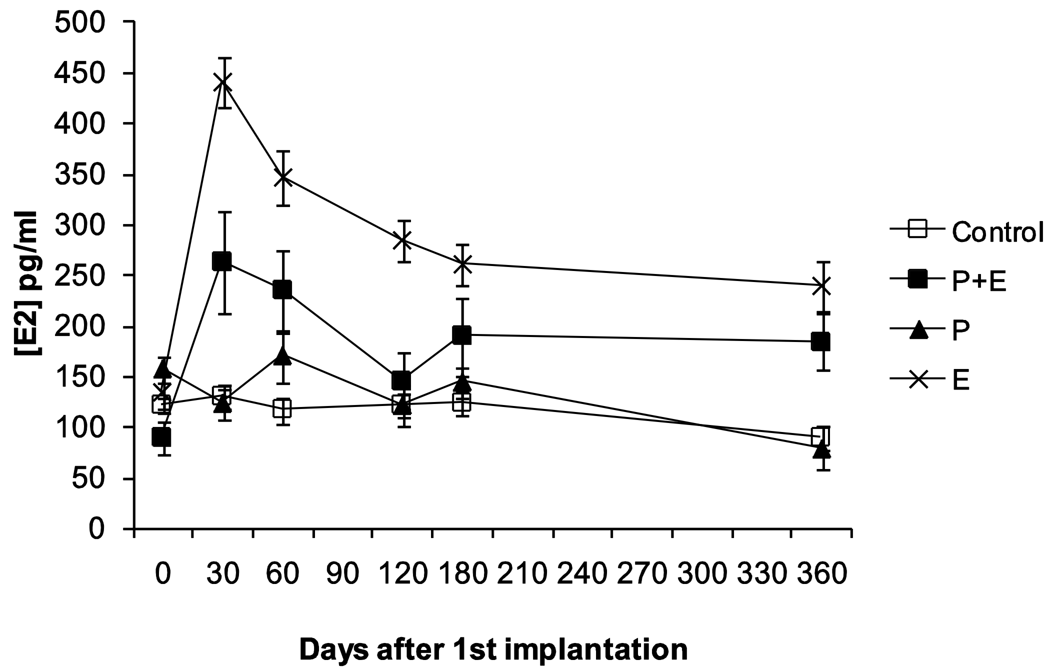

Figure 3.4 Effect of treatment on plasma estradiol levels (n=8-14 hens per treatment). E ($p<0.01$) or P+E ($p<0.05$) significantly elevated plasma estradiol levels compared to control. Treatment with P had no effect on plasma estradiol levels compared to control.

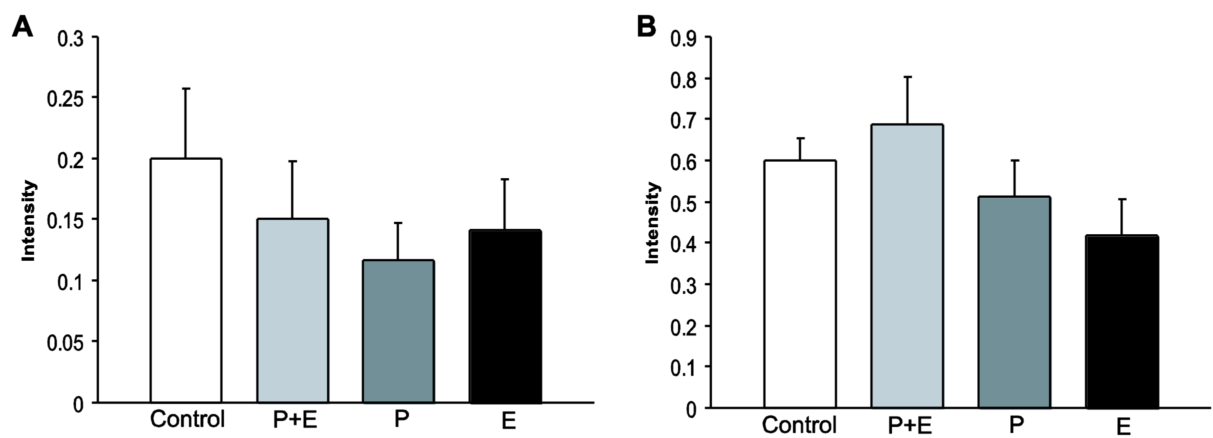

Figure 3.5 Effect of treatment on apoptosis in the OSE (A) and the ovarian body (B). There was no significant effect of treatment on apoptosis in the OSE or the ovarian body (n=6 per treatment).

Hormones are thought to affect the development and/or progression of ovarian cancer. Progesterone has been proposed to protect against ovarian tumor development [19]. This protective effect might be independent of the effect of progestin on ovulation. Interestingly, women on progestin-only formulations of oral contraceptives are also at reduced risk of ovarian cancer even though ovulation is only suppressed in about 40% of users [20]. In vitro studies have shown that progesterone inhibits cell growth and induces apoptosis in ovarian cancer cell lines [4], as well as inhibits growth of normal OSE [21]. One study reported that monkeys treated with a progestin exhibited increased apoptosis in the OSE compared to control and ethinyl-estradiol treated monkeys [22]. These studies suggest that the apoptotic effects of progestin might explain the protective effect observed with oral contraceptive use. In our study, however, we did not find a significant effect of progestin on apoptosis in the OSE (Figure 3.5A) or the ovarian body (Figure 3.5B) from normal hens. Therefore, it appears that, in the hen, induction of apoptosis does not account for the protective effect observed, although other mechanisms of progestin action may be involved. In addition to effects on apoptosis, progestins are also thought to affect proliferation, differentiation and invasiveness of cancer cells [23]. Further studies are needed to determine the mechanism of progestin action in the hen.

Interestingly, it appears that progestin administration hindered the development of tumors we have classified as "oviduct" type (Table 3.5). These tumors resemble normal chicken oviduct and are characterized by the presence of large amounts of secretory granules (Figure 3.2C). They may represent subtypes of the endometrioid or serous types also identified, higher degrees of tumor differentiation, or an alternative site of origin (like the oviduct). We observed nodules on the oviduct for seven out of the nine (78%) birds with the "oviduct" subtype, and this was similar to the percentage of hens with oviductal nodules in the other two subtype groups (Table 3.6). Since

most of the hens had ovarian tumors with involvement of the oviduct, it is not clear whether the tumors in any of the particular subtypes originated in the oviduct and then spread to the ovary. It should be noted that morphology alone is not sufficient to type ovarian tumors. Pathologists also use immunohistochemistry for molecular markers such as estrogen receptors, p53 and WT1 to distinguish between the subtypes [24, 25]. A similar approach could be taken to determine whether the "oviduct" type tumors are subtypes of the endometrioid and serous ovarian tumors in the hen. In contrast to other studies in the hen which identified all of the four ovarian tumor subtypes seen in women [15, 16, 26] we did not observe clear cell or mucinous tumors. This may reflect a strain-dependent susceptibility to different types of tumors, similar to the observation that some strains have a higher prevalence of ovarian cancer [7]. In addition, associations for some ovarian cancer risk factors differ by subtype in women [27], and this may be the case in hens as well. The high prevalence of endometrioid and serous tumors in the hen, make it a good model to study the etiology and progression of these subtypes.

In contrast to progesterone, estrogens are thought to promote ovarian tumor progression [28]. Studies have shown that women who undergo estrogen-only hormone replacement therapy have an increased risk of developing ovarian cancer [29-31]. In the current study, hens treated with estrogen (either alone or in combination with progestin) exhibited significantly increased plasma estradiol levels compared to control hens (Figure 3.4), but there was no effect of estrogen treatment on cancer prevalence (Table 3.4). We did, however, observe a significant increase in the number of hens with tumors that involved the intestines with estrogen treatment, either alone or in combination with MPA (Table 3.2). In addition, although not significant, a larger percentage of hens treated with estrogen exhibited serous type tumors (83%) versus hens that were not treated with estrogen (17%; Table 3.5). In humans, serous

tumors are considered more aggressive than the other subtypes and the association of estrogen treatment with intestinal nodules and the serous subtype suggests that estrogen may promote tumor progression in the hen. These results are similar to those seen in a recent study where exogenous estrogen was shown to accelerate the onset of ovarian tumor development and decrease survival in a mouse model of the disease [32]. Although we did not directly measure tumor onset in the current study, hens treated with estrogen alone exhibit a decline in egg production before the hens in the control group (Figure 3.3). Egg production has been shown to decline in hens with ovarian cancer [7] and it is possible that this decline in egg production signifies an earlier onset of the disease in these hens. Interestingly, we observed pre-neoplastic lesions in H&E sections from three hens treated with the combination of MPA and estrogen, but none in the other treatment groups, further supporting a role for estrogen in ovarian tumor progression (data not shown). Estrogens have been shown to promote cellular proliferation and inhibit apoptosis in ovarian cancer cells [23]. Similar to our results with progestin alone, we did not observe an effect of estrogen on apoptosis in the OSE (Figure 3.5A) or the ovarian body (Figure 3.5B) from normal hens. In our previous study (Chapter 2), we identified estrogen-regulated genes that are over-expressed in chicken ovarian tumors. Those results, combined with this current study, suggest that estrogen affects the progression of ovarian cancer in the hen.

Another study from our lab indicated an association between the number of ovulatory events and the risk of ovarian cancer. Giles et al reported a decreased incidence of ovarian cancer in restricted ovulator (RO) hens compared to wild-type (WT) hens [17]. RO hens have a mutation that affects their ability to incorporate yolk into the developing follicle and consequently ovulate significantly fewer times than their WT counterparts [33]. Interestingly, RO hens have a hormone profile associated

with high risk of ovarian cancer (low progesterone, high estrogen; [34, 35]) further suggesting that ovulation is linked to ovarian cancer risk. There was no difference, however, in overall ovulation rate between WT hens that were diagnosed with ovarian cancer versus normal WT hens [17]. This is similar to a previous report in the hen suggesting that ovulation rate was not the differentiating factor in high producing hens experiencing very frequent ovulatory events [5]. In the current study, we also did not find a significant difference in overall egg production in hens that did or not have ovarian cancer within the control group (data not shown). These findings suggest that other factors may modify the risk of ovarian cancer.

In the 1970's Fathalla proposed the "incessant ovulation" hypothesis [36]. This hypothesis is based on the idea that ovulation results in the repeated rupture and repair of the OSE. Over time, this cyclical damage could result in genetic mutations that predispose these cells to become cancerous. Women with high lifetime ovulation numbers would therefore have an increased risk of developing ovarian cancer. Epidemiological studies in women support this hypothesis. Pregnancy and oral contraceptive use are associated with decreased risk of ovarian cancer and both reduce the number of ovulatory events [37]. Our results in the hen also support this hypothesis since oral contraceptives (progestin alone or combined with estrogen) decreased the prevalence of ovarian cancer as well as egg production. Other studies in the hen have tested alternative chemopreventive agents, including aspirin [7] and flaxseed [8]. Administration of these agents resulted in a decrease in ovarian cancer stage, but no difference in prevalence. Interestingly, no effect on egg production was observed with these agents. This is in contrast to the significant decrease of ovarian cancer prevalence and egg production with progestin treatment, but no effect on stage. Our results indicate that ovulatory events might set the level of risk of the disease and other factors (such as hormones) may modulate this risk. Importantly, our results

support the use of oral contraceptives as chemopreventive agents for ovarian cancer, as well as the use of the hen as a model for chemoprevention research.

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CHAPTER 4

CHARACTERIZATION OF ESTROGEN RECEPTOR EXPRESSION IN AN ANIMAL MODEL OF OVARIAN CANCER

Abstract

There is growing evidence that estrogens may promote tumor progression, including ovarian tumors. Studies have shown that hens develop ovarian cancer spontaneously, therefore providing a suitable animal model for the disease. We have previously shown that estrogen-dependent genes are up-regulated in chicken ovarian tumors and that estrogen modulates tumor progression. Estrogens exert their actions in tissues through two different receptor subtypes (ESR1 and ESR2); therefore, our aim was to determine the expression of mRNA and protein for the estrogen receptor subtypes in ovaries of normal hens and ovaries from hens with ovarian cancer. Quantitative real-time PCR results showed that the relative mRNA expression of ESR1 and the ratio of ESR1/ESR2 are significantly greater, while relative ESR2 mRNA expression tended to be decreased in tissue from hens with ovarian cancer when compared to normal ovarian tissue. We also found differential ESR2 expression by tumor subtype in hens with metastatic cancer. By Western blot, we detected a single main transcript for chicken ESR1 and chicken ESR2 at approximately the expected size in normal ovarian tissue and tissue from hens with ovarian cancer. Immunohistochemical analysis showed abundant ESR1 in tumor tissue and limited ESR2 protein expression in tumors as compared to normal. Finally, we did not find a significant difference in plasma estradiol levels between normal hens and hens with ovarian cancer. These results suggest that, in the hen, ESR1 may be mediating a proliferative response in ovarian cancer cells. This similarity to human ovarian cancer supports the utility of the hen for testing possible endocrine therapies.

