Cancer Treatment and Research
Steven T. Rosen, M.D., Series Editor

Robert H. Lurie Comprehensive Cancer Center
Northwestern University Medical School

Ovarian Cancer

Second Edition

edited by
M. Sharon Stack
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As we struggle to understand, treat, and cure this deadly disease, this book is dedicated to the women who suffer with ovarian cancer and those who love them.

Preface

This volume provides a set of comprehensive reviews from experts in the field on key clinical, translational, and basic research issues in ovarian cancer for clinicians and scientists. Chemoprevention, staging, and novel therapeutic targets are addressed in Part I with a series of reviews highlighting prevention strategies, surgical treatments, and translation of novel targets into clinical practice. Part II is focused on tumorigenesis and biomarkers. Reviews highlight genetic and epigenetic changes active in transformation of ovarian surface epithelium and biomarkers currently under investigation as diagnostic/prognostic indicators or therapeutic targets. Part III includes comprehensive overviews of tumor progression, metastasis, and translational research models. These reviews evaluate key signal transduction pathways in ovarian cancer, describe the novel adhesive microenvironment unique to ovarian tumors, and provide a comprehensive description of in vitro organotypic and in vivo murine models used to study ovarian cancer onset, progression, and metastasis.

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Chapter 1
Chemoprevention of Ovarian Cancer

Anna Hoekstra and Gustavo C. Rodriguez

Introduction

Epithelial ovarian cancer remains a highly lethal malignancy. It is the fourth to fifth leading cause of cancer deaths among women in the United States and causes more than 140,000 deaths annually in women worldwide.[1] Despite intensive research efforts over the past decade directed toward improved detection and treatment of ovarian cancer, the long-term survival of women with ovarian cancer has only improved modestly. Progress in the fight against ovarian cancer has been hampered by a number of factors, including late diagnosis, the absence of highly curative chemotherapy, and a high degree of molecular heterogeneity in ovarian tumors, a finding that is a direct consequence of the large tumor burden typical in most patients at the time of presentation.

The unusually large tumor burden that characterizes most advanced ovarian cancers at diagnosis makes ovarian cancer uniquely different than other solid tumors. Most women (75%) diagnosed with ovarian cancer have disseminated intra-abdominal disease at diagnosis, often characterized by a tumor volume comprising many cubic centimeters.[1] Considering that each cubic centimeter of tumor contains as many as 1 billion cells, numerous cell doublings have occurred by the time an ovarian cancer is typically diagnosed, providing an immense opportunity for tumors to develop molecular and genetic heterogeneity. The Goldie-Coldman hypothesis suggests that the number of tumor cells and the length of time they are present are directly correlated with the likelihood of emergence of chemotherapy-resistant clones that may fail to respond to conventional chemotherapy.[2] The markedly increased probability of chemoresistance in advanced bulky ovarian tumors readily explains the guarded clinical

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course of women with ovarian cancer, as many will recur and succumb to the disease as chemoresistant clones of ovarian cancer cells emerge.

Clearly, a novel approach is needed if the fight against ovarian cancer is to be won. An alternative and promising approach is to target the early steps in ovarian carcinogenesis when cellular heterogeneity is less likely and the myriad genetic mutations present in advanced tumors have not had an opportunity to evolve.[3] This is possible through the administration of chemopreventives that specifically arrest or reverse the early steps in ovarian carcinogenesis.

Ovarian Epithelium: A Potential Target for Ovarian Chemopreventives

The pathogenesis of epithelial ovarian cancer is not completely understood. It is commonly believed, however, that the process of recurrent ovulation (incessant ovulation) causes genetic damage in ovarian epithelial cells and that sufficient genetic damage can lead to ovarian cancer in susceptible individuals.[4] Under this model, it has been suggested that reproductive and hormonal factors, such as pregnancy and oral contraceptive (OC) use, decrease ovarian cancer risk mainly via their inhibitory effects on ovulation.[5–9] The incessant ovulation model is attractive in that it is supported by a large volume of epidemiologic evidence linking ovulation with ovarian cancer risk in humans and by the observation that poultry hens, which ovulate daily, have a high incidence of spontaneous ovarian cancer.[10] The model falls short in that it fails to explain the markedly protective effect conferred by pregnancy and OC use against ovarian cancer. OC use for 3 years, which inhibits less than 10% of the number of ovulatory cycles in a woman's lifetime, confers as high as a 50% reduction in risk of ovarian cancer, rather than 10%. One pregnancy, which is associated with approximately 1 year of anovulation, is associated with a 30% to 35% decrease in ovarian cancer risk. These data suggest that there may be biologic effects unrelated to ovulation that mediate the influence of reproductive factors on ovarian cancer risk.

There is mounting evidence that the ovarian epithelium is a hormonally responsive target organ whose biology can be strongly affected by the local hormonal environment. The normal ovarian epithelium expresses receptors for most members of the steroid hormone superfamily, including estrogen, progestin, retinoids, vitamin D, and androgens. In addition, the ovarian epithelium contains cyclooxygenase. There is the potential for reproductive and environmental factors to have an impact on ovarian cancer risk via a direct biologic effect of hormonal and nonhormonal agents on the ovarian epithelium. Studies have shown that reproductive hormones can have potent biologic effects directly on the ovarian epithelium, thus affecting ovarian cancer risk. Progestins induce apoptosis, one of the most important molecular pathways in vivo for the prevention of cancer and a pathway that mediates the action of many

known chemopreventive agents. Progestin-mediated apoptotic effects may be a major mechanism underlying the ovarian cancer protective effects of pregnancy (a high progestin state) and OC use. Similarly, retinoids, vitamin D, and nonsteroidal anti-inflammatory drugs (NSAIDs) may have biologic effects on the ovarian epithelium that are cancer preventive, whereas androgens may have stimulatory effects on the ovarian epithelium, leading to an increased ovarian cancer risk.

Carcinogenesis and Chemoprevention

Epithelial carcinogenesis refers to the process by which cells undergo neoplastic transformation. Carcinogenesis is a multistep process, characterized by the sequential accumulation of somatic mutations in a single cell, ultimately leading to sufficient molecular alterations that result in phenotypic progression from normal to a fully malignant phenotype.[11] The different stages of carcinogenesis include (1) *initiation*, in which carcinogenic factors directly damage the DNA, (2) *promotion*, which involves the gradual accumulation of alterations in proliferating premalignant cells, often influenced by epigenetic mechanisms, and (3) *progression*, which is due to additional genetic alterations that facilitate the final transformation to cancer. These stages typically evolve over many years, often decades, explaining the frequent finding of early age of onset of cancer in individuals with a hereditary predisposition, as these individuals are born with inherent genetic alterations and thereby require fewer additional somatic mutations to develop cancer. The number of major genetic alterations required to achieve full transformation to malignancy is variable but is likely to number at least five to six and typically involves alterations in genes involved in regulating the cell cycle, signal transduction, and transcription. These include oncogenes, tumor suppressor genes, apoptosis genes, as well as DNA repair genes.[12]

Cancer chemoprevention is defined as the use of chemical agents (natural, synthetic, or biologic) to arrest or reverse the process of carcinogenesis.[11,13] First described by Sporn in 1976, cancer chemoprevention is intended to abrogate the multistep process of carcinogenesis.[13] As such, cancer chemoprevention arrests or reverses carcinogenesis while cells are dysplastic or preneoplastic. The benefits of chemoprevention are obvious, as the costs and morbidity associated with treatment of clinically evident cancers are immense, as is the social cost associated with treatment failure.

Significant challenges, however, have limited progress in the field, especially with regard to drug development and the regulatory process. Pharmaceutical drug development typically follows a rigorous stepwise algorithm, including preclinical, toxicology, and pharmacokinetic studies, as well as phase I, II, and III studies to optimize dosing and demonstrate safety and efficacy. These steps can often take 5–15 years to complete, thereby consuming most of the patent life and thus profit potential of novel emerging therapies. If the same standards for

development of therapeutic agents are applied to chemopreventive agents, the cost and time required for development would be prohibitive. Cancer-prevention trials addressing the outcome variable of cancer incidence are costly and require many years to complete, potentially outlasting the scope of patent protection and thus profit potential. To address these concerns, the National Cancer Institute (NCI), Food and Drug Administration (FDA), and the pharmaceutical and research community are striving to collaborate in order to develop an alternative road map to drug development for chemopreventives.[11,14] It has been proposed that preclinical evaluation and phase I clinical trials might potentially be omitted if the toxicity and pharmacokinetics of the agent of interest are already known from animal and human studies and the agent is thought to be safe. Phase I studies can further be accelerated for drugs already approved for other indications and shown to be safe at dosages considered for chemoprevention. Phase II randomized trials can be directed toward individuals at high risk of malignancy, with outcome measures being alteration of surrogate end-point biomarkers thought to be predictive of cancer risk. Finally, Phase III clinical trials with cancer incidence as an end point can be considered even as the preventive agents are brought to the clinic. To date, this novel approach to drug development has yet to be fully implemented. Of concern is the adverse experience from prior failed chemoprevention trials, such as the ATBC Cancer Prevention Study, where intervention with beta-carotene, shown in epidemiologic studies to reduce lung cancer risk, was associated with an increased risk of lung cancer as well as overall deaths in smokers.[15] Even simple over-the-counter supplements such as vitamin E and A, thought by many to enhance wellness, have recently been shown to increase overall mortality.[16] These adverse outcomes make it critically important that chemopreventive agents be carefully vetted prior to implementation in the general population.

Molecular Targets for Chemoprevention

Study of late-stage cancers has produced the majority of evidence on the genetic and protein alterations that result in the development of cancer. This evidence has demonstrated that numerous genetic alterations must occur to achieve a clinically recognized tumor, affecting multiple pathways known to affect cellular homeostasis, including control of cellular attachment, proliferation, invasion, and implantation, as well as the complex factors associated with angiogenesis. Several molecular targets within these protective pathways have been characterized in carcinogenesis and are thus potential targets for preventive interventions, although no one target has yet to be critically validated in ovarian cancer. Specific molecular targets include the HER-2/neu receptor and the epidermal growth factor receptor (EGFR),phosphatidylinositol 3-kinase (PI3K)/Akt, p53, activator protein 1 (AP-1), and nuclear factor kappa B (NF-κB). A broader and perhaps more attractive target may be the apoptosis pathway, which has the potential to nonspecifically clear a broad scope of cells with genetic damage.

HER2/neu and EGFR

The HER family of type I tyrosine kinase receptors includes EGFR (HER1/Erb1), HER2 (encoded by the proto-oncogene neu), HER3, and HER4. HER2 promotes growth and is overexpressed in 20% to 30% of ovarian cancers.[17] Overexpression is associated with advanced tumors and a worse prognosis. EGFR is a transmembrane receptor that is also overexpressed in a proportion of ovarian cancers and similarly associated with poor outcomes.[18] The inhibition of EGFR kinase modulates signal transduction pathways without significant cytotoxicity. Several synthetic, small-molecule tyrosine kinase inhibitors (gefitinib, erlotinib) and an EGFR-specific monoclonal antibody (trastuzumab) have been developed to target EGFR clinically. Erlotinib, an EGFR inhibitor, is currently FDA approved for the treatment of non–small cell lung cancer, breast cancer, and pancreatic cancer, and its preventive potential is being studied.[19] It has been investigated in advanced recurrent ovarian cancer as well, showing a modest response rate of 6% with 44% of patients achieving stable disease.[20] Dietary phytochemicals identified to inhibit this receptor in vitro include curcumin, genistein, resveratrol, and catechins.

PI3K/Akt

The PI3K/Akt pathway transduces extracellular growth regulatory signals to intracellular mediators of growth and cell survival. Akt is an important regulator of cell proliferation and cell survival and contributes to tumor growth and progression.[21] It is activated in response to activation of a variety of growth factors. Akt, in turn, inhibits apoptosis through phosphorylation of downstream transcription factors such as Bad, caspase-9, the forkhead transcription factor family, and IκBa kinases. Phosphate and tensin homologue (PTEN) is a well-known tumor suppressor gene that negatively regulates the PI3K/Akt signaling pathway.[22] Mutation of PTEN and upregulation of Akt has been found in endometrial and prostate tumors and in preinvasive bronchial lesions.[23–25] Upregulation of this pathway is also found in 30% of ovarian tumors.[26] Several dietary phytochemicals have been shown to suppress and inactivate Akt, including genistein (soybean), indole-3-carbinol, diosgenin (fenugreek), curcuminoids (turmeric), black raspberries, and the polyphenol epigallocatechin-3-gallate in green tea.[27]

p53

p53 is a tumor suppressor gene and transcription factor. It plays a critical role in many cellular processes vital to normal homeostasis, including genomic stability, cell cycle control, and regulation of apoptosis, all of which are integral to abrogating the propagation of cells with damaged DNA and malignant

potential. With regard to ovarian cancer, a mutation in p53 is the most common genetic alteration, present in more than 50% of cases. In addition, the frequency of alterations in p53 in human ovarian cancers has been shown to correlate with the number of lifetime ovulatory events in women. Importantly, the majority of these mutations are transitions and are thus likely to represent spontaneous errors in DNA replication rather than mutations induced by carcinogens.[28–34] Thus, alterations in p53 are likely to play an important role in the stepwise progression of ovarian carcinogenesis.

NF-κB

NF-κB is a family of protein dimers that bind to a common DNA sequence called the κB site.[35] NF-κB dimers typically reside in the cytoplasm until activated by a variety of stimuli (free radicals, inflammatory molecules, cytokines, carcinogens, tumor promoters, endotoxins, radiation, ultraviolet light, and x-rays). Upon activation, it relocates to the nucleus where it induces the expression of a variety of genes that have activity in the suppression of apoptosis and induce cellular transformation, proliferation, invasion, metastasis, inflammation, and resistance to chemotherapy and radiation. Among the genes it targets are cyclin D, apoptosis suppressor proteins such as Bcl-2 and Bcl-XL, matrix metalloproteinases, and vascular endothelial growth factor (VEGF; required for metastasis and angiogenesis).[36] These target genes are critical to the establishment of early and later stages of neoplastic tumor growth. Inhibition of the NF-κB pathway may occur via blockage of activation of the NF-κB signaling cascade, translocation of NF-κB into the nucleus, DNA binding of the dimers, or interactions with transcription. Dietary agents have been found to be potent inhibitors of NF-κB, including curcumin, resveratrol, green tea catechins, and a variety of others.[37–39] In vivo studies in a mouse model have shown that blockage of this pathway decreases the tumorigenicity of ovarian cancer cell lines, possibly through VEGF and interleukin-8 downregulation.[40]

AP-1

Many of the activated pathways in carcinogenesis lead to AP-1, which, in turn, regulates other genes involved in carcinogenesis including cyclooxygenase-2 (COX-2), making it an optimal molecular target for chemoprevention. AP-1 is a transcription factor that regulates many cellular processes, including proliferation, inflammation, differentiation, and apoptosis. It is activated by extracellular stimuli at both transcriptional and posttranslational levels by growth factors, hormones, stress, cytokines, and ultraviolet radiation. Modulation of this transcription factor prevents carcinogenesis via antioxidant and anti-inflammatory effects. AP-1 induces expression of proinflammatory genes, including COX-2 and

inducible nitric oxide synthase (iNOS). Inhibitors of AP-1 have not yet been identified, but genetically engineered inhibitors have been created. TAM67 is a dominant negative inhibitor of AP-1 that has been shown to reverse carcinogenesis.[41,42] In addition, a variety of natural chemopreventive agents have been shown to inhibit AP-1, including curcumin, epigallocatechin gallate, resveratrol, COX-2 inhibitors, and caffeic acid phenethyl ester.

Apoptosis

The apoptosis pathway is arguably one of the most important in vivo mechanisms for cancer prevention. Activation of apoptosis leads to the efficient disposal of cells that have undergone irreparable genetic damage and that are thus prone to neoplastic transformation.[43] It is therefore a key molecular pathway for the elimination of premalignant cells in vivo. It is a biologic mechanism associated with many known chemopreventive agents,[44–51] and pharmacologic agents that selectively enhance apoptosis have been shown to lower the risk of a variety of cancers in animals and in humans.[52] In addition, in animal models of cancer as well as in humans, the efficacy of cancer-preventive agents has been shown to correlate with the degree of apoptosis induced by these agents in the target organ for prevention.[52–55] Conversely, mutations in the genes involved in the apoptosis pathway have been shown to be associated with enhanced cancer risk.[56] Among the various molecular targets for reversal of early ovarian carcinogenesis, the apoptosis pathway holds great promise. This pathway is markedly activated by the OC, a potent ovarian cancer preventive, suggesting that agents that selectively activate apoptosis in the ovarian surface epithelium may be potent ovarian chemopreventives.[57,58]

From a clinical perspective, the apoptotic pathway is a uniquely attractive target for cancer prevention. Importantly, apoptosis-inducing agents are likely to be more effective in cells harboring genetic damage, thereby sparing normal cells.[59] In addition, because the apoptotic mechanism has a cytocidal rather than a cytostatic effect on precancerous cells, apoptosis-inducing agents would be expected to destroy premalignant cells, rather than just arrest or slow their growth. As a result, it may not be necessary to administer apoptosis-inducing chemopreventives continuously to achieve effective cancer prevention as would be the case for chemopreventives that have a cytostatic effect. Periodic or pulsed administration of an apoptotic chemopreventive would lessen the long-term exposure to the agent, thereby decreasing toxicity, as well as the risk of chemoresistance.[52] Taking this approach even further, a pharmacologic strategy that uses periodic administration of two or more apoptosis-inducing agents that have synergistic potency may enhance the therapeutic ratio even more. This strategy not only has the potential for increased cancer-preventive efficacy, but also has the potential to further decrease long-term side effects by allowing a lowering of the dose of each preventive administered.[60]

Candidate Ovarian Cancer Chemopreventive Agents

Progestins

There is strong evidence suggesting that progestins may be potent chemopreventives for ovarian cancer. Routine use of estrogen-progestin combination OC preparations markedly lowers subsequent ovarian cancer risk. The protective effect increases with the duration of use and persists for as long as 20 years after discontinuation of use.[61–65] The underlying protective mechanism of action of OCs is not fully elucidated but is likely to involve a chemopreventive effect of progestins on the ovarian surface epithelium.

