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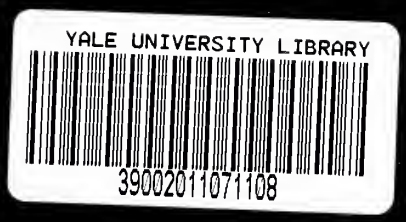

The Role and Regulation of Fas and Fas Ligand in the Ovary: Implications for Ovarian Cancer

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The Role and Regulation of Fas and Fas Ligand in the Ovary: Implications for Ovarian Cancer

A Thesis Submitted to the
Yale University School of Medicine
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Degree of Doctor of Medicine

by

Wendi Diane Brown

2000

THE ROLE AND REGULATION OF FAS AND FAS LIGAND IN THE OVARY: IMPLICATIONS FOR OVARIAN CANCER. Wendi D. Brown, Eva Sapi, and Gil Mor. Department of Obstetrics and Gynecology, Yale University, School of Medicine, New Haven, CT.

While ovarian cancer is the leading cause of gynecologic death in the U.S., its pathogenesis remains unclear. In this study we investigate the expression and regulation of Fas and FasL in the carcinogenesis of ovarian epithelial cell transformation.

The studies were done using fresh ovarian surgical specimens of varying degrees of malignancy. Tissues were immediately frozen or used for the *in vitro* organ culture system. Organ cultures and ovarian cancer cell lines were grown in the presence or absence of sex hormones. Fas and FasL expression was determined at the RNA and protein level by RT-PCR and Western Blot Analysis, respectively. The cells expressing Fas and FasL were characterized by immunohistochemistry. In addition, estrogen receptors α and β were identified in the mRNA and protein of the tissue specimens.

The normal, primary, and metastatic ovarian tissue showed a pattern of increasing FasL expression with increasing severity of the tumor as demonstrated by immunohistochemistry, RT-PCR, and Western Blot Analysis. Another pattern that emerged in these samples was the decreasing expression of ER-β with increasing malignancy. Fas receptor remained the same among these tissues. In the untreated organ cultures, the inclusion cysts and granulosa layer of the follicle were mildly immunoreactive for FasL. With the addition of estrogen, there was a general up-regulation of FasL expression, especially in the follicular cells. RT-PCR and Western Blot analysis of the tissue showed a similar result with the largest band detected in the

estrogen-treated group. Ovarian cancer cell lines showed variable FasL expression in response to estrogen and tamoxifen stimulation, depending on the type and proportion of estrogen receptors present.

We propose that the over-expression of FasL by epithelial cells could serve as the mechanism by which abnormal ovarian surface epithelium could escape immune surveillance, allowing tumor growth and metastasis and that this event is regulated by estrogen differentially acting through ER α and β.

III. ACKNOWLEDGEMENTS

After deciding to devote an extended year of study to scientific research, I set several goals for myself which I hoped to achieve in an admittedly short period of time. I wanted to thoroughly understand how to intelligently ask a medically relevant question and then form my own hypothesis. I also wanted to learn how to then answer that question by designing and performing the appropriate experiment. I even hoped to present some of my results in a scientific article or to share my conclusions at a conference. Finally, I planned to graduate from Yale Medical School having completed a thesis of which I could be proud. With the help of the many wonderful people surrounding me, I was able to accomplish these goals and so many more.

My principal investigator and thesis advisor, **Dr. Gil Mor**, was one of the most outstanding teachers that I encountered in my five years here at Yale. His patience and persistence were encouraging during difficult times, while his enthusiasm and sense of humor were the best reward for a job well done. I thank him so much for allowing me to be a part of such an exciting laboratory.

I began working with **Dr. Eva Sapi** via a collaborative effort with Gil. She served as an inspirational role model, being a successful woman on a traditionally male-dominated career path. I thank her for her long hours of instruction and her interesting perspective on research, medicine, and life in general. I would also thank her for introducing me to **Karrie Tartaro** who provided millions and millions of cells for my experiments.

I would also like to thank the many extraordinary, though transient, people in my lab. I thank **Jon Nilsen** for answering all of my many questions, **Santiago Brown** for

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I would also like to acknowledge **Dr. Frederick Naftolin** and the **Yale Obstetrics and Gynecology Department**. In particular, I would like to thank **Dr. Tom Rutherford** for his endless supply of affordable tissue for my experiments and **Dr. Setsuko Chambers** for taking the time to read and critique my thesis.

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V. INTRODUCTION

V.A. Epithelial Ovarian Cancer

V.A.1. The Epidemiology of Epithelial Ovarian Cancer

Epithelial ovarian cancer is the leading cause of death from gynecologic cancer in the U.S. However, the etiology of this fatal disease remains unknown. Researchers have identified possible reproductive or environmental factors associated with ovarian cancer and have implicated various genes in the pathogenesis of this disease, but none have determined the specific events leading to neoplastic transformation.

The demographic patterns of ovarian cancer have remained stable over the past few decades. Approximately one in 70 American women will develop ovarian cancer in her lifetime with an average annual incidence of 15.0 per 100,000 women (26,000 new cases diagnosed in the U.S. in 1995) (1). This incidence increases with advancing age, peaking in the eighth decade. The median age at diagnosis is 63, while it is infrequently diagnosed in women under 40 years of age. Because of late diagnosis, this disease carries a high mortality rate of 7.9 per 100,000 women (14,500 deaths in the U.S. from ovarian cancer in 1995) with a 5-year survival rate of approximately 30% (1).

Although epithelial ovarian cancer claims the lives of so many women, the molecular events responsible for carcinogenesis have yet to be determined. Epidemiologists have found associations between the development of ovarian cancer and endocrine/reproductive, environmental, and genetic factors. One of the foremost of the reproductive theories is that of "incessant ovulation" increasing risk of epithelial ovarian cancer.

This hypothesis that ovarian activity leads to neoplastic changes is based on the premise that excess or inappropriate proliferation is a critical component in the early pathogenesis of cancer in any system. During each ovulatory cycle, the surface epithelium is ruptured and repaired, resulting in increased proliferation of the epithelial cells. This elevated level of mitotic activity increases the likelihood of genetic mutations that lead to malignant transformation (2).

This hypothesis is supported by numerous studies showing the protective effect of parity, multiple births, history of breastfeeding, and oral contraceptive use – all of which limit the number of ovulations. Compared to nulliparous women, those who were ever pregnant have a 30-60% less risk of ovarian cancer (3). One to two pregnancies reduce the relative risk even further to 0.49-0.97. Although trends involving months of breastfeeding are lacking, some studies have shown a reduction in risk in those women who breastfed compared to those who did not (4). Studies have consistently shown a compelling ovarian cancer risk reduction of 30-60% in users of oral contraceptives (5). In a World Health Organization study, women who had ever used oral contraceptives had a relative risk of 0.75 (6). Other evidence in support of the "incessant ovulation" theory are studies linking increased ovarian cancer rates with use of ovulation-inducing infertility drugs (7).

In addition to a proposed role for ovulation in ovarian cancer, familial clusters of ovarian cancer also suggest a genetic component. While the lifetime risk of a woman in the general population developing ovarian cancer is 1.6%, this risk climbs to 4-5% if a single family member is affected by ovarian cancer, and it increases to 7% if two relatives are affected (8). Surprisingly, only 7% of all patients report a first-degree

relative with ovarian cancer (9), demonstrating that the sporadic form of the disease is much more common.

Three genotypes of hereditary ovarian cancer are recognized: breast-ovarian cancer syndrome (linked to the susceptibility locus BRCA1 and BRCA2), ovarian cancer only syndrome, and Lynch Type II syndrome which combines colorectal, endometrial, and ovarian cancer (10). These are transmitted from either parent in the classic Mendelian autosomal dominant pattern with incomplete penetrance and variable prognosis. The predominant histologic type of hereditary ovarian cancer is serous adenocarcinoma, and it is often diagnosed two decades earlier than those with sporadic disease.

In addition to internal host factors, external factors from the environment may also play a role in the development of epithelial ovarian carcinoma. Some have speculated that dietary habits influence the risk of ovarian cancer. Animal fat and meat intake have often been thought to increase the risk of many cancer types (11). In contrast, Byers and others found no effect from fat intake on ovarian cancer risk (12). Other proposed dietary risk factors include lactose-rich dairy products, coffee, and alcohol, although there is no clear consensus regarding the effect of these products (13). Other environmental risk factors have been investigated including talc exposure increasing risk and mumps infection decreasing risk of ovarian cancer, but neither theory has been proven.

Although epidemiologic studies have not offered clear answers regarding the risk factors and precise etiology of ovarian cancer, scientists have extensively studied the

pathologic changes occurring at the cellular level of the normal and neoplastic ovary in order to address some of these questions.

V.A.2. Histopathology of the Ovary and Ovarian Cancer

Although ovarian tumors arise from a single organ, they are not considered a single entity. The pathologic details of ovarian cancers have been extensively examined and classified into more descriptive categories.

In order to understand fully the pathologic variants of ovarian carcinoma, one must first appreciate the histologic characteristics of the normal ovary. The human ovary is a 3-5 centimeter ovoid shaped organ, comprised of stroma and follicles enveloped by serosa. During embryonic development, the mesothelial lining of the coelomic cavity gives rise to Müllerian ducts, from which the fallopian tubes, uterus, vagina, and ovarian serosa are derived (10). The exterior of the ovary is covered by the tunica albuginea and also by a single layer of cuboidal or columnar epithelial cells, which is continuous with the mesothelium of the peritoneal cavity (14). As the ovary develops, the surface epithelium extends into the stroma to form inclusion glands and cysts that are found as early as the 12th week of fetal life (15).

In addition to fetal origin, inclusion cysts may be acquired throughout life. During the postovulatory repair process, proliferating epithelial cells may be sequestered as inclusion cysts where they are in turn eliminated by apoptosis. It is postulated that an altered elimination process could result in the persistence within the stroma of these entrapped epithelial cells (16). In this environment, the epithelial cells are exposed to estrogen-rich follicular fluid and other proliferative stimuli, thus, leading to further generation and propagation of mutations and potentially to carcinogenesis. In a study

comparing the contralateral ovaries from patients with unilateral ovarian cancer to ovaries of age-matched controls, there was an increase in the number of inclusion cysts among the patients with ovarian cancer. Also, an age-related increase of inclusion cysts was noticed in the study group, but not in the control group (17).

Assuming that the mitogenic microenvironment of an inclusion cyst does promote gene mutations, it is important to identify exactly what mutations are responsible for tumorigenesis. Considerable research has shown the p53 gene to be one such gene. Alterations in this tumor suppressor gene are associated with negative cell cycle control, as demonstrated in 50% of ovarian cancers (18). The *erb*B-2 (HER-2/*neu*) oncogene also plays an important role in ovarian cancer pathogenesis, but study results are inconsistent in regards to the frequency with which this alteration is found among ovarian cancer patients (18). Also found to be involved in tumor formation is the oncogene family *ras*, which is one of the most frequently mutated genes in human malignancy (10). The K-*ras* mutation occurs most often (26-75%) in the mucinous type of ovarian cancer. Also, the *myc* gene family codes for a nuclear transcription factor that has been shown in four small studies to be amplified in epithelial ovarian cancer (19). Another gene under examination is *fms*, which encodes for M-CSF receptor. This receptor is produced by ovarian cancer cell lines and is expressed by many ovarian cancers (20). Although many genes have been implicated in ovarian carcinogenesis, there are many that remain unidentified. In addition, the precise order and degree of accumulation necessary for ovarian transformation has yet to be elucidated.

While the exact mutations responsible for ovarian cancer have not been identified, it is certain that epithelial ovarian cancer accounts for 80-90% of all ovarian tumors and

is responsible for the majority of ovarian cancer deaths (2). In the process of carcinogenesis, the ovarian epithelium differentiates into cells resembling other Müllerian-derived tissues. The most common subtype of common epithelial ovarian cancer is termed serous (46%), similar to fallopian tube histology. Mucinous carcinoma (36%) mimics the endocervix, while the endometroid type (8%) reflects endometrial tissue. Finally, clear cell ovarian cancer (3%) is composed of glycogen-rich cells that appear as endometrial glands would in pregnancy. Other less common classes include transitional-cell tumors, mixed epithelial tumors, undifferentiated carcinoma, and unclassified epithelial tumors (10).

Among the various categories of epithelial ovarian tumors, there is also a range of behavior. Benign tumors, most commonly serous or mucinous in origin, are often large and cystic. Ovarian neoplasms labeled "borderline" share an excellent prognosis, but they also share histologic features suggestive of cancer. Fatalities from this type of cancer are not usually seen until many years after the diagnosis. These women tend to be older than the average benign case and younger than the average malignant case (21). Malignant tumors are often discovered as solid masses with areas of necrosis and hemorrhage. They can be distinguished from borderline tumors by destructive growth features, such as disorganized cellular growth patterns, or sharp borders that transcend stromal planes.

The natural history and patterns of ovarian cancer metastasis oftentimes follow a predictable model of intraperitoneal or lymphatic spread. The transformation usually takes place in the epithelium of the ovary, although 10% of cases arise as primary peritoneal cancers. Upon further growth, the tumor ruptures the ovarian surface and

enters the peritoneal cavity. From here, they may implant as surface nodules throughout the peritoneum, especially on the omentum. The other mode of spread is through the retroperitoneal lymphatics that drain the ovary, resulting in lymph node metastasis (10). These patterns of spread are often appreciable at the time of diagnosis.

V.A.3. Diagnosis of and Screening for Ovarian Cancer

At diagnosis, two-thirds of ovarian tumors are in advanced stages (beyond the pelvis) because of late, non-specific signs and symptoms (2). In the early stages, women may have irregular menses, or complaints due to pelvic mass effect including urinary frequency, constipation, dyspareunia, and lower abdominal distention and discomfort. Later symptoms such as increased abdominal girth, nausea, constipation, anorexia, fatigue, and dyspnea may occur as a result of ascites, omental or small bowel metastases, or pleural effusions (2).

If ovarian cancer is diagnosed early, a relative survival rate of 90% can be achieved (22). However, there is a high fatality rate because the majority of women are not diagnosed until they have advanced disease (23). Unfortunately, there is currently no widespread, reliable screening modality for the early detection of ovarian cancer.

The screening tests available are bimanual rectovaginal pelvic examination, CA-125 testing, and ultrasonography (10). The most common of these tests, the annual physical examination, has not been shown to be adequate for detection in that it has insufficient sensitivity and specificity (23).

Although transabdominal ultrasound was once thought to be a promising noninvasive technique of ovarian cancer detection, it has since proven to be inadequately specific. In an abdominal ultrasound study of 5479 women, 338 had positive screens

followed by laparotomies. Of those, only 5 were found to have Stage I ovarian cancer (24). Researchers have placed new hope on transvaginal ultrasound, which is purported to be a more specific test. In one study of TVS, the PPV was reported as 6.7% (25) – that is 15 laparotomies for every one cancer. The most recent advancement in this area is the addition of color flow Doppler to reduce the high rate of false positives. This would distinguish malignancies from benign disease or from normal ovaries by analyzing the neovascularization and pulsatility of a mass (10).

Finally, the cell surface glycoprotein CA-125 has been used as a serum tumor marker. The normal physiologic function of CA-125 is unknown, but in healthy adults, it is found on the fallopian tube lining, endometrium, endocervix, peritoneum, pleura, pericardium, and bronchus (26). Very little CA-125 can be detected in the normal ovary, although it is sometimes found in inclusion cysts. It is elevated in 80-90% of all patients with epithelial ovarian cancer and in one-half of women with Stage I and Stage II ovarian cancer. Levels are also increased in patients with benign conditions such as peritonitis, pancreatitis, renal failure, and alcoholic hepatitis (27). The high false-positive rate combined with the low incidence of epithelial ovarian cancer makes a single CA-125 assay ineffectual (10).