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 U.S. women. The American Cancer Society estimates that approximately 21,880 women in the U.S. will be diagnosed with ovarian cancer in 2010. Furthermore, ovarian cancer will account for more than 13,850 deaths among U.S. women in 2010. Currently, only 20% 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%. The fact that most ovarian cancers are diagnosed at later stages illustrates our lack of understanding of the etiology of the disease. This may be due, in part, to the limited availability of a suitable animal model for ovarian cancer.

One animal model, the domestic hen, has been shown to spontaneously develop ovarian cancer [1, 2]. Fredrickson (1987) found that approximately 24% of 466 hens developed malignant ovarian adenocarcinomas. He also observed that tumor development was age-dependent and uncommon in birds less than 2 years of age. We have also observed an increase in percentage of birds with ovarian cancer after 2 years of age [2]. Furthermore, hen tumors are positive for antibodies frequently used to characterize human ovarian tumors, including some useful in chemoprevention trials [3]. A previous study from our lab has also shown expression of an oviductal protein in hen ovarian tumors, suggesting that these tumors acquire characteristics of Müllerian-duct derived epithelia, similar to serous ovarian adenocarcinoma in women [4]. The hen thus provides a suitable animal model for the study of ovarian cancer.

Although the molecular mechanisms of ovarian cancer remain unclear, there is growing evidence that estrogens may be involved in tumor progression. Estrogens directly promote proliferation in ER positive ovarian cancer cell lines [5-7], with effects possibly being associated with cellular levels of estrogen receptors [5]. More

recently, one study confirmed that ovarian cancer cell lines with functional ER are growth responsive to estrogen and that estradiol mediates expression changes in genes that regulate the cell cycle, apoptosis, transcription and signaling [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 estrogen receptor (ESR1 and ESR2) encoded by separate genes, that are expressed in a tissue specific manner and thought to regulate differential gene functions. Interestingly, some studies have shown that the loss of ESR2 may be a common step in estrogen dependent ovarian tumor progression [10, 11]. Increased expression of ESR1 [10, 11] and an increase in the ratio of ESR1/ESR2 are also associated with tumor progression [12-14]. 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 [15]. In tumor cells, however, this balance is disrupted and cells are allowed to proliferate abnormally [15]. The importance of this disruption is evident in a recent study which reported that ESR1, but not ESR2, mediates estrogen-driven growth of epithelial ovarian cancer [8]. Combined, these studies lend support to the idea that the relative levels of ESR1 and ESR2 may be important regulators of cell growth.

To date, no studies have addressed estrogen receptor subtype mRNA and protein expression in ovarian cancer of the hen, the only animal model that develops a high incidence of the disease spontaneously. Our objective was to determine mRNA

and protein expression of ESR1 and ESR2 in normal ovarian tissue samples and tissue samples from hens with ovarian cancer. We also determined whether there were differences in plasma estradiol levels of normal hens and hens with ovarian cancer.

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 RNAlater (Ambion, Inc., Austin, TX) for real-time PCR analysis or were fixed and paraffin-embedded and used for immunohistochemistry. Normal oviduct tissue samples from a 3 year-old White leghorn hen was also collected, paraffin-embedded, and used as a positive control for immunohistochemistry. Blood samples from a subset of the normal hens (n=8) and hens with ovarian cancer (n=11) used for real-time PCR and immunohistochemistry, were collected at the time of tissue collection for radioimmunoassay (RIA) analysis of plasma levels of estradiol. Ovarian and oviduct tissue was collected from 3-4 year-old White leghorn hens, frozen on dry ice, and used as a control for Western blot analysis. Diagnosis was made by gross observation and confirmed by histological analysis of hematoxylin and eosin (H&E) stained sections. The H&E stained sections were also used to categorize the tumors based on the three types we have previously identified (unpublished data). These types include endometrioid tumors which are solid, with a dense stromal component, serous tumors with very little stroma associated with the tumor cells, and “oviduct” tumors that are associated with the production of secretory granules and resemble normal chicken oviduct. 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 into cDNA and the cDNA was used for quantitative PCR 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 control and run for each sample. Total volume of reactions was 50 μL with a final concentration of 1X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 900 nM of ER primer pairs (ESR1+2), 50 nM of 18S primer pair, and each respective TaqMan probe (at 250 nM of ER probes (ESR1+2) and 200 nM of 18S probe). 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 (also run in duplicate). The relative amount of mRNA in a particular sample was determined by the comparative threshold cycle (C_T) 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 from ovarian tissue samples was added to sample buffer and samples, including 25 ug of protein from oviduct as a positive control, were loaded onto 10% Tris-HCl gels (Pierce Biotechnologies, Rockford, IL) and run under denaturing conditions at 100V for 50 minutes. 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 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 in stripping solution (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. SDS-PAGE and Western blot were performed in duplicate.

Immunohistochemistry

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.

Estradiol radioimmunoassay (RIA)

Plasma isolated from blood samples 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%.

Statistics

Data were analyzed for significance with SAS using proc GLM with a significance level of $P < 0.05$. Duncan's multiple range tests were used to analyze means.

Results

Real-time quantitative PCR

Figures 4.1A and 4.1B show data for real-time quantitative PCR for ESR1 and ESR2, respectively. Results indicate that ESR1mRNA expression is significantly increased in ovarian tumor samples as compared to normal ovarian samples ($P < 0.01$). 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 ($P = 0.07$). Figure 4.1C shows that the ratio of ESR1/ERSR2 mRNA expression is significantly higher in ovarian tumor samples as compared to normal ovarian samples ($P < 0.02$).

Figure 4.2 shows ESR2 mRNA expression across for the three ovarian tumor subtypes previously identified by our lab: endometrioid, serous, and “oviduct”. We found that there is significantly more ESR2 expression in “oviduct” tumors compared to endometrioid and serous tumors ($p<0.05$). There was no difference in ESR1 expression by tumor subtype.

Western blot and immunohistochemistry

Figure 4.3A is a representative Western blot of ESR1 protein expression. A primary band appears close to the expected size (~67 kDa) in ovarian tissue samples from hens (lanes 1 and 2), as well as the positive control (oviduct; lane 3). Figure 4.3B illustrates representative immunohistochemistry 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. There is 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

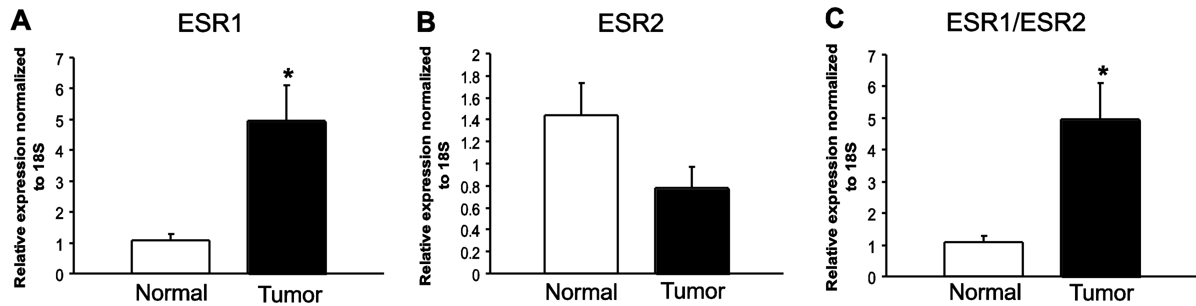

Figure 4.1 Estrogen receptor mRNA expression in ovaries with tumors (n=13) and normal ovaries (n=11). A. ESR1 mRNA expression. Asterisks indicate a significant increase in ovaries with tumors as compared to normal ovaries ($p < 0.01$). B. ESR2 mRNA expression. No significant difference was found ($p = 0.07$). C. Ratio of ESR1/ESR2 mRNA expression. Asterisks indicate a significant increase in ovaries with tumors as compared to normal ovaries ($p < 0.02$). Bars indicate mean ± standard error.

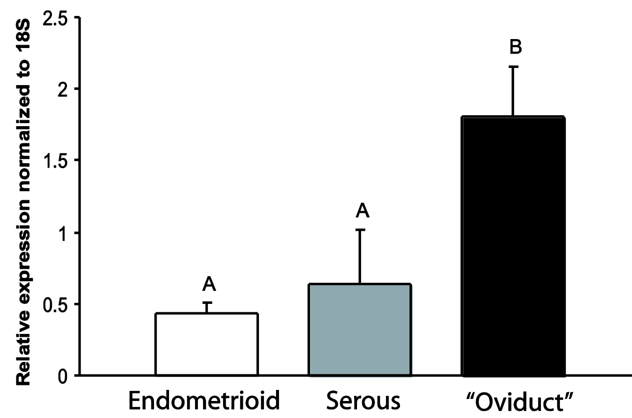

Figure 4.2 ESR2 mRNA expression in endometrioid, serous and "oviduct" tumors. Different letters indicate significant differences ($p<0.05$). Bars indicate standard error.

expression in the stroma and theca of normal ovarian tissue (Panel E arrows). ESR1 protein expression is seen 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. Panels G, H, and I are rabbit IgG controls for oviduct tissue, normal ovarian tissue, and tumor ovarian tissue, respectively. There is no specific staining evident in these sections, although there is some autofluorescence associated with red blood cells (arrowheads).

Figure 4.4A shows a representative Western blot of ESR2 protein expression. There is a single band at slightly higher than the expected size (~55 kDa) in ovarian tissue samples from hens (lanes 1 and 2), and in the positive control (oviduct; lane 3). Figure 4.4B illustrates representative immunohistochemistry 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 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). 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. Panels G, H, and I are rabbit IgG controls for oviduct tissue, normal ovarian tissue, and tumor ovarian tissue, respectively. Once again, there is no specific staining evident in these sections, although some autofluorescence of red blood cells is seen (arrowheads).

Estradiol radioimmunoassay (RIA)

Figure 4.5 shows that plasma estradiol levels are not significantly different between normal hens and hens with ovarian cancer ($p = 0.45$).