As discussed above, there has been widespread belief that the ovarian cancer protective effect of OC use is due to the ability of these agents to inhibit ovulation. We challenged this presumption because routine OC use results in a disproportionately greater protective effect than that which can be solely attributed to ovulation inhibition. We hypothesized that the marked protective effect conferred by OCs might be due to a potent chemopreventive biologic effect of contraceptive hormones on the ovary. To test this hypothesis, we performed a study in primates (*Cynomolgus macaques*) designed to evaluate the long-term biologic effect of the contraceptive Triphasil (levonorgestrel/ethinyl estradiol; Wyeth, Madison NJ) on the ovaries. The remarkable similarity of the *Cynomolgus macaque* to humans, particularly in regard to its 28-day menstrual cycle, makes this primate model ideal for designing experiments pertinent to human ovarian and reproductive biology. The purpose of our study was to search in the ovaries of contraceptive-treated monkeys for molecular changes that had the potential to be responsible for the known chemopreventive effects of OCs. Given the importance of the apoptosis pathway in vivo for cancer prevention, we elected to investigate whether long-term OC exposure induced apoptosis in the primate ovarian epithelium.[57,58] Eighty animals were prospectively randomized into four groups including a control group, a group treated with Triphasil (which contains the estrogen ethinyl estradiol and the progestin levonorgestrel), and one group each treated either with ethinyl estradiol or levonorgestrel alone on the same dosage and schedule as those animals receiving Triphasil. The animals were maintained on the monthly contraceptive hormone schedule for 3 years. During the third week of the last month of the study, the animals were sacrificed; the ovaries were removed, formalin-fixed, sectioned, and then examined for morphologic and immunohistochemical evidence of apoptosis by observers blinded to treatment group. For each ovarian section, the percentage of epithelial cells undergoing apoptosis was quantified. The results are summarized in Table 1.1. Compared with control and ethinyl estradiol–treated monkeys, a striking and statistically significant increase in apoptosis was noted in the ovarian epithelium of monkeys treated with Triphasil ($p < 0.01$) or levonorgestrel ($p < 0.001$), with the maximal effect (sixfold) seen in the group treated with levonorgestrel alone. The degree of apoptosis was not different between ethinyl estradiol–treated monkeys and

Table 1.1 Apoptotic effect of hormone treatment on macaque ovarian epithelium

Study Group	Number of Animals/ Group	Median Percent of Apoptotic Epithelial Cells (%)	Range of Percent of Apoptotic Cells (%)
Control	20	3.9	0.1–33.0
Hormone treated			
Ethinyl estradiol	20	1.8	0.1–28.6
Combination pill	17	14.5	3.0–61.0
Levonorgestrel	18	24.9	3.5–61.8

Multiple comparisons: control, levonorgestrel ($p < 0.001$); combination pill, ethinyl estradiol ($p < 0.001$); ethinyl estradiol, levonorgestrel ($p < 0.001$); control, combination pill ($p = 0.01$).

controls. These data demonstrate the novel finding that OC exposure markedly induces apoptosis in the ovarian epithelium and that the progestin component of the pill is responsible for this effect.

The discovery that progestin markedly induces apoptosis in the ovarian epithelium led us to search for factors that regulate this effect. Transforming growth factor (TGF)-β has been implicated in the apoptotic pathway of a variety of cell types including hormonally sensitive epithelia such as the breast and prostate. In addition, well-known cancer-preventive agents such as the retinoids and the antiestrogen tamoxifen have been shown to induce TGF-β expression in the target tissues that they protect from malignant change, including epithelial cells in the upper aerodigestive tract and breast.[66] Interestingly, multiple members of the steroid hormone superfamily including the retinoids, vitamin D, and estrogens have been shown to modulate expression of TGF-β, and the promoter region for specific TGF-β isotypes contains features suggesting hormonal control. Given the known importance of TGF-β as a regulator of apoptosis and as a potential mediator of action of other chemopreventives, we decided to examine whether progestins regulate TGF-β expression in the ovaries of primates from the trial described above.[58] Primate ovarian sections from the four treatment groups noted above were stained immunohistochemically with monoclonal antibodies reactive with either TGF-β1 or TGF-β2 and TGF-β3 (TGF-β2/3). The ovarian sections were examined by two independent sets of reviewers, all of whom were blinded to the hormone administration data. Staining for TGF-β was evaluated in four separate ovarian compartments of each study slide (ovarian surface epithelium, primordial oocyte cytoplasm, granulosa cells of tertiary follicles, and endothelium in ovarian hilar vessels) and graded according to degree of staining intensity from 0 to 3+ (TGF-β1) and 0 to 4+ (TGF-β2/3). High expression of TGF-β1 was defined by the slide reviewers as 2+ to 3+ staining intensity, whereas high expression of TGF-β2/3 was defined as 3+ to 4+ staining intensity. The quantitative results are summarized in Tables 1.2 and 1.3. Progestin treatment, either combined with estrogen (Triphasil group) or administered alone (levonorgestrel group), was associated with a striking and highly statistically significant decrease in

expression of TGF-β1 in the ovarian epithelium ($p < 0.001$) and a moderate decrease in expression of TGF-β1 in the oocyte cytoplasm ($p = 0.002$; Table 1.2). In contrast, progestin treatment was associated with a marked increase in expression of TGF-β2/3 in the ovarian epithelium ($p < 0.001$). Without exception, TGF-β2/3 expression in the ovarian epithelium was high (3 to 4+ staining) in every monkey on progestin ($N = 34$). Similarly, there was a significant increase in TGF-β2/3 expression in the ovarian hilar endothelial cells in monkeys on progestin ($p < 0.001$). In contrast, progestin treatment was associated with a marked decrease in TGF-β2/3 expression in granulosa cells ($p < 0.001$; Table 1.3).

Within the ovarian epithelial compartment, comparison of the apoptotic index with the degree of change in the expression of the TGF-β isoforms revealed a significant correlation between changes in TGF-β expression and apoptosis (Table 1.4). The Pearson correlation between the proportion of high TGF-β expression and the mean proportion of apoptotic cells across treatments was –0.998 ($p = 0.002$) for TGF-β1 and 0.973 ($p = 0.03$) for TGF-β2/3. Finally, overall, there was a negative association between TGF-β2/3 overexpression and TGF-β1 overexpression (kappa = –0.62; $p < 0.001$). Taken together, these data demonstrate the novel

Table 1.2 Hormone regulation of TGF-β_1 expression in the macaque ovary. Number (%) of ovaries/treatment group with high TGF-β_1 expression (2 to 3+) in each ovarian compartment

Treatment Group	Epithelium	Granulosa Cells	Oocytes	Endothelium
Control	18 (90)	7 (35)	7 (35)	0 (0)
Ethinyl estradiol	16 (84)	4 (21)	2 (13)	0 (0)
Triphasil	3 (19)	2 (13)	0 (0)	0 (0)
Levonorgestrel	1 (6)	2 (12)	0 (0)	0 (0)
Overall approximate exact test:	$p < 0.001$	0.31	0.002	1.00
Triphasil/ levonorgestrel versus control/ ethinyl estradiol	$p < 0.001$	0.15	0.003	1.00

Table 1.3 Hormone regulation of TGF-β2/3 expression in the macaque ovary. Number (%) of ovaries/treatment group with high TGF-β expression (3 to 4+) in each ovarian compartment

Treatment Group	Epithelium	Granulosa Cells	Oocytes	Endothelium
Control	6 (32)	12 (63)	14 (74)	5 (26)
Ethinyl estradiol	2 (10)	8 (38)	17 (81)	3 (23)
Triphasil	17 (100)*	1 (6)*	16 (94)	16 (94)*
Levonorgestrel	17 (100)*	1 (6)*	14 (82)	16 (94)*

*$p < 0.001$, approximate exact test. Pairwise comparisons of Triphasil/levonorgestrel versus control/ethinyl estradiol groups were statistically significant ($p < 0.001$) for all compartments except oocytes, except for the granulosa comparison with ethinyl estradiol ($p = 0.03$).

Table 1.4 Relationship between treatment, TGF-β expression, and apoptosis in the macaque ovarian epithelium

	N	**TGF-β_1** Percent 2 to 3+ (%)	Mean Proportion of Apoptotic Cells in Ovarian Epithelium (SE)	*N*	**TGF-$\beta_{2/3}$ Percent 3 to** 4+ (%)	Mean Proportion of Apoptotic Cells in Epithelium (SE)
Control	20	90	6.3 (1.6)	19	32	6.4 (1.7)
Ethinyl estradiol	19	84	6.2 (2.1)	20	10	4.5 (1.6)
Triphasil	16	19	22.3 (4.1)*	17	100	21.2 (4.0)**
Levonorgestrel	17	6	25.1 (4.3)*	17	100	26.4 (4.1)*

SE, standard error.
*$p < 0.001$ by Dunnett's test of mean apoptotic index for treatment with control.
**$p = 0.002$ by Dunnett's test of mean apoptotic index for treatment with control.

finding that progestin-induced apoptosis in the ovarian epithelium is associated with an isotype switch in expression of TGF-β. The finding that progestins activate these two critical cancer-preventive–related pathways in the ovarian epithelium raises the possibility that progestin-mediated chemopreventive effects, and not solely ovulation inhibition as has been previously assumed, may underlie the reduction in ovarian cancer risk associated with routine OC use.

A growing body of published human data is supportive of the notion that a biologic effect related to progestins may be a major mechanism underlying the cancer-preventive effect for both the OC as well as pregnancy, which confers potent protection against subsequent ovarian cancer and which is associated with high serum progesterone levels:

(a) An analysis of the data from the Cancer and Steroid Hormone Study (CASH) has demonstrated that progestin-potent OCs confer greater protection against ovarian cancer than do OCs containing weak progestin formulations.[67] When comparing OCs categorized by estrogen and progestin potency in 400 ovarian cancer cases and 3000 controls, use of OC formulations with increased progestin potency conferred twice the reduction in risk of ovarian cancer as that of those with lower progestin potency, irrespective of the estrogen content ($p < 0.001$). The analyses also demonstrated a significant reduction (60% to 70%) in risk of ovarian cancer associated with exposure to high-progestin-potency OCs even among women who used OCs for a relatively short duration (less than 18 months).

(b) Further support for progestins as ovarian cancer preventives has come from an analysis of data from the World Health Organization (WHO) by Risch, demonstrating a 60% reduction in the risk of nonmucinous ovarian cancer in women who have only ever used depo-medroxyprogesterone acetate, a progestin-only contraceptive.[68] Progestin-only contraceptives do not reliably inhibit ovulation. Thus, the 60% reduction in ovarian cancer risk from a progestin-only contraceptive is further evidence that progestins have a direct chemopreventive effect on the ovary.

(c) In addition, epidemiologic evidence has shown that twin pregnancy is more protective against subsequent ovarian cancer than is singleton pregnancy. Previously, it was presumed that women who have twins would be at greater risk of ovarian cancer, presumably due to an increased likelihood of more lifetime ovulatory events compared with that in women who do not have twins, and the notion that increased ovulation would confer greater risk of ovarian epithelial damage. Because women with twin pregnancy have higher progesterone levels than do women with singleton pregnancy, it has been proposed that the human epidemiologic evidence regarding twin pregnancy is supportive of the notion of a biologic effect of progesterone as conferring ovarian cancer protection and that the effect is dose dependent.[69]
(d) Finally, pregnancy at a later age is more protective than is pregnancy early in life. In fact, a pregnancy after the age of 35 years is twice as protective against ovarian cancer as is a pregnancy prior to the age of 25 years. It has been proposed that this would suggest a protective effect of pregnancy that is unrelated to effects on ovulation, supporting the notion that pregnancy may clear premalignant or damaged cells from the ovary.[69,70]

Taken together, these data provide a strong rationale for an investigation of progestins as chemopreventive agents for ovarian cancer and potentially opens the spectrum of use of these agents as ovarian cancer preventives to all women, including the elderly, who have the highest age-specific incidence of ovarian cancer. In addition, confirmation of this hypothesis could lead to the development of a highly effective pharmacologic chemopreventive approach that can be applied to those women with hereditary predisposition to ovarian cancer that are at greatest risk of developing ovarian cancer. It is interesting to speculate that if routine OC use can reduce ovarian cancer risk by 50%, a pharmacologic strategy that exploits the mechanism of action underlying the protective effects of OCs could achieve even greater protective effects, leading to improved longevity and quality of life for women and effective ovarian cancer prevention. The Gynecologic Oncology Group (GOG) has recently opened a protocol (GOG 214) that will examine the chemopreventive biologic effect of the progestin levonorgestrel on surrogate endpoint biomarkers in the ovarian surface epithelium in women at increased risk of ovarian cancer who have decided to undergo risk-reducing salpingo-oophorectomy.

Vitamin D

Vitamin D has a spectrum of biologic effects in epithelial cells that may lead to cancer prevention, including growth retardation, induction of cellular differentiation, induction of apoptosis, and stimulation of TGF-β.[71] A schematic representation of vitamin D production and the variety of targets for beneficial vitamin D action is shown in Fig. 1.1.[72] The beneficial effects of vitamin D are due to the activity of its dihydroxylated metabolite, $1,25(OH)_2D_3$ (calcitriol), the active form of the molecule.

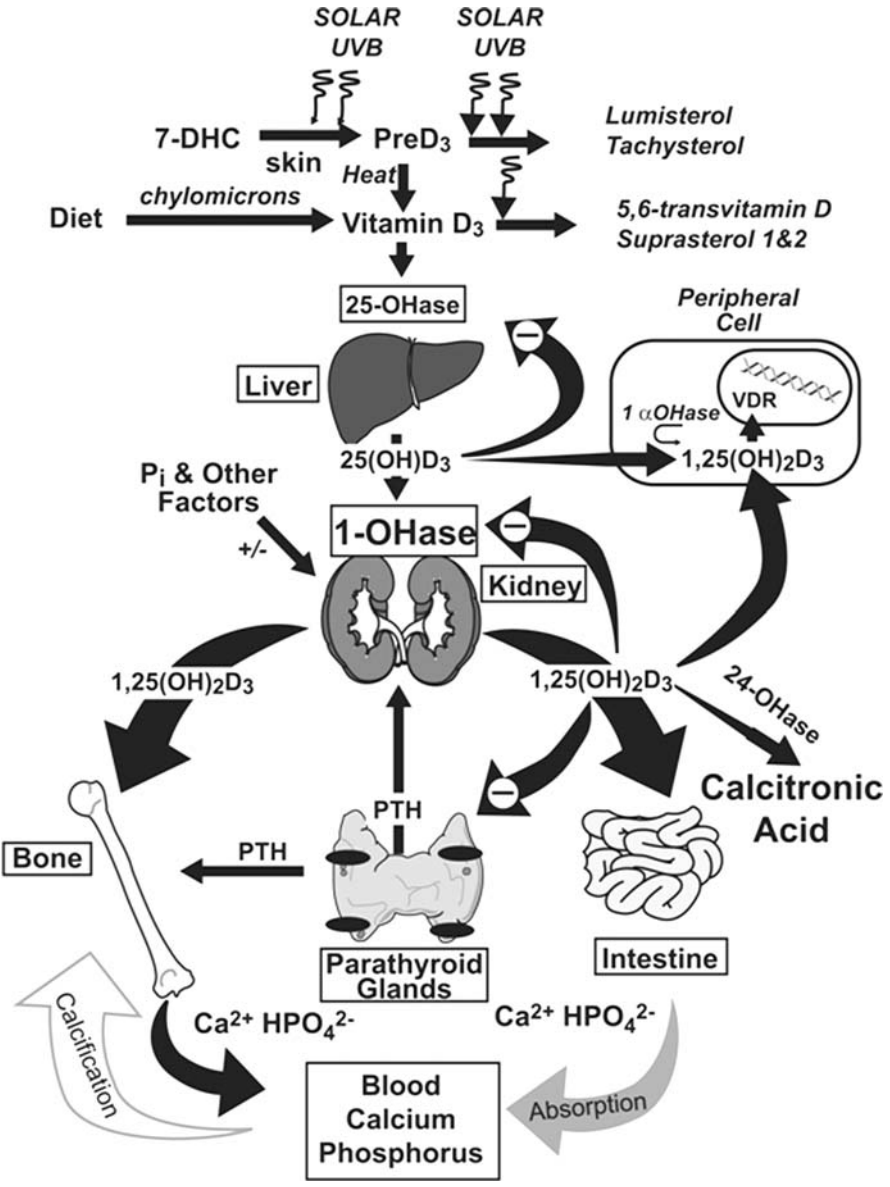

Fig. 1.1 Synthesis and targets of $1,25(OH)_2D_3$. Adapted from Holick[75]

The human body obtains vitamin D (specifically vitamin D_3, or cholecalciferol) through synthesis by skin tissue exposed to sunlight or orally through the diet. Of these two sources, however, very little is supplied by the diet as few foods contain appreciable amounts of vitamin D_3. During exposure to sunlight, 7-dehydrocholesterol (7-DHC), which is present in abundance in the skin, is

converted by ultraviolet B (UVB) radiation to previtamin D_3 (preD_3). Once formed, preD_3 undergoes thermally induced transformation to vitamin D_3. Both previtamin D_3 and vitamin D_3 can be further converted by UVB radiation to a number of degradation products (e.g., Fig. 1.1, upper right, lumisterol and suprasterol), thereby limiting the excess production of vitamin D_3 in skin. Thus, there have been no reported cases of vitamin D intoxication related to excess sun exposure. Vitamin D_3 from either dietary or skin sources enters the circulation and is metabolized in the liver by vitamin D_3-25-hydroxylase (25-OHase) to 25-hydroxyvitamin D_3 (25(OH)D_3). 25(OH)D_3 reenters the circulation and is converted in the kidney by 25-hydroxyvitamin D_3-1-alpha-hydroxylase (CYP27B1) to 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the active hormone. A variety of factors, including serum phosphorus (Pi), parathyroid hormone (PTH), calcium, and circulating levels of 1,25$(OH)_2D_3$, regulate the renal CYP27B1. The enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24) catabolizes both 25(OH)D_3 and 1,25$(OH)_2D_3$ into biologically inactive metabolites including calcitroic acid.