Other antibody serum markers with different antigenic determinants for ovarian cancer are under investigation in hopes of improving the sensitivity and specificity. Some of these include ovarian carcinoma-associated antigen (OCA) and macrophage colony stimulating factor (M-CSF).

While each of these screening tests alone cannot offer adequate specificity or PPV, some researchers have looked at a combination of the methods. In a study of

22,000 women without a family history of disease, CA-125 levels were determined first. Only if there were an elevation did the patient receive a transabdominal ultrasound. The overall PPV increased to 26.8% when modalities were combined. Although improvement was seen with the multi-modality approach, the PPV for detection of early stage disease remained below 10% (28).

The National Institutes of Health Consensus Conference on Ovarian Cancer determined based on the above findings that there was "no evidence available yet that the current screening modalities of CA-125 and transvaginal ultrasonography can be effectively used for widespread screening to reduce mortality from ovarian cancer nor that their use will result in decreased rather than increased morbidity and mortality" (23). Therefore, there are currently no ovarian cancer screening recommendations.

Because there are no widespread screening programs for ovarian cancer, patients are often diagnosed with advanced disease, requiring exhaustive treatment plans.

V.A.4. Treatment of Ovarian Cancer

Once ovarian cancer is diagnosed, there are several treatment options - cytoreductive surgery, chemotherapy, and radiation therapy – but the precise sequence and complex combination of therapies is determined by the surgical stage of disease.

Therefore, the first step in surgical management is to stage the disease (see next section). The next goal is to remove any large or visibly metastatic disease in order to facilitate diffusion of cytotoxic agents into the tumor, to limit the number of resistant clones, to remove any slow-growing masses that would not respond to chemotherapy, and to improve discomfort in the patient (2). A tumor that is amenable to optimum debulking has been shown to carry a better prognosis (29).

The chemotherapeutic regimen has undergone many changes over the last few decades. Before the late 1970's when both combination chemotherapy and cisplatin were introduced, patients were treated with single agents such as melphalan. Cisplatin combination therapy (with doxorubicin and cyclophosphamide) quickly became the regimen of choice. Currently, carboplatin has become the preferred platinum compound due to fewer ototoxic, neurotoxic, and nephrotoxic effects than cisplatin. In 1996, the utility of cisplatin/paclitaxel combination therapy was demonstrated in patients with sub-optimally debulked Stage III and IV tumors (30). Another study found a survival advantage in certain patients who received cisplatin via an intraperitoneal route rather than intravenous administration (31). Newer drugs under investigation include docetaxel (a member of the taxane family), gemcitabine (a pyrimidine antimetabolite), topotecan (a topoisomerase-1 inhibitor), buthionine-sulfoximine (an inhibitor of glutathione biosynthesis), and cyclosporin (an antiinflammatory that that increases cisplatin toxicity) (32).

The use of radiotherapy in the treatment of ovarian cancer is a controversial topic due to early trials involving inappropriate techniques, doses, and patients. In the past decade, advancements have been made in the strategy of radiation therapy. For example, the knowledge of intraperitoneal dissemination led to techniques involving radiation of the entire peritoneal cavity rather than the pelvis alone. The ideal patient has also been better characterized as one with no macroscopic residual disease after primary surgery (10). Long-term, relapse-free survivals have been reported for Stages II and III after optimal debulking and postoperative radiotherapy (23). This modality of management continues to be debated although supportive data exists.

Despite these advances in ovarian cancer therapy, the prognosis remains dismal.

V.A.5. Ovarian Cancer Prognosis

The most important factor in determination of epithelial ovarian cancer prognosis is the tumor stage. Therefore, careful staging of ovarian cancer is of critical importance. The stage, or the extent of disease at the time of diagnosis, is determined by exploratory laparotomy for ovarian cancer. The general procedure is as follows: vertical rather than low transverse incision is made; multiple cytologic washings are performed; the tumor is removed intact; the abdomen is completely explored; the remaining ovaries, uterus, and tubes are removed; the omentum is removed, lymph nodes are sampled, and random peritoneal sites are biopsied. The results of this comprehensive surgery will decide the anatomic stage of disease and will direct all postoperative therapy and prognosis, so the skill and experience of the surgeon is of critical importance (10).

When studies evaluated the frequently inadequate surgical staging of early ovarian cancer and corrected for the apparent understaging of patients, the 5-year survivals improved. Stage I disease, previously reported to have a 60-80% survival was more accurately shown to reach 5-year survivals of 90% (22). Similarly, Stage II disease, originally reported as 0-40% survival had rates as high as 80%. Stage III and IV disease was not misstaged as often and therefore the survivals remained at 15-20% and 5%, respectively (33).

There is a myriad of other factors contributing to the prognosis of ovarian cancer. The diminished volume of residual disease after an optimal cytoreductive can improve patient survival by 22 months as compared to a patient with sub-optimal debulking (10). Histologic grade is another predictor of survival, especially in patients with Stage I

disease. Although the preoperative CA-125 level is often obtained, it only reflects the volume of disease, and it is not as predictive of survival as the postoperative levels (34). Some physicians feel that the CA-125 concentration after 2-3 courses of chemotherapy is the most important predictor of survival (35), while others believe that CA-125 levels should not be used for treatment decisions in individual patients (36).

Some investigational approaches to ovarian cancer prognosis are being explored. Among these are platinum-DNA adduct levels as a sign of responsiveness to chemotherapy, epidermal growth factor receptor and gene levels as a risk factor of progression, elevated p53 levels as an association with an unfavorable prognosis, and ploidy analysis as a marker of differentiation.

While the epidemiology, histopathology, diagnosis/screening, treatment, and prognosis of ovarian cancer differs from that of most other tumors, **the fundamental defect underlying all cancers appears to be the same – resistance to apoptosis**. This represents the main focus of the present study. Specifically we evaluate the role of the Fas/FasL system (a main apoptotic pathway) in the regulation of programmed cell death in normal ovarian tissue as well as during neoplastic transformation..

V.B. Programmed Cell Death and the Ovary

V.B.1. Fas/ FasL and the Immune System

The Fas and Fas ligand (FasL) system plays a dual role – protector and assassin - in the regulation of cellular proliferation and cell death in tissues. However, it is only recently that researchers have begun to study and understand the relevance of these actors (37).

Fas (Apo-1/CD95) is a type I cell surface protein responsible for the initiation of events leading to apoptosis when ligated by its natural ligand (38). The Fas receptor shares homology with members of the tumor necrosis factor (TNF)/ nerve growth factor (NGF) receptor family, and it is widely expressed in a variety of cells in the immune system, including T cells, B cells, and monocytes (37). In addition, the Fas receptor can be found throughout the body in such tissues as the liver, heart, and kidney (39).

FasL, the natural ligand for the Fas receptor, is a type II membrane protein belonging to the TNF superfamily. It is predominantly expressed in activated T cells and activated NK cells, but is also found on some macrophages/monocytes, and on the cells of immune privileged tissues (37).

Apoptosis is a pattern of programmed cell death responsible for many physiologic and pathologic events including: embryogenesis, following hormone dependent involution (endometrial cell shedding, regression of the lactating breast), cell death in proliferating cell populations (intestinal crypt epithelia), tumor cell death, immune cell death, pathologic atrophy, and cell death due to cytotoxic T cells or certain viruses' infection (40). The Fas/FasL system plays an important role in many of these processes.

During apoptosis, cells undergo a specific sequence of morphologic events. The first step is cell shrinkage, followed by chromatin condensation, formation of apoptotic bodies (blebs), and finally phagocytosis of apoptotic bodies or cells (41). This process, unlike necrosis, does not elicit inflammation. This allows for elimination of a cell without destruction of its surrounding tissue.

The mechanism of apoptosis in the Fas/FasL system involves a cascade of proteases triggered by the binding of Fas ligand with and the subsequent trimerization of the Fas receptor (Fig. 1). This enables the Fas receptor's death domain to interact with FADD (Fas-associating protein with death domain) (42). FADD then cleaves the first of a series of cysteine proteases (caspases), FLICE (FADD-like interleukin-1 beta-converting enzyme). This initiates the protease cascade and results in apoptosis of the cell (43).

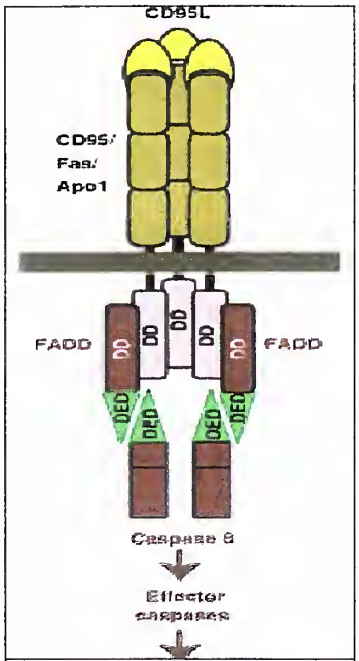

Figure 1. Model of internal events in apoptotic cell.

Among the many roles attributed to this mechanism of apoptosis is that of immune system development and function. It is well known that cytotoxic T cells act by inducing target cells to undergo apoptosis (44). The principal mechanism by which T cells mediate cell death is via cytotoxic proteins, which they release upon recognition of the target cell. These proteins include perforins, granzymes (fragmentins), and lymphotoxins. In addition to granule-mediated killing, membrane proteins of CD8 T cells can also activate apoptosis. This mechanism is believed to involve the activation of Fas in the target cell membrane, which interacts with FasL on the cytotoxic T cell membrane, resulting in cell death (44).

More recently, the Fas/FasL system has been shown to be involved in peripheral tolerance in the immune system. During maturation, T cells recognizing self-peptides are usually deleted in the thymus before they can migrate to the periphery (44). Once they have migrated to peripheral tissues, T cells responding to a strong antigenic stimulus

(such as self-peptides) decrease in number over time. Chronic activation of T cells leads to proliferation and expression of both Fas and FasL, resulting in apoptotic death of the T cells (45) thereby preventing destruction of tissue.

Finally, in the process of activation-induced cell death (AICD), the Fas/FasL system appears to regulate the expansion of lymphocytes by acting in an autocrine fashion to delete recently activated T cells (42). This T cell suicide enables the immune system to mount an attack without continually expanding the immune response at the site of infection. In patients with mutations in the Fas receptor, this expansion is unregulated, and they develop disfiguring lymphadenopathy due to an autoimmune lymphoproliferative syndrome (46).

In addition to playing a crucial role in immune system maintenance, Fas/FasL has also been shown to regulate apoptosis in other systems of the body and even serve to protect certain organs from immune system attack.

V.B.2. Fas/FasL and Immune Privilege

The role of the Fas/FasL system in the regulation of cellular homeostasis is not confined to the immune system. This system also allows for certain sites and tissues in the body to evade immune system attack and therefore to be immunologically privileged.

Privileged sites are those regions in the body that offer special protection to grafts, by preventing immune reactions and graft rejection in response to histocompatibility differences. Such locations include the brain, anterior chamber of the eye, cornea, retina, cartilage, matrix of hair follicles, testis, ovary, prostate, mammary and subcutaneous fat pads, and pregnant uterus, (47). From the evolutionary standpoint, preventing an

inflammatory response in these specialized tissues is beneficial as such responses in these sites could have fatal consequences for the individual organism or for the species.

It was once thought that this privileged status was due to separation of the antigens and the immune system. Some believed that physical barriers caused sequestration of the antigens from T cells. For example, some of these sites possess a blood-tissue barrier, making it difficult for immune cells to penetrate (47). Also, most of these sites lack conventional lymphatic drainage (47). Others believed that the antigens failed to leave these sites in order to elicit an immune response. However, studies have shown that antigens do in fact leave and do interact with T cells (44).

Current theories are more complex, focusing less on mechanical barriers and more on active mechanisms of immune evasion. Some of these theories include reduced expression of classical MHC glycoproteins (48) or the local secretion of immunosuppressive cytokines and neuropeptides (38). One hypothesis involves the preferential stimulation of the anti-inflammatory TH2 cell response as opposed to the inflammatory responses of TH1 cells and complement-fixing antibodies (47).

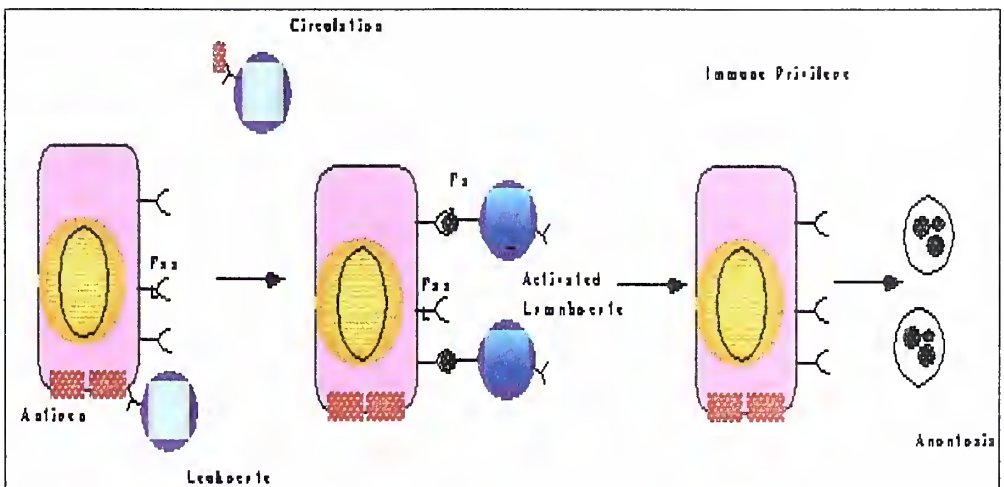

Figure 2. Model of Fas/FasL interaction in immune privileged tissues.

Recently, an additional mechanism of immune privilege was realized after researchers determined that both the eye and the testis constitutively express FasL (Fig. 2) (38). After inoculation with virus, the anterior chamber of the eye incurs no inflammatory injury, because the FasL on the epithelial surfaces induce apoptosis of the invading lymphocytes. This is not the case in *gld* mutants, mice with defective FasL. Instead, the recruited lymphocytes are not killed, the inflammatory response is uncontrolled, and the eye tissue is destroyed (45).

In a study by Bellgrau et al., this same protective effect was seen in the context of the testicle. This group transplanted allogenic mice testes into the kidney capsules of recipient mice. The graft was successful if both Fas and FasL were functional. If, however, the donor lacked functional FasL (*gld* mouse) or the recipient expressed defective Fas (*lpr* mouse), the graft was vigorously rejected (49).

The Fas/FasL system has also been shown to be involved in immune regulation at the site of placental implantation. Mor et al. localized FasL to the maternal-trophoblast interface and identified apoptotic leukocytes at this junction (50), suggesting that FasL triggers programmed cell death in immune cells which affords the placenta an immune privileged state.

While FasL provides immune protection to certain organs, its interaction with Fas accounts for the normal cellular turnover inherent to tissues undergoing continual proliferation and cell death. It is in this capacity that the Fas/FasL system acts in the development of normal tissues.

V.B.3. Fas/FasL in Normal Reproductive Tissue

As previously mentioned, the process of apoptosis is responsible for normal cellular turnover in numerous tissues throughout the body. The cyclic nature of the female reproductive system, imposed on these tissues by the episodic release of ovarian steroids, is particularly dependent on this means of cell death. The variable coexpression of Fas and FasL is believed to be in part responsible for the proliferative/degradative changes occurring in these tissues.