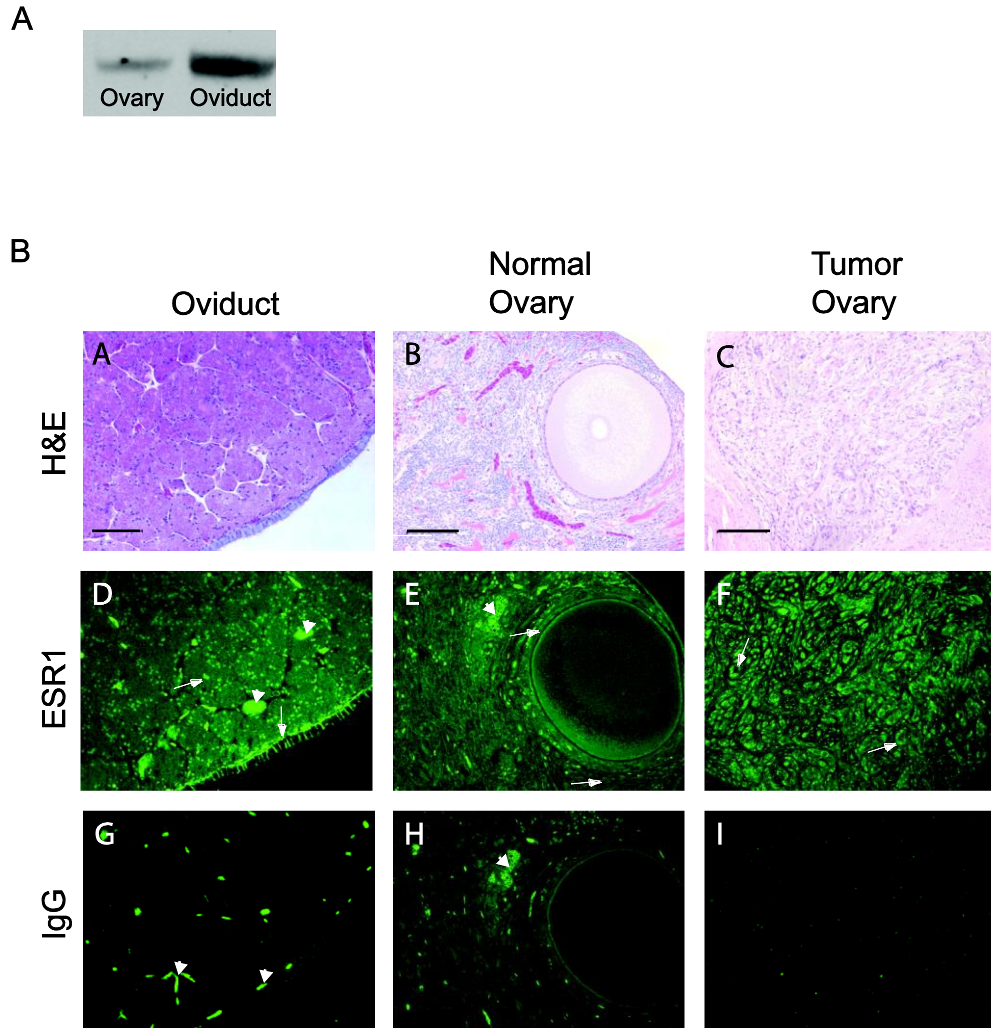

Figure 4.3 There is abundant ESR1 protein expression in chicken ovarian tumor tissue. A. Western blot of chicken ovarian tissue and oviduct confirming specificity of the antibody. B. Immunohistochemistry with ESR1 antibody. H&E staining of oviduct tissue (A), normal ovarian tissue (B) and ovarian tissue 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.

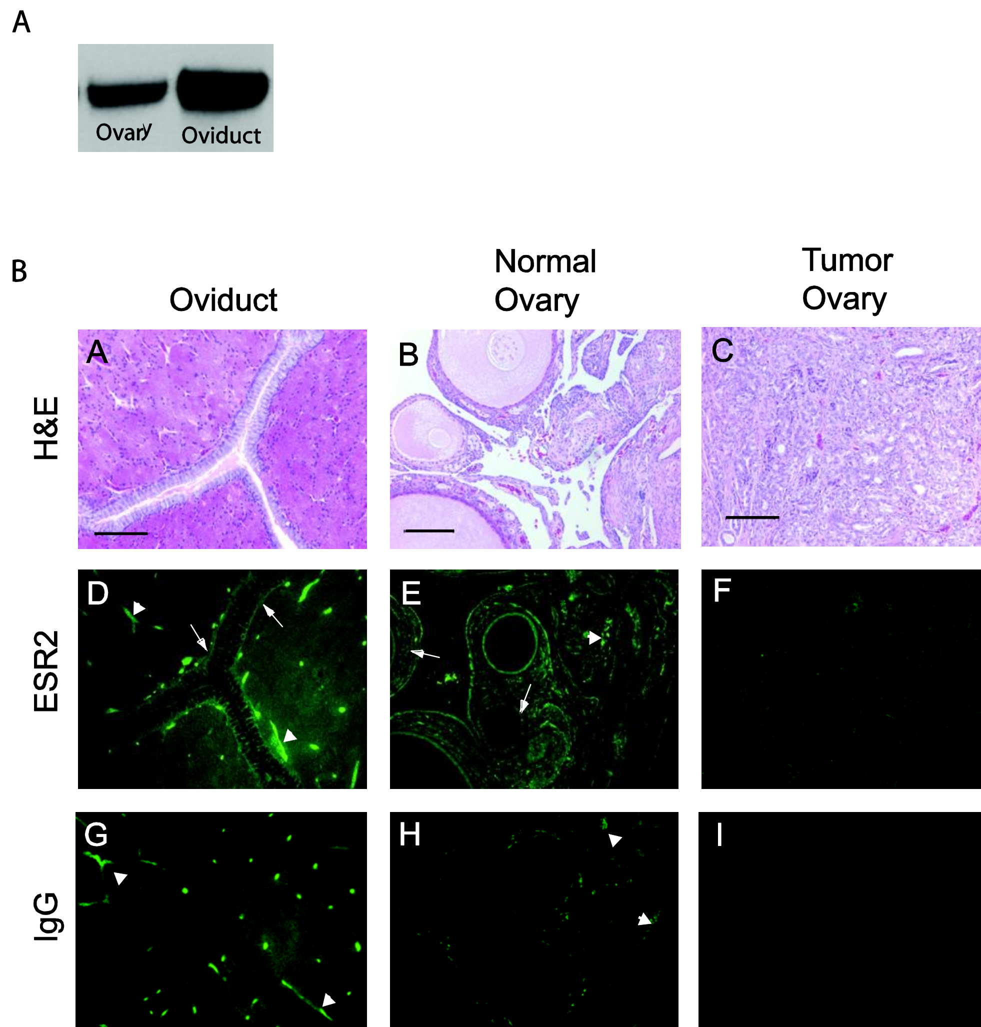

Figure 4.4 There is little to no expression of ESR2 protein expression in chicken ovarian tumor tissue. A. Western blot of chicken ovarian tissue and oviduct confirming specificity of the antibody. B. Immunohistochemistry with ESR2 antibody. H&E staining of oviduct tissue (A), normal ovarian tissue (B) 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.

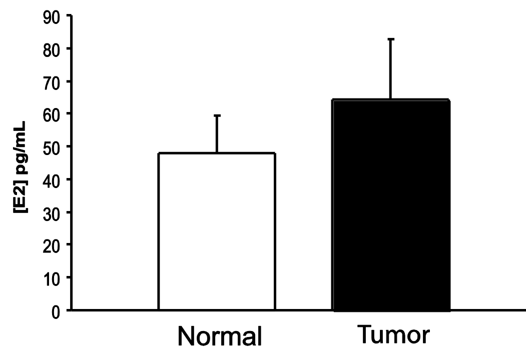

Figure 4.5 Plasma estradiol level of normal hens (n=8) and hens with ovarian cancer (n=11). No significant difference was found ($P = 0.45$). Bars indicate mean ± standard error.

Discussion

Our data indicate that ESR1 mRNA is significantly increased in ovaries from hens with ovarian cancer as compared to those from normal hens. This result has been seen in similar studies utilizing ovarian cancer tissue samples from women [10, 11]. Studies in women have also shown a significant decrease in ESR2 mRNA in ovarian tissue from women with ovarian cancer [11-13]. There is a trend toward decreased ESR2 mRNA in ovarian tissue from hens with ovarian cancer as compared to tissue from normal hens, but this trend does not reach significance. Our data also reveal that the ratio of ESR1/ESR2 mRNA is significantly increased in ovarian tissue from hens with cancer compared to tissue from normal hens. This result is similar to what has been previously reported in women [13, 14].

Estrogen receptor expression has been reported to be variable based on tumor subtype, with higher expression in serous and endometrioid subtypes [16-18]. We have observed a difference in ESR2 mRNA expression based on tumor subtype, with greater expression in "oviduct" tumors compared to endometrioid and serous types in hens with metastatic disease. This result is interesting because ESR2 expression is associated with longer disease-free survival and overall survival in women with ovarian cancer [19]. In addition, ESR2 has been shown to be a predictor of response to therapy in breast cancer [20]. These results suggest that "oviduct" tumors in the hen may be more responsive to endocrine therapies. Further characterization of this subtype is needed; however, it is clear that ovarian cancer subtypes and grades exhibit differences in receptor expression levels that may be exploited when developing therapeutic strategies.

It should be noted that 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) [21]. A conserved domain search through NCBI also identifies conserved domains of chicken ESR2 for ligand-binding and DNA-binding [22]. These conserved regions suggest that transcriptional action and regulation are similar between human and chicken estrogen receptor subtypes. This is an important consideration when using the hen to study the role of estrogen/estrogen receptor signaling in ovarian cancer because the different receptor subtypes signal in unique ways dictated by their interaction with different co-activators and co-repressors. Sequence similarity between human and chicken estrogen receptor subtypes suggest that these unique interactions would be conserved in the chicken.

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 ESR1 in avian oviduct [23, 24]. Our immunohistochemistry analysis illustrates the expression of ESR1 and ESR2 protein in the chicken. ESR1 and ESR2 protein expression is primarily nuclear, although some cytoplasmic expression is also seen (data not shown). Although estrogen receptor protein expression in normal ovarian tissue and tissue from hens with cancer was not quantified, ESR1 protein expression was widespread throughout the glandular areas comprised of tumor cells in the ovaries from hens with cancer. We observed limited ESR2 protein expression in ovaries from hens with cancer. These results are consistent with what is seen in humans.

We performed RIA analysis to determine whether estradiol levels may be regulating the increase in ESR1 mRNA and protein in hens with ovarian cancer. It is possible that estrogen may upregulate its receptor. We found no significant difference in plasma estradiol levels between normal hens and hens with ovarian cancer although we cannot completely rule out the possibility that estradiol does regulate ESR1 because of the variation in estradiol levels seen in our sample population. This variation is due to the fact that hens may have been at different points of their ovulatory cycle when they were euthanized. Estradiol levels fluctuate throughout the cycle, and increase 4-6 hours before the hen ovulates [25]. One would expect laying hens to have higher basal levels of circulating estradiol than non-laying hens. In fact, because they were older hens, only 38% (3/8) of normal birds were laying, while 9% (1/11) of the hens with ovarian cancer were ovulating. Our data would suggest that hens with ovarian cancer have a disproportionately higher level of plasma estradiol considering that a smaller percentage was ovulating when compared to normal hens.

Our study is the first to show mRNA and protein expression of estrogen receptor subtypes in ovarian cancer of the hen. We find ESR1 mRNA levels are significantly increased in ovarian tissue from hens with ovarian cancer as compared to tissue from normal hens. 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. It has been shown that ESR2 inhibits estradiol-stimulated breast cancer cell proliferation [26]. ESR2 is thought to play a protective role against the mitogenic activity of ESR1 [27] and has been proposed to be a possible tumor suppressor in ovarian carcinogenesis [28]. Interestingly, one study reported that ESR2 may modulate the transcriptional activity of ESR1, suggesting that the relative expression levels of these subtypes may determine cellular responses to agonists and antagonists [29]. 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 carcinogenesis.

Due to the complicated nature of estrogen receptor signaling, it is clear that further studies investigating the role and nature of ESR1 in ovarian cancer are warranted. We have previously shown that estrogen-dependent genes are up-regulated in chicken ovarian tumors (Chapter 2) and that exogenous estrogen modulates tumor progression (Chapter 3). These studies, combined with the altered estrogen receptor subtype expression reported here highlight the importance of estrogen/estrogen receptor signaling in ovarian cancer of the hen. 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.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

We provide evidence that ovulation is important for the initiation of ovarian cancer, and that tumors possibly arise from the epithelial cells of the oviduct. Furthermore, our studies support a role for steroid hormones, particularly estrogen, in mediating the progression of the disease. Figure 5.1 incorporates these data into a model for ovarian tumor development and progression of ovarian cancer of the hen.

Ovulatory events are important for the initiation of ovarian tumor development in the hen. We (Chapter 3) and others [1, 2] have shown that suppression of ovulation is associated with a decreased risk of the disease. Furthermore, other chemoprevention studies in the hen showed no reduction in the prevalence of ovarian cancer when there was no decrease in egg production, although there was a decrease in stage (progression) of the disease [3, 4]. Ovulation itself has been described as a pro-inflammatory process characterized by the local production and global recruitment of inflammatory factors to the site of ovulation [5]. These inflammatory factors are proposed to be mutagenic for OSE [6]; however, epithelial cells of the oviduct (either the fallopian tube in women or the infundibulum in chickens) can also be affected by these factors [5]. Figure 5.1A and Figure 5.1B illustrate the effect of ovulation on the oviduct epithelium and OSE, respectively.