Factors that limit the amount of UVB radiation to which skin is exposed significantly curtail the amount of vitamin D_3 that is produced. This includes covering of the skin with sunscreens or clothes. In addition, the ozone layer absorbs UVB radiation, thereby decreasing the amount of UVB radiation available for the skin to produce previtamin D_3. Thus, during the winter months at latitudes distant from the Equator, where the angle of sunlight is tangential, sunlight passes through more ozone, severely limiting the amount of UVB radiation that reaches the earth's surface and thus the cutaneous production of vitamin D_3. In northern latitudes such as in mid–North America or Europe, the skin is incapable of producing sufficient vitamin D_3 during the winter, even with adequate exposure to the sun. In addition to geographic factors, populations in industrialized countries generally avoid sunlight by remaining mainly indoors and going outside only when fully clothed and/or protected by sunblocking products. As a result, it is now well accepted that vitamin D deficiency is widespread and chronic in most industrialized countries, predisposing individuals to an increased risk of a number of diseases, including cancer.[72–78]

Historically, it was assumed that the most important role of 1,25$(OH)_2D_3$ was the regulation of calcium metabolism and promotion of bone health via interaction with its major target tissues, the bone, kidney, intestine, and parathyroid gland. Recently, however, it has been shown that vitamin D is important not just for bone health but also for overall health and well-being. Research over the past decade has demonstrated that the risk of a host of chronic diseases and cancer are increased in individuals who live in higher latitudes or have vitamin D deficiency. In addition, the vitamin D receptor has been shown to be expressed ubiquitously throughout most epithelia as well as in cells of the immune system. Via interaction with the vitamin D receptor (VDR) in these tissues, 1,25$(OH)_2D_3$ is now known to confer potent biologic effects that include protection against diabetes, hypertension, autoimmune diseases, and cancer.[75] Moreover, it has been recently elucidated that these same tissues express CYP27B1 and thereby have the capacity to produce

the active form of vitamin D_3 ($1,25(OH)_2D_3$) from circulating $25(OH)D_3$ (Fig. 1.1, middle upper right, peripheral cell). It has been suggested that it is the $1,25(OH)_2D_3$ produced locally in tissues rather than systemic $1,25(OH)_2D_3$ produced in the kidney that is responsible for the nonskeletal benefits of vitamin D nutrition including its cancer-preventive effect.[79–91] This is supported by the finding that it is the serum level of $25(OH)D_3$ and not that of $1,25(OH)_2D_3$ that has been consistently shown to be inversely correlated with cancer risk. Systemic levels of $1,25(OH)_2D_3$ are typically highly regulated and held within a tight range, whereas $25(OH)D_3$ levels vary markedly relative to overall vitamin D nutrition. Thus, in the setting of vitamin D deficiency, low levels of circulating $25(OH)D_3$ substrate may significantly curtail local production of $1,25(OH)_2D_3$ in tissues, whereas systemic $1,25(OH)_2D_3$ levels may remain normal.[75,78]

The mechanism underlying the cancer-protective effect of vitamin D nutrition may involve the activation by $1,25(OH)_2D_3$ of a number of biologic effects related to cancer prevention in tissues, including retardation of growth, induction of cellular differentiation, induction of apoptosis, and upregulation of TGF-β.[71,92–95] Induction of these cancer-preventive biologic effects in healthy tissue may cause genetically damaged cells to be efficiently eliminated rather than to persist to transform into cancers.

As described above, the source of $1,25(OH)_2D_3$ for the diverse health benefits of the vitamin, including cancer prevention, is likely to be local production of the active hormone via conversion by 1-alpha-hydroxylase (CYP27B1) of circulating $25(OH)D_3$. Locally produced vitamin D then interacts with the vitamin D receptor, leading to transcriptional events within the cell. Local levels of $1,25(OH)_2D_3$ are held in delicate balance between the activity of CYP27B1, which promotes $1,25(OH)_2D_3$ synthesis, and the catabolic deactivation of vitamin D, which occurs primarily via the action of CYP24, which hydroxylates 25-hydroxyvitamin D compounds, thereby decreasing their potency.[75] Factors that modify the activity of the vitamin D receptor, CYP27B1, and CYP24 have the potential to impact vitamin D signaling, synthesis, and catabolism, thereby having the potential to significantly modify the biologic effect and potency of the vitamin. In this regard, a growing body of evidence has linked polymorphisms in the vitamin D receptor, CYP27B1, and CYP24 with the risk of diseases known to be beneficially affected by vitamin D.[96–105]

Vitamin D and Cancer. It has been estimated by vitamin D experts that as many as 50,000–63,000 individuals in the United States and 19,000–25,000 in the United Kingdom die annually from cancer due to insufficient vitamin D, at a cost to society of $40 billion to $50 billion.[106] Strong epidemiologic evidence has linked vitamin D deficiency (as measured by either lower serum $25(OH)D_3$ levels or lower ultraviolet exposure) to an increased risk of a number of cancers including those especially relevant to women (such as breast, ovarian, colon, and uterine cancer).[107–111] There is a geographic distribution for most of these cancers that favors a higher risk in northern than in southern latitudes in the United States. For example, as shown in Fig. 1.2, there is a geographic variation

in incidence and mortality of ovarian cancer, with higher levels in the north. Patterns for breast, colon, and prostate cancer all show a similar pattern essentially exhibiting higher rates in the northern states. Similarly, some of the highest cancer incidence rates in Europe occur in the extreme northern part of the continent where for several months each winter, the significant lack of ultraviolet light essentially confers a vitamin D "holiday."

With regard to human ovarian cancer, a study has correlated population-based data regarding ovarian cancer mortality in large cities across the United States with geographically based long-term sunlight data reported by the National Oceanic and Atmospheric Administration. The study demonstrated a statistically significant inverse correlation between regional sunlight exposure and ovarian mortality risk.[112] Given that sunlight induces production of pre-vitamin D in the skin, it is interesting to speculate that vitamin D might confer protection against ovarian cancer via direct biologic effects in vivo on nonmalignant ovarian epithelium similar to that induced by progestins. For example, through induction of apoptosis/and or TGF-β in the ovarian epithelium, vitamin D may cause the selective removal of nonmalignant but genetically damaged ovarian epithelial cells.[113,114]

Recently published case-control data also support the notion that vitamin D confers ovarian cancer prevention at dosages of vitamin D easy to achieve through the diet. Compared with a low dietary intake of vitamin D, a high dietary intake of vitamin D was associated with a 50% reduction in ovarian

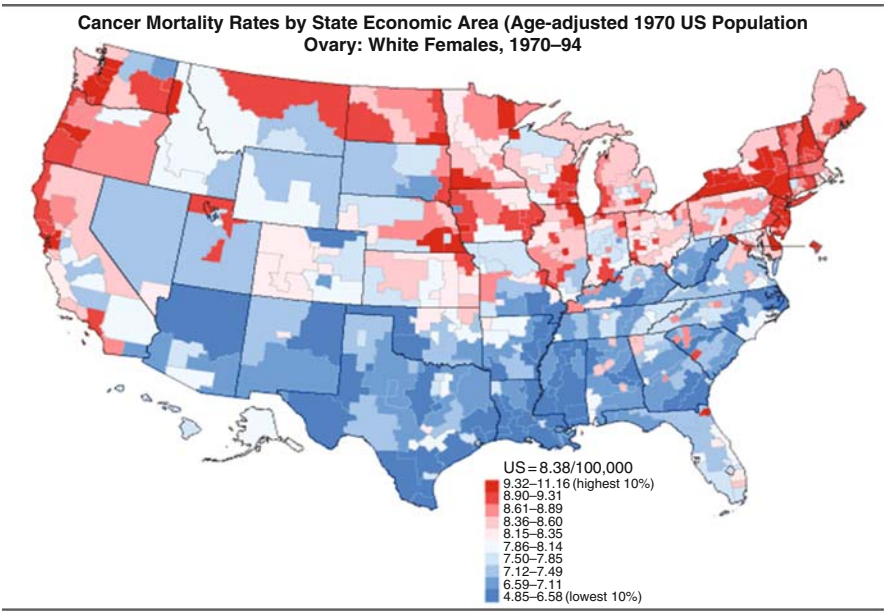

Fig. 1.2 Ovarian cancer mortality rates by state economic area. Data source: American Cancer Society

cancer risk.[115] Given the limitations of a dietary questionnaire, and the fact that the most important source of vitamin D is not dietary but rather via sunlight exposure, the data from this study provide powerful evidence that it may be possible to achieve ovarian cancer–preventive effects through doses of vitamin D that can be administered safely via the oral route.

NSAIDs

Epidemiologic and laboratory evidence suggests that NSAIDs and other anti-inflammatory agents may have chemopreventive effects against a variety of tumors.[116] The data on NSAID use and ovarian cancer has been somewhat inconsistent, but on balance the evidence suggests a protective effect of NSAID use against ovarian cancer.[117–122] In addition to NSAIDs, limited evidence also suggests a possible protective effect of acetaminophen.[117,120] The molecular basis for the protective effect of these agents has not been well defined but may involve an inhibitory effect of these agents on ovulation, modulation of local inflammatory mediators in the ovarian environment, enhancement of the immune response, or possibly a direct chemopreventive biologic effect on the ovarian epithelium.[123–125] It has been shown, for example, that NSAIDs induce apoptosis in cells derived from human ovarian epithelium, similar to progestins.[126–128] In addition, the structure of acetaminophen contains an acetyl group that has similarity to the chemical structure of progesterone, raising the possibility that acetaminophen may have progesterone-like biologic effects on the ovarian epithelium.

Retinoids

Retinoids are natural and synthetic derivatives of vitamin A. They have great potential for cancer prevention due to a broad range of important biologic effects on epithelial cells, including inhibition of cellular proliferation, induction of cellular differentiation, induction of apoptosis, cytostatic activity, and induction of TGF-β. Retinoids have been shown to alter the activity of several key signaling pathways relevant to carcinogenesis, including AP-1, mitogen-activated protein kinase (MAPK), and the PI3K/Akt pathway.[129]

The use of vitamin A analogues has been limited by the requirement for large pharmacologic doses to reach therapeutic efficacy. In addition, high dosages of naturally occurring retinoids produce significant side effects. By modifying the basic retinoid structure, analogues with reduced toxicity have been developed. An example of such a compound is fenretinide (*N*-4-hydroxy-phenyl retinamide [4-HPR]), a retinamide derivative of vitamin A, which is a promising chemopreventive compound with therapeutic efficacy in a variety of carcinogenesis models. 4-HPR currently is being evaluated in clinical trials as a chemopreventive agent for oral leukoplakia and breast and lung cancer.

Epidemiologic and laboratory evidence suggests a potential role for retinoids as preventive agents for ovarian cancer.[130] A high dietary intake of 3-carotene has been associated with a decreased ovarian cancer risk, whereas low serum retinol levels have been associated with an increased risk of ovarian cancer. In vitro, it has been reported that the growth of human ovarian carcinoma cell lines and normal human ovarian epithelium is inhibited by retinoids. The mechanism underlying this effect may involve induction of TGF-β or apoptosis in ovarian epithelial cells.[131–138] The most significant evidence supporting a rationale for retinoids as chemopreventives for ovarian cancer is that of an Italian study suggesting an ovarian cancer–preventive effect from the retinoid 4-HPR. Among women randomized to receive either 4-HPR or placebo in a trial designed to evaluate 4-HPR as a chemopreventive for breast carcinoma, significantly fewer ovarian cancer cases were noted in the 4-HPR group compared with that in controls.[139,140]

Resveratrol and Red Wine

Resveratrol is a red wine polyphenol synthesized by a variety of plant species in response to injury, ultraviolet irradiation, and fungal attack. It is a phytoestrogen that has been found to have activity in carcinogen metabolism, cellular proliferation, inflammation, cell cycle regulation, and apoptosis.[141] It activates the antioxidant response element (ARE) of a variety of genes, turning on de novo synthesis of these genes that encode detoxification or antioxidant enzymes/proteins. Studies using animal models have confirmed its inhibitory role in carcinogenesis, showing a decrease in incidence of skin, breast, and esophageal cancer when the animals were pretreated with reseveratrol.[142]

An Australian case-control study examined the relationship between alcohol, wine, and ovarian cancer. Prior epidemiologic studies had shown an increased, decreased, or null association of ovarian cancer with alcohol intake. However, many of these studies had limited power to detect an effect because of small numbers, particularly for higher levels of alcohol intake, and did not differentiate between the effects of types of alcohol. Six hundred ninety-six women with epithelial ovarian cancer and 786 controls were interviewed, and information about diet and alcohol consumption was elicited.[143] Compared with nondrinkers, the odds ratio (OR) for women who drank an average of = 2 standard drinks/day was 0.49 (95% confidence interval [CI] = 0.30 to 0.81) and was 0.56 (95% CI = 0.33 to 0.93) for women who drank = 1 glass/day versus nondrinkers. This effect was restricted to wine, as no effect was seen for beer (OR = 1.26, 95% CI = 0.65 to 2.46) or sherry/spirits (OR = 1.07, 95% CI = 0.59 to 1.95). Combining these results with the six previous population-based studies gave a pooled OR of 0.72 (95% CI = 0.54 to 0.97) for the highest alcohol intake group versus nondrinkers. These data suggest that the benefit of wine for protection from ovarian cancer was a consequence of antioxidants

and/or phytoestrogens in wine rather than the effect of alcohol itself. The relationship between consumption of wine and beer was further confirmed in the Swedish Mammography Cohort.[144] This cohort of 61,084 women was followed for more than 13 years, and 266 women were diagnosed with epithelial ovarian cancer during that time. Information on consumption of alcoholic beverages was obtained through a mailed food frequency questionnaire. There was a significant decrease in the risk of ovarian cancer with = 1 glass/week of wine with a relative risk (RR) = 0.37 (95% CI, 0.15 to 0.93) and a trend toward an increase in risk of ovarian cancer with = 1 glass/week of beer, with RR = 1.35 (95% CI, 1.00 to 1.81).

Soy and Other Dietary Phytochemicals

Observations of decreased rates of a variety of diseases (i.e., breast and prostate cancer, osteoporosis) in populations with high dietary intake of soy products has led to the study of the impact of isoflavones and other dietary phytochemicals on human health. Dietary phytochemicals, including phytoestrogens and isothiocyanates, are found in fruits, soy, and vegetables. Isoflavones, a class of phytoestrogens found in soy-based foods, have antiestrogenic and antiproliferative effects.[145] The three major forms of soy isoflavones are genistein, glycitein, and daidzein. The literature suggests that genistein offers the most significant health benefits associated with the isoflavones. Isothiocyanates, precursors of which are found in cruciferous vegetables, can inhibit metabolic activation and enhance detoxification of carcinogens,[146] alter apoptosis, protect against oxidative damage, and exert antiestrogenic effects,[147] possibly in synergy with isoflavones.[148] The mechanism of action of most phytochemicals involves the targeting of signal transduction pathways such as the NF-κB, COX-2, Akt, MAPK, p53, androgen receptor, and estrogen receptor pathways.

Both epidemiologic and laboratory data support the role of phytochemicals in the prevention of ovarian cancer. Block et al., in a review of more than 200 epidemiologic studies, found a significant protective effect of fruit and vegetable intake against many cancer types, including ovarian cancer.[149] Block and others found up to 1000 different phytochemicals, including selenium, alkaloids, organosulfur compounds, nitrogen-containing compounds, phenolics, and carotenoids, that may have chemopreventive activity in foods such as avocado, cranberry, apple, grape, pomegranate, tea, peanuts, soy products, milk, and in many other fruits and vegetables.[150–152] In vitro studies in a variety of cancer cell lines, including ovarian cancer, have shown that these natural chemopreventive agents lead to inhibition of proliferation and apoptosis.[150,153–156]

The role of isoflavones and isothiocyanates, as well as other dietary factors, in the development of ovarian cancer was examined in the prospective California Teachers Study. The California Teachers Study cohort analyzed dietary factors

and incidence of ovarian cancer among 97,275 women. Those who consumed 3 mg per day of total isoflavones had a 44% lower risk of ovarian cancer than that of women who consumed less than 1 mg (RR = 0.56, CI = 0.33 to 0.96). Typical soy foods such as tofu or soymilk contain, on average, about 20 to 50 mg per serving depending on processing.