The uterus shows prominent steroid-dependent cyclic changes in structure and function in preparation for pregnancy. After a proliferative and secretory phase, steroid support is withdrawn and the uterine epithelium is shed. While menstruation was once thought to be the result of ischemic necrosis, Hopwood and Levision later demonstrated the presence of apoptosis in the human endometrium (51). Fas and FasL have been reported to be consistently co-expressed in the human endometrium throughout the menstrual cycle (52). During the late proliferative phase, these proteins are primarily located on the Golgi apparatuses and vesicles, and therefore are unable to interact and cause apoptosis. In contrast, the proteins are incorporated into the cellular membranes during the secretory phase when FasL can bind Fas and signal apoptosis. In addition, on Western Blot analysis, it was demonstrated that FasL exhibits a cyclic expression pattern

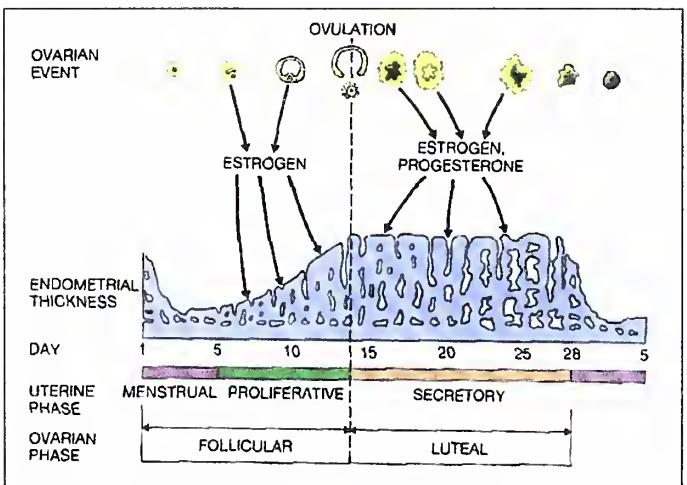

Figure 3. Model of cyclic changes in female reproductive tissues.

in the endometrium with a peak during the secretory phase (Fig. 3) (52).

The hormonally regulated, dynamic tissue of the breast also undergoes cyclic reorganization. During pregnancy, the glandular epithelium experiences a cycle of lobulo-alveolar development and maturation in preparation for milk production, followed by mammary involution after cessation of lactation. Studies involving the pregnant and postpartum breast indicate a role for apoptosis during the involution of mammary gland tissue. Quarrie et al. demonstrated the presence of apoptotic cells in mouse mammary tissue by the technique of nick-end DNA labeling. They found laddering both before and then increasingly after litter removal, suggesting that while apoptosis is a normal physiological event in the lactating mammary gland, it is also rapidly induced by milk stasis (53). Furthermore, this apoptotic event in the mammary gland has been divided into two distinct phases. The first phase is characterized by apoptosis of fully differentiated mammary epithelial cells without visible degradation of the extracellular matrix. The last phase involves remodeling of the extracellular matrix, altered mesenchymal-epithelial interactions, and apoptosis of cells that are losing differentiated functions (54).

Recently, Song and others have linked this pattern of apoptosis in the pregnant breast to the changing expression of Fas and FasL in the mammary epithelium. They have shown the absence of FasL in the breast tissue of non-pregnant/virgin mice, followed by the increasing appearance of FasL expression during pregnancy and lactation. Corresponding to this rise in FasL, Fas expression is diminished, allowing for growth of mammary tissue without induction of apoptosis. Once the puppies are weaned,

high levels of both Fas and FasL are observed in the maternal breast, which coincides with the emergence of apoptotic cells in the ducts and glands (Song:submitted to *PNAS*).

Similarly, the Fas/FasL system may play a prominent role in the ovary by mediating cellular turnover, as exemplified by follicular atresia and by post-ovulatory surface epithelial repair. The ovarian cycle consists of follicular and luteal phases (Fig. 3). As estrogens are derived from the follicles, the estrogen-dominant period prior to ovulation is known as the follicular phase while the progesterone-dominant phase is termed the luteal phase as the corpus luteum, the postovulatory remnant, secretes an abundance of progesterone. It is the ovarian secretion of estrogen and progesterone that coordinates the cyclic activities of the genital tract and breast with the ovulatory cycle. During the follicular phase, 15-20 early antral follicles are rescued from atresia and recruited for development at the start of each menstrual cycle. Of these 15-20 early antral follicles, there is usually only one successful follicle that will undergo subsequent preovulatory growth. It is this single follicle that develops LH receptors on its granulosa layer and is therefore able to ovulate in response to the LH surge. Meanwhile, the majority of the follicles are eliminated by the process of atresia in order to maintain the cell mass and homeostasis of the ovary.

The method by which follicles degenerate had long remained a mystery, until recent reports identified apoptosis as the underlying mechanism of follicular selection (55). Previous studies have shown the following physiological markers associated with follicular atresia: detachment and degeneration of the granulosa layer (56), fragmentation of the basal lamina (57), reduced DNA synthesis (58), decreased estrogen production (59), and decreased gonadotropin binding (60). Recent evidence demonstrates that the

internucleosomal fragmentation of DNA that is characteristic of cells undergoing apoptosis can be detected in ovarian atretic follicles (55). Atresia is therefore achieved by the self-destruction of thecal and granulosa cells that comprise the follicle, by this process of apoptosis.

Upon realizing that follicular regression was dependent on apoptosis, many researchers began searching for the underlying mechanism of apoptotic regulation in the ovary. The Fas/FasL system has been implicated in such a role. Guo et al. found the expression of both Fas and FasL in the murine ovarian follicle. They localized Fas to the oocytes (61) while FasL appeared to be restricted to the area of the granulosa cells by indirect immunofluorescence (IIF) test. In addition, FasL expression was regulated by the administration of pregnant mare's serum gonadotropin (PMSG) (61). Hakuno et al. also detected Fas in the ovary, but they localized this protein to granulosa cells in secondary and tertiary follicles at an early stage of atresia through immunohistochemical and *in situ* hybridization analyses. Furthermore, on Western Blot analysis of the oocytes' lysate, they identified a 31- kDa band specific for FasL (62). In another study of the rat ovary, Fas and FasL were found to be coexpressed in granulosa cells (63). In an attempt to localize the Fas antigen in the infant and adult human ovary, Kondo et al. found moderate Fas expression in the oocytes of primordial and primary oocytes. This expression was only weak in the oocytes of secondary and antral follicles, and there was a complete absence of staining in preovulatory follicles. In addition, they detected an abundance of Fas antigen in the regressing corpora lutea, suggesting Fas/FasL participation in this aspect of the ovulatory cycle as well (64).

Another cyclic pattern of apoptosis in the ovary occurs in the ovarian surface epithelium (OSE). At the time of ovulation, these cells on the surface of the preovulatory follicle undergo apoptosis. Once the follicle has ruptured, this layer of cells rapidly proliferates in order to repair and cover the surface of the developing corpus luteum (65). Researchers studied the effect of adding Fas monoclonal antibody to mouse corpus luteum cultures and to enriched cultures of mouse OSE to determine the role of Fas/FasL in this apoptotic event. They concluded that OSE cells were triggered to undergo apoptosis in response to Fas mAb when they were pretreated with IFN (66) and, therefore, Fas/FasL likely plays a role in the apoptosis of ovulation.

If a problem arises in this normal cellular removal process, it may enable certain cells to proliferate without control, potentially resulting in a transformational event. Theoretically, these cells would then be recognized as abnormal or "foreign" by a functional immune system and promptly eliminated. In fact, many components of the immune system do identify these pre-neoplastic cells and do attempt to clear them from the body.

V.C. Fas/FasL in the Normal and Neoplastic Ovary – A Role for Tumor Escape

V.C.1. The Immunology of Ovarian Cancer

Basic research in the field of immunology has revolutionized our understanding of the cellular and molecular basis of immunity and the principles of tumor immunology. It is now known that tumors have chemically defined antigens on their surface that differ in quality and quantity from those on normal host cells (10). Some of these tumor-associated antigens are recognized by the host's immune system, and treated in a similar

manner as an infection. The traditional components of the immune system – various leukocytes, cytokines, complement cascades, and MHC antigens - all play a significant role in stimulating and regulating this immune response to cancer.

Just as leukocytes perform an integral role in defending the body from infection, T and B lymphocytes, macrophages and monocytes, and natural killer cells all cooperate to combat tumors. T cells are the primary effectors of cell-mediated immunity with offspring that both regulate other leukocytes through cytokines and become cytotoxic cells capable of lysing malignant cells. Both CD4+ and CD8+ T cells have been observed in ovarian carcinomas (26). T lymphocytes from ovarian cancer patients have been shown to inhibit allogeneic tumor cells of the same tissue type (68); however, the overall number of T cells seems to be slightly decreased in this disease (69).

The B cells of the humoral immune system may also help attack cancer by production of antibodies that bind to specific tumor antigens, leading to inactivation, uptake, digestion, and elimination of these antigens (10). Antibodies against tumor cells have been detected in the sera and ascites of patients with ovarian cancer, but have not been shown to mediate tumor resistance (70).

Macrophages appear to have both a beneficial and harmful effect in their response to tumors. Activated macrophages can distinguish transformed from benign cells by recognizing alterations in the cell membrane, and then they destroy tumor cells through direct ingestion (71). In addition to phagocytosis of cancer cells, macrophages also produce cytokines. Unfortunately, these cytokines (IL-1, IL-6, and TNF-α) have been shown *in vitro* to stimulate the growth of ovarian carcinoma cell lines (72). Furthermore,

most ovarian cancers produce M-CSF, attracting circulating monocytes and activating macrophages (73).

Natural killer cells can also control the growth of tumors. These cells can mediate the death of neoplastic cells without antibody or specific immunity. The NK cell can do so without having previous contact with the target cell (10).

Although many human cells are resistant to complement-mediated killing, the binding of antibodies to certain tumor-associated antigens can trigger the cascade and result in membrane attack complex insertion and impaired osmotic integrity. This method of defense also activates neutrophils, basophils, and mast cells to create an inflammatory response (10).

In addition to mediating graft rejection after tissue transplantation, MHC molecules also trigger an immune response to most antigens recognized by T cells. An individual's MHC complex is able to bind particular peptides and to recognize a tumor cell. Fragments of antigens synthesized within a tumor cell may become associated with Class I MHC antigens on a tumor cell surface, making an effective target for cytotoxic T cells. Also, inducer T cells can be stimulated by tumor peptides bound to Class II MHC antigens (10). Although it has not been correlated with survival (74), it is interesting to note that about 80% of both normal ovary and epithelial ovarian carcinomas express Class I MHC determinants, while only 40% of ovarian cancers and no normal ovaries express Class II antigens (26).

Despite the many attempts of an active immune response, the ovarian tumor is often able to "escape" the immune system. This process of evasion has been suggested to result from the inability of the immune system to react to the tumor either because of

non-recognition of these antigens or due to non-reactivity secondary to insufficient co-stimulation, anergy, tolerance, or immunosuppression. Recently, the expression of FasL in neoplastic cells has been suggested as a potential mechanism of tumor immune privilege.

V.C.2. FasL in Cancer

Although the immune system is often capable of identifying and attacking transformed cells, some cancers have discovered a successful means of retaliation – FasL expression. This model postulates that lymphocytes, constitutively expressing Fas, recognize and infiltrate a neoplasm, encounter the FasL expressing tumor cells, and undergo death via apoptosis signaled by the Fas-FasL interaction (45).

Numerous tumors have been reported to express FasL, including hepatocellular carcinoma, melanoma, colon cancer, astrocytoma, and lung carcinoma (42). Furthermore, these tumor cells were shown to induce apoptosis in Fas-sensitive but not in Fas-resistant Jurkat lymphocyte cell lines (75). In addition to these sites, a variety of reproductive tissue tumors escape immune surveillance via FasL expression.

In an extension of their work on FasL and placental implantation, Mor et al. investigated the role of FasL expression in the context of gestational trophoblastic disease and immune evasion. They assert that the same mechanism by which the placental trophoblast cells subvert the maternal immune system during implantation is utilized by neoplastic choriocarcinoma cells during uterine invasion. Using immunohistochemistry and reverse transcriptase-polymerase chain reaction, a high expression of FasL was found in both complete hydatidiform moles and in choriocarcinoma. In the vicinity of these invading tumor cells were apoptotic leukocytes, suggesting that FasL acts as an effector

signal to kill activated lymphocytes, thereby offering escape from the host's immune system (50).

A similar mechanism of uncontrolled growth and invasion has been proposed for breast carcinoma. Breast tumors are often associated with a large, seemingly ineffective lymphocytic infiltrate that is unable to suppress or eliminate the "foreign" cells to which it is directed. This immune incompetence may be a result of lymphocyte apoptosis induced by the Fas/FasL system. In a study of human breast carcinomas, Gutierrez et al. found strong membranous and cytoplasmic FasL staining in ductal carcinomas and in hyperplastic breast tissue while there was no staining in normal glands in non-tumor quadrants. As in the choriocarcinoma study, TUNEL analyses and CD3+ staining demonstrated apoptosis predominantly among the lymphocytic population. There also appeared to be an up-regulation of FasL expression in hyperplastic and normal breast ducts close to the tumor, suggesting a possible role in early neoplastic transformation and proliferation (76).

Currently, the literature on the relationship between Fas/FasL and ovarian carcinoma is sparse. One group obtained lymphocytes from ascitic fluid of women with ovarian cancer and showed an alteration in T cell receptor function related to tumor-induced apoptosis (77). Other groups have focused on the treatment of ovarian cancer by administering anti-Fas mAb (FasL) to induce apoptosis of Fas-bearing cell lines (16,78). Overall, this is a research area lacking in clear answers regarding the role of FasL in the transformation and growth of ovarian cancer.

We propose that FasL overexpression could result in escape of tumor cells from immune surveillance. Furthermore, this abnormal expression of FasL may

represent a precursor stage of ovarian epithelial carcinoma. In this study, we investigate the expression of FasL in the carcinogenesis of ovarian epithelial cancer.

V.D. Hormonal Regulation of FasL

V.D.1. Effect of Estrogen on FasL

Several hormones are involved in human reproductive activity, among those being the steroid hormones. Included in this class are progestagens, androgens, and estrogens, all of which are derived from the common sterol precursor cholesterol. While the three classes of sex hormones share a common biosynthetic pathway and even have similar basic structures, the functional properties of each class are vastly different (79). In general, the progestagens are associated with preparations for pregnancy and its maintenance, the androgens are responsible for the development of masculine characteristics and fertility, and the estrogens promote development and maintenance of female qualities and fertility. Specifically, estrogens stimulate female secondary sex characteristics, prepare the uterus for spermatozoal transport, increase vascular permeability and tissue edema, stimulate growth and activity of mammary glands and endometrial tissue, prepare the endometrium for progestagen action, stimulate calcification, regulate secretion of gonadotropins, and support pregnancy (80).