In humans, high grade ovarian tumors exhibit mutations in p53 [7] and are hypothesized to originate in the fallopian tubes [8]. Interestingly, hen ovarian tumors also exhibit p53 mutations, suggesting that tumors in the hen may arise from the oviduct as well [9]. Our microarray analysis (Chapter 2) provides further support for an alternative site of origin of chicken ovarian tumors. We found that chicken ovarian

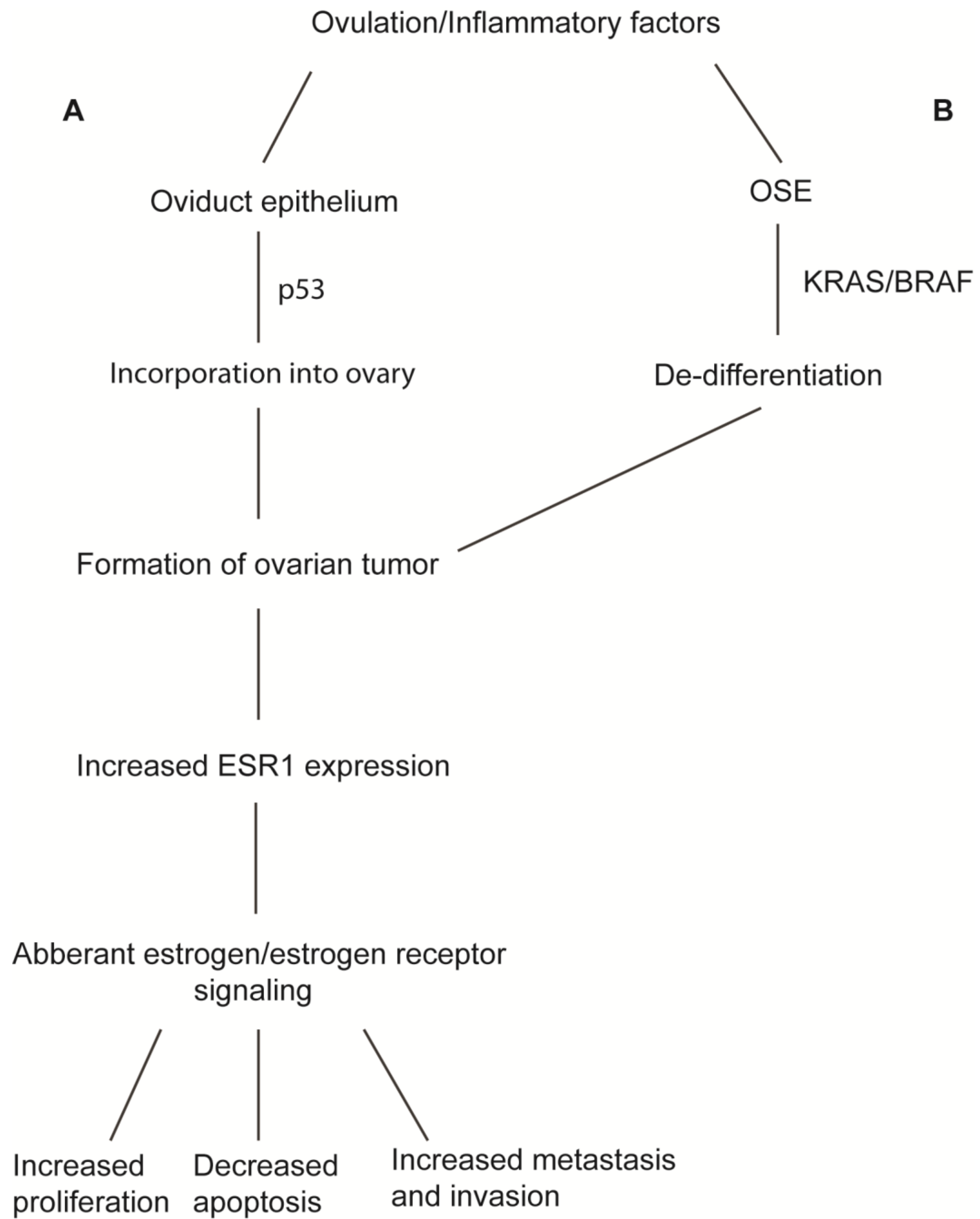

Figure 5.1 Model of ovarian tumor development and progression in the hen.

tumors express oviduct-related genes and that early stage ovarian tumors, but not OSE, express oviduct-related genes. If the oviduct is a site of origin, it is possible that inflammatory factors produced during ovulation induce mutations in p53 in oviduct epithelial cells (Figure 5.1A). These cells can then become incorporated into the ovary due to the intimate association between the infundibulum and ovarian surface, resulting in the development of an ovarian tumor (Figure 5.1A). Alternatively, inflammatory factors may induce mutations of genes, such as *Kras* and *Braf* (as seen in women) [7], which result in the de-differentiation of OSE and the formation of an ovarian tumor (Figure 5.1B). Cessation of ovulation could therefore reduce the local inflammatory reaction, possibly resulting in decreased formation of precursor lesions in the oviduct (or the de-differentiation of OSE) and a concomitant decreased prevalence of ovarian cancer.

Once ovarian cancer has developed, hormones, particularly estrogen, may modulate the growth of ovarian tumors. Tumor cells express estrogen receptors, in particular ESR1, and become responsive to estrogen produced locally in the ovary. Estrogen/estrogen receptor signaling can then mediate downstream effects such as proliferation, apoptosis, metastasis and invasion. We have shown increased expression of *Esr1* in chicken ovarian tumors compared to normal ovary (Chapter 4) and in early stage tumors compared to the surrounding stroma (Chapter 2). Interestingly, estrogen is thought to mediate its growth stimulatory effects through ESR1 [10]. We have also shown that estrogen-regulated oviduct-related genes are up-regulated in chicken ovarian tumors, even at early stages (Chapter 2). These results suggest that estrogen/estrogen receptor signaling is aberrant in ovarian cancer of the hen. This aberrant signaling may contribute to a variety of downstream effects. For instance, estrogen may play a role in the development and/or progression of specific subtypes of ovarian cancer. We observed that exogenous administration of estrogen

resulted in an increased prevalence of serous type tumors compared to control treatment, although this did not reach statistical significance (Chapter 3). Interestingly, studies have reported that serous ovarian tumors in women are more commonly estrogen receptor positive (along with endometrioid tumors) [11] and hence may be responsive to estrogen stimulation. Estrogen has also been shown to affect multiple cellular pathways in ovarian cancer cells, including proliferation, apoptosis, as well as metastasis and invasion [12]. Figure 5.1 summarizes the possible role of estrogen/estrogen receptor signaling in ovarian tumor progression.

Our studies have yielded information about ovarian tumor development and progression in the hen, but there are still questions to be answered. Future studies in the hen should focus on determining the site of origin of ovarian cancer. It is possible that chicken ovarian tumors may develop along two distinct pathways with different sites of origin, similar to tumors in women. These distinct tumor types may exhibit different gene mutations, behavior, and response to therapeutics; therefore, determining the site of origin would have implications for the diagnosis and treatment of ovarian cancer in women. Our study suggests that the oviduct may be an alternative site of origin, but we cannot rule out the possibility of de-differentiation of the OSE (Chapter 2). One way to examine whether ovarian tumors in the hen derive from the oviduct would be to study the infundibulum. In humans, there is an association between the presence of tubal intraepithelial carcinomas (TICs) in the fallopian tube and serous ovarian carcinoma [13]. In our study (Chapter 3), ovarian tumors are frequently accompanied by nodules in the oviduct, but we did not examine the infundibulum histologically to determine whether TICs exist. These studies could provide correlative evidence for the oviductal origin of chicken ovarian cancer.

Direct evidence for an oviduct source of ovarian tumors could be derived from studies in which normal oviduct epithelial cells (possibly tagged for visualization) are

incorporated into the ovary. The viability and proliferative capacity of these cells, as well as the ability of the cells to embed into the ovarian stroma and respond to steroid hormone signals could be monitored. Ultimately, it would be interesting to see if transplanted oviduct epithelial cells can form ovarian tumors, but this ability may be dependent on the nature of the transplanted cells. For instance, oviduct cells that exhibit p53 mutations (the "p53 signature") are proposed to represent the precursor of ovarian serous carcinomas [14] and these cells might prove to represent the precursor of chicken ovarian tumors as well. This type of experiment may not definitively determine whether chicken ovarian tumors do arise from the oviduct, but it can determined whether oviduct epithelial cells have the capacity to survive and thrive in the ovarian environment.

Since we have not ruled out the OSE as a source of chicken ovarian tumors at this time, further analysis of chicken OSE can be conducted in parallel. Studies of human ovarian cancer suggest that genetic alterations of the OSE may create a distinct population of differentiated cells that undergo metastasis (stochastic model) or a cancer stem cell population that initiates tumor development (hierarchical model) [reviewed in 15]. There is increasing evidence that cancer initiating cells, or cancer stem cells derived from somatic stem cells, may play a role in the initiation of ovarian cancer [reviewed in 15] and putative cancer stem cells have been identified in the OSE [reviewed in 16]. Furthermore, a recent study has shown that the OSE expresses genes associated with adult stem cell maintenance, supporting the idea that these cells are multipotent and capable of serving as a precursor for the multiple subtypes of ovarian cancer [17]. To date, only one study characterizing chicken OSE has been published [18], therefore it is clear that more research is needed to examine the possibility of the OSE as a site of origin of chicken ovarian cancer.

We have also provided evidence that ovulation is important for the initiation of ovarian tumor development (Chapter 3). Our study design, however, does not allow us to separate the effect of ovulation suppression from that of hormones on the prevalence of ovarian cancer in the hen. We have used hormones to decrease egg production and cannot rule out a direct effect of these hormones on cellular processes. Alternative methods, such as feed restriction or manipulation of photoperiod, can be used to decrease ovulatory events and assess the effect of ovulation on the prevalence of ovarian cancer. We also have not determined the mechanism of oral contraceptive action in the chicken ovary. As mentioned previously, administration of progestin in monkeys was associated with an increase of apoptosis in the OSE [19], and this has been proposed as the mechanism for the protective effect of oral contraceptives. Our studies suggest that there is no direct effect of progestin on ovarian cancer prevalence since we did not observe an effect on apoptosis in the OSE of hens treated with progestin (Chapter 3). We measured only one indicator of apoptosis (TUNEL), however, and did not examine other cellular pathways. It is possible that progestin administration affects other cellular pathways, such as wound healing. Proper wound healing would prevent the accumulation of cells (either from the oviduct or OSE) with inflammation-induced mutations. Interestingly, studies in sheep have shown that progesterone plays a role in PARP-mediated DNA repair [20], as well as DNA base excision repair [21] at the site of ovulation. Future studies in the hen are needed to determine the effect of progestins on ovarian cancer development.

Finally, we have provided evidence of a role for estrogen receptor signaling in ovarian cancer of the hen (Chapter 4), but it is currently not clear what the downstream effects of this signaling are. Studies could be designed to determine what cellular processes and pathways are affected by estrogen in order to identify possible therapeutic targets. For instance, global gene expression analysis could be conducted

utilizing the ovarian samples from our *in vivo* study (Chapter 3). Estrogen-regulated genes could be identified by comparing the ovarian tissue from control hens and those treated with estrogen. Once these genes are identified, they can be tested for oncogenic or tumor suppressor effects and the hen could be used to test putative therapeutics targeting these genes. Additional studies could be conducted to examine the nature of the estrogen receptor in chicken ovarian tumors. Estrogen receptors can signal through a variety of mechanisms including genomic and non-genomic pathways. Genomic effects are long-lasting and result in the transcription of target genes. Non-genomic pathways are rapid and result in the activation of membrane signaling pathways. Studies in women have shown that estrogen can initiate membrane signaling pathways that regulate cell proliferation and prevent apoptosis in breast cancer [reviewed in 22]. No studies to date, have examined the role of non-genomic estrogen signaling in ovarian cancer. Non-genomic effects are mediated by a membrane-associated estrogen receptor, so the cellular localization of the estrogen receptor in chicken ovarian tumors could be determined. If estrogen receptors are present on the membrane in tumors, the functionality of these receptors could be tested *in vitro*. In addition, the rapid responses mediated by estrogen receptor could be investigated. If non-genomic signaling in response to estrogen exists in ovarian cancer, these pathways represent other possible therapeutic targets as has been proposed for breast cancer [23].

In conclusion, ovarian cancer is a deadly disease that is only just beginning to be better understood. Studies in the hen can provide valuable information about the origin, development, and progression of ovarian cancer that may unlock the mysteries of ovarian cancer in women.