Omega-3 Fatty Acids

Omega-3 fatty acids (OM-3FAs) are polyunsaturated fats that have been referred to as essential fatty acids because they cannot be synthesized by mammals and must be obtained from the diet.[158] In early human diets, the ratio of omega-6 fatty acids (OM-6FAs) to OM-3FAs was approximately equal. The evolution of the modern Western diet has altered the ratio of OM-6FAs/OM-3FAs such that the OM-6FAs are in greater proportion than the OM-3FAs. The altered ratio of OM-3FAs to OM-6FAs is thought to be partially responsible for the increased risk of cardiovascular disease as well as cancers such as breast and colon. In fact, the increase in breast cancer risk in Japanese women over the past four decades is thought to be related to a change in diet favoring a high OM-6FAs/OM-3FAs ratio.[158] Inversely, changing the ratio of the OM-6FAs/OM-3FAs to favor the OM-3FAs has been shown to have suppressive growth effects on cancerous cells within in vitro systems and in vivo animal models.[158]

Epidemiologic studies indicate that populations that consume high amounts of OM-3FAs have a lower incidence of breast, prostate, and colon cancers.[159–161] Population-based studies have also shown that diets rich in polyunsaturated fatty acids (PUFAs) such as linolenic acids (an omega-3 fatty acid) and fish (an important source of omega-3 fatty acids) have a protective effect against ovarian cancer.[159–164] Recently published data suggest that OM-3FAs have definitive growth-suppressive effects on epithelial ovarian cancer cell lines and that this effect may be mediated, in part, by p53 status.[165] There is also evidence that OM-3FAs may work by inducing the TGF-beta/Smad signaling pathway as well as downregulating the COX-1 enzyme, both important molecular cancer-preventive pathways.[166]

Combinatorial Chemoprevention

The process of carcinogenesis is complex and is heterogeneous with regard to the numerous combinations of genetic and epigenetic events that can occur in an individual cell leading to neoplastic transformation. Thus, optimal strategies for cancer treatment involve simultaneous administration of multiple chemotherapeutic agents to overcome chemoresistance. Administration of multiple agents, ideally working via different mechanisms of action, increases the dose intensity and the likelihood that cancer cells will be susceptible to treatment. In addition, the likelihood of treatment failure is decreased in that the acquisition

of chemoresistance requires that resistant clones of cancer cells adapt to more than one therapeutic agent.

Similar to chemotherapy treatment of full-fledged cancers, it is likely that an optimal cancer-preventive strategy can be best achieved using a combination of chemopreventive agents. A combinatorial chemopreventive strategy could achieve enhanced cancer-preventive efficacy while minimizing the risk of failure due to dysplastic cells that are resistant to one or more preventive agents.[167,168] Fortunately, compared with fully malignant cells, the dysplastic cells targeted by a chemopreventive approach are less likely to harbor multiple genetic alterations, decreasing the likelihood for resistance. Importantly, if the combination of preventive agents chosen has a synergistic effect on cancer-preventive molecular pathways and non-overlapping toxicities, it may be possible to achieve both increased cancer-preventive efficacy while reducing the dosage of each agent, thereby lessening the long-term risks associated with chemoprevention.

Animal Models for Ovarian Cancer: Relevance of the Domestic Fowl for Chemoprevention Research

The lack of a valid ovarian cancer animal model has been a major obstacle to ovarian cancer prevention research. In order to develop effective chemopreventive strategies for ovarian cancer in a timely fashion, animal models that closely mimic human ovarian cancer are desperately needed. Human prevention trials are costly, requiring large numbers of subjects and many years to complete. Development of an animal model for ovarian cancer prevention research would represent a significant breakthrough allowing the expedited evaluation of numerous agents. Ideally, this would lead to the rapid identification of a select number of agents with the greatest potential for ovarian cancer prevention and treatment that could then be evaluated in human trials.

Great progress has been made over the past two decades in the development of animal models for ovarian cancer. Each generation of animal model has had its advantages and limitations. The earliest models employed a xenograft approach in which human ovarian tumor cells or tissues were grown in immunodeficient mice.[169–173] The xenograft model preserved the complex interactions that occur between cancers cells and their microenvironment, including stromal-epithelial cell interactions, as well as influences of matrix proteins, growth factors, and angiogenesis. Thus, this model was a great advance over cell culture model systems and advanced the study of therapeutic interventions. However, an important weakness in the xenograft model was the lack of host immunity, which severely limited the ability to reliably predict the impact of noncancer immune-host influences on outcomes. In addition, tumors were introduced in the xenograft model rather than arising as primary lesions in the ovary thus allowing the investigation of therapeutic, but not chemopreventive, interventions.

Very recently, the advent of several genetically engineered mouse models has facilitated the investigation of ovarian cancer pathogenesis and pharmacologic interventions in the context of both an intact tumor microenvironment as well as a host immune system.[174–178] These genetically engineered models feature both the development of ovarian cancers in situ in the ovary and a metastatic pattern similar to that of human ovarian cancer. In addition, some of these models even have one or more oncogene or tumor suppressor gene alterations similar to that of human ovarian cancer.[175,177] However, even these models have features that significantly limit their usefulness for studying human ovarian carcinogenesis. Importantly, genomic alterations occur in the germ line and thus may confer abnormal influences during embryonic development, in contrast with human ovarian cancer, which occurs spontaneously in the mature ovary. An additional limitation is the induction of ovarian tumors by genomic alterations that occur infrequently in human ovarian cancer.[174,176] Thus, some of these models may have utility for studying interventions directed against specific ovarian cancer genotypes but may not be useful for studying the natural evolution of the disease at it occurs in humans.

In order for an ovarian cancer animal model to yield insights that are likely to have a meaningful impact on the prevention or treatment of women with the disease, it is important that the model recapitulates as many aspects of human ovarian cancer as possible. Ideally, ovarian cancers in the model should be adenocarcinomas that arise in the ovarian surface epithelium and have an intraperitoneal spread pattern similar to that of human ovarian cancer. Preferably, the neoplastic process should arise in the mature ovary and not be subject to abnormal influences present during embryologic development. In order to account for the impact of the host on tumor growth and response to therapy, the animal should have intact immunity. In addition, the tumors that develop should have a genetic profile similar to that of human ovarian cancer. Finally, for the purpose of chemoprevention research, it is ideal that the tumors have a long latent phase.

The chicken ovarian cancer animal model poses an attractive alternative to genetically engineered mouse models and has features that make it ideal for ovarian chemoprevention research. The most relevant feature of the domestic hen is its high incidence of *spontaneous* ovarian cancer, which ranges from 11% to 35% between 4 and 6 years of life.[179,180] This makes the laying chicken unique relative to other animals that require either experimental induction or genetic engineering to induce the development of ovarian tumors.[169–178,181–193] Thus, the developing ovary in the chicken is not subject to the abnormal influences associated with tissue-specific promoter-driven oncogenes. In addition, the chicken model shares many characteristics of human ovarian cancer. Ovarian cancers arise in the adult, mature ovarian surface epithelium in animals that have intact immunity. Tumors develop after a long latent phase, making the model well suited for investigation of chemopreventive strategies. Similar to human ovarian cancer, tumor incidence is impacted by the number of lifetime ovulatory events, and progestins confer chemopreventive effects.[194] Finally, we

have recently observed a number of genetic features in chicken ovarian adenocarcinomas that parallel those in human ovarian cancer, including frequent alterations in p53, and HER-2/neu, and infrequent alterations in ras.[195] We have shown alterations in p53 in 48% of chicken adenocarcinomas. In addition, similar to human ovarian cancers, we found the prevalence of p53 alterations in chicken ovarian cancers correlated with the number of lifetime ovulations. Thus, in chickens as in women, the process of ovulation with repeated cycles of rupture and then repair of the ovarian epithelium may increase the number of proliferative events and genetic errors in the ovarian surface epithelium, leading to more p53 mutations. Overall, in the chicken, no mutations were seen in H-ras, and only 2 of 172 tumors (1.1%) had K-ras mutations. Thus, mutations in the ras oncogene are rare in chicken ovarian cancers, similar to that in human ovarian cancers. Finally, 10 of 19 chicken ovarian adenocarcinomas (52.6%) demonstrated significant HER-2/neu staining, and significant expression of HER-2/neu was associated with the larger ovarian tumors, suggesting that it may be a marker of cancer aggressiveness in the chicken. This is similar to what has been shown in human ovarian carcinomas, where HER-2/neu overexpression has been reported in as high as 50% of human ovarian cancers and has been associated with tumors that are more aggressive.[196,197] Clearly, the chicken, like any other animal model, is far removed from humans and thus suffers from this great limitation. Nonetheless, the clinical and genetic findings in the chicken model bear a resemblance to that in human ovarian carcinomas, thereby providing support for the chicken ovarian cancer animal model. More work is needed to further validate the model and importantly to test whether chemopreventive interventions in the chicken can be reliably translated to humans.

Although the best use of the chicken model remains to be determined, we believe that the model is ideally suited for chemoprevention research. By 2 years of age, young birds will have had more ovulatory events than occur in 1–2 lifetimes in women. These young birds, who rarely have malignancy, but who presumably have incurred initiating events in the ovary, can be randomized to chemopreventive interventions with the goal of determining the impact of these interventions on ovarian cancer incidence 12–24 months later.

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Chapter 2
Staging and Surgical Treatment

Mario M. Leitao, Jr., and Richard R. Barakat

Introduction

The treatment of ovarian cancer truly involves a multimodal approach with surgery as the cornerstone. Surgery serves many roles in addition to providing a definitive diagnosis. In disease grossly confined to the ovary and/or pelvis, surgery can provide accurate and comprehensive staging based on the known patterns of spread. Many patients with clinically apparent early-stage ovarian cancer will be upstaged after comprehensive surgical staging. Accurate staging is crucial for a credible prognosis and as a guide for additional therapeutic approaches. The role of surgery for grossly advanced ovarian cancer is cytoreduction (debulking) of large tumor volume, which results in improved outcomes. Surgical cytoreduction also may improve the survival in select patients with recurrent disease. Surgery is considered in the palliation of many symptoms of advanced ovarian cancer. Patients with ovarian cancer will benefit from being treated by physicians with special expertise in the surgical management of this disease. Invasive epithelial ovarian cancers account for the vast majority of ovarian cancers. This chapter primarily refers to this type of ovarian cancer unless otherwise specified.

Staging and Early-Stage Ovarian Cancer

Staging System

Ovarian cancer is staged primarily using criteria defined by the International Federation of Gynecology and Obstetrics (FIGO).[1] The FIGO staging system for ovarian cancer is depicted in Table 2.1 and mandates surgical exploration for

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Table 2.1 International Federation of Gynecology and Obstetrics (FIGO) staging system for ovarian cancer (Pecorelli et al.[1])

I	Limited to the ovaries
IA	Limited to one ovary, capsule intact, no tumor on ovarian surface, no malignant cells in the ascites or peritoneal washings.
IB	Limited to both ovaries, capsule intact, no tumor on ovarian surface, no malignant cells in the ascites or peritoneal washings.
IC	Limited to one or both ovaries, with any of the following: ovarian capsule ruptured, tumor on ovarian surface, presence of malignant cells in the ascites or peritoneal washings.
II	Tumor involves one or both ovaries with pelvic extension.
IIA	Extension and/or implants in uterus and/or tubes, no malignant cells in the ascites or peritoneal washings.
IIB	Extension to other pelvic organs, no malignant cells in the ascites or peritoneal washings.
IIC	Above with positive malignant cells in the ascites or positive peritoneal washings.
III	Tumor involves one or both ovaries with confirmed peritoneal metastasis outside the pelvis and/or regional lymph node metastasis.
IIIA	Microscopic peritoneal metastasis beyond the pelvis.
IIIB	Macroscopic peritoneal metastasis beyond the pelvis $\leq$2 cm in greatest dimension.
IIIC	Macroscopic peritoneal metastasis beyond the pelvis $\leq$2 cm in greatest dimension, and/or regional lymph node metastasis (including inguinal nodal metastasis), and/or superficial liver capsular metastasis.
IV	Distant metastasis beyond the peritoneal cavity, and/or parenchymal liver metastasis, and/or malignant pleural effusion.

patients without obvious extrapelvic disease. There are correlates of the FIGO staging system within the American Joint Committee on Cancer (AJCC) TNMsystem (a staging system for classifying malignant tumors. T describes the size of the tumor, N describes the regional lymph nodes that are involved, and M describes metastasis). However, the majority of physicians treating patients with ovarian cancer use the FIGO system, and almost all published literature relies on this system. Patients with obvious extrapelvic disease at presentation will already be either FIGO stage IIIC or IV, and in these cases the goal of surgery is cytoreduction and not staging.

Comprehensive surgical staging of ovarian cancer appearing to be grossly confined to the ovaries or pelvis includes total abdominal hysterectomy (TAH), bilateral salpingo-oophorectomy (BSO), bilateral pelvic and para-aortic lymphadenectomy to the renal vessels, omental biopsy, peritoneal washing, and systematic peritoneal surface biopsies. The results of a complete para-aortic lymphadenectomy for ovarian cancer are shown in Fig. 2.1. This procedure traditionally has been performed via laparotomy using a large vertical midline abdominal incision.

Minimally invasive approaches with laparoscopy have been preliminarily described and may be safely performed by experienced surgeons.[2–9] Trocar site placement for laparoscopic surgical staging of ovarian cancer is depicted in

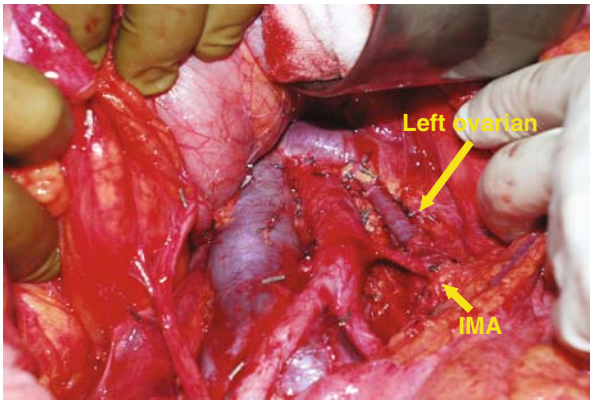

Fig. 2.1 Appearance of the para-aortic nodal region after a comprehensive para-aortic lymphadenectomy. IMA = inferior mesenteric artery

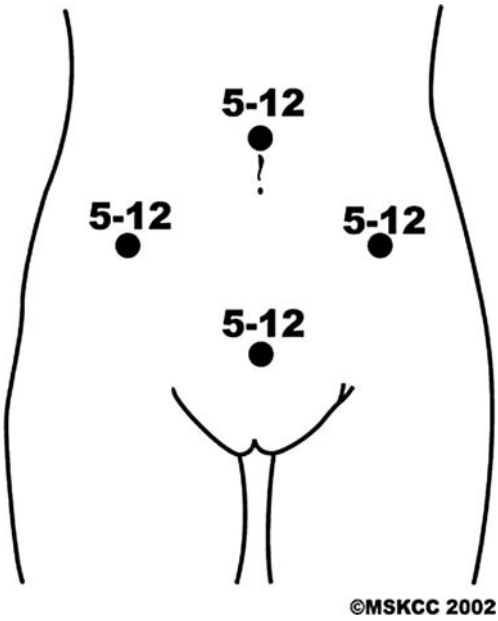

Fig. 2.2 Trocar site placement for laparoscopic surgical staging of ovarian cancer

Fig. 2.2. A minimum of four 5- to 12-mm trocars are used to allow for passage of various laparoscopic instruments. Additional ports are sometimes helpful for retraction of bowel. Multiple instruments are available. We have found that using an argon beam coagulator (ABC) and various graspers facilitates the lymphadenectomy. Typically, the para-aortic lymphadenectomy is performed with the laparoscope in the suprapubic trocar and the ABC in the umbilical trocar. The lymphadenectomy is then started by incising the peritoneum just lateral to the upper common iliac artery and then extended cephalad to the duodenum. The duodenum is then mobilized and the space developed to the

right renal vein. The lymphatic tissue overlying the inferior vena cava from the aorta to the psoas fascia is removed in its entirety. The ureter courses nearby and should be identified so as not to injure it. The left para-aortic lymphadenectomy is performed in a similar fashion by developing the space across the aorta to the left psoas. The inferior mesenteric artery may be ligated and transected if necessary to allow better visualization of this space. The lymphatic packages may be removed directly through one of the port sites with or without specimen retrieval bags. The omentectomy can be performed with the assistance of a vessel-sealing device and also delivered through a trocar site with a specimen retrieval bag.

The pelvic lymphadenectomy and TAH & BSO is then performed by exchanging the entry sites for the laparoscope and ABC. The lymphatic tissue overlying the external iliac artery from the circumflex iliac vein back to the mid common region is then removed with the lateral boundary being the genitofemoral nerve. The dissection is carried to the lymphatic tissue overlying the internal iliac artery and the tissue within the obturator space superior to the obturator nerve. A total laparoscopic or laparoscopic-assisted vaginal hysterectomy is performed either before or after the pelvic lymphadenectomy based on surgeon preference. Descriptions of laparoscopic hysterectomies may be found in various surgical texts and atlases.