The ovary is the main source of estrogen, synthesized in granulosa cells from androgenic precursors of the theca (80). Estrogen regulates tissue function by modulating gene transcription (Fig. 4) (81). The hormone-activated estrogen receptor binds to specific estrogen-recognizing elements (ERE) located in the promoter region of estrogen-regulated genes. Mor et al. reported the presence of ERE-like motifs

(AGTCANNNTGACC) in the promoter region of the human FasL gene at nucleotides 543-552 (82). This finding suggested that estrogen could have a direct effect on FasL expression. Further studies confirmed this hypothesis. MCF-7 breast cancer cells treated with 17-β-estradiol ($1x10^{-9}$M) induced a three-fold increase in FasL expression at both the mRNA and protein levels after 24 and 48 hours of incubation (82). In addition to

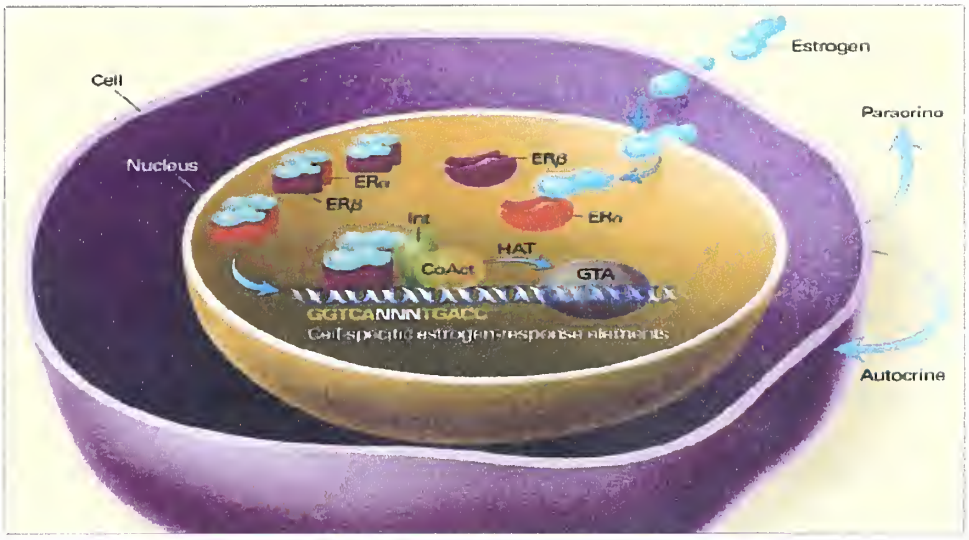

Figure 4. Model of intracellular actions of estrogen

estrogen influencing the expression of FasL in the breast, its antagonist, tamoxifen, has also been implicated in such a regulatory role.

V.D.2. Effect of Tamoxifen on FasL

Antiestrogens block the action of estrogen usually by binding to receptors and interfering with their normal function. These antagonists are traditionally divided into three groups: short-acting antagonists (estriol), long-acting antagonists (tamoxifen, clomiphene), and physiological antagonists (progesterone, androgens, glucocorticoids).

Of most current clinical relevance are the long-acting antagonists, such as tamoxifen, which are derived from triphenylethylene. They have both agonist and

antagonist effects on estrogen action (83). As a mixed agonist-antagonist, tamoxifen partially inhibits the action of estrogen but partially mimics the response of estrogen. The species, organ, tissue, or cell type ultimately determines the overall effect. For example, when administered alone, tamoxifen stimulates growth of the rat uterus by causing hypertrophy of the epithelial cells in the endometrium while having little effect on the stroma or myometrium. Unlike tamoxifen, estradiol stimulates growth of all three tissue layers. If tamoxifen is given simultaneously with estradiol, it will inhibit the overall estrogenic growth effect through antagonism in the stromal and myometrial compartments although it is an agonist in the epithelial layer (84).

The mechanisms underlying this tissue-selective action of mixed agonist-antagonists are not fully understood. The physiochemical characteristics of antiestrogen-receptor and estradiol receptor complexes may differ and thereby undergo contrasting conformational changes (85). Tamoxifen induces an intermediate conformation, balancing between active and inactive states. The final result depends on the intracellular concentration of coactivators and corepressors, providing a potential means of differential cell stimulation (86).

Antiestrogens have been used in the management and now in the prevention of breast cancer. The main indication for their use arises from the observation that estrogen is a mitogen in mammary carcinoma (82). Tamoxifen appears to have an antiestrogen effect in human breast tissue, inducing a therapeutic response in about one third of women and men with breast cancer (87). Alternatively, chronic tamoxifen use is associated with an increased risk of endometrial cancer, owing to its agonistic effect in the epithelial cells of the uterus (88).

In addition to demonstrating that estrogen up-regulates FasL expression in breast tissue, Mor et al. also found that tamoxifen inhibits FasL expression, thereby preventing tumor escape from immune surveillance. When co-administered with estrogen, tamoxifen inhibited the stimulatory effect of estrogen. This supported the idea that estrogen's action on FasL expression is receptor-mediated. When MCF-7cells were incubated with increasing concentrations of tamoxifen alone, there was a dose-dependent down-regulation of FasL mRNA expression (82). To confirm that the effect of tamoxifen on FasL is estrogen receptor-dependent, similar experiments were performed on the Jar choriocarcinoma cell line that is negative for both α and β estrogen receptors. Tamoxifen did not have any effect on the expression of FasL under these circumstances.

In contrast to breast cancer, ovarian cancer appears to be hormone-unresponsive. Although 40-60% of ovarian cancers express ER-α (89), only 7-18% of these clinically respond to antiestrogen treatment (90), although it does seem effective in disease stabilization. Schwartz et al. used tamoxifen to treat 13 patients with recurrent ovarian epithelial cancer and had no complete responses, one patient with a partial response, and four patients with prolonged stabilization of disease (91). While preliminary results of hormonal therapy in ovarian cancer seem unsuccessful, further studies are necessary in order to confirm this.

We hypothesize that similar to the breast, the expression of FasL in the ovary may also be regulated by estrogen and tamoxifen. Because both estrogen and tamoxifen affect cells by binding to their intracellular estrogen receptors, investigation into these receptors could help explain the variable responses of different tissues to these hormones.

V.E. ER alpha and beta Expression in the Ovary

V.E.1. Estrogen Receptors

Estrogen receptors are members of the nuclear steroid receptor superfamily and act as transcriptional factors, targeting organs such as bone, brain, and reproductive tissue (92). The classic ER-α was thought to be the only receptor to which estrogen could bind, until 1996 when researchers cloned a novel estrogen receptor, ER-β, expressed in rat prostate and ovary (93). In addition, other estrogen target tissues have been shown to coexpress ER-α and the second isoform ER-β (Fig. 5), including breast, endometrium, and testis (94). The ERα is translated from a 6.8-kilobase mRNA that contains 8 exons derived from a gene on the long arm of chromsome 6 and has a molecular weight of about 66,000 with 595 amino acids. The more recently discovered ERβ is encoded by a gene localized to chromosome 14q22-q24, close to genes related to Alzheimer's disease (95), and encoding some nine to 10 differently sized ERβ isoforms in the range of 485, 503 and 530 amino acids (95, 96). The affinity for the 17β-estradiol and other known estrogen receptor ligands was found to vary between the ERα and the different ERβ isoforms (97). Although, they respond in a comparable manner to the same hormones, there are differences. For example, phytoestrogens have a greater affinity for ERβ than ERα (98). Whether all the ERβ isoforms will turn out to have any significant biological and physiological role is yet undetermined. This recent characterization of ER-β

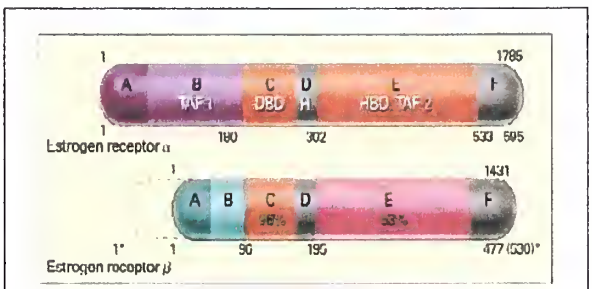

Figure 5 Model of estrogen receptors α and β.

provides a new means for understanding the pathophysiology of estrogen-regulated tissues.

V.E.2. ER Expression in Cancer

For many years, ER-α expression in breast cancer has been correlated with tumor progression, prognosis, and the prediction of hormone sensitivity (99); however, the significance of ER-α and ER-β in ovarian cancer has only recently been appreciated. In the normal human ovary, both ER isoforms are present. Hillier et al. showed that both ER-α and ER-β PCR products were weakly detectable by Southern analysis in cultured human ovarian surface epithelial (OSE) cells and readily detectable in freshly isolated granulosa cells and granulosa-lutein cells (100). In another study of normal ovary ER expression, Lau et al. found coexpression of ER-α and ER-β in four primary cultures of human OSE cells (101). Brandenberger et al. also demonstrated the expression of both estrogen receptors in normal, pre-, peri-, and postmenopausal ovaries at comparable levels in all age groups. In contrast, human fetal ovary predominantly expressed ER-β mRNA. In analysis of specific cell types, however, they found that the human OSE cell line IOSE-Van expressed a low level of ER-α and no ER-β and that granulosa cells expressed high levels of ER-β (102).

Further estrogen receptor studies of the ovary indicate a role for differential ER-α and ER-β expression in ovarian carcinogenesis. Brandenberger et al. found that ER-α mRNA levels were equal or slightly higher in carcinomas of the ovary while there was a decrease in the level of ER-β in the tumor samples (102). Pujol et al. also analyzed the relative expression of ER-α and ER-β in malignant human ovarian epithelial tumors. They reported a similar finding – an increased level of expression of ER-α mRNA in

comparison to ER-β mRNA in ovarian cancers. Furthermore, their study showed a difference in estrogen receptors between cysts and tumors (103). Thus, the increased ER-α:ER-β ratio may represent an early step in carcinogenesis and a potential marker of aggressiveness.

To evaluate the potential role of ER in ovarian cancer, we analyzed the relative expression of ERα and ERβ mRNA and protein in normal ovarian tissue and primary and metastatic tumors.

V.F. Rationale

Our lab and others have identified FasL as a key mediator of tumor immune escape by induction of apoptosis in invading T cells. In particular, this phenomenon has been demonstrated in such tumors as breast cancer and gestational trophoblastic disease (50,76). While the Fas/FasL system has been shown to underlie apoptosis in follicular atresia, it is unclear if FasL is also the mechanism by which ovarian cancer escapes immune surveillance.

It is known that hormones influence growth of normal and neoplastic reproductive tissues including breast, uterus, and ovary, and we have even shown that FasL expression in breast tissue is responsive to estrogen stimulation (82). However, this same regulation of FasL by estrogen has yet to be demonstrated in the ovary.

Estrogen is known to assert its effect via two estrogen receptor subtypes, ER alpha and ER beta (93). In breast cancer, estrogen receptor status has been associated with variable tumor characteristics and prognoses (87). Only recently have researchers questioned the significance of estrogen receptors in ovarian tumors, specifically the relative expression of ERα and ERβ (100, 101, 102, 103).

There are numerous unanswered questions surrounding the role and regulation of the Fas/FasL system in the ovary and how this system may be involved in ovarian carcinogenesis. The purpose of this study is to answer some of these questions.

VI. Statement of Purpose and Hypothesis

Year after year, ovarian cancer claims the lives of more women in the U.S. than any other gynecologic cancers yet many questions remain regarding this fatal disease. **It is our hypothesis that ovarian tumors may elude immunological surveillance by inducing, via the Fas/FasL system, the apoptosis of activated lymphocytes that would have otherwise mediated rejection of these tumors. Furthermore, we propose that the expression of FasL in the ovary may be regulated by sex hormones, differentially acting through various hormone receptors.** The questions that we seek to answer are the following: How is the Fas/FasL system involved in the normal and neoplastic ovary and does this system mediate tumor escape from immune surveillance? Do sex hormones regulate the expression of FasL in the ovary? Does the differential expression of estrogen receptor subtypes correspond to ovarian carcinogenesis?

VII. METHODS

I actively performed the following methods described below in the laboratory of Gil Mor, M.D., Ph.D or Eva Sapi, Ph.D: Acquisition and storage of all clinical material; all immunohistochemistry for Fas, FasL, and cytokeratin; all preparation of RNA and protein using TRIzol®; all RT-PCRs and Western Blots (excluding the estrous cycle); and all hormonal studies with both organ and cell cultures. The author did not perform the TUNEL assays or the rat experiment.

From my work, I have written several articles and abstracts that were edited and reviewed by Gil Mor, Tom Rutherford, and/or Eva Sapi. Those that have been accepted for publication include:

Rutherford, T., Brown, W.D., Sapi E., Aschkenazi S., Munoz, A., Verwer, K.M.A., Naftolin, F., Mor, G. 2000. Absence of estrogen receptor-β expression in metastatic ovarian cancer. *Obstetrics and Gynecology*. In press.

Sapi, E., Brown, W.D., Aschenkenazy, S., Tartaro, K., Lim, C., Munoz, A., Kacinski, B.M. Rutherford, T., Mor, G. 2000. Regulation of Fas Ligand Expression by Estrogen in Normal Ovary. *International Journal of Cancer*. Submitted.

Rutherford, T., Sapi, E., Brown, W., Verwer, K, Munoz, A., Mor, G. 2000. Fas and Fas Ligand Expression in Normal and Pathologic Ovarian Tissue. Society for Gynecologic Investigation (Abstr).

Brown, W., Mor, G., Rutherford, T., Tartaro, K., Sapi, E. 2000. Fas Ligand Expression Is Regulated by Estrogens in Normal and Neoplastic Ovarian Epithelia. Society for Gynecologic Investigation. (Abstr).

Brown, W.D., Sapi, E., Verwer, K.M.A., Mor, G. 1999. Fas Ligand Regulation in an Ovarian Organ Culture System:Implications for Ovarian Cancer. *Reproductive Immunology*. #P-10-NI-5. (Abstr.).

VII.A. Clinical Material – Human Ovarian Tissue Specimens

Ovarian specimens were obtained from patients at the time of surgery in the Department of Obstetrics and Gynecology, Yale University School of Medicine (HIC #10425). The tissue was divided and then a portion was transported in DMEM for later use in organ culture, snap-frozen in liquid nitrogen and stored at -70°C, and sent for histologic examination. Primary tumor and metastatic tumor were obtained from unmatched patients. Two of the primary and metastatic tumors were matched.

VII.B. Immnohistochemistry (IHC) for FasL, Fas, and Cytokeratin

Detection of FasL and Fas expression was performed using an anti-human FasL rabbit polyclonal antibody and a mouse monoclonal anti-human Fas antibody, (clones N-20 and B-10 respectively, Santa Cruz Biotechnology, Santa Cruz, CA). Detection of cytokeratin positive cells was performed using mouse anti-human monoclonal antibodies. All antibodies were added to dilution buffers consisting of PBS, 0.1% saponin, and 1% BSA before application to slides (50).

Following surgical removal or organ culture, ovary specimens were fixed in 4% paraformaldehyde and paraffin embedded using standard procedures. Five micrometer sections were deparaffinized using graded xylene and ethanol, and the endogenous peroxidase activity of the cells was quenched using 2% hydrogen peroxide in methanol for 30 minutes. The sections were then rehydrated through graded ethanol and washed with phosphate-buffered saline (PBS) for 5 minutes. Slides were incubated first with diluted whole goat serum (1:200, Organon Teknika Corp, Westchester, PA) for FasL and with diluted normal horse serum (1:200, Vector Laboratories, Burlingame, CA) for Fas and cytokeratin for 20 minutes at room temperature. Then they were incubated with

primary antibody overnight at 4°C in a humidified chamber (104). Slides were then rinsed with 1% Triton-PBS and washed with PBS for 15 minutes. Slides were incubated with biotin-conjugated goat anti-rabbit IgG (1:100, Vector Laboratories, Burlingame, CA) for FasL or with biotin-conjugated horse anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA) for Fas and cytokeratin for one hour and rinsed again in PBS for 10 minutes. Immunoperoxidase staining was carried out using Vectastain ABC (Vector Laboratories, Burlingame, CA) and 3'3-diaminobenzidine (DAB) HCl peroxidase substrate kits (Vector Laboratories, Burlingame, CA), following the manufacturer's instructions. Before development with DAB, slides were placed in 0.25% Triton for 30 seconds. After DAB development was completed (4-7 minutes) slides were washed for 5 minutes in water, counterstained with Harris hematoxalin solution (7.5g/L, Sigma, St. Louis, MO), and mounted (Permount, Fischer Scientific, Fair Lawn, NJ).