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APPENDICES

Appendix 1 Differentially expressed genes between ovarian tissue from hens with cancer (n=3) and tissue from normal hens (n=3). Asterisks indicate oviduct-related genes selected for further analysis. Fold change >1 indicates up-regulated genes and fold change <1 indicates down-regulated genes in chicken ovarian tumors compared to normal ovary.

Gene ID	Gene symbol	Gene name	Fold change	p-value
Gga.623.1.S1_at	SERPINB14*	Ovalbumin (OVAL)	267	0.00199
Gga.5085.1.S1_at	OVM*	Ovomucoid (OVM)	223	$8.69x10^{-5}$
GgaAffx.8146.1.S1_at	SERPINB14B*	Serpin peptidase inhibitor, clade B (ovalbumin), member 3 (SERPINB3)	134	0.00025
Gga.5527.1.S1_at	TSPAN1	Tetraspanin 1	102	0.00077
Gga.6723.1.S1_at	LOC422031	Similar to meleagrin	74	0.00017
Gga.8175.1.S1_x_at	RAP2B	RAP2B, member of RAS oncogene family	71	0.00029
Gga.7493.1.S1_at		Transcribed locus	69	0.00032
GgaAffx.7417.1.S1_at	LOC428451	Similar to prostatic acid phosphatase precursor	65	0.00219
Gga.6496.1.S1_at	GAL11	Gallicin 11	60	0.00021
Gga.2952.S1_s_at	TF*	Transferrin (lactotransferrin; LTF)	51	$4.49x10^{-5}$
Gga.2982.1.S1_at	CLDN3	Claudin 3	46	0.00538
Gga.2982.1.S1_at	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	40	0.00198
Gga.125.1.S1_at	LOC396449*	Riboflavin-binding protein (RD)	39	0.00522
Gga.6520.1.S1_at	KRT20	Keratin 20	37	0.00329
GgaAffx.3635.1.S1_at	LOC427400	Hypothetical LOC427400	36	0.00103
Gga.5848.3.S1_a_at	CLDN10	Claudin 10	35	0.00943
Gga.713.1.S1_at	LYZ	Lysozyme (renal amyloidosis)	28	0.00551
Gga.7931.1.S1_at		Transcribed locus	26	0.00527
GgaAffx.21785.1.S1_s_at	FOXA2	Forkhead box A2	19	0.00182
GgaAffx.21835.1.S1_s_at	GAL12	Beta-defensin 12	18	0.00229
Gga.4977.1.S1_at	MSX2	Msh homeobox 2	16	0.00893

Gga.313.1.S1_at	PAX2*	Paired box 2	16	0.00636
Gga.7195.1.S1_at	CMTM8	CKLF-like MARVEL transmembrane domain containing 8	13	0.00601
Gga.5856.1.S1_at	SLC9A2	Solute carrier family 9 (sodium/hydrogen exchanger), member 2	8	0.00037
Gga.16523.1.S2_s_at	ATP2A3	ATPase, Ca2+ transporting, ubiquitous	8	0.00910
Gga.7466.1.S1_at	FAM123A	Family with sequence similarity 123A	8	0.00830
Gga.2807.1.S1_at		EST,clone ChEST945a6	8	0.00515
Gga.2739.1.S1_at		EST,clone ChEST748p24	7	0.00453
GgaAffx.7428.1.S1_at	ACPP	Acid phosphatase, prostate	7	0.00577
Gga.8264.1.S1_at		Transcribed locus	7	$2.41x10^{-5}$
Gga.318.1.S1_at	KCNN2	Potassium intermediate/small conductance calcium activated channel, subfamily N member 2 SK2	7	0.00154
Gga.18351.1.A1_at	SULF1	Sulfatase 1	7	0.00274
Gga.19929.1.S1_at		EST,clone ChEST821a11	7	0.00552
GgaAffx.3636.1.S1_at	LOC427400	LOC427400	7	0.00680
Gga.2072.1.S1_a_at	CA8	Carbonic anhydrase 8	6	0.00235
GgaAffx.9042.3.S1_s_at	LOC427942	Similar to alpha-2-macroglobulin	6	0.00615
GgaAffx.7879.1.S1_at	LOC418038	Similar to IP4/PIP3 binding protein-like protein	5	0.00573
Gga.8064.1.S1_at	C9orf150	Chromosome 9 open reading frame 150	5	0.00259
GgaAffx.12270.1.S1_at	ATP13A4	ATPase type 13A4 RCJMB04_11o9	4	0.00296
GgaAffx.21637.1.S1_s_at	HNF1B	HNF1 homeobox B	4	0.00981
GgaAffx.24025.3.S1_at	SERPINB1	Serpin peptidase inhibitor, clade B (ovalbumin) pseudogene 1 SERPINB11	4	0.00910
Gga.12073.1.S1_a_at	KRT23	Keratin 23	4	0.00507
Gga.243.3.S1_a_at	NTM	Neurotrimin CEPU	4	0.00615
GgaAffx.20877.1.S1_at	SOX7	SRY (sex determining region Y)-box 7	4	0.00093
Gga.15641.1.S1_at		Transcribed locus	4	0.00796
Gga.10566.1.S1_at	CHRM5	Cholinergic receptor, muscarinic 5 CHKM5MR	3	0.00219
GgaAffx.24029.1.S1_s_at	MARVELD3	MARVEL domain containing 3	3	0.00577
Gga.15371.1.S1_at	LOC768772	LOC768772	3	0.00398
Gga.481.1.S1_at	FMOD	Fibromodulin	3	0.00887
Gga.7425.1.S1_at	ANXA8	Annexin A8	3	0.00658
GgaAffx.25068.3.S1_s_at	TBC1D8	TBC1 domain family, member 8	3	0.00771
GgaAffx.1913.1.S1_at	HOXB5	Homeobox B5	3	0.00721
Gga.14878.1.S1_at		EST,clone ChEST289i12	3	0.00978
Gga.3242.1.S1_at	LOC769185	Similar to Breast Carcinoma amplified sequence 1 (BCAS1)	3	0.00154
Gga.7207.1.S1_at		Transcribed locus	3	0.00910

Gga.7777.1.S1_at		Transcribed locus; strongly similar to NP_001161383.1	3	0.00361
Gga.14691.1.S1_at	TUFT1	Tuftelin 1	3	0.00230
Gga.6096.1.S1_at	EPB41L5	Erythrocyte membrane protein band 4.1 like 5	3	0.00535
Gga.5494.1.S1_at	CPE	Carboxypeptidase E	3	0.00328
Gga.8076.2.S1_a_at	GNAL	Guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type	3	0.00241
Gga.14228.1.S1_at		EST,clone ChEST496p9	3	0.00706
Gga.7379.1.S1_at		EST,clone ChEST53p22	3	0.00386
Gga.4730.1.S1_at	NAPB	N-methylmaleimide-sensitive factor attachment protein, beta	2	0.00795
GgaAffx.2745.1.S1_at	LUZP2	Leucine zipper protein 2	2	0.00109
Gga.9994.1.S1_at	RNF146	Ring finger protein 146	2	0.00023
GgaAffx.354.1.S1_at	LOC421173	Similar to leucine-rich repeat-containing G protein-coupled receptor 6	2	0.00240
Gga.12667.1.S1_at	FILIP1	Filamin A interacting protein 1	2	0.00837
Gga.10429.2.S1_a_at		EST, clone ChEST178l15	2	0.00834
GgaAffx.5710.2.S1_s_at	NPDC1	Neural proliferation, differentiation and control, 1	2	0.00341
Gga.2899.1.S1_at		EST,clone ChEST380f7	2	0.00499
Gga.9519.1.A1_at	LOC420552	Hypothetical gene supported by BX929555	2	0.00420
Gga.14236.1.S1_at		EST, clone ChEST123g6	2	0.00903
Gga.10365.1.S1_at		EST, clone ChEST662h4	2	0.00209
GgaAffx.12013.1.S1_s_at	CAT	Catalase RCJMB04_1j22	2	0.00447
GgaAffx.3186.1.S1_at	DRD4	Dopamine receptor D4	2	0.00774
GgaAffx.4323.1.S1_at	TMC5	Transmembrane channel-like 5	2	0.00691
GgaAffx.20372.1.S1_at		EST, clone ChEST915k14	2	1.93×10^{-5}
GgaAffx.7275.1.S1_at	RPS6KA2	Ribosomal protein S6 kinase, 90 kDa, polypeptide 2	2	0.00576
GgaAffx.8253.1.S1_at	CACNA1C	Calcium channel, voltage-dependent, L type, alpha 1C subunit CHCACHA1C	2	0.00842
Gga.8900.1.S1_a_at	ZNF711	Zinc finger protein 711 ZNF6	2	0.00022
GgaAffx.21089.1.S1_at	LMO7	Lim domain 7	2	0.00821
Gga.9403.1.S1_a_at	CCDC109B	Coiled-coil domain containing 109B	2	0.00130
Gga.14898.1.S1_at		EST, clone ChEST649o12	2	0.00306
Gga.12534.1.S1_at	LXN	Latexin	2	0.00112
Gga.13321.2.S1_a_at	PHB	Prohibitin	2	0.00856
Gga.14559.1.S1_at		EST, clone ChEST47i6	2	0.00048
GgaAffx.20465.1.S1_at		EST, clone ChEST765p22	2	0.00556
GgaAffx.25114.3.S1_s_at	MBNL2	Muscleblind-like 2 (Drosophila)	2	0.00010
GgaAffx.1062.1.S1_at	CAMKK1	Calcium/calmodulin-dependent protein kinase kinase 1, alpha	2	0.00625
Gga.15005.1.S1_at		EST, clone ChEST1030n3	2	0.00495
Gga.5576.2.S1_at	LOC770142	Similar to histone H4	2	0.00233