The surgical and oncologic outcomes of laparoscopic surgical staging of ovarian cancer appear to be very similar to those seen with surgical staging via laparotomy (Table 2.2).[2–9] However, it should be recognized that all of these series are small retrospective reports and not all had a comparative group. The lymph node counts as well as omental specimen size obtained laparoscopically

Table 2.2 Comparison of laparoscopy and laparotomy for the surgical staging of ovarian cancer

	Chi et al.[7]	*p* Value	Ghezzi et al.[8]	*p* Value	Park et al.[9]	*p* Value
N						
LRS	20		15		19	
LAP	30		19		33	
Mean BMI, kg/m^2						
LRS	24.6		23.8		23.2	
LAP	25.4	NS	25.8	0.05	22.7	NS
Mean pelvic lymph node counts						
LRS	5.8 (L); 6.5 (R)		25.2		27.2	
LAP	7.1 (L); 7.6 (R)	NS	25.1	NS	33.9	NS
Mean para-aortic lymph node counts						
LRS	2.9 (L); 3.8 (R)		6.5		6.6	
LAP	4.8 (L); 4.4 (R)	NS	7	NS	8.8	NS
Mean omental specimen, cm^3						
LRS	186		—		159.5	

Table 2.2 (continued)

	Chi et al.[7]	*p* Value	Ghezzi et al.[8]	*p* Value	Park et al.[9]	*p* Value
LAP	347	NS	—	—	274.2	NS
Mean operative time, min						
LRS	321		377		220.7	
LAP	276	0.04	272	0.002	274.7	0.01
Mean estimated blood loss, ml						
LRS	235		250		240	
LAP	367	0.003	400	NS	568.2	0.005
Blood transfusions, *N* (%)						
LRS	—		1(6.7)		1(5.3)	
LAP	—	—	2(10.5)	NS	10(30.3)	0.04
Mean length of hospital stay, days						
LRS	3.1		3		8.9	
LAP	5.8	<0.001	7	0.001	14.5	0.002
Complications, N (%)						
LRS	0(0)		2(13.3)		—	
LAP	2(7)	NS	8(42.1)	NS	—	—

LRS, laparoscopy; LAP, laparotomy; BMI, body mass index; NS, non-significant.

appear to be similar to those obtained via laparotomy.[7–9] Operative times for laparoscopy were noted to be significantly longer in two series.[7–8] Park et al., however, reported a significantly shorter time for laparoscopic staging.[9] Mean estimated blood loss, numbers of complications, and length of postoperative hospital stays all appear to diminish when surgeons use a laparoscopic approach compared with use of laparotomy.[7–9] Lecuru and colleagues reported similar oncologic outcomes regardless of initial surgical approach.[6] The preliminary results reported thus far for laparoscopy will require further long-term follow-up. Ideally, a randomized trial is needed to truly compare laparoscopic and laparotomic surgical staging for ovarian cancer in terms of both perioperative outcomes as well as long-term oncologic outcomes. There also existed a theoretical concern about port-site metastasis after laparoscopy. This rate of port-site metastasis is exceedingly low (0.64%) and is not seen as an isolated event.[10]

The importance of comprehensive surgical staging was first reported by Young and colleagues in 1983.[11] Systematic restaging was prospectively performed in a cohort of patients referred to Ovarian Cancer Study Group institutions with a diagnosis of "early" ovarian cancer. Upstaging occurred in 31% of cases, with 23% of the entire cohort being upstaged to FIGO stage III due to extrapelvic and retroperitoneal nodal metastases. Le et al. reported a 36% rate of upstaging after comprehensive staging of disease grossly confined to the ovary, with 60% of those upstaged reclassified as FIGO stage III.[12] Isolated nodal metastases were present in 12% of the cases in this series. Additional studies demonstrated that microscopic lymph node metastases are detected in 9%–23%

of patients with clinical stage I or II disease.[13–15] Comprehensive and optimal staging is strongly associated with outcomes and affects treatment decisions.[12,16]

Bilateral pelvic and para-aortic lymph node dissection to the level of the renal vessels should be performed in all patients undergoing staging. Isolated para-aortic nodal and isolated contralateral nodal metastases are frequently detected. Cass and colleagues found that 36% of patients with disease confined to the ovary and nodal metastases had involvement of the para-aortic nodes alone.[14] In addition, 30% of the patients with nodal metastases had contralateral involvement alone. Tsumura et al. reported only para-aortic nodal metastases without pelvic nodal metastases in a similar group of patients.[15] A very large portion of the para-aortic metastases were found cephalad to the inferior mesenteric artery. Palpation alone by specialists in the treatment of ovarian cancer, even with the opening of the retroperitoneal spaces, will miss up to 36% of all microscopic metastases, deeming it inadequate.[17,18]

Comprehensive staging with bilateral pelvic and para-aortic lymphadenectomy is not necessary in patients who have a final pathologic diagnosis of ovarian borderline tumor as it is neither prognostic nor therapeutic if there is no macroscopic disease. In a large review of the Surveillance, Epidemiology, and End Results (SEER) database, the 10-year survival for stage III ovarian borderline tumors was 96% compared with 99% for stage I.[19] Similarly, the risk of recurrence or death was reported by Winter and colleagues to be exactly the same regardless of whether patients were staged or unstaged.[20]

Fertility Preservation

The "standard" approach for surgical staging in ovarian cancer will obviously eliminate any possibility of future childbearing. Fertility-preserving surgical approaches are not usually considered for patients with advanced disease and in those with obvious involvement of both ovaries or uterus. However, it is the preferred approach in young patients, desirous of fertility preservation, with ovarian borderline tumors and those with germ cell and sex cord–stromal tumors of the ovary with disease grossly confined to the ovary.[21–28] Fertility preservation may even be possible for patients with advanced-stage ovarian borderline tumors. The rate of recurrence is slightly higher in patients with ovarian borderline tumors who undergo fertility-sparing procedures, but the overall survival is the same, regardless of stage, compared with that of patients who undergo a TAH & BSO.[21–28]

Fertility-preserving procedures may also be an option in selecting young patients with ovarian carcinoma grossly confined to only one ovary.[29–36] All of the above- mentioned procedures for staging are still performed except that the contralateral normal-appearing ovary and normal-appearing uterus are left in place. Biopsy of a normal contralateral ovary is not needed. Table 2.3 describes the reported outcomes in patients with ovarian carcinoma that underwent

Table 2.3 Reported outcomes after fertility-sparing procedures in patients with ovarian carcinoma

Series	*N*	FIGO stages (*N*)	Median follow-up, months (range)	Recurred, *N* (%)	Recurred after radical surgery,* *N* (%)	5-year DFS, %	5-year OS, %	Fertility outcomes (*N*)
Colombo et al.[29]	56	IA (36)	75 (NR)	3 (5.4)	5/43 (11.6)	NR	NR	Attempted conception (17)
		IB (1)						Conceived (17)
		IC (19)						"Healthy" babies (16)
								Ectopic (2)
								SAB (4)
								ETOP (4)
Zanetta et al.[30]	56	IA (32)	94 (34–175)	5 (9.6)	5/43 (11.6)	NR	NR	Attempted conception (NR)
		IB (2)						Conceived (20)
		IC (22)						"Normal" pregnancies (17)
								Ectopic (2)
								SAB (4)
								ETOP (4)
Raspagliesi et al.[31]	10	IA (2)	70 (24–138)	0(0)	NR	NR	NR	Attempted conception (NR)
		IC (2)						Conceived (3)
		IIIA (2)						Full-term pregnancies (2)
		IIIC (4)						Ectopic (0)
								SAB (1)
								ETOP (0)
Morice et al.[32]	25	IA (19)	47 (6–201)	7 (28)	NR	64	82	Pregnant at diagnosis (1)

Table 2.3 (continued)

Series	*N*	FIGO stages (*N*)	Median follow-up, months (range)	Recurred, *N* (%)	Recurred after radical surgery,* *N* (%)	5-year DFS, %	5-year OS, %	Fertility outcomes (*N*)
		IC (1) II (2) Unk (3)						Attempted conception (NR) Conceived (4) Full-term pregnancies (3) Ectopic (0) SAB (1) ETOP (0)
Schilder et al.[33]	52	IA (42) IC (10)	78 (3–423)	5 (9.6)	NR	NR	98	Pregnant at diagnosis (1) Attempted conception (24) Conceived (17) Full-term pregnancies (26) Ectopic (0) SAB (5) ETOP (0)
Morice et al.[34]	34	IA (30) IC (3) II (1)	47 (11–224)	11 (32.4)	NR	63	84	Attempted conception (NR) Conceived (9) "Normal" pregnancies (6) Ectopic (0) SAB (1) ETOP (0) Ongoing (1)

Table 2.3 (continued)

Series	*N*	FIGO stages (*N*)	Median follow-up, months (range)	Recurred, *N* (%)	Recurred after radical surgery,* *N* (%)	5-year DFS, %	5-year OS, %	Fertility outcomes (*N*)
								Outcome unknown (2)
Kajiyama et al.[35] (all clear cell)	10	IA (4)	35.4 (21.7–153.2)	1(10)	NR	NR	NR	Attempted conception (NR)
		IB (6)						Conceived (4)
								Full-term pregnancies (2)
								35-week pregnancy (1)
								Ectopic (0)
								SAB (2)
								ETOP (0)
Park et al.[36]	62	IA (36)	56 (6–205)	11 (17.7)	NR	80	88	Attempted conception (19)
		IB (2)						Conceived (15)
		IC (21)						Full-term pregnancies (22)
		IIB (1)						Ectopic (0)
		IIIA (1)						SAB (2)
		IIIC (1)						ETOP (0)

FIGO, International Federation of Obstetrics and Gynecology; NR, not reported; SAB, spontaneous abortion; ETOP, elective termination of pregnancy; Unk, unknown.

*Some series reported and compared the outcomes between their cohort that had fertility-sparing procedures and those who had "radical surgery," meaning total abdominal hysterectomy and bilateral salpingo-oophorectomy, at initial diagnosis.

fertility-sparing procedures. The best candidates would be patients with FIGO stage I (preferably FIGO stage IA). The reported oncologic outcomes appear to be similar to those reported in other series of traditionally treated patients with early-stage ovarian carcinoma. The rates of recurrence for patients with fertility preservation and those without were reported in two series in Table 2.3; the rates for both groups were similar. All of these reports have small numbers of cases.

Surgery for Advanced-Stage Ovarian Cancer

Stage is apparent in patients with grossly metastatic intraperitoneal disease. The goal of surgery in the setting of advanced disease is tumor cytoreduction (debulking). Tumor cytoreduction of advanced ovarian cancer has both theoretical and clinical benefits. The optimal approach to the management of advanced ovarian cancer is an attempt at optimal cytoreduction followed by postoperative chemotherapy. Randomized trials addressing the value of initial cytoreduction have not been completed and reported. However, there is an overwhelming body of nonrandomized literature supporting tumor cytoreduction for ovarian cancer. Interval cytoreduction after a course of neoadjuvant chemotherapy may be an option for select patients. Cytoreductive, as well as video-assisted thoracoscopic surgery (VATS) procedures may also be useful in the management of patients with stage IV ovarian cancer.

Theoretical Benefits Supporting Surgical Cytoreduction

A key concept in understanding the potential benefits of tumor cytoreduction is the "Gompertzian" cell growth curve. Tumor cell numbers tend to increase exponentially over time, and the rate of growth is faster in the earlier part of the curve when tumors are relatively small.[37] Log-kill of tumors with chemotherapy is therefore probably greater in tumors of smaller volume.[37] Surgical cytoreduction of tumor volume is thought to offer patients a greater chance of response to chemotherapy based on this concept. Surgical cytoreduction is generally not attempted without a plan for administering postoperative chemotherapy, as surgery alone is rarely curative.

The elimination of potentially chemoresistant cells is another potential benefit of surgical cytoreduction. The probability of spontaneous mutations and drug-resistant phenotypes increases as tumor size and cell numbers increase according to the mathematical model of Goldie and Coldman.[38] It is reasonable to think that primary cytoreductive surgery will remove existing resistant tumor cells and decrease the spontaneous development of additional resistant cells. In addition, surgery has the potential to remove large tumor masses with poor blood flow, allowing better distribution of intratumoral chemotherapy. These theoretical benefits are supported by the many reported clinical benefits.

Clinical Benefits Supporting Surgical Cytoreduction

No prospective trials have been completed and reported addressing the benefit of primary surgical cytoreduction prior to chemotherapy. Unsuccessful attempts have been made to conduct such trials in the United States. In the absence of a prospective trial, an overwhelming body of retrospective experience has been reported supporting the benefit of primary surgical cytoreduction. The immediate clinical benefits of surgical cytoreduction are improved patient comfort, gastrointestinal function, nutrition, and quality of life.[39] Optimal surgical cytoreduction has been shown to improve both progression-free and overall survival.

The concept of "maximal surgical effort" for patients with ovarian cancer was introduced in the 1930s but was first quantified and correlated with outcome in a report by Griffiths in 1975.[40] This report was published before the current practice of using platinum- and taxane-based regimens as standard therapies. Single-agent melphalan was used after primary surgery in a series of patients reported by Griffiths. The amount of residual tumor was an independent predictor of survival. The respective mean survival times for patients with no gross residual, gross residual $\leq$0.5 cm, gross residual of 0.6–1.5 cm, and gross residual $>$1.5 cm were 39 months, 29 months, 18 months, and 11 months, respectively (Table 2.4).[11,41,42] These findings have been subsequently confirmed by many authors.

The value of surgical cytoreduction was further supported by a landmark publication by Hoskins and colleagues.[41] A subset analysis was performed of two Gynecologic Oncology Group (GOG) randomized therapeutic trials. The 4-year survival was greatest for patients with no gross residual disease (60%) compared with that for those with gross residual $\leq$1 cm (35%), gross residual

Table 2.4 Select reports of primary surgical cytoreduction prior to chemotherapy administration in patients with advanced epithelial ovarian cancer

Author	Year	Residual disease (cm)	*N*	Mean/median survival (months)	5-year survival (%)
Griffiths[40]	1975	0	29	39	
		0–0.5	28	29	
		0.6–1.5	16	18	
		>1.5	16	18	
Hoskins et al.[41]	1994	0	97		60
		≤1	246		35
		1–2 cm	31		35
		≥2	263		<20
Chi et al.[42]	2006	0	67	106	
		≤0.5	70	66	
		0.6–1	99	48	
		1–2	53	33	
		>2	176	34	

1–2 cm (35%), and gross residual ≥2 cm (Table 2.1).[11,41,42] There was no difference in survival, regardless of increasing tumor residual, in the group of patients with gross residual ≥2 cm. The greatest advantage was observed in patients with tumor residual <2 cm, although those with no gross residual appeared to have the best survival advantage. The group of patients with gross residual of 1-2 cm and the group with no gross residual was small compared with the other groups. This may account for the lack of statistical significance between no gross residual and any gross residual or gross residual of ≤1 cm and 1–2 cm. The GOG went on to establish the current definition of "optimal" cytoreduction as largest residual ≤1 cm based on these and other subsequent reports, and the ≤1 cm cutoff point remains the currently accepted definition of optimal cytoreduction. Recent reports support aggressive attempts to achieve complete gross resections. A benefit is still maintained for an optimal cytoreductive effort short of a complete gross resection and should include extensive upper-abdominal procedures as needed.

The benefit of aggressive surgical cytoreduction has been questioned.[43,44] Opponents suggest that aggressive surgery may only benefit patients with less advanced disease and that the ability to achieve an optimal cytoreduction is merely a reflection of tumor biology. Some argue that an optimal cytoreduction was most beneficial in the era of less effective chemotherapeutic agents prior to platinum and taxanes. A recent large meta-analysis incorporating 53 studies and 6885 patients clearly demonstrated that one of the most powerful determinants of survival among patients with stage III or IV ovarian cancer treated with adjuvant platinum-based therapies was maximal cytoreduction (Fig. 2.3).[45] Patient cohorts with ≥75% maximal cytoreductive efforts had a median survival of nearly 37 months compared with a median survival of only 23 months for cohorts with ≤25% maximal effort.

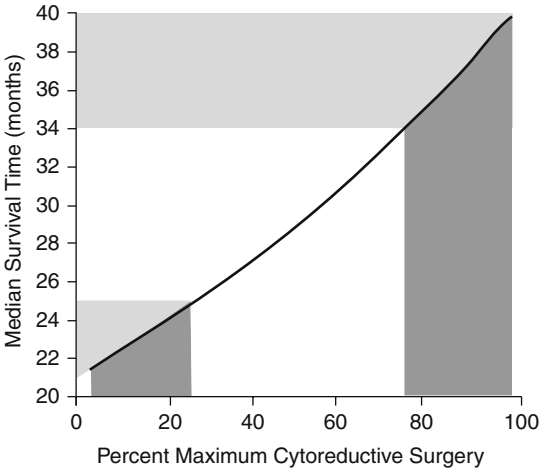

Fig. 2.3 Median survival time plotted against percent maximal cytoreductive surgery in patients with advanced ovarian cancer. Gray area, maximal cytoreductive surgery<25%and>75% crosshatched area, corresponding range of median survival times (Bristow et al.[45])

Extensive upper-abdominal disease was the most frequent reason for suboptimal cytoreduction, and many centers reported optimal rates of less than 50%. A recent change in surgical approach has led to the routine use of extensive upper-abdominal procedures.[46] The following procedures, in addition to hysterectomy, bilateral salpingo-oophorectomy, and omentectomy, are now performed and should be considered in order to obtain an optimal cytoreduction: enterectomy, colectomy, rectal resection, resection of retroperitoneal nodal disease, diaphragm peritonectomy/resection, splenectomy, distal pancreatectomy, liver resection, resection of porta hepatis disease, and cholecystectomy.[46] Optimal cytoreduction rates significantly improved at our institution (from 50% to 76%) after the incorporation of aggressive upper-abdominal procedures.[46]

The addition of extensive upper-abdominal surgery has improved the survival in patients with stages IIIC to IV epithelial ovarian cancer (EOC).[47,48] Patients who require extensive upper-abdominal procedures to achieve optimal cytoreduction have the same progression-free, disease-specific, and overall survival compared with that of patients who do not require an extensive resection to achieve an optimal cytoreduction.[47,48] These findings do not support the notion that bulky upper-abdominal disease indicates poor tumor biology. Patients with EOC who are seen by physicians who frequently perform radical abdominal procedures to achieve optimal cytoreduction will have improved survivals.[48] The incorporation of these radical procedures at our institution has resulted in a significant improvement of overall survival (unpublished abstract). The overall median survival increased from 43 months during the period 1996–1999 to 58 months during the period 2001–2004 ($p = 0.04$).