VII.C. In situ 3' End Labeling of DNA for Cell Death Detection

The presence in ovary tissue sections of single strand DNA breaks indicating apoptosis was assessed using the TUNEL technique (In Situ Cell Death Detection Kit, Fluorescent, Boehringer Manneheim, Indianapolis, IN) according to the manufacturer's instructions. Briefly, following deparaffinization and rehydration, slides were incubated with proteinase K (20mg/ml in 10mM Tris/HCL, pH 7.4) for 30 minutes at 37°C. Samples were then treated with terminal deoxynucleotidyl transferase enzyme for 60 minutes at 37°C in the dark. After washing with PBS, an alkaline phosphatase-antifluorescein antibody was added and nitro-blue-tetrazolium alkaline phosphatase kit (Vector) was used for color development. The TUNEL assays were performed by Eva Sapi.

VII.D. Preparation of Total RNA and Protein Samples for RT-PCR and Western Blot Analysis

Total RNA and protein were prepared from HEY cells, from ovarian organ culture tissue, and from frozen ovarian surgical specimens using TRIzol® reagent (GIBCO BRL, Gaithersburg, MD) according to manufacturer's instructions. This method allows for extraction of RNA and protein from the same samples. Protein concentrations were measured by the BCA Protein Assay (Pierce), according to the manufacturer's instructions.

VII.E. ReverseTranscription-PolymeraseChainReaction (RT-PCR) Analysis

While RT-PCR is considered to only be semiquantitative, we have characterized each system such that the reaction does not occur at the plateau phase. In addition each RT-PCR is also performed for β-actin, which is constitutively expressed. In doing so, we are able to compare the ratio of β-actin to the experimental bands to help quantify the results.

RT-PCR was performed using the RT-PCR kit from Pharmacia BioTech (Piscataway, NJ), according to the manufacturer's protocol. cDNA synthesis was carried out with 0.2 μg of pd $(N)_6$ and 5 μg of total RNA. Seven μl of the cDNA was then put in a reaction mix for PCR. The reaction mix consisted of 15 pmol of each primer, 5 μl PCR Buffer (10X), 2 μl Rediload, 2 mM $MgCl_2$, and 2.5 Units Taq polymerase (Gibco, BRL). The PCR reaction mix was then thermal-cycled on a Perkin-Elmer Thermalcycler for the indicated cycle programs.

The primers used for amplification of human FasL have been recently described (75) and have the following sequence: upstream 5'-

ATAGGATCCATGTTTCTGCTCCTTCCACCTACAGAAGGA-3' and downstream 5'–ATAGAATTCTGACCAAGAGAGAGCTCAGATACGTTGAC-3'.

The human Fas primers, described by Otsuki et al are as follows: forward 5'–AAGGAGTACACAGACAAAGCCC–3' and reverse 5'-AAGAAGAAGACAAAGCCACCC-3' (105). Each PCR cycle consisted of the following: a denaturation step at 94°C, for 30 seconds, annealing at 52°C (for FasL) or at 57°C (for Fas) for 30 seconds, and elongation at 72°C for 1 minute for 10 cycles, followed by 35 cycles modified by a cumulative 5 second increase of extension time per cycle.

In order to quantify the ratio of ERα:ERβ expression we amplified both receptors in the same sample using one primer common for both receptors and one specific for each receptor type. The principle of this approach has been previously described by Pujol et al (103). This competitive PCR is possible due to the homology between the two receptors, and involves the use of 3 separate primers: 5' sense oligonucleotide was 5'-AAGAGCTGCCAGGCCTGCC-3', 3'antisense for ER-α was 5'-TTGGCAGCTCTCATGTCTCC-3', and the 3' antisense oligonucleotide for ER-β was 5'-GCGCACTGGGGCGGCTGATCA-3'. 25 cycles: 94°C 30 sec, 62°C 30 sec, 72°C 1 min. All of the PCR products were analyzed on a TAE 1% agarose gel with ethidium-bromide.

The amplified PCR products were electrophoresed on a 1-2% agarose gel and visualized with ethidium bromide (2μg/70-ml gel) under UV light. Images of the gels were input into a computer through a video camera (Eagle Eye). The resulting digitized images were used for densitometeric analysis of the appropriate bands. The intensity of

each band was normalized against its corresponding ß-actin band in order to compare values between samples.

VII.F. Western Blot Analysis

Proteins were separated by SDS-PAGE using 10% polyacrylamide gels. Twenty µg of total cellular protein was loaded per well and run at 80V for 3 hours. The proteins were then transferred to nitrocellulose membranes at 32V overnight. The membranes were washed with ddH_2O and stained with Ponceau Red to ensure efficient transfer and equivalent loading of proteins. The membranes were destained with 0.5N NaOH and blocked with 5% milk for 1 hour. The blots were first incubated with the primary antibody (FasL monoclonal antibody, clone 33, Transduction Laboratories, Lexington, KY) at 1:1000 dilution for 1 hour. The primary antibody for Fas, a polyclonal antibody, (M-20, Santa Cruz Biotechnology, Santa Cruz, CA) was diluted at 1:500 for 1 hour. ERα antibody (NCL-ER-611 Novocastra) was diluted at 1:50 and incubated for 4 hours, while ERβ antibody (Ab-1 Calbiochem) was diluted at 1:500 and incubated for 1 hour. After washing with PBS-Tween, the membranes were incubated with the secondary antibody, peroxidase labeled horse anti-mouse gamma globulin (Vector Laboratories, Burlingame, CA) for 1 hour for the FasL membrane or for 4 hours with the Fas membrane. The antibodies were visualized by developing with the TMB Peroxidase substrate kit (Vector Laboratories, Burlingame, CA). The intensity of the bands was analyzed by densitometry and standardized against the total amount of protein present in the gel after staining with Coomassie blue using a digital imaging analysis system (AlphaEase, Alpha Innotech Corporation, San Leandro, CA, USA).

VII.G. Ovarian Organ Culture

Organ culture is a relatively new technique in which surgical tissue specimens are maintained in short term culture for experimental purposes. Because this method is in its infancy, it is not currently accepted by all scientists, and therefore continues to undergo further exploration.

Ovarian organ culture was performed, with few modifications, as previously described by Sapi et al. in which they cultured breast tissue (104). To monitor cellular proliferation, they performed immunohistochemical studies on the paraffin sections of the tissues for a cell cycle protein, Ki67. The sections stained positively for Ki67, demonstrating active cellular proliferation in the cultured tissues, thus showing the continued growth of the cultured tissue.

Thus, human ovary biopsy tissue was obtained from normal cycling women (less than 50 years old) who had undergone surgery at Yale-New Haven Hospital for benign conditions or for prophylaxis because of high risk for ovarian carcinoma. Immediately after excision, the tissue was washed with minimal essential medium, DMEM (Life Technologies, Grand Island, NY) supplemented with antibiotics and was dissected free of necrotic tissue. Under a sterile hood and using sterile instruments, each specimen was then cut into pieces approximately 1-2 mm^3 and then washed in PBS. 20 of these explants were then immersed in 5 ml of DMEM F12 without phenol red supplemented with 1% charcoal-stripped serum (to avoid steroid contaminants) and epidermal growth factor (100ng/ml) in 6-well culture dishes. For hormonal studies, the culture media contained 10^{-8}M 17 ß-estradiol, determined by dose response studies in the physiologic range to be the most effective concentration of estrogen (Sigma Chemical Co., St. Louis, MO). For

chemoprevention studies, culture media contained 10^{-7}M 4-hydroxy-tamoxifen (Sigma Chemical Co, St. Louis, MO). Cultures were then incubated at 37°C in a 5% CO_2 incubator on a shaking platform for 7-8 days, as the breast organ cultures were originally characterized. The culture media was renewed every 2 days. As a control, one sample was cultured without additional chemicals. The cultured tissues were then fixed in 4% paraformaldehyde for 6 hours, embedded in paraffin, and sectioned at 5μm.

VII.H. Cell Lines

HEY cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in McCoy's media supplemented with 5% fetal bovine serum (Life Technologies, Grand Island, NY) and with antibiotics/antimycotics. The HEY cell line was propagated in Dulbecco's modified essential medium (DMEM) Ham's F12 medium supplemented with 1% calf bovine serum. The cell line was cultured at 37°C humidified chamber with 5% CO_2 in air.

Cells were passed by standard methods of trypsinization, grown in 75 ml flasks, and allowed to replicate to 80% confluence before experiments were performed.

VII.I. In Vitro Hormonal Studies

HEY cells were incubated in serum-free, phenol red-free media for 24 hours before the addition of hormones/antihormones. Treatments were carried out in serum-free, phenol red-free media supplemented with 10^{-8} M β-estradiol (Sigma Chemical Co., St. Louis, MO), 10^{-7} M tamoxifen (Sigma Chemical Co., St. Louis, MO), or both treatments for three, six, or 24 hours. A sample with no additional chemicals served as a control. After the appropriate treatment times, the media was removed from each 75 ml

tissue culture flask and replaced with 7.5 ml of TRIzol® (GIBCO BRL, Gaithersburg, MD). RNA and protein were isolated following manufacturer's instructions and analyzed using RT-PCR and Western Blot Analysis, respectively.

VII.J. Animal Experiment-Specimens from Rats

All studies involving animals were approved by Yale Institutional Review Boards for Animal Care or for Human Experimentation. Animals were evaluated by vaginal smears during three consecutive cycles. Those that showed regular cycling were sacrificed in the morning, following deep ether anesthesia. Each ovary specimen was excised and frozen in liquid nitrogen for RNA and protein extraction, or fixed in 4% freshly made paraformaldehyde and paraffin-embedded for immunohistochemistry. Tissue blocks were stored at room temperature until 5μm sections were cut and mounted.

VIII. RESULTS

VIII.A. Fas/FasL in the Normal and Neoplastic Ovary

VIII.A.1. Immunohistochemical Localization of FasL in Normal Human Ovarian Tissue

Paraffin sections from normal ovarian tissue were studied for FasL expression using a specific polyclonal antibody (clone N-20, Santa Cruz Biotechnology, Santa Cruz, CA). The normal human ovary shows weak immunoreactivity for FasL in the granulosa layer of the follicles (Fig. 6A) and in some prominent inclusion cysts (Fig. 7A), as determined by colocalization of cytokeratin (Fig. 10). These inclusion cysts stain most intensely in the preneoplastic areas where the epithelial layer has lost its capacity to grow as a monolayer. The ovarian stroma appears to be negative for FasL, aside from the occasional macrophage within the stroma.

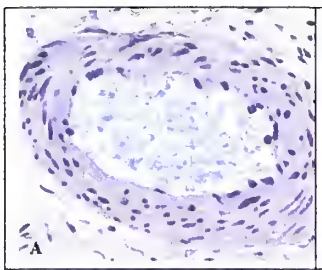

Figure 6A. A normal human ovarian follicle stained for FasL

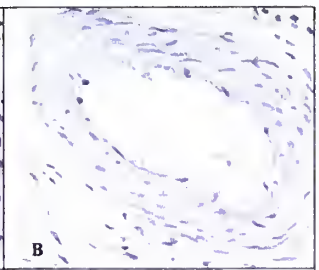

Figure 6B. A normal human ovarian follicle stained for Fas.

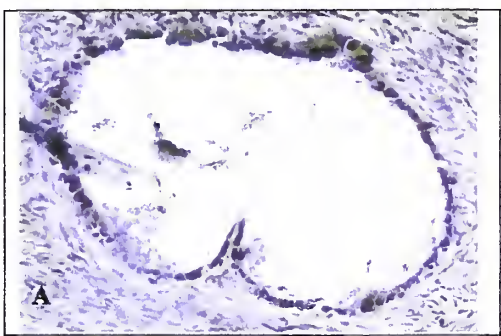

Figure 7A. An ovarian inclusion cyst stained for FasL.

Figure 7B. The same ovarian inclusion cyst stained for Fas.

VIII.A.2. Immunohistochemical Localization of FasL in Malignant and in Metastatic Human Ovarian Tissue

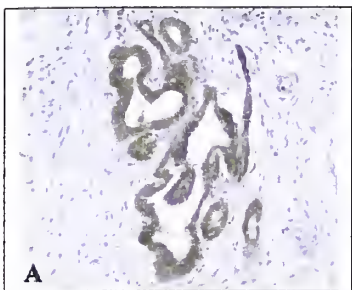

Figure 8A. Localization of FasL in primary ovarian cancer.

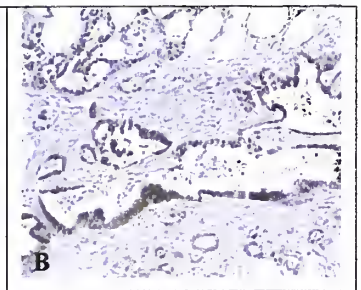

Figure 8B. Localization of FasL in metastatic ovarian cancer.

While normal human ovarian tissue shows only weak FasL immunoreactivity surrounding the follicles, immunohistochemistry of human ovarian tumors shows discrete, intense staining in the clusters of neoplastic cells, including both cytoplasmic and membranal distributions (Fig. 8A). Sections of peritoneal metastases were examined also and were found to be strongly positive for FasL within the neoplastic implants throughout the peritoneal tissue (Fig. 8B).

VIII.A.3. Immunohistochemical Localization of Fas in Normal, Malignant, and Metastatic Human Ovarian Tissue

As with FasL, paraffin sections of normal and metastatic ovarian tissue were studied for the presence of Fas using a specific monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). There did not appear to be a variation in Fas immunoreactivity among the different tissue samples. The stroma appears to be moderately immunoreactive for Fas while the follicles are not (Fig. 6B). The metastatic implants were weakly positive for Fas (Fig. 9), but Fas staining was only detectable in normal tissue in the inclusion cysts (Fig. 7B), corresponding to the areas of FasL expression in inclusion cysts.

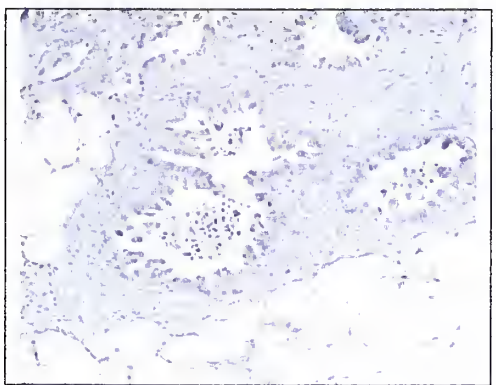

Figure 9. Localization of Fas in metastatic ovarian cancer.