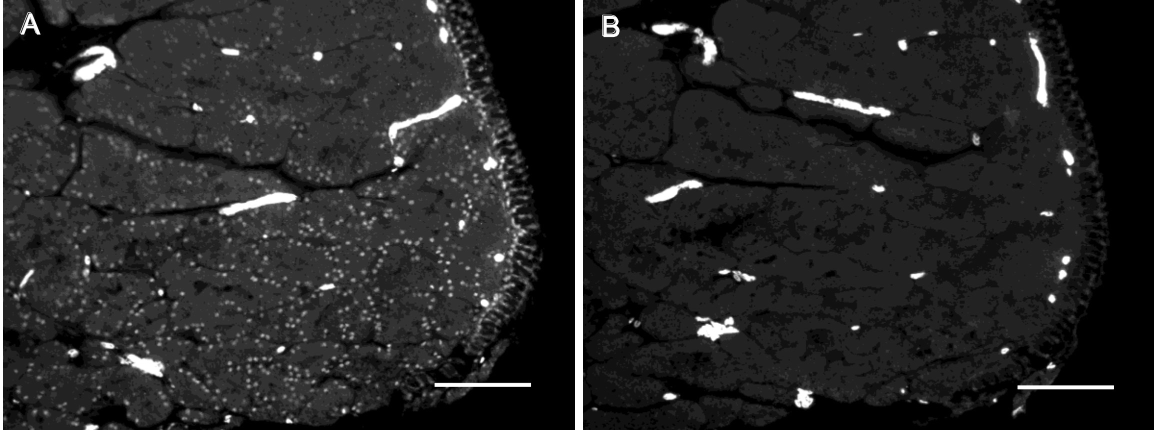
GgaAffx.5604.1.S1_at	PLCB4	Phospholipase C, beta 4	2	0.00861
Gga.18457.1.S1_at		EST, clone ChEST629l13	2	0.00649
Gga.183.1.S1_at	UGT8	UDP glycosyltransferase 8	2	0.00207
Gga.324.1.S1_at	LOC395589	Myeloid ectopic viral insertion site-1a protein	2	0.00309
Gga.8900.2.S1_a_at		EST, clone ChEST984i16	2	0.00991
Gga.10682.1.S1_at		EST, clone ChEST964e13	2	0.00293
Gga.2733.1.S1_at	CNR1	Cannabinoid receptor 1 (brain)	2	0.00087
Gga.12356.2.S1_a_at		EST, clone ChEST870g24	2	0.00637
Gga.15231.1.S1_at	C21orf70	Chromosome 21 open reading frame 70	2	0.00617
Gga.12036.1.S1_at	EHF	ETS homologous factors	2	$5.79x10^{-5}$
GgaAffx.10063.1.S1_at	C6orf165	Chromosome 6 open reading frame 165	2	0.00366
Gga.8542.3.S1_a_at	ATP50	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	2	0.00573
GgaAffx.7270.1.S1_at			2	0.00653
Gga.10212.1.S1_at		EST, clone ChEST1026b10	2	0.00564
Gga.5218.1.S1_at	GKAP1	G kinase anchoring protein	2	0.00336
GgaAffx.20956.1.S1_at	C1orf34	Chromosome 1 open reading frame 34	2	0.00856
GgaAffx.21665.1.S1_at		EST, clone ChEST743l10	2	0.00459
GgaAffx.12102.1.S1_s_at	PMS1	Postmeiotic segregation increased 1	2	0.00018
GgaAffx.22140.1.S1_at	LOC425659	LOC425659	2	0.00487
Gga.17189.1.S1_at		EST, clone ChEST536g4	2	0.00786
GgaAffx.1456.1.S1_at			2	0.00071
GgaAffx.858.1.S1_s_at	ZNF652	Zinc finger protein 652	2	0.00415
Gga.15876.1.S1_at	LRIG3	Leucine-rich repeats and immunoglobulin-like domains 3	2	0.00560
Gga.12927.1.S1_at	DNAJC11	DnaJ (Hsp40) homolog, subfamily C, member 11	2	0.00214
Gga.17291.1.S1_at	C1orf168	Chromosome 1 open reading frame 168	2	0.00503
GgaAffx.2157.2.S1_s_at	CA12	Carbonic anhydrase XII	2	0.00323
GgaAffx.11083.1.S1_at	FGF23	Fibroblast growth factor 23	2	0.00026
GgaAffx.3994.1.S1_s_at	PANK1	Panthothenate kinase 1	2	0.00224
Gga.979.1.S1_at	PCK2	Phosphoenolpyruvate carboxykinase PEPCK-M	2	0.00967
Gga.279.1.S1_at	DTX4	Deltex homolog 4 (Drosophila)	2	0.00304
Gga.7002.1.S1_at	FBXW8	F-box and WD repeat domain containing 8	2	0.00684
GgaAffx.7745.3.S1_s_at	PDE1C	Phosphodiesterase 1C, calmodulin-dependent 70 kDa	2	0.00796
Gga.10214.1.S1_at	MAPK12	Mitogen-activated protein kinase 12	2	0.00029
GgaAffx.8959.1.S1_s_at	TEC	Tec protein kinase RCJMB04_29e6	2	0.00504
Gga.9636.1.S1_at	SNORA32	Small nucleolar RNA, H/ACA box 32 JOSD3	2	0.00839
Gga.6364.1.S1_at	CDCA7L	Cell division cycle associated 7-like RCJMB04_35j20	2	0.00338
GgaAffx.26459.1.S1_s_at	USP54	Ubiquitin specific peptidase 4	2	0.00532

GgaAffx.26404.1.S1_at	NDST2	N-deacetylase/N-sulfotransferase (heparin glucosaminyl) 2	2	0.00713
Gga.16148.1.S1_at		Transcribed locus	2	0.00184
Gga.15463.1.S1_at	LOC769636	LOC769636	2	0.00093
Gga.1743.1.S1_at	LOC769944	LOC769944	2	0.00164
GgaAffx.6654.1.S1_at	NRXN3	Neurexin 3	2	0.00171
GgaAffx.20862.1.S1_s_at	PPAP2B	Phosphatidic acid phosphatase type 2B	2	0.00492
GgaAffx.21067.1.S1_at		EST, clone ChEST867d17	2	0.00467
Gga.5301.1.S1_at	FAHD1	Fumarylacetoacetate hydrolase domain containing 1	2	0.00171
Gga.9005.2.S1_a_at	LOC422424	Similar to growth and transformation-dependent protein	2	0.00213
Gga.3136.1.S1_at	SDC3	Syndecan 3	2	0.00863
Gga.10477.1.S1_at	TNFRSF19	Tumor necrosis factor receptor superfamily, member 19	2	0.00672
Gga.6284.2.S1_a_at	ECHDC3	Enoyl Coenzyme A hydratase domain containing 3	2	0.00928
GgaAffx.8204.2.S1_s_at	ZBTB2	Zinc finger and BTB domain containing 2 RCJMB04_22k16	2	0.00676
Gga.9292.1.S1_at	EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2 PKR	2	0.00279
		EST, clone ChEST321m13	0.66	0.00824
Gga.1647.1.S1_at		EST, clone ChEST712h12	0.66	0.00557
Gga.9917.1.S1_at	CBLL1	Cas-BR-M (murine) ectopic retroviral transforming sequence-like 1 RCJMB04_31p12	0.66	0.00648
GgaAffx.11134.1.S1_at	SPSB4	SplaA/ryanodine receptor domain and SOCS box containing 4	0.66	0.00155
Gga.19160.1.S1_s_at	SLC10A7	Solute carrier family 10, member 7 RCJMB04_18a5	0.66	0.00212
GgaAffx.12454.1.S1_s_at	PPT1	Palmitoyl-protein thioesterase 1 RCJMB04_15o4	0.66	0.00528
GgaAffx.11537.1.S1_s_at	LGTN	Ligatin RCJMB04_2e17	0.65	0.00664
GgaAffx.7884.1.S1_at	DDHD1	DDHD domain containing 1	0.65	0.00899
GgaAffx.1335.2.S1_s_at	RNF157	Ring finger protein 157	0.65	0.00083
Gga.9574.1.S1_at	LOC419390	Similar to enhancer of split related protein-7	0.65	0.00152
Gga.10127.1.S1_at	LOC423499	Similar to SERTA domain-containing protein 2	0.65	0.00197
Gga.14609.1.S1_at	HERC4	Hect domain and RLD 4 RCJMB04_3p16	0.64	0.00314
GgaAffx.6768.1.S1_s_at	SDCCAG8	Serologically defined colon cancer antigen 8	0.64	0.00499
Gga.12287.1.S1_at		EST, clone ChEST209e2	0.64	0.00604
GgaAffx.21118.1.S1_s_at	LOC770402	Similar to long microtubule-associated protein 1A	0.63	0.00109
GgaAffx.12114.1.S1_at	HAUS6	HAUS augmin-like complex, subunit 6 RCJMB04_9g9	0.63	0.00443
GgaAffx.12080.1.S1_s_at	DCK	Deoxycytidine kinase RCJMB04_2e2	0.63	0.00344
GgaAffx.5670.1.S1_at	WNT16	Wingless-type MMTV integration site family, member 16	0.62	0.00853
Gga.17993.1.S1_at	ZC3HAV1	Zinc finger CCCH-type antiviral 1 RCJMB04_23i8	0.62	0.00681

GgaAffx.11665.1.S1_at	ITGA9	Integrin, alpha 9 RCJMB04_3g3	0.62	0.00978
Gga.11861.1.S1_at	TRNT1	tRNA nucleotidyl transferase, CCA-adding, 1	0.62	0.00457
GgaAffx.10761.1.S1_s_at	SH3RF3	SH3 domain containing ring finger 3 SH3MD4	0.62	0.00490
GgaAffx.20976.1.S1_at		EST, clone ChEST755g14	0.62	0.00622
Gga.5528.1.S1_at	CTBP2	C-terminal binding protein 2	0.62	0.00565
Gga.16897.1.S1_at	ETAA1	Ewing tumor-associated antigen 1	0.62	0.00185
Gga.9048.1.S1_at		Transcribed locus	0.62	0.00993
Gga.7465.1.S1_at		Transcribed locus	0.62	0.00166
Gga.2498.1.S1_at		EST, clone ChEST730j18	0.61	0.00316
GgaAffx.5168.2.S1_s_at	TRIM36	Tripartite motif-containing 36	0.61	0.00586
Gga.5692.1.S1_at	NDUFB4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 4, 15kDa	0.61	0.00844
GgaAffx.11745.1.S1_s_at	BIN1	Bridging integrator 1 RCJMB04_4n21	0.61	0.00245
GgaAffx.26778.1.A1_s_at			0.61	0.00341
GgaAffx.21368.1.S1_at	NPTN	Neuroplastin SDFR1	0.61	0.00258
GgaAffx.3618.1.A1_at	LOC776960	LOC776960	0.61	0.00029
GgaAffx.24513.9.S1_s_at	EPB41L3	Erythrocyte membrane protein band 4.1-like 3	0.61	0.00349
Gga.16540.1.S1_s_at	HELLS	Helicase, lymphoid specific RCJMB04_11c16	0.61	0.00243
GgaAffx.9985.1.S1_s_at	ROD1	Regulator of differentiation 1 (S. pombe)	0.60	0.00813
Gga.7965.1.S1_at	RAMP3	Receptor (G protein-coupled) activity modifying protein 3	0.60	0.00813
GgaAffx.20451.1.S1_s_at		EST, clone ChEST905o6	0.59	0.00696
Gga.13301.1.S1_at	GDPD5	Glycerophosphodiester phosphodiesterase domain containing 5 GDE2	0.59	0.00029
Gga.249.1.S1_at	SLC6A2	Solute carrier family 6, member 2	0.59	0.00585
GgaAffx.3906.1.S1_s_at	GALNS	Galactosamine (N-acetyl)-6-sulfate sulfatase	0.59	0.00663
Gga.9326.2.S1_at	LOC768982	LOC768982	0.59	0.00946
GgaAffx.20545.1.S1_at		EST, clone ChEST1015n20	0.59	0.00646
GgaAffx.12860.1.S1_at	TXNDC5	Thioredoxin domain containing 5 RCJMB04_24o2	0.58	0.00274
GgaAffx.20498.1.S1_s_at	MMD	Monocyte to macrophage differentiation-associated RCJMB04_11o17	0.58	0.00406
GgaAffx.9461.1.S1_at			0.58	0.00724
Gga.20014.1.S1_s_at	DNER	Delta/notch-like EGF repeat containing	0.58	0.00105
Gga.14884.1.S1_s_at	CCDC88A	Coiled-coil domain containing 88A RCJMB04_18n22	0.57	0.00423
Gga.5537.1.S1_at	FUNDC1	FUN14 domain containing 1	0.57	0.00632
GgaAffx.9094.1.S1_s_at	RELL1	RELT-like 1 RCJMB04_24l24	0.57	0.00884
Gga.4724.1.S2_at	HSP90B1	Heat shock protein 90kDa beta (Grp94), member 1 hsp108	0.57	0.00183
Gga.1182.1.S1_s_at	MOV10	Mov10, Moloney leukemia virus 10, homolog RCJMB04_11i10	0.57	0.00698
Gga.20082.1.S1_at		EST, clone ChEST846a5	0.56	0.00801