We have also demonstrated that maximal removal of tumor provides the greatest chances of long-term survival.[42] Similar findings were reported by Aletti and colleagues.[48] In our series, the median survival for patients with no gross residual was 106 months compared with 66 months for those with gross residual $\leq$0.5 cm, 48 months for gross residual 0.6–1 cm, 33 months for gross residual 1–2 cm, and 34 months for gross residual $>$2 cm (Table 2.4).[11,41,42] No gross residual disease was a strong independent predictor of survival compared with that for gross residual disease $\leq$1 cm or gross residual $>$1 cm. The difference seen for gross residual disease $\leq$0.5 cm compared with that for gross residual 0.6–1 cm was not statistically significant, but there was still an 18-month difference.

The benefit of tumor cytoreduction for stage III ovarian carcinoma was again recently supported by an ancillary analysis of 1895 patients that participated in published GOG protocols.[49] Patients with no gross residual tumor after initial cytoreduction prior to randomization to a platinum-taxane regimen had the longest progression-free survival (PFS) and overall survival (OS). The median PFS and OS for these patients were 33 months and 72 months, respectively. In contrast, the median PFS and OS were 17 months and 42 months, respectively, for patients with 0.1- to 1-cm residual and 14 months and 35 months, respectively, for those with $>$1-cm residual. The amount of residual

disease was the strongest independent predictor of outcome on multivariate analysis. Age and histologic cell type also were independent predictors to a lesser degree.

All patients who have undergone an optimal cytoreduction should receive platinum- and taxane-based adjuvant chemotherapy. Intraperitoneal chemotherapy should be discussed with all patients preoperatively. Adjuvant intraperitoneal and intravenous chemotherapy improves survival in patients with optimally debulked stage III EOC compared with that for intravenous therapy alone.[50] Intraperitoneal catheters may be placed at the time of optimal cytoreduction.

Stage IV Ovarian Carcinoma

Optimal cytoreduction has also been associated with an improved survival in patients who have stage IV EOC (Table 2.5).[51–57] The median survival for "optimally" debulked stage IV disease, however, is nearly half of that for optimally debulked stage IIIC EOC. It is important to be able to appropriately select those patients with stage IV EOC who may have the greatest benefit from an aggressive surgical cytoreduction.

Table 2.5 Outcomes for patients with FIGO stage IV ovarian cancer who underwent primary surgical cytoreduction followed by adjuvant chemotherapy

Series	Year	Residual (cm)	No. patients	Median survival (months)
Goodman et al.[51]	1992	<2	23	28
		>2	12	22
Curtin et al.[52]	1997	<2	41	40
		>2	51	18
Liu et al.[53]	1997	<2	14	37
		>2	33	17
Munkarah et al.[54]	1997	<2	31	25
		>2	61	15
Bristow et al.[55]	1999	<1	25	38
		>1	59	10
Akahira et al.[56]	2001	<2	155	32
		>2	70	16
Aletti et al.[57]	2007	<1	24	38
		1–2	10	22
		>2	15	11

VATS is now commonly used at our institution for patients with moderate to large pleural effusions.[58] We define a moderate to large pleural effusion as the presence of layering fluid occupying greater than or equal to one-third of the pleural cavity on upright chest radiograph. VATS will identify macroscopic disease in 65% of these patients. In addition, 48% of patients with moderate to

large pleural effusions will have macroscopic pleural-based disease measuring >1 cm. Computed tomography (CT) scanning will miss 36% of these patients. Primary chemotherapy ("neoadjuvant") is given to patients with stage IV EOC who have pleural-based disease that cannot be optimally debulked. An attempt at interval cytoreduction is then considered after at least three to four cycles of chemotherapy.

Aletti and colleagues have proposed a treatment strategy for patients with stage IV EOC.[57] Patients with unresectable extensive pleural disease or multiple hepatic metastases are not the best surgical candidates and may be best treated with either neoadjuvant chemotherapy or novel strategies. We would add that VATS evaluation should be performed in patients with a moderate to large pleural effusion. Careful selection of patients with stage IV EOC for attempted cytoreduction is important and will offer patients the most favorable outcomes.

Interval Cytoreduction and Neoadjuvant Chemotherapy

Many patients are left with suboptimal disease despite aggressive attempts at optimal cytoreduction. These patients will receive chemotherapy but have a markedly worse outcome compared with that of optimally debulked patients. Surgery may still be beneficial in select patients who cannot be optimally debulked prior to chemotherapy. There are two primary groups of "suboptimal" patients. One group comprises patients that are left with suboptimal disease despite an aggressive surgical attempt. These patients will go on to receive chemotherapy, and it is unclear whether a second attempt at optimal cytoreduction is beneficial. Another group comprises patients who do not undergo an aggressive surgical attempt due to preoperative imaging or medical status, or because they are deemed unresectable at the time of a diagnostic laparotomy or laparoscopy. Interval cytoreduction after three to four cycles of neoadjuvant chemotherapy followed by additional chemotherapy may be beneficial in some of these patients.

Small retrospective and nonrandomized prospective reports have suggested that outcomes are similar for those patients who receive primary chemotherapy instead of undergoing a primary attempt at surgical cytoreduction.[59] These same studies have reported higher optimal cytoreduction rates after neoadjuvant chemotherapy. These reports, however, made comparisons with cohorts of patients that had initial optimal cytoreduction rates much lower than the rates now reported in cohorts that have undergone aggressive surgical procedures by specialists in the surgical treatment of advanced EOC.

A meta-analysis of primary platinum-based chemotherapy instead of primary cytoreduction was recently published.[60] This meta-analysis included 21 studies with a total of 853 patients. The mean weighted median overall survival for all cohorts was 24.5 months. The median survival in patients suboptimally debulked and then treated with cisplatin and paclitaxel therapy is much greater

(38 months).[61] The reported outcomes using a neoadjuvant approach are significantly inferior to optimal cytoreduction prior to chemotherapy administration. There will be select patients, however, who cannot undergo an initial extensive surgical procedure as described previously. A neoadjuvant approach with interval cytoreduction may be the appropriate treatment for these patients. The estimated median survival for cohorts undergoing 100% maximal interval cytoreduction after neoadjuvant chemotherapy is 31 months compared with 12.5 months for cohorts with a 0% maximal interval cytoreduction.[60]

An interval cytoreduction after an initial aggressive attempt at maximal debulking resulting in suboptimal residual is not likely to be beneficial. This is slightly different from an interval cytoreduction for patients who had not undergone an aggressive initial procedure as described above. A European Organization for Research and Treatment of Cancer (EORTC) trial demonstrated a significant survival benefit to an interval surgery after three cycles of cyclophosphamide and cisplatin for suboptimally debulked patients.[62] The median overall survival was 26 months for patients who underwent an interval surgery after three cycles of chemotherapy compared with 20 months for those who went on to only receive three more cycles of chemotherapy. A similar study conducted in the United States by the GOG did not have the same results.[63] An interval cytoreduction did not result in an improved survival compared with merely completing 6 cycles of chemotherapy. The median survival for both groups was 34 months. There are significant differences between the two trials that explain the different results. The most important difference was that nearly all of the patients enrolled in the GOG trial had their initial surgical procedures performed by gynecologic oncologists with maximal attempts at optimal cytoreduction. In addition, 72% of the patients in the EORTC trial were left with initial residual disease greater than 5 cm compared with only 44% of those in the GOG trial. Table 2.6 summarizes these two trials. Interval cytoreduction is unlikely to provide a significant benefit in patients who are initially treated by

Table 2.6 Randomized trials of interval cytoreduction

	EORTC[62]	GOG[63]
N		
Surgery*	140	216
No surgery	138	208
Median PFS (months)		
Surgery	18	10.5
No surgery	13	10.7
HR (recurrence)	$p = 0.01$	1.07 (95% CI: 0.87, 1.31)
Median OS (months)		
Surgery	26	33.9
No surgery	20	33.7
HR (death)	0.69 ($p = 0.01$)	0.99 (95% CI: 0.79, 1.24)

Table 2.6 (continued)

	EORTC[62]	GOG[63]
Stage IV	22%	6%
Initial residual disease >5 cm	72%	44%
Initial cytoreduction by specialist	Not indicated	95%
Interval cytoreduction by specialist	Not indicated	99%
Completed six cycles of chemotherapy	84%	95%

EORTC, European Organization of Research and Treatment of Cancer trial; GOG, Gynecologic Oncology Group trial; PFS, progression-free survival; HR, hazard ratio; OS, overall survival; CI, confidence interval.
* Refers to attempted interval cytoreduction followed by three additional cycles of chemotherapy versus just three additional cycles of chemotherapy.

specialists in the surgical management of patients with advanced EOC and who are truly suboptimal after an aggressive surgical effort prior to chemotherapy.

Surgical Cytoreduction for Recurrent Ovarian Cancer

Repeated attempts at surgical cytoreduction at the time of diagnosis of recurrent ovarian cancer may be beneficial in select patients. There have been multiple retrospective and some prospective nonrandomized series published that have reported an association with improved survival and optimal secondary surgical cytoreduction.[64] There have been no completed trials that have randomized patients with recurrent ovarian cancer to have secondary cytoreduction followed by chemotherapy or to chemotherapy alone. The GOG currently has an active trial that will randomize patients with platinum-sensitive recurrent ovarian carcinoma to either surgery followed by chemotherapy or just chemotherapy alone.

Berek and colleagues first reported on a cohort of patients who underwent optimal debulking ($\leq$1.5 cm) at the time of secondary cytoreduction.[65] They reported a median survival of 20 months compared with 5 months for those who were suboptimally debulked. Treatment-free interval, presence of symptoms, presence of ascites, and tumor mass at recurrence were all important prognostic factors in their review.

Table 2.7 summarizes the series of secondary cytoreduction reporting more than 50 cases each that underwent attempts at surgical cytoreduction without novel additional treatment approaches.[66–77] Secondary cytoreduction is considered beneficial if an optimal cytoreduction is achieved. This is commonly defined as the maximal size of residual tumor measuring <1 cm, which includes complete gross resection of all tumor. However, many authors suggest that the greatest benefit of secondary cytoreduction is seen if all grossly visible recurrent tumor is resected.[68,70,72,73,75,76] Cytoreductive surgery beyond the secondary

Table 2.7 Series with more than 50 cases of recurrent EOC that underwent attempted secondary cytoreduction

Series	Year	*N*	Definition of optimal cytoreduction	Percent optimally cytoreduction (%)	Percent complete gross resection (%)	Median overall survival (months)		
						No gross residual*	Optimal*	Suboptimal*
Segna et al.[66]	1993	100	<2 cm	61	—	—	27	9
Lichtenegger et al.[67]	1998	63	<2 cm	65	22	24	N/A	17
Eisenkop et al.[68]	2000	106	No gross	82	82	44	—	19
Zang et al.[69]	2000	60	<1 cm	43	—	—	20	8
Scarabelli et al.[70]	2001	149	<1 cm	70	36	See note	See note	See note
Zang et al.[71]	2003	60	≤1 cm	38	—	—	18	13
Zang et al.[72]	2004	117	≤1 cm	62	9	61%	21%	4.5%
Ayhan et al.[73]	2006	64	≤1 cm	83	44	39	19	18
Chi et al.[74]	2006	153	≤0.5 cm	52	41	—	56	27
Harter et al.[75]	2006	567	<1 cm	76	50	45	20	20
Salani et al.[76]	2007	55	<1 cm	89	75	50‡	—‡	7.2‡
Tebes et al.[77]	2007	85	<1 cm	86	—	—	30	17

Note: Provided hazard ratios (HR) only. HR for death was 2.65 (95% CI: 1.43–4.92) for gross residual = 1 cm and 5.79 (95% CI: 2.99–11.21) for gross residual >1 cm compared with no gross residual.

*In series where survival was assessed based on no gross residual, the optimal category includes only optimal but gross residual disease.

†5-year overall survival.

‡Median overall survival in the suboptimal category is for any gross residual regardless of size.

setting may also benefit select patients.[78,79] The exact size of residual disease that should be considered optimal is still debatable, but we seek to achieve a complete gross resection in the secondary or tertiary setting. If this is not possible, cytoreduction to <1 cm may also be of benefit. Aggressive surgical attempts that will leave residual tumor >1 cm are not warranted except in the palliative setting.

Multiple tumor, disease, and patient characteristics have been reported to be associated with outcome after secondary or tertiary cytoreduction. The only consistent factor almost uniformly associated with an improved survival has been "optimal" cytoreduction, however defined.[66–79] The real challenge has been selecting patients preoperatively for whom a secondary or tertiary cytoreduction would most likely offer the greatest benefit. The other factors that have also been associated with outcome by many authors, but not all, have been disease-free interval (DFI) prior to recurrence, number of sites of recurrence, presence of carcinomatosis, and ascites.[68–72,74,75,77–79]

We have recently published recommendations that may help in the process of deciding if secondary cytoreduction should be offered (Table 2.8).[74] These recommendations were based on our multivariate analysis of survival incorporating multiple factors. DFI and number of sites of recurrent disease were the only other independent predictors of survival in addition to size of residual tumor. The presence of ascites was significant on univariate analysis but not multivariate. The median survival for patients with ≤0.5 cm of residual tumor after secondary cytoreduction was 56 months compared with 27 months for those with >0.5 cm of residual tumor. The median survival after secondary cytoreduction was 60 months for patients with a single site of recurrence compared with 27.5 months for patients with carcinomatosis (defined as ≥20 tumor nodules). The optimal cut-point method for prognostic variables and applied smoothing techniques were used to look at survival as a function of DFI. There was little change in survival in patients with a DFI of 6 to 12 months. There was a significant increase in survival as the DFI increased from 13 to 30 months and then plateaued after 30 months.

Table 2.8 Recommendations for secondary cytoreduction (Chi et al.[74])

Disease-free interval	Single site of recurrence	Multiple sites of recurrence but no carcinomatosis	Carcinomatosis
6–12 months	Offer SC	Consider SC	No SC
12–30 months	Offer SC	Offer SC	Consider SC
>30 months	Offer SC	Offer SC	Offer SC

SC, secondary cytoreduction.

Based on our analysis, secondary cytoreduction should be offered to all patients with a single site of recurrent disease regardless of DFI as well as all patients with a DFI of >30 months regardless of number of sites of disease (Table 2.8). Patients with carcinomatosis and a DFI of <12 months should not

be considered for secondary cytoreduction. This is not to say that other algorithms are not valid, and it is important to understand that these decisions must be individualized based on the patient's goals, performance status, operative risk, and available therapeutic options.

Results from the DESKTOP OVAR trial, a large, multicenter, retrospective review, led to the design of an algorithm that will be used in a nonrandomized prospective trial.[75] The algorithm will have patients with a good performance status and a DFI >6 months undergo an attempted secondary cytoreduction. In addition, these patients must have had no residual after initial surgery and also currently not have large-volume (>500 ml) ascites. Patients with recurrent disease who do not meet the above criteria but in whom surgery is still being considered will undergo a diagnostic laparoscopy. If there is no peritoneal carcinomatosis, secondary cytoreduction via laparotomy will be attempted. Patients with carcinomatosis will not undergo a laparotomy and will go on to platinum-based chemotherapy. Platinum-based chemotherapy will also be given to all patients after secondary cytoreduction.

Predicting Optimal Cytoreduction

Another challenging aspect is the ability to accurately predict, preoperatively, which patients can be optimally cytoreduced. Limited data exist that address the role of CT scanning to predict for optimal resectability. No single feature has been uniformly associated with unresectability. A model for predicting surgical outcome in patients with advanced ovarian carcinoma undergoing primary surgical cytoreduction was proposed by Bristow and colleagues.[80] Thirteen CT features were identified that had good specificity, positive predictive value, negative predictive value, and accuracy based on their criteria. Points were assigned to these criteria. In addition, GOG performance status of $\geq$2 was also included due to its predictive value. A Predictive Index score was then created by adding up the individual point assignments. A Predictive Index score of $\geq$4 had the highest overall accuracy (92.7%) in predicting surgical outcome using receiver operating characteristic (ROC) curve analysis. The likelihood of having a suboptimal resection was 87.5% with a Predictive Index score $\geq$4. Additionally, 85% of the patients who were truly optimally cytoreduced were identified correctly.