VIII.A.4. In situ Cell Death Detection-Terminal Deoxy (d)-UTP Nick End-Labeling (TUNEL)

To determine the possible role of Fas and FasL coexpression in the induction of cell death of epithelial cells lining inclusion cysts, we studied the presence of apoptosis in these ovarian structures. Apoptotic cells were detected by staining of DNA fragments with the TUNEL technique. TUNEL-stained nuclei were mainly found in the cells lining the inclusion cysts where Fas and FasL are colocalized (Fig. 10). No, or very few, apoptotic nuclei were detected within the stroma surrounding these cysts. Further characterization of these cells with anticytokeratin antibody identified these apoptotic cells as epithelial cells.

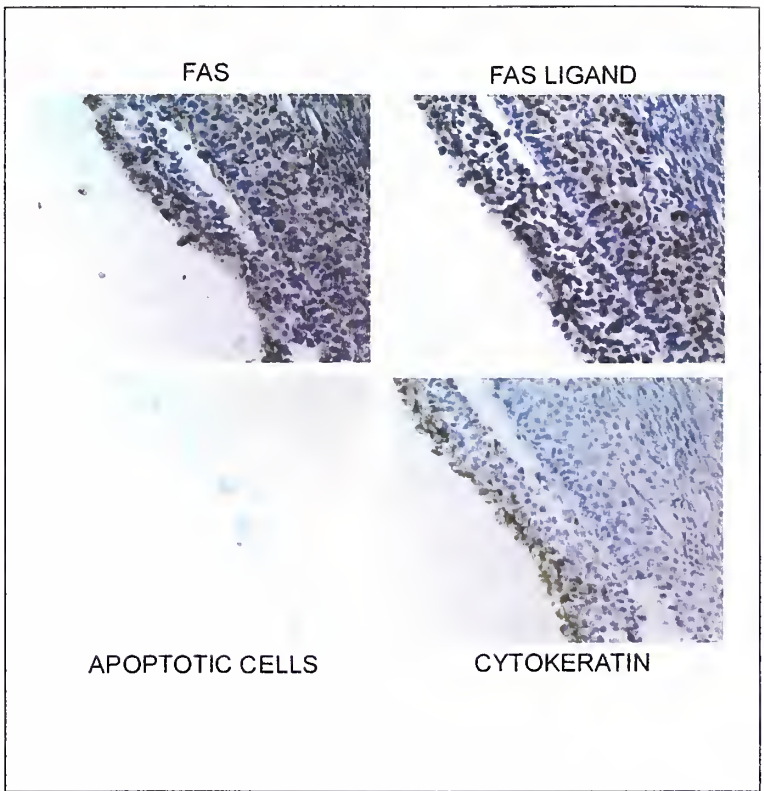

Figure 10. Localization of Fas/FasL and cytokeratin compared to location of apoptosis.

VIII.A.5. Expression of FasL mRNA in Normal, Malignant, and Metastatic Human Ovarian Tissue

To confirm the results obtained from the immunohistochemical studies, we then evaluated the expression of FasL mRNA in normal, primary malignant, and metastatic human ovarian tissue obtained from surgical specimens. Total RNA was extracted and tested using RT-PCR. We found that normal ovary expresses a weak amount of FasL,

while primary ovarian tumors express a variable amount of FasL. Upon analysis of the metastatic ovarian tumor RNA, we found this tissue to consistently express FasL at extremely high levels compared to the other tissues (Fig. 11).

Figure 11. Expression of FasL mRNA in normal ovary, primary ovarian cancer, and metastatic ovarian cancer.

VIII.A.6. Expression of FasL Protein in Normal, Malignant, and Metastatic Human Ovarian Tissue

In addition to examining the RNA of normal, primary malignant, and metastatic ovarian tissue from surgical specimens, we also compared the levels of FasL protein expression among these samples. As with the RNA, there appears to be a progression of increasing FasL protein expression as the tissue becomes more aggressive in nature. The primary ovarian tumor tissue expresses more FasL than the normal tissue, while the metastatic tissue expresses the largest amount (Fig. 12). Interestingly, the metastatic tissues are the only samples that demonstrate expression of soluble FasL upon Western Blot analysis (Data not shown).

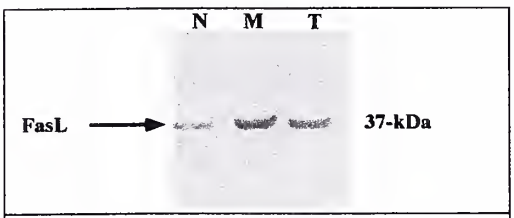

Figure 12. Expression of FasL protein in normal ovary, primary ovarian cancer, and metastatic ovarian cancer.

VIII.B. Hormonal Regulation of FasL

VIII.B.1. FasL Expression During the Normal Rat Cycle

In order to evaluate the effect of hormonal changes in the ovary on FasL expression, we extracted protein from the rat ovary in each stage of the estrous cycle. Western blot analysis of these proteins showed dramatic fluctuations in FasL expression throughout the cycle. The highest levels of FasL protein expression were detected during the estrous phase, followed in decreasing order by metestrous and diestrous phases. FasL protein expression was not detected during proestrous (Fig. 13).

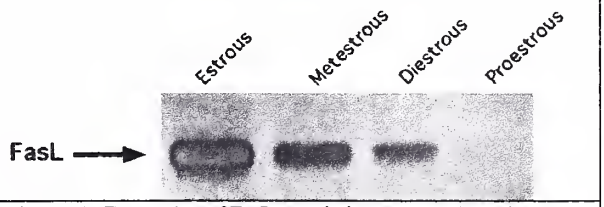

Figure 13. Expression of FasL protein in rat ovary throughout the cycle.

VIII.B.2. Estrogen Regulation of FasL Expression in Normal Ovarian Tissues

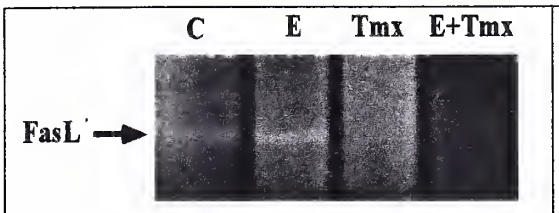

Figure 14A. Effect of estrogen on FasL mRNA expression in ovarian organ culture.

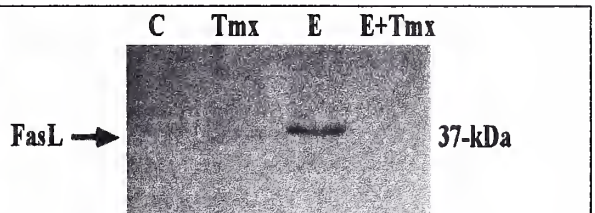

Figure 14B. Effect of estrogen on FasL protein expression in ovarian organ culture.

We investigated the hormonal regulation of FasL expression in normal human ovarian tissue using an organ culture system. FasL mRNA and protein expression increases under the influence of estrogen, as determined by RT-PCR and Western Blot analysis (Fig. 14A, 14B). Furthermore, we found by IHC of paraffin sections that this increase in FasL is localized to the cells immediately surrounding the follicles in the

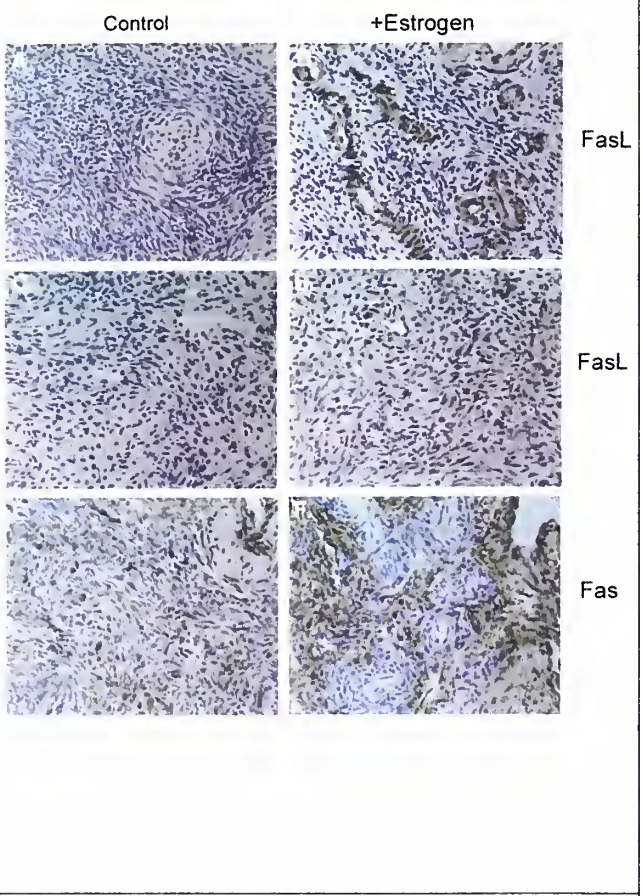

Figure 15. Effect of estrogen administration on Fas and FasL immunoreactivity in human ovarian organ culture immunohistochemistry.

granulosa layer (Fig. 15). However, not every ovary responded to estrogen treatment. In some of our organ cultures' slides, we did not detect an increase in FasL in inclusion cysts following estrogen treatment. This lack of response seems to be related to the level of expression. Thus, there was no increase in FasL expression in the group receiving treatment as compared to the inclusion cysts in the control cultures if they were already expressing high amounts of FasL (Data not shown).

VIII.B.3. Estrogen Regulation of Fas Expression in Normal Ovarian Tissues

We also examined the role of estrogen stimulation in the regulation of Fas expression in human ovarian organ cultures by immunohistochemical analysis (Fig. 15). Estrogen administration did not have any effect on Fas expression suggesting that Fas expression is not under direct control of estrogen. .

VIII.B.4. Estrogen Regulation of FasL in Ovarian Cancer Cell Line (HEY)

In addition to examining FasL regulation in organ cultures, we also evaluated the effect of estrogen on ovarian cancer cell lines. Similar to our results reported for the whole tissue, we found that FasL expression in tumor cells is susceptible to hormonal regulation.

In the human ovarian epithelial carcinoma cell line, HEY, estrogen increases FasL mRNA expression, demonstrated by RT-PCR (Fig. 16). When HEY cells were treated with estrogen at different time intervals, a peak in FasL mRNA expression was detected after 6 hours of estrogen treatment.

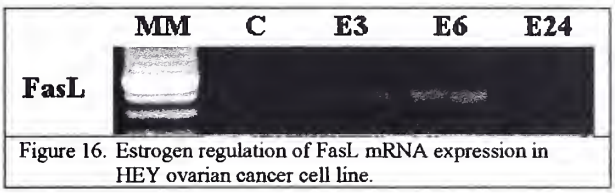

Figure 16. Estrogen regulation of FasL mRNA expression in HEY ovarian cancer cell line.

VIII.B.5. Effect of Tamoxifen on FasL Expression in Normal Ovarian Tissue

In order to determine that the effect of estrogen on FasL expression was estrogen-receptor mediated we studied the effects of Tamoxifen on FasL expression in ovary organ cultures. When we examined the ovarian tissue cultured with Tamoxifen, we found a decrease in FasL mRNA and protein expression (Fig. 14A, 14B). This difference was not apparent, however, under immunohistochemical analysis (Data not shown).

VIII.B.6. Effect of Tamoxifen on FasL Expression in Ovarian Cancer Cell Line (HEY)

Tamoxifen had an agonist effect on FasL expression in HEY cells as determined by RT-PCR analysis. Similar to estrogen, there was a gradual increase in FasL expression, peaking at 6 hours of tamoxifen stimulation (Fig. 17).

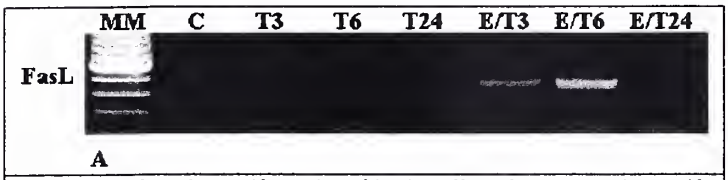

Figure 17. Effect of tamoxifen and combination effect of estrogen and tamoxifen on FasL mRNA expression in HEY ovarian cancer cell line.

A synergistic effect of estrogen and tamoxifen was observed. Thus, the addition of both hormones together induce FasL expression levels higher than those observed with estrogen or tamoxifen alone (Fig. 17).

VIII.B.7. Effect of Estrogen and Tamoxifen on Fas Expression in Ovarian Cancer Cell Line (HEY)

Figure 18A. Estrogen regulation of Fas mRNA expression in HEY ovarian cancer cell line.

Figure 18B. Effect of tamoxifen and combination effect of estrogen and tamoxifen on Fas mRNA expression in HEY ovarian cancer cell line.

When we analyzed the effect of estrogen and tamoxifen on HEY cells' Fas expression no effects were found. Estrogen had no effect on Fas expression at 3, 6, and 24 hours. Tamoxifen alone or together with estrogen had no apparent effect on the expression of Fas in HEY cells either (Fig. 18A, 18B).

VIII.C. ER Alpha and Beta Expression in the Ovary

VIII.C.1. mRNA Expression of ER alpha and beta in Normal Human Ovarian Tissue, Primary Human Ovarian Tumor, and Metastatic Disease

In order to study the potential role of the two ERs in ovarian carcinogenesis, we evaluated the relative levels of ER-α and ER-β mRNA expression in 9 normal ovarian samples, 8 primary ovarian cancers and 8 metastatic ovarian cancers. Details of each patient's age and histologic diagnosis are described in Table 2.

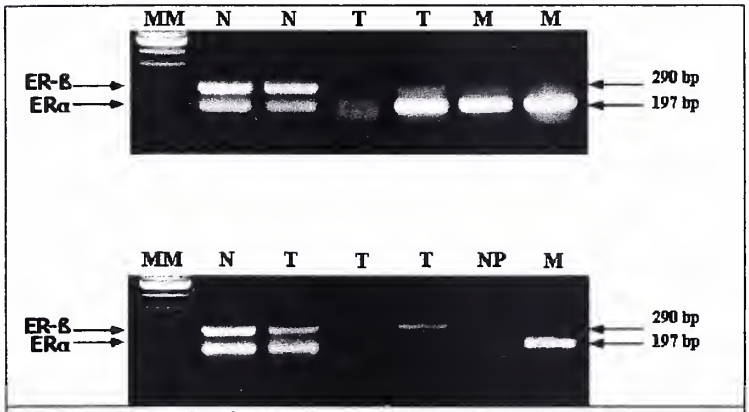

Figure 19. ERα and ERβ mRNA expression in human ovarian samples.

In the non-neoplastic ovary, both estrogen receptors alpha and beta are expressed although ERβ is the predominant ER subtype. Quantification of the ERα:ERβ ratio showed a ~1:2 relationship. When we further analyzed ER expression in primary ovarian tumors, a high variation in the ER mRNA expression and ratio was detected between the different samples (Fig. 19). As shown in Table 2, 37.5 % of the cases expressed both ERα and ERβ although the level of

expression for ERα was higher than ERβ. 37.5% primary tumors expressed only ERα and 25% had only ERβ (Table 2).

On the other hand, metastatic tumors expressed only ERα mRNA with complete absence of ERβ mRNA. In one case, expression of ERα and ERβ were identified although ERβ mRNA level was markedly lower than ERα (data not shown). However, this sample was, according to the pathologic diagnostic, heterologous malignant mixed Müllarian tumor.