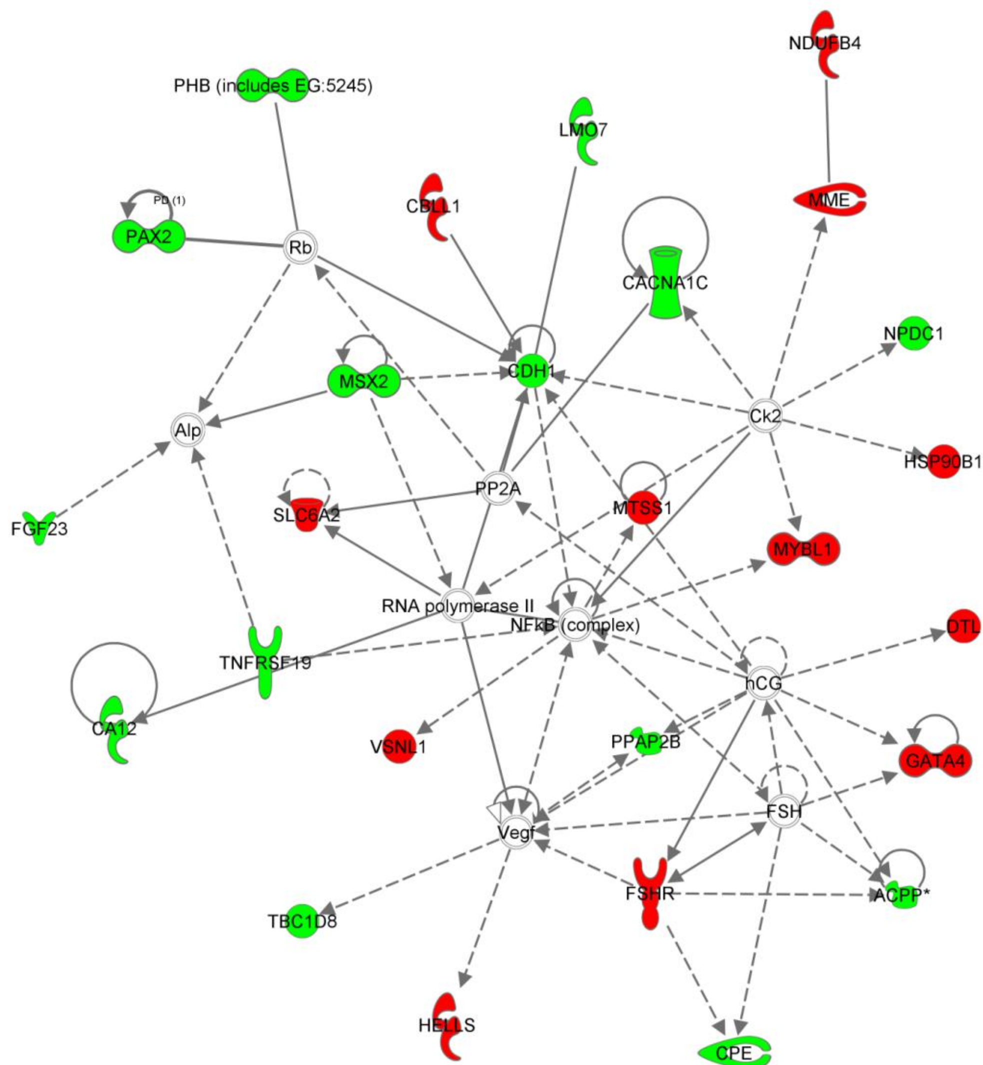
Gga.18136.1.S1_at	ZDHHC21	Zinc finger, DHHC-type containing 21 RCJMB04_10p19	0.56	0.00637
GgaAffx.6392.1.S1_at	DMRT3	Doublesex and mab-3 related transcription factor 3	0.56	0.00066
Gga.9228.1.S1_at	CAMSAP1L1	Calmodulin regulated spectrin-associated protein 1-like 1	0.56	0.00495
Gga.14837.1.S1_at		EST, clone ChEST14i12	0.56	0.00302
GgaAffx.1633.1.S1_at	BDP1	B double prime 1, subunit of RNA polymerase III transcription initiation factor IIIB	0.55	0.00224
GgaAffx.20969.1.S1_at		EST, clone ChEST322a24	0.55	0.00524
GgaAffx.20558.1.S1_at	CHD1	Chromodomain helicase DNA binding protein 1	0.55	0.00363
GgaAffx.20217.1.S1_at	LOC418900	LOC418900	0.55	0.00691
Gga.19070.1.S1_s_at		Transcribed locus	0.54	0.00662
Gga.6321.1.S1_a_at	UBE2T	Ubiquitin-conjugating enzyme E2T	0.54	0.00716
GgaAffx.25800.4.S1_at	LOC776927	Similar to HBxAg transactivated protein 2	0.54	0.00909
GgaAffx.5271.1.S1_at	MAGI2	Membrane associated guanylate kinase, WW and PDZ domain containing 2	0.53	0.00030
Gga.5416.1.S1_at		EST, clone ChEST601e1	0.53	0.00937
Gga.12594.1.S1_at	LOC424461	Similar to TGF-beta type II receptor	0.53	0.00580
GgaAffx.5267.1.S1_at	LMCD1	LIM and cysteine-rich domains 1	0.53	0.00187
GgaAffx.22895.1.S1_at	EPB41L1	Erythrocyte membrane protein band 4.1-like 1	0.52	0.00637
Gga.475.1.S2_at	FSHR	Follicle stimulating hormone receptor	0.52	0.00933
Gga.487.1.S1_at	SEMA3A	Sema domain, immunoglobulin domain (IgG),short basic domain, secreted, (semaphoring) 3A	0.51	0.00228
Gga.8061.1.S1_at	HSCB	HscB iron-sulfur cluster co-chaperone homolog (E. Coli)	0.51	0.00654
Gga.795.1.S1_at	GATA4	GATA binding protein 4 GATA-4	0.51	0.00212
Gga.7011.1.S1_a_at	LOC419195	Similar to cAMP-dependent protein kinase inhibitor gamma	0.51	0.00444
Gga.18592.1.S1_at		EST, clone ChEST194e10	0.51	0.00191
GgaAffx.7674.1.S1_at	AOAH	Acyloxyacyl hydrolase (neutrophil)	0.51	0.00032
GgaAffx.25748.2.S1_s_at	COL4A3	Collagen, type IV, alpha 3	0.51	0.00868
GgaAffx.7149.1.S1_at	PLCL2	Phospholipase C-like 2	0.50	0.00764
Gga.8200.1.S1_s_at	SLC16A10	Solute carrier family 16, member 10	0.50	0.00337
Gga.19591.1.S1_at	LOC428770	Prematurely terminated mRNA decay factor-like	0.50	0.00757
GgaAffx.21823.1.S1_s_at	LOC414835	cHz-cadherin	0.50	0.00638
Gga.12991.1.S1_at		EST, clone ChEST397k15	0.49	0.00892
Gga.15184.1.S1_at		EST, clone ChEST843h5	0.49	0.00925
Gga.8510.1.S1_at		Transcribed locus, strongly similar to NP_009016.1	0.49	0.00936
Gga.18929.1.S1_s_at	WIPF1	WAS/WASL interacting protein family, member 1 RCJMB04_9m19 WASPIP	0.48	0.00638
GgaAffx.12599.1.S1_s_at	TMEM68	Transmembrane protein 68 RCJMB04_19b17	0.48	0.00115
GgaAffx.12419.1.S1_s_at	DTL	Denticleless homolog (Drosophila) RCJMB04_15a2	0.47	0.00863

Gga.8786.1.S1_at	LGR4	Leucine-rich repeat-containing G protein-coupled receptor 4	0.46	0.00105
Gga.4505.1.S2_at	MYBL1	Myeloblastosis oncogene-like 1	0.45	0.00543
GgaAffx.6177.1.S1_at	GNS	Glucosamine (N-acetyl)-6-sulfatase	0.44	0.00304
Gga.15893.1.S1_at		EST, clone ChEST114c13	0.44	0.00102
GgaAffx.5110.6.S1_s_at	COL4A5	Collagen, type IV, alpha 5	0.43	0.00489
GgaAffx.11375.1.S1_at	MTSS1	Metastasis suppressor 1 RCJMB04_1a13	0.43	0.00820
Gga.10375.1.S1_at	KCNK9	Potassium channel, subfamily K, member 9 KCNK3	0.42	0.00457
Gga.13092.1.S1_at		EST, clone ChEST254e9	0.42	0.00091
Gga.13341.1.S1_at	PLEKHH1	Pleckstrin homology domain containing, family H, member 1	0.42	0.00137
Gga.16573.1.S1_at		EST, clone ChEST537d16	0.42	0.00110
Gga.4583.2.S1_x_at	RPL24	Ribosomal protein L24	0.42	0.00862
GgaAffx.12513.1.S1_s_at	DCTD	dCMP deaminase RCJMB04_17c11	0.41	0.00915
Gga.1964.1.S1_s_at	MYO10	Myosin X	0.40	0.00069
GgaAffx.8024.1.S1_at	LOC771624	LOC771624 OCC-1	0.40	0.00340
GgaAffx.6079.2.S1_s_at	KCNK2	Potassium channel, subfamily K, member 2	0.39	0.00585
Gga.5883.1.S1_at	PARP8	Poly (ADP-ribose) polymerase family, member 8	0.39	0.00724
Gga.17311.1.S1_at		EST, clone ChEST604k23	0.35	0.00298
Gga.6379.4.S1_a_at	CEL	Carboxyl ester lipase (bile salt-stimulated lipase)	0.35	0.00072
Gga.195.1.S1_at	GPR149	G protein-coupled receptor 149	0.32	0.00162
Gga.8880.1.S1_at	MME	Membrane metallo-endopeptidase	0.31	0.00630
Gga.1530.1.S1_at		Transcribed locus	0.27	0.00958
Gga.9239.1.S1_s_at	LOC768350	MHC-like class 1 Y	0.20	0.00494
Gga.10425.S1_s_at	VSNL1	Visinin-like 1	0.20	0.00056
Gga.14454.1.S1_at	ZPD	Zona pellucida protein D	0.19	0.00152
Gga.7210.1.S1_at	ZP3	Zona pellucida glycoprotein 3 (sperm receptor) ZPC	0.09	0.00247



Appendix 2 Immunohistochemistry with PAX2 antibody. Nuclear PAX2 protein expression in chicken oviduct (A). No PAX2 protein expression in the control section (B). Paraffin embedded sections of oviduct (n=3) were boiled in citrate buffer for antigen retrieval and blocked in 10% goat serum in PBS for 30 min. Sections were then incubated with rabbit anti-human PAX2 (Abcam, Cambridge, MA) at a dilution of 1:50 overnight at 4°. Control slides were incubated without primary antibody. This was followed by incubation in AlexaFluor 488 goat anti-rabbit IgG (0.24 ug/ml) for 1h at 39°. Slides were viewed using a Nikon eclipse E600 and pictures were taken with a Spot RT Slider camera. Scale bar = 100 um.

Appendix 3A Cell morphology, cellular development, and embryonic development pathway created by the Ingenuity Pathway Analysis (IPA) program. Green indicates genes that are up-regulated and red indicates genes that are down-regulated in chicken ovarian tumors compared to normal ovary.



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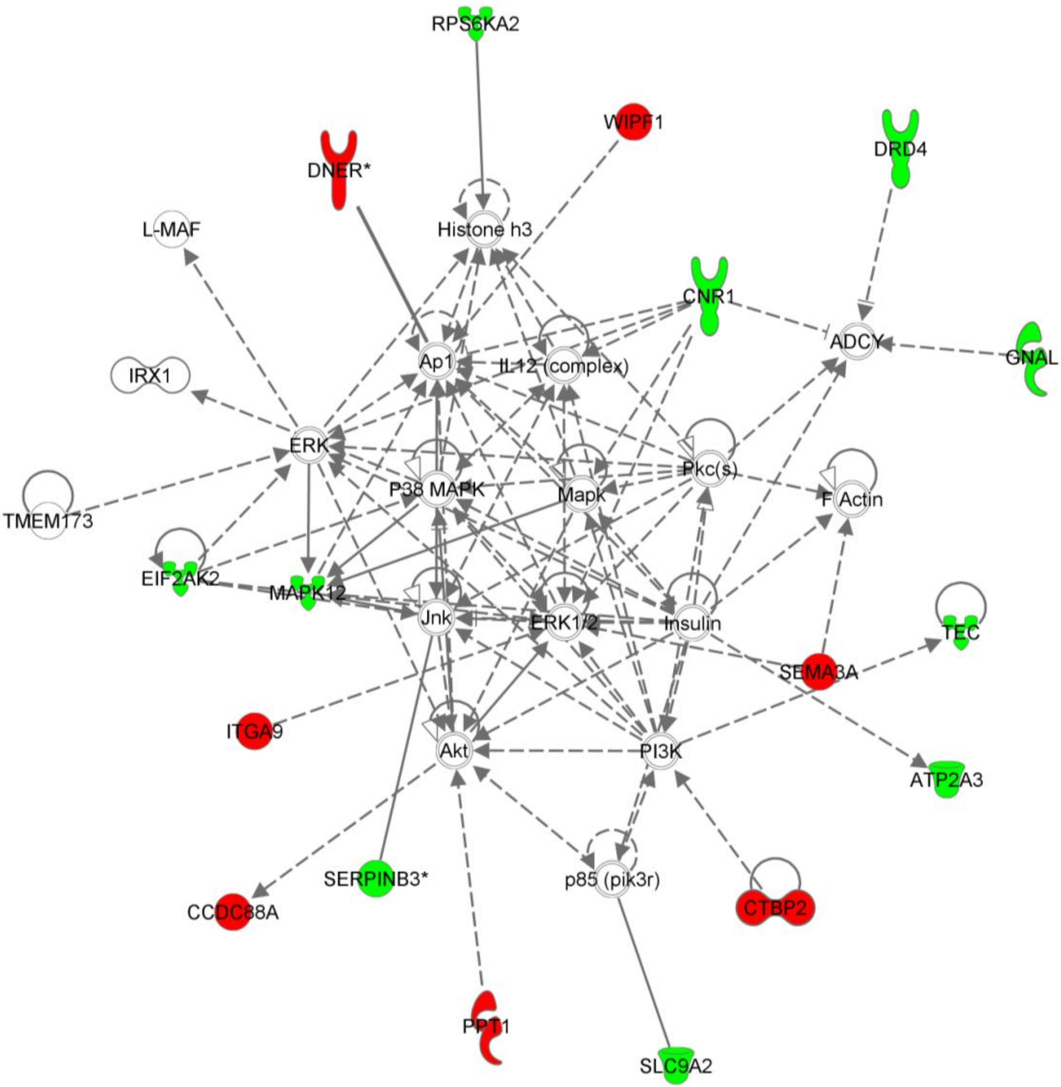
Appendix 3B Amino acid metabolism, behavior, cell-to-cell signaling, and interaction pathway created by the Ingenuity Pathway Analysis (IPA) program. Green indicates genes that are up-regulated and red indicates genes that are down-regulated in chicken ovarian tumors compared to normal ovary.