Funt et al. attempted to correlate CT findings with surgical outcome in patients undergoing secondary cytoreduction.[81] Two radiologists unaware of surgical outcomes retrospectively reviewed CT images and tried to assess for resectability using many of the features described by Bristow and colleagues. Pelvic sidewall invasion and hydronephrosis were significant independent predictors of suboptimal resectability. Large- or small-bowel obstruction, nodal or perihepatic liver metastasis, ascites, peritoneal carcinomatosis, involvement of bladder, rectum, sigmoid, or vagina, or infrarenal para-aortic lymphadenopathy

were not strong indicators of tumor resectability. This was a small study of only 36 cases; therefore, some of the non-significance noted for other CT findings in predicting optimal cytoreduction may be related to these small numbers. Despite these reports, there are no single CT findings that alone predict suboptimal resectability with 100% accuracy. It is not entirely clear why pelvic sidewall invasion would be unresectable in the recurrent setting as it is often not found to be unresectable in the primary setting. However, this may be explained by the hypothesis that tumor cells become fibrin-entrapped on previously traumatized peritoneal surfaces.[82]

It should be kept in mind that the retrospective series published on the many aspects of secondary and tertiary cytoreduction include heterogeneous groups of patients and surgeons, involve tumors with different biological behavior, and have strong selection biases in the authors' criteria for surgical interventions. The GOG has recently initiated a multi-institutional randomized trial attempting to define the role of surgery in patients with their first recurrence. This trial will randomize patients who have recurred more than 6 months after completion of initial therapy to either undergo surgery or not. There will then be a second randomization to one of two chemotherapeutic regimens. This trial will provide important information once it is completed.

Intraoperative Heated Chemotherapy

An interesting treatment modality being investigated in the setting of recurrent ovarian carcinoma limited to the peritoneal cavity is hyperthermic intraperitoneal chemotherapy (IPHC, HIPEC).[83,84] Hyperthermia has been shown to increase the response to cytotoxic agents in human cell lines and animal models.[85,86] Small series in optimally cytoreduced recurrent ovarian carcinoma have shown some promise.[83,84] However, the infusion must be done over 90 minutes and, therefore, increases operating room times. A mean operating room time of nearly 10 hours was reported by Helm and colleagues.[83] The preliminary reported median survivals do not appear to be strikingly better than those seen in Tables 2.3, 2.4, 2.5, 2.6, and 2.7. HIPEC is an interesting concept but must undergo further rigorous investigation before it can be offered to patients outside of a clinical trial.

Surgery for Malignant Bowel Obstruction

Another potential role of surgery is in the palliative setting, most often in the management of malignant bowel obstruction (MBO).[87–92] Tumor removal alone for symptom control is rarely attempted. MBO is the most common reason for hospital admission during the last year of life in patients with ovarian carcinoma.[93] The available data are from limited small series of highly selected cases. The median survival seen in patients who undergo attempted surgical correction seems to be better than that for those who have a drainage

gastrostomy placed with or without total parenteral nutrition (TPN).[89,91,94,95] Surgical correction and successful palliation is possible in 84% and 71% of patients with MBO, respectively, if appropriately selected.[91] However, nearly 25% of patients will have a grade 3 or 4 perioperative complication, and there is a perioperative mortality of 6%.[91] Many factors must be considered when discussing management options with patients who have a MBO. In a review by Ripamonti et al., the following factors were believed to be absolute contraindications for attempted surgical correction of MBO: ileus secondary to diffuse carcinomatosis, ascites requiring frequent paracentesis, diffuse palpable intra-abdominal masses with liver involvement, recent laparotomy with unsuccessful correction of MBO, previous surgery revealing diffuse metastatic cancer, and involvement of the proximal stomach (Table 2.9).[89] Surgical management of MBO is controversial, challenging, and highly individualized.

Table 2.9 Prognostic indicators of low likelihood of clinical benefit from surgery for MBO (modified from Ripamonti and Bruera[89])

Absolute contraindications
1. Intestinal motility problems due to diffuse intraperitoneal carcinomatosis.
2. Ascites requiring frequent paracentesis.
3. Diffuse palpable intra-abdominal masses and liver involvement.
4. A recent laparotomy that demonstrated that further corrective surgery was not possible.
5. Previous abdominal surgery that showed diffuse metastatic cancer.
6. Involvement of proximal stomach.
Other indicators
1. Obstruction secondary to cancer.
2. Widespread tumor.
3. Patients over 65 years old with cachexia.
4. Low serum albumin and low serum prealbumin.
5. Previous radiotherapy of the abdomen or pelvis.
6. Patients with nutritional deficits.
7. Distant metastases, pleural effusion, or pulmonary metastases.
8. Multiple partial bowel obstruction with prolonged passage time on radiographic imaging.
9. Elevated blood urea nitrogen levels, elevated alkaline phosphatase levels, advanced tumor stage, short diagnosis to obstruction interval.
10. Poor performance status.
11. Extra-abdominal metastases producing symptoms that are difficult to control.

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Chapter 3
Novel Therapeutic Targets

John Farley and Michael J. Birrer

Introduction

Ovarian cancer remains an important health problem for women in the United States. It is estimated that there will be 21,650 cases of invasive ovarian cancer resulting in 15,520 deaths in 2008.[1] Ovarian cancer has the highest case fatality rate of any gynecologic cancer,[1] and it is the most common cause of death from cancers of the female genital tract.[1–3] The high case fatality rate results from the frequent diagnosis of epithelial ovarian cancer at an advanced stage: 75% of all cases are diagnosed as stage III or IV, where the disease has spread throughout the abdomen.[1,4,5] Patients with advanced-stage disease have a 5-year survival of only 29%. Despite years of clinical trials, there is a only a limited number of chemotherapeutic agents with activity against epithelial ovarian cancer. Further, all patients with this disease (regardless of histology and tumor grade) are treated up-front in the same fashion with the standard of the combination of platinum and paclitaxel (Taxol, Bristol-Myers Squibb Co, Corporate Headquarters, 345 Park Avenue, New York, New York 10154). Epithelial ovarian cancer is initially a chemoresponsive tumor with response rates in the 80% range.[1–5] Unfortunately, most patients develop recurrent disease, which rapidly acquires chemoresistance.

The identification and characterization of the genes and their protein products that contribute to the malignant phenotype has greatly increased our understanding of human carcinogenesis. There are a variety of cell surface receptors, signaling pathways, and nuclear proteins that stimulate cellular proliferation, or inhibit cell death, and are possible targets for novel therapeutic

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agents. These new agents that target these pathways usher in a new era of rational therapeutics, which promises to revolutionize our approach to cancer patients. The first and best example of successfully targeted molecular therapy is Gleevec (STI571; imatinib; Novartis Pharmaceuticals; One Health PlazaEast Hanover, NJ 07936-1080) in the treatment of gastrointestinal stromal tumors and chronic myeloid leukemia (CML).[6–8]

Oncologists are beginning to investigate a variety of new biologic agents for the treatment of ovarian cancer.[9,10] The ideal molecular target for clinical therapeutic applications should be differentially expressed by the tumor, have a potential drugable molecular site, and be necessary for the viability of the cancer cell.[11] The molecular heterogeneity of ovarian cancer compared with other disease sites, such as hematologic malignancies, has made the successful transfer of molecular agents into the ovarian cancer treatment armamentarium problematic.[11] As opposed to the singular molecular abnormality observed in gastrointestinal stromal tumor (GIST) (c-kit) or chronic lymphocytic leukemia (CLL) (BCR-ABL) for which imatinib is effective, ovarian cancer possesses a multitude of molecular abnormalities any of which may play a pivotal role in ovarian cancer proliferation and survival. An appreciation and understanding of the complex pathways of growth deregulation in gynecologic cancers is providing a framework for the rational application and testing of novel therapies.[9,10] This review will summarize the emerging biologic therapies with an emphasis on the molecular pathways they affect and their relevance to gynecologic malignancies.

Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) provide attractive targets for anticancer therapy. Members of this family that are frequently activated in cancer cells include the epidermal growth factor receptor family and vascular endothelial growth factor receptors. Inhibitory compounds fall into two broad categories: monoclonal antibodies and small-molecule inhibitors.[12]

Endothelial Growth Factors: Vascular Endothelial Growth Factor

Angiogenesis is the formation of new blood vessels, required by many biologic processes including the development of cancer.[13] New vessel formation can be stimulated by a variety of factors including vascular endothelial growth factor (VEGF).[14,15] VEGF mediates angiogenic signals to the vascular endothelium through high-affinity RTKs that are thought to activate the mitogen-activated protein kinase (MAPK) pathway. There are six known VEGF proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta-derived growth factor (PlGF) 1 and 2.[16] There are three VEGF receptors designated VEGFR-1, VEGFR-2, and VEGFR-3. VEGFR-1 and VEGFR-2 are mainly limited to vascular endothelium and VEGFR-3 is restricted to the lymphatic endothelium.[17] Although many stimulators and inhibitors of angiogenesis have been

identified, the trigger that causes a dormant tumor to transform into a proangiogenic tumor remains elusive.[18,19] Tumor growth beyond 2–3 mm is dependent on new vessel formations.

Microvessel density (MVD) is an independent prognostic factor for overall survival in primary tumors of the breast and in malignancies of the female genital tract.[20] A direct correlation has been observed between MVD with higher grade and depth of invasion in stage I adenocarcinomas of the endometrium and with prognosis in stage I and II and recurrent disease.[20] In early-stage ovarian cancer, increased VEGF protein expression by immunohistochemistry was associated with a decreased disease-free survival: 18 months versus greater than 120 months for VEGF nonexpressor.[21] In a multivariate analysis, only VEGF expression was associated with poorer survival in these early-stage ovarian cancer patients. In ovarian epithelial adenocarcinoma, higher microvessel counts in the primary ovarian tumor or omental metastases may serve as a prognostic indicator for survival.[20] The dependence of tumor growth on new vessel formation is further supported by the observed tumor stabilization or regression when endothelial cell proliferation is inhibited.[22,23]

There have been three main approaches to targeting angiogenesis in the treatment of cancer (Table 3.1). The first has been to target VEGF itself, the second to block the VEGF binding site on its cell surface receptors, and finally to inhibit tyrosine kinase activation and downstream signaling with small molecules at the intracellular level.[16,24] Several naturally occurring angiogenesis inhibitors have been identified in preclinical assays including platelet factor-4 (PF-4), thrombospondin (TSP), angiostatin, and endostatin. Angiostatin and endostatin synergistically inhibit both endothelial cell proliferation and ovarian tumor growth in preclinical models.[25] In two phase I trials, angiostatin has been well tolerated with no dose-limiting toxicity.[24]

Table 3.1 Current anti-angiogenic agents under evaluation for the treatment of epithelial ovarian cancer (Martin and Schilder[16])

Type	Agent	Target
Ligand binding		
	Bevacizumab	VEGF-A
	VEGF-Trap	VEGF A, B, C, D, E; PlGF 1 and 2
Receptor binding		
	Pertuzumab	HER2
	Volociximab	A5β1
	Imc-1121b	VEGFR-2
RTK inhibition	Valatanib	VEGFR 1, 2, 3; PDGFR; c-kit
	Sunitinib	PDGFR; VEGFR 1, 2, 3; c-kit; FLT-3
	AMG-706	VEGFR 1, 2, 3; PDGFR; c-kit
	Erlotinib	EGFR
	Sorafenib	Raf; VEGFR 2, 3; FLT-3; c-kit; PDGFR-β

VEGF, vascular endothelial growth factor; PlGF, placental-derived growth factor; HER, human epidermal growth factor; PDGFR, platelet-derived endothelial cell growth factor receptor; EGFR, epidermal growth factor; mTOR, mammalian target of rapamycin; PKC, protein kinase C; RTK, receptor tyrosine kinase.

Bevacizumab is a humanized monoclonal antibody that targets angiogenesis by binding to VEGF-A, effectively blocking the interaction of VEGF with its receptor. Bevacizumab has been fairly well tolerated by patients. In the earliest trials of bevacizumab in combination with chemotherapy in patients with colorectal cancer, there was a 1.5% to 3% risk of bowel perforation.[16,26,27] Grade 3 and 4 toxicities related to the use of bevacizumab to date in patients with ovarian cancer include hypertension, thrombosis, proteinuria, and bowel perforation.[14,28]

Bevacizumab is the first targeted biologic molecule to show significant single-agent activity in ovarian carcinoma.[16] This agent was evaluated in patients with recurrent advanced epithelial ovarian cancer who had disease progression through multiple prior chemotherapeutic regimens.[29] A 16% response rate was observed, with 62.5% of patients demonstrating stable disease for greater than 6 months. The median overall survival (OS) was 6.9 months, and the median progression free survival (PFS) was 5.5 months. The Gynecologic Oncology Group (GOG) has evaluated the efficacy of bevacizumab in recurrent ovarian cancer, and early results showed a PFS in some that was at least 6 months.[28] Because of the early promising results of the phase II trial in ovarian cancer, the GOG is evaluating bevacizumab in a phase III trial, protocol 218, in combination with first-line chemotherapy for advanced stage III or IV epithelial ovarian cancer.

The efficacy and safety of bevacizumab in combination therapy with cyclophosphamide in heavily pretreated patients with recurrent ovarian cancer has been investigated.[30] Two patients (13.3%) had a complete response and six patients (40.0%) had a partial response. The median duration of this response was 3.9 months.[30] In this population of very heavily pretreated patients, with at least five prior regimens, bevacizumab in combination with cyclophosphamide had significant activity with a response rate of 53%, without significant toxicity. Despite being heavily pretreated and having confirmed intra-abdominal cancer, no gastrointestinal perforations developed. Bevacizumab in combination with sorafenib is currently under phase II evaluation for efficacy against relapsed ovarian cancers (Annunziata and Kohn, personal communication).

Bevacizumab has shown anecdotal activity in recurrent fallopian tube carcinoma: a case of a complete response in a woman with refractory metastatic fallopian tube carcinoma treated with bevacizumab was reported.[31] At the present time, more than 50 different anti-angiogenic agents are under clinical study in cancer patients (Table 3.1).[24] VEGF-Trap (AVE0005) binds VEGF-A similarly to bevacizumab, but also binds VEGF-B, PlGF1, and PlGF2. Valatanib (PTK787) targets all VEGFR tyrosine kinases as well as platelet derived growth factor receptor (PDGFR) and the c-kit protein tyrosine kinase.[16] Sunitinib targets PDGFR, VEGFR, c-kit, and FLT-fms-related tyrosine kinase and has shown activity in one patient with ovarian cancer in a phase I clinical trial. GOG protocols 229B and 230B are evaluating thalidomide in the treatment of recurrent or persistent endometrial carcinomas and sarcomas. These protocols are too early for assessment of activity.

The Epidermal Growth Factor Receptor Family (Erb Family)

One of the best characterized pathways driving malignant changes in epithelial cells is the epidermal growth factor receptor (EGFR) family of membrane proteins.[32–36] The EGFR family consists of four structurally similar receptor tyrosine kinase (RTK) proteins including ErbB-1 (EGFR), ErbB-2 (HER2/Neu), ErbB-3, and ErbB-4.[35] These receptors are activated by binding of the ligands EGF, transforming growth factor alpha, amphiregulin, and the neuregulins. Upon binding to one of these ligands, the receptors form homodimers or heterodimers at the cell surface. Dimerization initiates tyrosine kinase activation through autophosphorylation of serine residues and is accompanied by recruitment of downstream signal transduction molecules including steroid receptor coactivators 2 (src2), growth factor receptor-bound protein 2 (GRB2), and src homology 3 (SH3).[32,35] This complex induces downstream signaling via activation of Ras, Raf, and MAPK, ultimately activating gene transcription.[37,38] The broad biologic effects mediated by the EGF family of receptors include cell proliferation, development, differentiation, and migration.[39–43]

The ErbB family of receptors may be amplified, mutated, or overexpressed on the protein level in cancers of the female genital tract.[44,45] EGFR was overexpressed in 19% to 77% of all epithelial ovarian cancers, and ErbB-4 was overexpressed in 94% of epithelial ovarian tumors.[46] Clinical trial evaluation of ErbB inhibitors in gynecologic malignancies is progressing, albeit at a slightly slower pace than that in lung or colon cancer.

Effective inhibitors of ErbB family receptors target their extracellular and intracellular domains (Table 3.2). One strategy has used monoclonal antibodies that recognize the receptor's extracellular domain and compete for binding of endogenous ligands or induce receptor downregulation from the cell surface.[47,48] Cetuximab (C225) is a recombinant chimeric humanized version of a mouse monoclonal antibody that targets EGFR1.[49] Cetuximab has been approved for use in metastatic colon cancer and unresectable head and neck cancer. It is being evaluated in advanced cervical cancer in combination with cisplatin (GOG 76DD) and with radiation (GOG 9918).