VIII.C.2. Protein Expression of ER alpha and beta in Normal Human Ovarian Tissue, Primary Human Ovarian Tumor, and Metastatic Disease

In order to evaluate if the ERα and ERβ protein was expressed in the human ovarian tissues, protein extracted from the same cases were analyzed by Western blot analysis using a monoclonal antibody for ERα (clone NCL-ER-6F11, Novo Castra Laboratories, Claremont Place, UK) and a polyclonal antibody for ERβ (clone AB-1, Oncogene Research Products, Cambridge MA). The same results as described for the RNA were found with the western blot analyses. Thus, ERα and ERβ were present in normal ovary and primary tumor. Metastatic ovarian tumors were characterized by the expression of high levels of

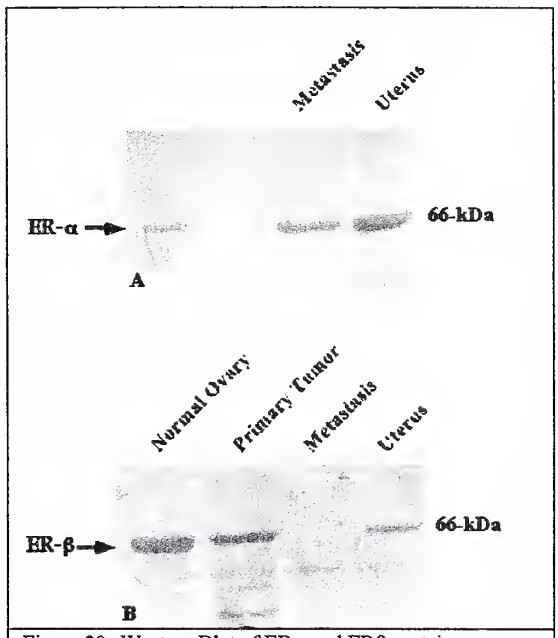

Figure 20. Western Blot of ERα and ERβ protein expression in human ovarian samples.

only ERα and the complete absence of ERβ protein expression (Fig. 20). Normal human endometrium was included as positive control for ERα. The normal endometrium expresses high levels of ERα and low levels of ERβ.

IX. DISCUSSION

IX.A. Fas/FasL in the Normal and Neoplastic Ovary

We began our investigation of Fas/FasL and the ovary by defining the presence and location of FasL in the normal human ovary. Our results indicate that the normal human ovary expresses FasL in two main areas – in the granulosa layer of the follicle and in the inclusion cysts (Fig. 6A, 7A).

We found that the granulosa layer of the follicle is weakly immunoreactive for FasL. This is consistent with previous studies reporting the presence of FasL in follicular cells of the ovary (61, 62, 63). In the murine ovarian follicle, FasL has been localized to the granulosa cells, while Fas seems to be restricted to the oocyte (61). Others have found the opposite in the rat ovary with the oocyte expressing FasL and the granulosa expressing Fas (62). Although there is no consensus on the exact cellular distribution of FasL in the human ovary, it is clear that the Fas/FasL system is present and does operate in some capacity.

Female reproductive tissues such as the ovary, breasts, and uterus undergo a normal, periodic cycle, resulting in cellular proliferation and death. In the ovary, this is exemplified by follicular growth and atresia. The ovarian cycle consists of follicular and luteal phases (Fig. 3), during which a single successful follicle undergoes ovulation and becomes a corpus luteum. Meanwhile, the unsuccessful follicles undergo atresia. It has been shown that this cellular reduction in the ovary occurs via apoptotic mechanisms (55). Apoptosis is influenced by a wide variety of stimuli. Among the known "death receptors" (TNF-R1, DR-3, TRAIL-R1, and 2), Fas is one of the most important.

Interaction between Fas and its ligand (FasL) induces receptor trimerization, which in turn results in recruitment of the adaptor protein FADD and activation of caspases, which leads to irreversible cell damage and death (Fig. 1) (37). Our results suggest that Fas/FasL is one of the underlying mechanisms of apoptosis in the cycling ovary.

A second role for the Fas/FasL system may be in post-ovulatory epithelial repair. In the ovarian surface epithelium, we found a moderate amount of both Fas and FasL. This is in agreement with a study showing that the addition of Fas mAb to enriched cultures of mouse OSE immediately stimulates apoptosis, suggesting the presence of FasL in the surface epithelium. These cells on the surface of the preovulatory follicle exhibit programmed cell death in preparation for ovulation (65); therefore, the Fas/FasL system could serve as the mechanism by which these cells undergo apoptosis and allow for release of the ovum.

During the postovulatory repair process, it is thought that proliferating epithelial cells invaginate, forming clefts, or become entrapped in the stroma, forming cysts (16). Within these structures, the cells are eliminated via apoptosis. We found co-localization of Fas and FasL in the epithelial cells lining these cysts. Furthermore, TUNEL assays of these same sections demonstrated apoptotic cells along this border of co-expression (Fig. 10), indicating a role for this system in controlling epithelial cell propagation. Although Fas and FasL appear to be distributed within the stroma as well, apoptosis seems to occur only along the epithelial areas of coexpression. This could be due to increasingly membranal expression of the molecules as they near the surface, allowing for more interaction between the receptor and its ligand.

In addition to the above-mentioned roles for Fas/FasL in the normal ovary, it may also serve as an immunological barrier. As Sertoli cells express FasL to defend the testicle from immunological invasion (38), perhaps the FasL-expressing granulosa cells surrounding each oocyte serve a similar function of immune protection. FasL expression may enable the follicular cells encircling the oocyte to kill invading immune cells, minimizing dangerous inflammatory reactions in this vital area. Interestingly, previous studies have shown that the age of an organ may affect its immune privileged status (107). They found that transplanted testicular tissue from young mice can resist rejection whereas tissue from adult mice may be rejected. If age is found to similarly down-regulate FasL expression in the ovary, this could render the ova susceptible to immune invasion and possibly be a factor in the initiation of menopause.

While examining Fas and FasL expression in inclusion cysts, we also observed a disproportionately strong immunoreactivity for FasL in those cysts that exhibited abnormal growth patterns, such as those developing into an epithelial cell mass rather than a monolayer. While Fas expression remained unchanged, FasL appeared to be overexpressed in these areas. Because inclusion cysts are exposed to concentrated levels of proliferative stimuli within the ovarian stroma, there is a high mitotic rate and a high probability of cellular mutation and clonal propagation (16), especially if that mutation disables a system of apoptosis such as Fas/FasL. This imbalance in Fas and FasL expression may indicate an early stage of neoplastic transformation. The over-expression of FasL by epithelial cells could serve as the mechanism by which abnormal ovarian surface epithelium escape immune surveillance, allowing tumor growth and metastasis.

This theory is supported by the subsequent observation that FasL expression in the human ovary increases in direct proportion to the degree of malignancy while Fas expression remains stable (Table 1). Upon light microscopic examination of normal human ovary tissue, primary ovarian cancer, and metastatic ovarian cancer sections stained for Fas and FasL, we found a dramatically different staining pattern among the various FasL samples (Fig. 8A, 8B) and no change among the Fas samples (Fig. 9). As compared to the normal ovary, an increase in FasL intensity was discovered in the primary tumor, and a wider distribution and even greater intensity was encountered in the metastatic disease. RT-PCR (Fig. 11) and Western blot analyses (Fig. 12) confirmed these results as they revealed enlarging FasL bands, particularly in the metastatic disease samples. The distribution and intensity of Fas expression among the tissues remained at a constant, low level as seen on IHC.

While FasL expression has been shown to be a major factor in immune privilege (Fig. 2), this same mechanism of immune evasion has been described in multiple cancers (42). Our results indicate that it may apply to ovarian carcinoma as well. If Fas-expressing activated lymphocytes invade an ovarian neoplasm that overexpresses FasL, the lymphocytes will undergo apoptosis induced by the interaction between Fas and FasL, enabling the cancerous cells to proliferate and metastasize.

IX.B. Hormonal Regulation of FasL

Once we established the presence of Fas and FasL in the ovary, we studied the physiological role of the Fas/FasL system in normal ovarian function and the factors that may regulate its expression and function. In the present study we demonstrate that FasL

expression is hormonally dependent and that estrogen has a direct effect on FasL expression.

The normal ovary both synthesizes and responds to hormones throughout the hormonal ovarian cycle; therefore, we speculated that if it is hormonally regulated, FasL expression within the ovary should also change during the cycle. Indeed, FasL expression fluctuates in accordance with the stage of the cycle (Fig. 13).

In order to identify the hormone(s) responsible for this phenomenon, we studied the effect of estrogen on FasL expression using two *in vitro* systems, an ovarian organ culture and a cell culture. Organ culture is a novel means of culturing tissue from surgical specimens for short-term experiments (104). In using this system, we were able to administer various sex hormones to pieces of ovarian tissue and observe their effects on the tissue as a whole. This system more closely resembles that of the human body than cell culture since different cell types (granulosa, theca, oocyte, stroma epithelial cells) are allowed to interact and affect surrounding cells as they would *in vivo*. A preculture control and the cultured control samples serve as comparisons. At the end of the experiment, the tissue may be put into paraffin blocks for immunohistochemical studies or processed for RNA and protein analysis.

We cultured 20 ovaries in this manner in the presence of 17-β-estradiol (10^{-8} M) and found that estrogen upregulates the expression of FasL. This is in accord with prior studies on estrogen and FasL in the breast in which an ERE-like motif was reported in the promotor region of the human FasL gene (82). In this study, estrogen increases FasL expression in the MCF-7 breast cancer cell line. In our study, both the preculture and culture controls stained similar to the normal ovarian tissue obtained directly from

surgical removal, with FasL present in the granulosa layer and in inclusion cysts (Fig. 15). With 17-β-estradiol administration, IHC studies demonstrated an increased FasL immunoreactivity in the granulosa layers of the follicles, suggesting an upregulation of FasL expression with estrogen addition (Fig. 15). At the RNA and protein levels, this same effect was detected. Analysis of the bands on RT-PCR and Western blot using a densitometer revealed a significant increase in FasL expression after estrogen treatment as compared to the control group (Fig. 14A, 14B).

When we examined the same sections for effects of estrogen on inclusion cysts, we found no difference in FasL expression between the treated and untreated tissues. Because FasL, along with Fas, is expressed at a continually high level in inclusion cysts to mediate apoptosis and assist in entrapped epithelial cell removal, estrogen administration cannot further increase FasL expression.

In order to determine the effect of estrogen and tamoxifen on ovarian epithelial cells in isolation, we studied the epithelial ovarian cancer cell line HEY. Previous investigators had reported a complete lack of estrogen receptors in the HEY cell line which was inconsistent with our findings (102). Our cultures of HEY cells have apparently evolved to have different ER characteristics and they express ERβ mRNA as determined by RT-PCR. Therefore, they theoretically are able to respond to estrogen stimulation. This cell line also displays a small baseline amount of FasL when grown under control conditions, making it an adequate model for determining the effect of estrogen on FasL expression in an ovarian cancer cell lines.

Our results show that the HEY cells respond to estrogen with increased FasL expression just as the organ culture tissue did and as the previously reported MCF-7

breast cancer cell line (82). This estrogenic effect was apparent as early as 3h of treatment, with a peak in FasL mRNA expression at 6 hours of estrogen treatment (Fig. 16). On the other hand, estrogen had no effect on Fas expression. Fas mRNA levels were similar throughout the time course of estrogen treatment (Fig. 18A).

Throughout the body, the overall effect of estrogen is mitogenic, stimulating growth and proliferation of tissues. In contrast, the estrogen agonist/antagonist tamoxifen has variable effects, depending on tissue type (86). Tamoxifen has been reported to have an antiestrogen effect on breast tissue and is, therefore, used in the treatment and prevention of estrogen-responsive breast cancers.

In contrast, ovarian cancer patients have not had the same success with tamoxifen treatment with only a 7-18% response rate (90). Some have postulated that ovarian tissue is hormonally unresponsive; however, the majority of ovarian cancers express estrogen receptors (89). We found differing results between organ cultured ovarian tissue and HEY ovarian carcinoma cells. When samples of the whole ovary were cultured *in vitro* and treated with tamoxifen, the effect was different than the previously reported inhibitory effect of tamoxifen on MCF-7 breast cancer cells (82). Moreover, the effect of tamoxifen treatment on HEY cells did not oppose that of estrogen. Instead, it was agonistic, increasing the expression of FasL mRNA over time (Fig. 17). Furthermore, when estrogen and tamoxifen were co-administered, there was a synergistic effect, with even greater enhancement of FasL expression than either treatment alone (Fig. 17). Fas expression did not change with either treatment. Further studies need to be done on cell lines that contain ERα alone and both estrogen receptors in order to determine if this difference in tamoxifen action is ER dependent. Also, the overall effect of tamoxifen in

the ovary may be dependent upon the interaction of the many different cell types in the complete ovary, since the organ cultures did show a down-regulation of FasL when incubated with tamoxifen.

IX.C. ER Alpha and Beta Expression in the Ovary

Upon learning of the role that FasL may play in ovarian carcinogenesis and after realizing the potential influence estrogen may have on the regulation of FasL expression, we undertook the task of characterizing the population of estrogen receptor subtypes in the ovary and in different stages of disease. We found that the normal ovary is characterized by the presence of both ERα and ERβ. This is in agreement with previous studies showing co-expression of ERα and β in the ovary, with ERα mRNA expression at a lower level than ERβ mRNA (102, 98, 106). The cellular distribution of ERβ in human and rodents, has been shown to be primarily in granulosa cells of small growing and preovulatory follicles while ERα is distributed throughout the normal ovary (106). Only recently, the ER status of normal ovarian surface epithelium (OSE) has been reported to show the presence of ERα and ERβ. After characterizing the ER status of normal ovary, we examined the ER subtypes in primary ovarian cancer and in metastatic disease. We demonstrate the presence of high levels of ERα mRNA expression in metastatic ovarian carcinoma with undetectable ERβ mRNA expression. This was further confirmed by Western blot analysis showing the presence of ERα protein but not ERβ in metastatic ovarian tumor (Fig. 19, 20).

Since the majority of ovarian cancer is presumed to originate in ovarian surface epithelium and 62.5% of the studied ovarian tumors expressed ERβ (Table 2) the

complete absence of ERβ expression in metastatic ovarian cancer could represent a characteristic of metastatic transformation. Another possibility is that cells expressing ERβ cannot survive in metastatic ovarian epithelial cancer.

Our work demonstrates a high variation in ER expression in primary ovarian tumors, with a predominant characteristic change in the ERα:ERβ ratio showing a higher level of ERα than ERβ compared to normal ovarian epithelium (Table 2). Further, we report the absence of ERβ expression at the mRNA and protein levels in metastatic ovarian tumors. These results, together with previous studies, provide strong evidence for an essential role of the ER type in the regulation of transformation and metastasis of epithelial ovarian cancer.

ERα expression has been extensively studied in ovarian cancer in order to correlate it to clinical behavior and prognosis. Despite that, no clear relationship between ER expression and tumor histology, patient age, or outcome has been noted in epithelial tumors (108). Of particular interest to normal ovarian physiology and malignant transformation is the observation that malignant tissues from epithelial origin express substantially higher levels of ERα than ERβ. Recent studies have shown that the relative levels of ERα and ERβ are important determinants of the biological response to ER agonists in specific target tissue and could be important in the pharmacology of antiestrogens (109). Therefore, future studies should focus on the use of a variety of selective estrogen receptor modulators (SERMs), including raloxifene, in the treatment of ovarian cancer. It has been shown that estrogen induces apoptosis in neuronal-like cells expressing ERβ but induces cell growth and proliferation in similar cells expressing both

alpha and beta ER (110). A lack of ERβ in malignant ovarian tissue could represent a state of resistance to inhibitory pathways.