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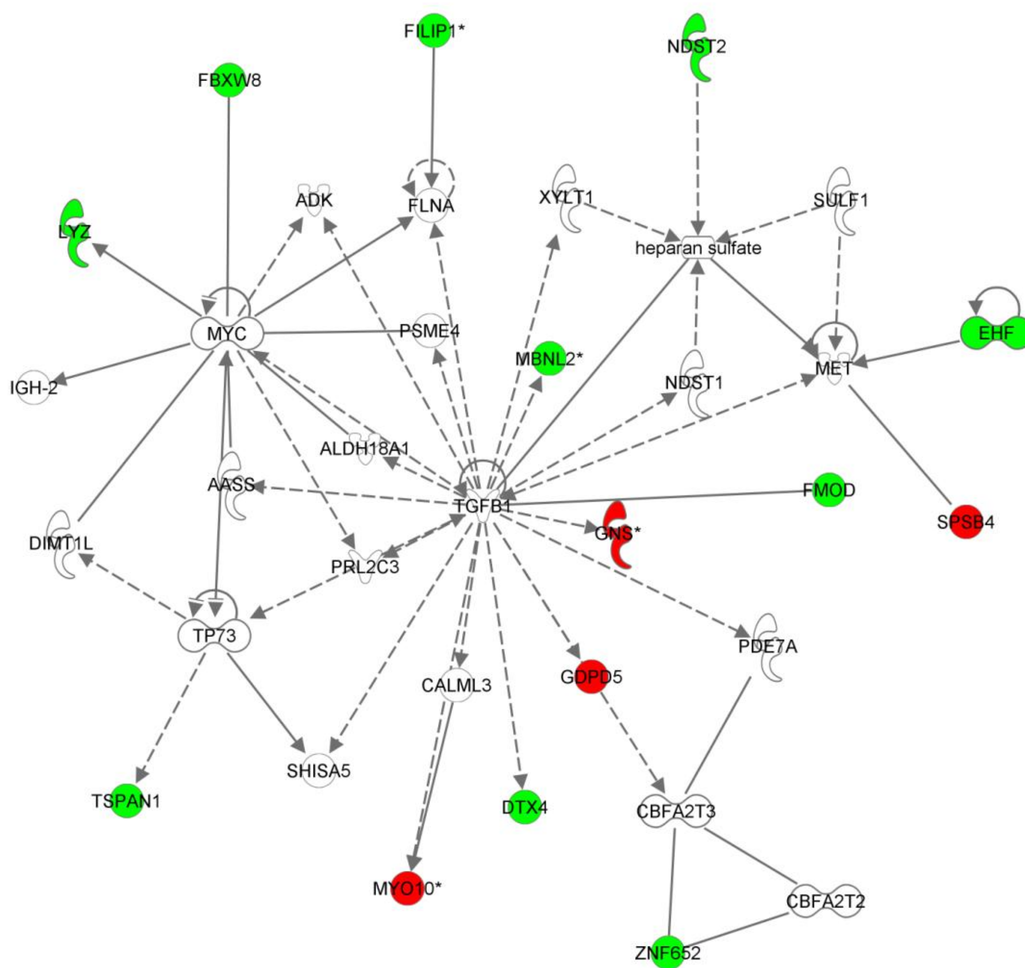
Appendix 3C Carbohydrate metabolism, molecular transport and small molecule biochemistry pathway created by the Ingenuity Pathway Analysis (IPA) program. Green indicates genes that are up-regulated and red indicates genes that are down-regulated in chicken ovarian tumors compared to normal ovary.



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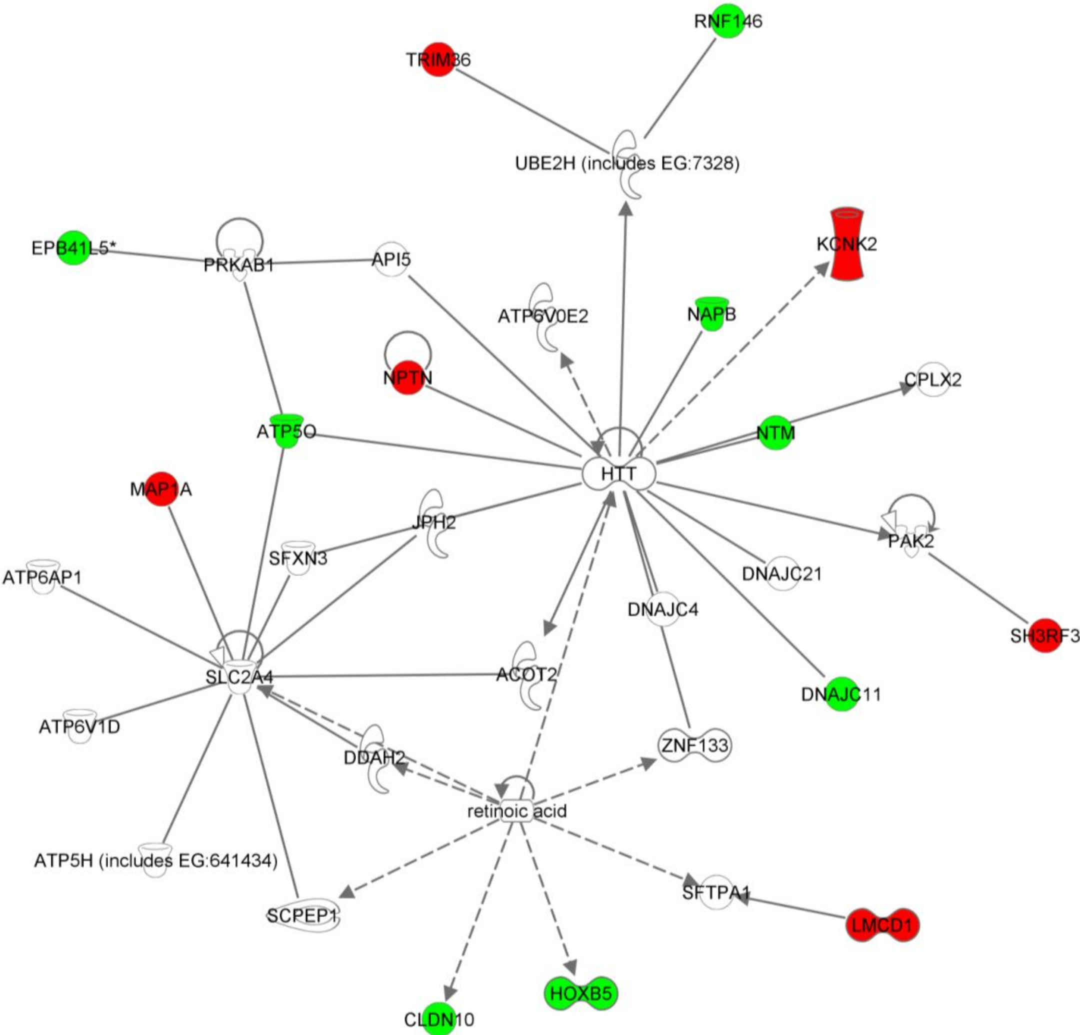
Appendix 3D Cell signaling, cellular function and maintenance, and molecular transport pathway created by the Ingenuity Pathway Analysis (IPA) program. Green indicates genes that are up-regulated and red indicates genes that are down-regulated in chicken ovarian tumors compared to normal ovary.



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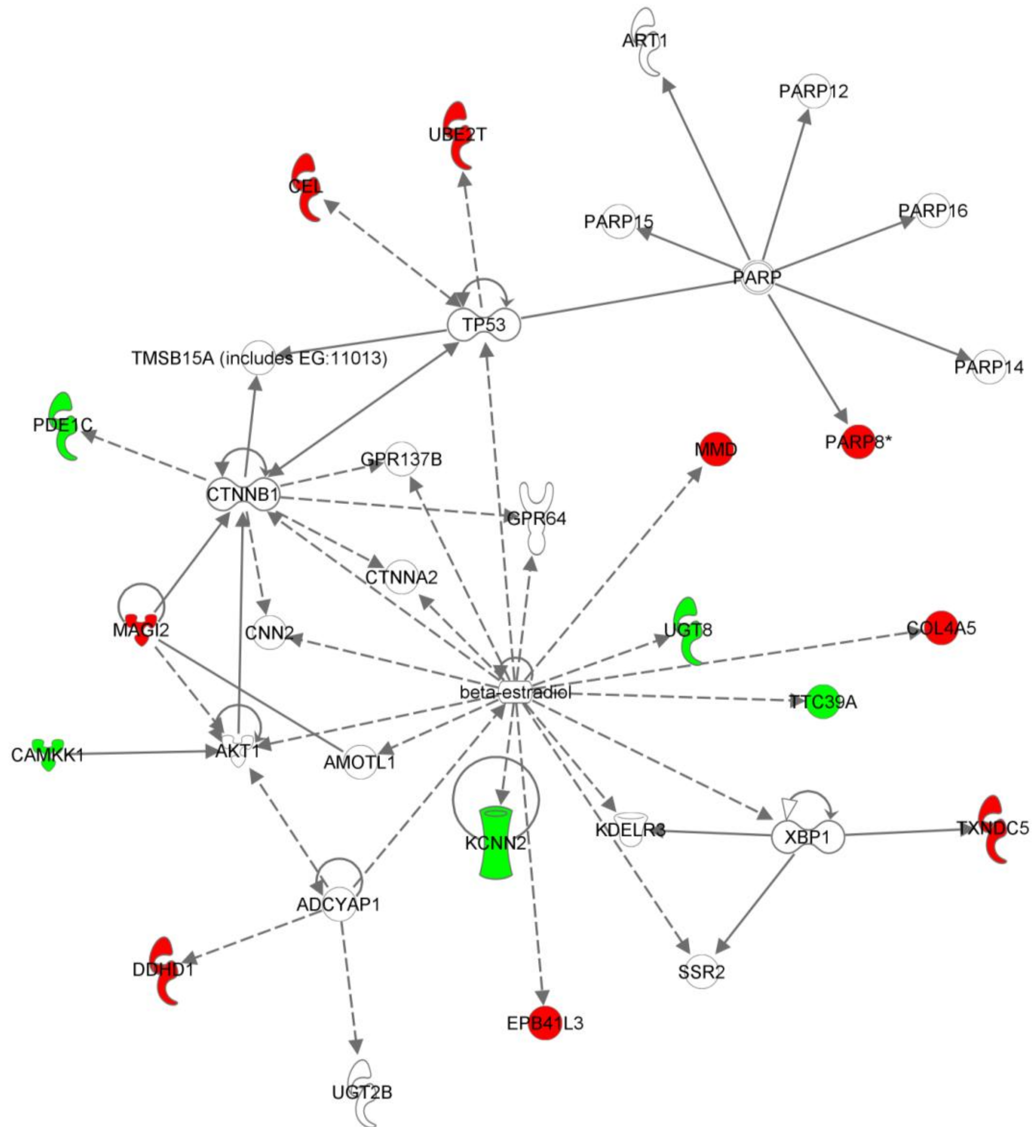
Appendix 3E Cell death, cancer, and developmental disorder pathway created by the Ingenuity Pathway Analysis (IPA) program. Green indicates genes that are up-regulated and red indicates genes that are down-regulated in chicken ovarian tumors compared to normal ovary.



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