In the primary ovarian tumors analyzed, we found a wide range in phenotypic ER expression with decreasing levels of ERβ expression being the only common denominator. These findings have two important implications. One is that the balance between ERα and ERβ receptors may be imperative to maintain normal cellular function. The second implication may be that, as the level of the ERβ decreases, uncontrolled cellular proliferation leads to a metastatic state. Therefore, the ER status could be used as a prognostic marker of ovarian carcinogenesis.

These findings indicate that a fundamental biological difference may exist between primary and metastatic ovarian cancer cells, which could be due to intrinsic, or extrinsic factors regulating gene expression. Understanding these factors may furnish insight into carcinogenesis, metastatic behavior and effects of treatment.

IX.D. Conclusion

Epithelial ovarian cancer is a leading cause of mortality among women in the United States, yet many questions remain regarding its etiology, prevention, detection and treatment. We believe that many of these questions can be answered by serious investigation into the Fas/FasL system of apoptosis and its relevance to ovarian tumorigenesis.

In the present study, we demonstrate the presence of Fas and FasL in ovarian tissue and offer several possible roles for the Fas/FasL system in the normal ovary. We also identify estrogen as a key regulator of FasL expression in the ovary. Furthermore, we demonstrate a gradient of increasing FasL expression along the pathway of cancer development and spread, along with a changing estrogen receptor distribution. From these observations, we conclude that a disruption in the Fas/FasL system in the ovary may underlie ovarian carcinogenesis.

An important question in tumor immunology involves the means by which a neoplasm is able to escape immune surveillance. We and others have demonstrated a role for FasL in this phenomenon of immune escape. This theory holds that Fas-expressing activated lymphocytes detect and invade a tumor, they contact the FasL-bearing cells of the tumor, and the Fas/FasL interaction induces cell death in the immune cells. Thus, an imbalance between Fas and FasL, with an overexpression of FasL, could lead to uncontrolled cellular proliferation and perhaps even to cellular transformation and tumor growth.

Although we believe that the Fas/FasL system plays an integral part in ovarian pathogenesis, it is likely that it represents only one of many other factors. The

development of ovarian carcinoma is the end result of a complex multistep process involving the complementary actions of different cancer causing genes (18), including p53, *erb*B-2, K-*ras*, *myc*, and *fms*.

One hypothesis for ovarian transformation links the p53 and Fas/FasL pathway (Fig. 21). The p53 gene has been implicated in ovarian cancer, with at least 50% of advanced stage ovarian cancers demonstrating point mutations (111). Recently, researchers have found that a functional p53 protein is necessary for proper Fas/FasL activity. Bennett and others found in human vascular smooth muscle cells that p53 transiently increases surface Fas expression by transport from the Golgi complex (112). By moving Fas to the cell surface, it allows for the interaction of Fas with FasL, which activates the death signal, and initiates apoptosis. Thus, through Fas transport, p53 can mediate programmed cell death.

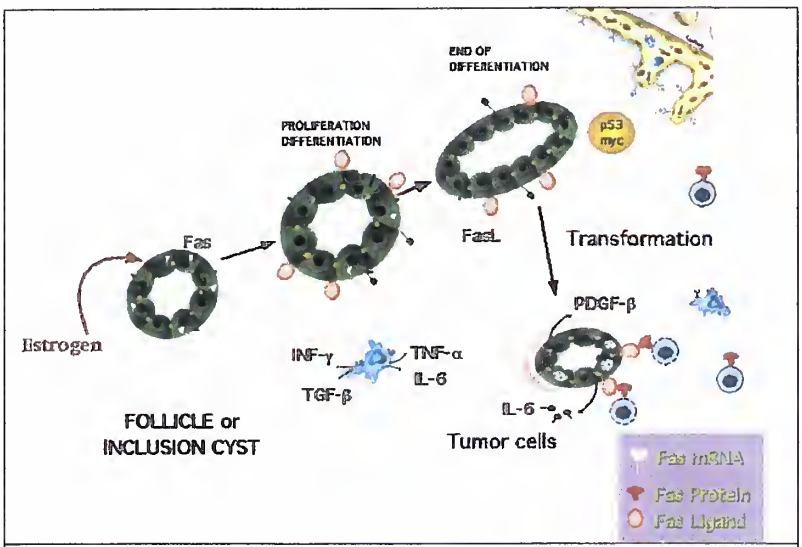

Figure 21. Model of proposed interactions among hormones, p53, and Fas/FasL in the ovary.

Repetitive injury and repair of the ovarian surface epithelium occurs secondary to continual ovulation, resulting in an increased mitotic rate and a high probability for mutations to occur. **If the p53 gene were mutated during the post-ovulatory repair process, this would prevent Fas movement to the cell surface, would allow for the sole expression of FasL, and would prevent the normal signal for death. If this**

abnormal cell was then exposed to a microenvironment rich in estrogen lining an inclusion cyst within ovarian stroma, FasL would be up-regulated, further exaggerating the Fas/FasL imbalance. The cell could then multiply without restraint due to a lack of apoptosis, creating a lineage of apoptosis-resistant cells. In addition, these cells would then have the capacity to evade an immune response due to their expression of FasL; thus, setting the stage for cellular transformation, growth, and metastasis.

By recognizing the role of Fas/FasL in the normal ovarian cycle and its potential role in neoplastic transformation and immune escape, we can offer new insights into the etiology of ovarian cancer. In addition, by understanding the factors that regulate this system, we can provide new targets for preventive and therapeutic interventions.

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XI. Figure References and Legends

Figure 1. Model of internal events in apoptotic cell (p. 14).

Interaction between Fas and its ligand (FasL) induces receptor trimerization, which in turn results in recruitment of the adaptor protein FADD and activation of caspases, which leads to irreversible cell damage and death. Model created by Gil Mor.

Figure 2. Model of Fas/FasL interaction in immune privileged tissues (p.16).

Immune privileged tissues can prevent serious inflammatory responses through the expression of FasL, which interacts with the Fas receptor on invading T cells and induces apoptosis. Model created by Gil Mor.

Figure 3. Model of cyclic changes in female reproductive tissues (p.18).

Serum hormone levels, follicular activity, and changes in the human endometrium during the menstrual cycle. Model created by Gil Mor.

Figure 4. Model of intracellular actions of estrogen (p.28).

Estrogen is a steroid hormone that acts on intracellular receptors to directly affect gene transcription. Model created by Gil Mor.

Figure 5. Model of estrogen receptors (p.31).

Comparison of the twoestrogen receptor subtypes alpha and beta. Model created by Gil Mor.

Paraffin sections of normal human ovarian tissue were stained for FasL or Fas. (**A**) A normal human ovarian follicle stained for FasL. (**B**) A normal human ovarian follicle stained for Fas.

Paraffin sections of normal human ovarian tissue were stained for FasL or Fas. (**A**) An ovarian inclusion cyst stained for FasL. (**B**) The same ovarian inclusion cyst stained for Fas.

Immune staining of paraffin sections of primary ovarian tumors and metastatic ovarian cancer. (**A**) Primary ovarian cancer. (**B**) Ovarian cancer metastatic to the peritoneum.

Fas expression in metastatic ovarian cancer.

Immune staining and TUNEL analysis of paraffin sections of human ovarian organ culture tissue. (**A, B**) IHC of inclusion cysts showing FasL and Fas expression

corresponding to cytokeratin staining (**D**). (**C**) TUNEL analysis of same area showing apoptosis where Fas and FasL overlap. Pictures taken by Eva Sapi.

Figure 11. Expression of FasL mRNA in normal ovary, primary ovarian cancer, and metastatic ovarian cancer (p.48).

Reverse transcriptase-polymerase chain reactions (RT-PCR) for FasL was performed with total RNA extracted from human ovarian tissue with various degrees of cancer invasion. **N**, normal ovarian tissue, **T**, primary ovarian tumors, and **M**, metastatic tumors, **MM**, molecular marker.

Figure 12. Expression of FasL protein in normal ovary, primary ovarian cancer, and metastatic ovarian cancer (p.48).

Western blot analysis for FasL was performed with protein samples obtained from the same samples described in Figure 12. **N**, normal ovarian tissue, **M**, metastatic tumor, **T**, primary ovarian tumor.

Figure 13. Expression of FasL protein in rat ovary throughout the cycle (p.49).

Western blot analysis for FasL was performed with protein samples obtained from cycling rat ovaries, showing a variation in FasL expression in the different stages of the cycle. Western blot and picture by Gil Mor.

Figure 14. Effect of estrogen on FasL expression in ovarian organ culture (p.49).

(**A**) RT-PCR for Fas and FasL were performed with a total RNA extracted from *in vitro* organ culture of human ovarian tissue. **C**, control tissue (no hormones), **E**, estrogen

treatment, **Tmx**, tamoxifen treatment, **E+Tmx**, estrogen and tamoxifen treatment. (**B**) Western Blot analysis for FasL was performed with protein samples obtained from *in vitro* organ culture of human ovarian tissue. **C** control (no hormonal treatment), **Tmx** tamoxifen treatment, **E** estrogen treatment, **E+Tmx** estrogen and tamoxifen treatment.

Figure 15. Effect of estrogen administration on Fas and FasL immunoreactivity in human ovarian organ culture immunohistochemistry (p.50).

Paraffin sections from ovarian organ cultures were studied for Fas and FasL expression using a specific monoclonal and polyclonal antibodies, respectively. When compared to the control group (**A**), estrogen-treated (17-β-estradiol, 10^{-8} M) human ovarian tissue (**B**) undergoes an upregulation of FasL expression in follicular cells. (**C**), (**D**) An ovary that is unresponsive to estrogen administration. There is little or no change in Fas expression when ovarian organ cultures are treated with estrogen (**E**, **F**). Pictures taken by Eva Sapi.

Figure 16. Estrogen regulation of FasL mRNA expression in HEY ovarian cancer cell line (p.51).

RT-PCR for FasL was performed with total RNA extracted from *in vitro* culture of HEY cells treated with estrogen for 3, 6, and 24 hours.

Figure 17. Effect of tamoxifen and combination effect of estrogen and tamoxifen on FasL mRNA expression in HEY ovarian cancer cell line (p.52).

RT-PCR for FasL was performed with total RNA extracted from *in vitro* culture of HEY cells incubated with tamoxifen or estrogen plus tamoxifen for 3, 6, and 24 hours. **C**, control; **T** tamoxifen; **E/T**, estrogen plus tamoxifen

Figure 18. Effect of estrogen and tamoxifen on Fas mRNA expression in HEY ovarian cancer cell line (p.52).

A. RT-PCR for Fas was performed with total RNA extracted from *in vitro* culture of HEY cells treated with estrogen for 3, 6, and 24 hours.

B. RT-PCR for Fas was performed with total RNA extracted from *in vitro* culture of HEY cells incubated with tamoxifen or estrogen plus tamoxifen for 3, 6, and 24 hours. **C**, control; **T** tamoxifen; **E/T**, estrogen plus tamoxifen

Figure 19. ERα and ERβ mRNA expression in human ovarian samples (p.53).

RT-PCR for ERα and ERβ was performed with a total RNA extracted from human ovarian samples. Results shown are representative of co-amplification for the human ERα and ERβ in: **N**, normal ovarian tissue, **T**, primary ovarian tumors, and **M**, metastatic tumors, **NP**, normal peritoneum, **MM**, molecular marker. PCR products were quantified by densitometry using a digital imaging and analysis system.

Figure 20. Western Blot of ERα and ERβ protein expression in human ovarian samples (p.54).

Western blotting for ERα and ERβ was performed with protein samples obtained from the same tissues analyzed for RNA. Proteins were separated by SDS-PAGE using 10% polyacrilamide gels, transferred into nitrocellulose membranes and immunoblotted with antibodies for ERα or ERβ. The secondary antibody (peroxidase-labeled horse anti-mouse or goat anti-rabbit) was developed with TMB peroxidase substrate kit. Normal

uterus was used as a positive control for ERα expression. Results shown are representative of at least three experiments. (**A**) ERα, (**B**) ERβ

Figure 21. Model of proposed interactions among hormones, p53, and Fas/FasL in the ovary (p.67).

Under proper hormonal stimulation, a functional p53 relocates Fas from a cytoplasmic to membranal site. Here, Fas can interact with FasL to induce apoptosis. If not properly regulated, however, Fas may fail to move to the surface, leaving sole expression of FasL, resulting in uncontrolled cellular growth due to failure of apoptosis. Furthermore, any attempt made by the immune system to combat this irregularity is thwarted when Fas-expressing T cells are induced to undergo apoptosis by the FasL expression of the abnormal cells. This scenario demonstrates how an imbalance in Fas and FasL expression could result in neoplastic transformation of cells, which can then avoid immune attack. Model created by Gil Mor.

XII. TABLES

Table 1.

Classification and characteristics of Fas/FasL mRNA.

	Fas	Fas Ligand	Histology	Age
Normal Ovary				
R039	(+)	(+/-)		45
R034	(+)	(+/-)		47
Primary				
R049	(++)	(++)	Papillary Serous, Stage IIIC, Grade III	50
R018	(++)	(+/-)	Papillary Serous, Stage IIIC, Grade I	61
Metastasis				
R031	(+++)	(+++)	Papillary Serous, Stage IIIC, Grade III	71
R048	(+++)	(+++)	Papillary Serous, Stage IIIB, Grade III	50

Table 2.

Classification and characteristics of ER alpha and beta mRNA.

	ER-α	ER-β	Histology	Age
Normal Ovary				
R035	(++)	(+++)		67
R036	(++)	(+++)		56
R039	(++)	(+++)		45
R034	(++)	(+++)		47
R026	(++)	(++)		80
OV17 PCC	(+)	(++)		60
R07	(++)	(+++)		71
R03	(++)	(+++)		53
R020	(++)	(++)		80
Primary Tumor				
R059*	(+++)	(-)	Papillary Serous, Stage IIIC, Grade III	48
R049*	(+++)	(-)	Papillary Serous, Stage IIIB, Grade III	50
R053	(-)	(+)	Papillary Serous, Stage IIIC, Grade III	53
R037	(-)	(++)	Mucinous Stage IIC, Grade I	56
R018	(+++)	(++)	Papillary Serous, Stage IIIC, Grade I	61
R061	(+)	(-)	Papillary Serous, Stage IIIC, Grade III	61
R041	(++)	(++)	Papillary Serous, Stage IIIC, Grade III	66
R054	(+++)	(++)	Borderline Mucinous, Stage IA, Grade I	38

Metastasis				
R060*	(+++)	(-)	Papillary Serous, Stage IIIC, Grade III	53
R048*	(++++)	(-)	Papillary Serous, Stage IIIB, Grade III	50
R031	(+++)	(-)	Papillary Serous, Stage IIIC, Grade III	71
R021	(++++)	(-)	Papillary Serous, Stage IIIC, Grade III	71
R058	(+++)	(-)	Papillary Serous, Stage IIIC, Grade III	48
R005	(++++)	(-)	Papillary Serous, Stage IIIC, Grade III	60
R052	(++++)	(-)	Papillary Serous, Stage IIIC, Grade III	30
R064	(++++)	(-)	Papillary Serous, Stage IV, Grade III	74

* Represents match primary to metastatic tumor. Pairs RO49/RO48 and RO59/RO60

Table 3.

Percentage of ERα and ERβ mRNA expression in normal, primary and metastatic ovarian tumors.

Tissue	ERα alone	ERβ alone	ERα/ERβ
Normal	0% (0/9)	0% (0/9)	100% (9/9)
Primary Tumor	37.5% (3/8)	25% (2/8)	37.5% (3/8)
Metastasis	100% (8/8)	0% (0/8)	0% (0/8)

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