A number of murine monoclonal antibodies (muMAbs) were also developed against the extracellular domain of ErbB-2 (HER2/Neu).[10,50] The most encouraging results were obtained using muMAb 4D5, which demonstrated direct antiproliferative effects in vitro against human breast cell lines that overexpress the HER2 receptor. The humanized version of muMAb 4D5, trastuzumab (Herceptin; Nomenclature Standards Committee no. 688097; Genentech Inc, South San Francisco, CA), binds the extracellular domain of HER2 with three times greater affinity than the parent muMAb 4D5. The GOG recently evaluated trastuzumab in the treatment of patients with recurrent ovarian cancer that had overexpression of ErbB-2. They found a 10% positivity of ErbB-2 expression in the patients analyzed with few responses.[51] To date, the usefulness of anti-ErbB-2 receptor therapy in ovarian cancer has been

Table 3.2 Inhibitors of EGFR family tyrosine kinase in preclinical and clinical development (Heymach et al.[53])

Type	Agent	Target	Source
EGFR TKI (reversible)	Erlotinib	EGFR	OSI/Genentech/Roche
	Gefitinib	EGFR	AstraZeneca
	Lapatinib	EGFR, ErbB2	GlaxoSmithKline
EGFR TKI (irreversible)	Canertinib (CI-1033)	EGFR, ErbB2, ErbB3	Pfizer
	EKB-569	EGFR, ErbB2	Wyeth
	HKI272	EGFR, ErbB2	Wyeth
VEGFR/EGFR TKI	ZD6474	EGFR, VEGFR-2	AstraZeneca
	AEE788	EGFR, VEGFR-2	Novartis
Monoclonal antibody	Cetuximab	EGFR	Imclone/Bristol-MyersSquibb/Merck
	Panitumumab (ABX-EGF)	EGFR	Abgenix/Amgen
	Matuzumab (EMD72000)	EGFR	EMD/Merck KgGA
	Pertuzumab (2C4)	EGFR-ErbB2	Genentech/Roche
	MDX214	EGFR	Medarex

TKI, tyrosine kinase inhibitor.

disappointing. Although trastuzumab is able to produce a low response rate as a single agent in pretreated ovarian cancer patients with overexpression of ErbB-2, its usefulness is likely limited due to the low frequency of strong ErbB-2 expression in ovarian cancer.[52]

Second-generation antibodies targeting the EGFR family are under development. Panitumumab (ABX-EGF) is a fully humanized monoclonal antibody directed against EGFR that binds with high affinity to the EGFR receptor. Panitumumab has been approved for therapy of refractory metastatic colon cancer.

Matuzumab (EMD72000) is a humanized monoclonal antibody directed against EGFR.[53] Matuzumab has been evaluated in patients with recurrent, EGFR-positive ovarian or primary peritoneal cancer.[54] Of 75 women screened for the study, 37 were enrolled and treated. Unfortunately, there were no objective responses, although 7 patients (21%) were on therapy for more than 3 months with stable disease.[54] In this population of very heavily pretreated patients with epithelial ovarian and primary peritoneal malignancies, there was no evidence of significant clinical activity.

Finally, pertuzumab (2C4) is the first in a new class of therapeutic agents designed to inhibit the dimerization of HER2 with EGFR and other ErbB tyrosine kinases (Table 3.2). A phase II evaluation of pertuzumab in refractory or recurrent ovarian cancer yielded somewhat promising results, with objective

tumor responses or CA125 responses in 15% of patients.[53,55] Median PFS for phosphorylated HER2 positive (pHER2+) patients was 20.9 weeks versus 5.8 weeks for pHER2 and 9.1 weeks for unknown pHER2 status.[55] Further studies with pHER2 as a diagnostic could be warranted.

A second approach to inhibition of activation of the ErbB family of receptors is based on the mutational analysis of the intracytoplasmic ATP binding site. Mutations that lack tyrosine kinase activity do not display the full range of biochemical responses.[39,42,56] Small molecules have been isolated that bind to and compete at the intracytoplasmic ATP binding site, thus blocking tyrosine kinase activity. These agents as a group are classified as small-molecule tyrosine kinase inhibitors (TKIs). The reversible TKIs include erlotinib, gefitinib, and lapatinib.[57–60] Irreversible inhibitors like CI1033 (canertinib) and EKB-569 have also been developed that specifically bind to the Cys residues in the ErbB receptor ATP-binding pocket. Theoretically, these irreversible inhibitors should achieve a longer in situ half-life at the ErbB receptor target site (Table 3.2).

A phase II trial by the GOG assessed the activity of the small-molecule TKI gefitinib (ZD1839; Iressa; Astrazeneca Pharmaceuticals; P.O. Box 15437 DE 19850-5437) in patients with recurrent or persistent epithelial ovarian or primary peritoneal carcinoma and explored the clinical value of determining the status of the EGFR.[61] The response rate for patients with EGFR-positive tumors was only 9% (1 of 11). EGFR expression was associated with longer progression-free survival ($p = 0.008$).[61] Interestingly, the patient with the only objective response had a mutation in the catalytic domain of the tumor's EGFR. Gefitinib, although well tolerated, had minimal activity in an unscreened patient population with recurrent ovarian or primary peritoneal carcinoma. Prescreening patients for activating mutations in EGFR may improve response rate to gefitinib.[61]

Erlotinib (OSI-774; Tarceva; Genetech and OSI Pharmaceuticals; One Antibody WayOceanside, CA 92056) monotherapy was evaluated in patients with refractory, recurrent, EGFR-positive epithelial ovarian tumors whose disease had progressed through prior taxane and/or platinum-based chemotherapy.[62] Only two patients were found to have partial responses, lasting 8 and 17 weeks, giving an objective response rate of 6%. The median overall survival was only 8 months indicating erlotinib had marginal activity in recurrent refractory ovarian cancer but was generally well tolerated.[62] The combination of erlotinib with chemotherapy or other targeted agents should be considered.

This raises the question of whether additional benefit may be derived from the use of newer EGFR inhibitors currently in development (Table 3.2). Advantages of these new agents include improved potency or pharmacokinetics of EGFR blockade, inhibition of multiple EFGR family members, activity against erlotinib- or gefitinib-resistant mutations, dual inhibition of VEGF, and additive or synergistic activity in combination with chemotherapy.[53] Some examples of second-generation EGFR inhibitors include lapatinib, an oral dual kinase inhibitor that targets both EGFR and Her2[53]; EKB-569, an oral, irreversible inhibitor of the EGFR and Her2 tyrosine kinases; HKI272, an irreversible tyrosine kinase inhibitor with dual activity against EGFR and Her2; canertinib

(CI-1033), an oral, irreversible inhibitor of all four members of the ErbB receptor family; and ZD6474 (vandetanib), an oral inhibitor of both EGFR and VEGFR. In vitro studies have shown activity of HKI272 in non–small cell lung cancer (NSCLC) cell lines with acquired resistance to gefitinib.[63] A phase II, open-label clinical trial evaluated canertinib in 105 patients with ovarian cancer who failed prior platinum-based therapy. No responses were observed.[64]

Although the currently available ErbB inhibitors erlotinib, gefitinib, and cetuximab represent important advances in EGFR-targeted therapy, the overall magnitude of this benefit has been modest. Furthermore, resistance to EGFR inhibitors eventually emerges in almost all cases, even in patients initially sensitive to treatment. The correlation between target expression and clinical response is unclear, as there has been no clear association between EGFR expression levels and response to EGFR target therapies, particularly with the intracellular domain inhibitors.[65] Additionally, there appears to be cross-talk between the ErbB family of receptors and downstream pathways initiated by the ErbB family of receptors. Until accurate quantitative assays for EGFR activity are developed, it is important not to limit enrollment in clinical trial by EGFR status and to collect tumor tissue for future evaluation of EGFR status.[65] Perhaps EGFR inhibitors in ovarian cancer can be better employed when combined with cytotoxic chemotherapy rather than as a single therapeutic agent.

c-kit

The transmembrane receptor tyrosine kinase KIT (CD117 antigen) is the product of the c-kit proto-oncogene.[7] Imatinib mesylate (Gleevec) blocks the kinase activity of the KIT tyrosine kinase. Imatinib mesylate also inhibits the BCR-Abl tyrosine kinase.[66] It has been well established that the Bcr-Abl protein, created as a consequence of a (9:22) chromosomal translocation, is critical to the development of CML.[66] As a result, patients with CML have achieved durable responses when treated with the drug.[8]

Expression of the c-kit proto-oncogene in the female genital tract has been described.[67] In a study by Inoue, one squamous cell carcinoma of the cervix, two small-cell carcinomas of the cervix, two serous adenocarcinomas of the ovary, and two immature teratomas of the ovary expressed the c-kit protein.[67] In another study, 92% of benign ovarian tumors and 71% of malignant ovarian epithelial tumors expressed c-kit.[67] Unfortunately, clinical application of c-kit in gynecologic malignancies has been disappointing.[20,68] In a phase II trail of stage III or IV platinum- and taxane-resistant ovarian cancer, whose tumor expressed c-kit (CD117) or platelet-derived growth factor receptor (PDGFR), no objective responders were found.[68] In another phase II evaluation, patients with recurrent epithelial ovarian cancer (EOC) who had received no more than 4 prior regimens and who had good end-organ function were administered imatinib without objective responses. The results of these studies indicate

imatinib likely has minimal activity as a single agent in EOC given the complex molecular abnormalities in epithelial ovarian cancers. Its ability to modulate its molecular targets suggests that it may be considered in combinatorial therapy.

Ras/Raf Pathway

The ras family of oncoproteins are integral modulators of signal transduction pathways, and its members are potent inducers of mitogenesis and invasion,[69,70] Mutant oncogenic forms of ras (H-ras, N-ras, K_A-ras, K_B-ras) have been found in up to 30% of all human cancers. K-ras mutations have been found in 50% of mucinous ovarian cancers and 40% of low malignant potential (LMP) tumors but are generally uncommon in serous ovarian cancers (4% of epithelial ovarian cancers).[69,70] In one extensive study of epithelial ovarian lesions, only 1 cystadenoma (5%), 6 LMP tumors (30%), and 1 ovarian carcinoma (4%) demonstrated an activated Ki-ras gene.[69] Approximately 10% to 30% of endometrial cancers, however, show K-ras mutations.[70] H-ras mutations have been associated with the progression of papillomavirus-induced lesions in the uterine cervix.[71]

The Ras proteins are low-molecular-weight guanine triphosphates (GTPases) that have been implicated in malignant transformation, invasion, and metastasis.[39] These GTPases require prenylation, a lipid posttranslational modification catalyzed by farnesyltransferase (FTase), which attaches them to the plasma membrane.[39] FTase inhibitors (FTIs) have been investigated as anticancer therapeutics.[72] One FTI, SCH66336, has been evaluated in a phase I trial evaluating the combination of paclitaxel (Taxol) and SCH66336 in adult patients with solid malignancies.[39] The maximum tolerated dose was found to be 175 mg/m^2 of Taxol every 3 weeks with SCH66336 at 100 mg twice a day. Clinical responses were remarkable, with a 43% response rate and only 24% progression rate after three cycles. Also of note, three patients with prior taxane exposure exhibited profound and durable responses to the combination of Taxol and SCH66336.

Ras signals are propagated through activation of Raf proteins. The presence of Raf mutations in gynecologic malignancies appears to be a rare event. Sorafenib (BAY 43-9006) was developed as a Raf kinase inhibitor but has also been found to inhibit VEGFR-2 and -3, FLT-3, c-kit, and PDGFR-β. This agent has shown striking activity in patients with renal cell carcinoma.[73] Preliminary results evaluating sorafenib in combination with gemcitabine in patients with recurrent ovarian carcinoma have been reported.[73] Of 18 assessable patients, six had demonstrated partial responses, one by decrease in tumor volume and five by declines in tumor marker, and an additional 10 were found to have stable disease. Phase II clinical trials are in progress to evaluate the efficacy of sorafenib in combination with bevacizumab for patients with recurrent epithelial ovarian cancer or renal cell carcinoma.

Mammalian Target of Rapamycin

Mammalian target of rapamycin (mTOR), a member of the phosphatidylinositol kinase–like kinases, is involved in regulation of membrane trafficking, protein turnover, transcription, translation, and maintenance of the cytoskeleton.[16,74,75] mTOR has a central role in controlling malignant cellular growth. The pathway from growth factor receptor stimulation to mTOR activation proceeds through and in parallel with phosphatidylinositol 3-kinase (PI3K) and Akt-protein kinase B.[75] In response to extracellular stimuli, phosphorylates the 3′-hydroxyl of phosphatidylinositol-4,5-bis-phosphate (PIP2), which leads to activation of Akt. Activated Akt phosphorylates and inhibits the tuberous sclerosis complex (TSC), which removes its inhibitory effect on mTOR. As a result, mTOR is viewed as an important target for anticancer drug development. Inhibitors of mTOR currently under evaluation in cancer clinical trials are rapamycin (sirolimus; Rapamune; Wyeth; 401 N. Middletown Roadpearl River, NY 10965) and derivatives temsirolimus (CCI-779; Wyeth; 401 N. Middletown RoadPearl River, NY 10965), everolimus, (RAD001; Novartis Pharma AG; One Health Plaza East Hanover, NJ 07936-1080), and AP23573 (Ariad Pharmaceuticals; Cambridge, MA).[75] Rapamycin and derivatives temsirolimus and everolimus bind to the FKBP12-rapamycin binding (FRB) domain adjacent to the kinase domain of mTOR. The resulting complex may alter the composition and/or conformation of the multiprotein mTOR complexes and impair upstream signaling that is necessary for mTOR activation.[75] They inhibit cell proliferation by arresting cells in G1 phase, induce apoptosis in selected models, and have limited normal tissue toxicity.

Dysregulation of the mTOR pathway has been demonstrated in ovarian cancer. Efforts to inhibit mTOR signaling to arrest progress of cells through the cell cycle can also lead to inhibition of angiogenesis. Temsirolimus has shown activity in patients with advanced renal cell carcinoma and is to be tested as a single agent in patients with recurrent ovarian carcinoma and in a phase I trial in combination with carboplatin and paclitaxel.[16] Everolimus has shown activity in multiple solid tumors in the phase I setting, and trials with this agent for patients with recurrent ovarian cancer are planned. Everolimus enhanced cisplatin-induced apoptosis in ovarian cancer cells with high Akt/mTOR activity in vitro, with minimal effect in cells with low Akt-mTOR activity.[76] Mouse xenografts of SKOV-3 cells revealed that everolimus inhibits tumor growth, angiogenesis, intraperitoneal dissemination of tumor, ascites production, and prolongs survival. It is likely that other regulators of cell function play a role in tumor growth and angiogenesis.

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc-containing proteolytic enzymes that are responsible for the breakdown of connective tissue proteins, invasion through stroma, establishment of metastases, and the promotion of

tumor-related angiogenesis.[77] MMPs have been associated with ovarian and cervical cancer carcinogenesis, invasion, and metastasis. MMP expression in ovarian cancer cell lines correlates with the invasiveness of the cells.[78,79] The hydroxamate peptidomimetic inhibitor batimastat and its orally bioavailable analogue marimastat were the first MMP inhibitors to be studied.[80] These molecules bind covalently to the zinc atom at the MMP active site. Marimastat (BB-2516) was the first matrix metalloproteinase inhibitor to enter clinical trials in the field of oncology and has reached a phase III trial in pancreatic cancer.[24] Batimastat potentiated the antineoplastic activity of cisplatin on ovarian cancer xenographs in nude mice.[77] Clinical trials of MMPs, however, have been largely disappointing with the notable exception of advanced gastric cancer.[78]

Heat Shock Proteins

Heat shock protein 90 (Hsp90) is an essential protein chaperone that mediates the stability and activity of a number of oncogenic signaling proteins, including mutant p53, hypoxia-inducible factor-1α protein (HIF-1α), Raf-1, HER2/Neu, and Akt.[81] Inhibition of a heat shock protein that regulates multiple oncogenic signaling proteins is attractive for epithelial malignancies where multiple pathways are affected. 17-Allylaminogeldanamycin (17-AAG), the modified derivative of the ansamycin antibiotic geldanamycin, binds to Hsp90 and displaces bound proteins causing them to be improperly folded and degraded. It specifically inhibits the chaperone function of Hsp90, resulting in simultaneous depletion of multiple oncogenic proteins.[82] Expression of Hsp90 protein in gynecologic malignancies has been found to correlate with certain clinicopathologic factors such as steroid receptor status and favorable prognosis in endometrial cancers.[83]17-AAG has been shown to enhance paclitaxel-mediated cytotoxicity and result in supra-additive growth inhibition effects in vitro and in vivo.[81] 17-AAG and its orally bioavailable counterpart 17-(Dimethylaminoethylamino)-17-Demethoxygeldanamycin are currently under evaluation in phase I trails.[84]

The proteasome is a large intracytoplasmic protease complex that degrades proteins conjugated with ubiquitin.[85] Ubiquitin-tagged proteins include those involved in cell cycle control, transcription, cyclin dependent kinase inhibitors (CKIs), apoptosis, and tumor growth.[86] PS-341 (bortezomib) is a specific and selective inhibitor of the 26S proteasome.[87] In a recent study, bortezomib induced responses in more than 50% of heavily pretreated patients with multiple myeloma, leading to its Food and Drug Administration (FDA) approval.[86] Bortezomib has shown activity in several tumor lines including ovarian cancer and enhanced the antitumor efficacy of chemotherapy and radiation therapy.[86] The combination of bortezomib and carboplatin was evaluated in a phase I trial in recurrent ovarian cancer patients.[88] Diarrhea, rash, neuropathy, and constipation were dose-limiting toxicities. Carboplatin had no effect on bortezomib pharmacodynamics as measured by percent inhibition of the 26S proteasome. The overall response rate to this combination was 47%, with two complete responses (CRs) and five partial

responses, including one CR in a patient with platinum-resistant disease.[88] In another phase I trial of bortezomib combined with carboplatin, 21 ovarian cancer patients with recurrent and platinum- and taxane-resistant disease had no responses, but 44% of patients experienced stable disease.[89]

Mitogen-Activated Protein Kinase Pathway

The mitogen-activated protein kinase (MAPK) kinase pathway is a major signal transduction pathway that clearly plays a central role in a variety of human cancers (Fig. 3.3).[90] The MAPK pathway receives stimuli from a variety of upstream receptors in pathways activated in cancer cells and transmits the downstream signals important for a number of biologic processes including proliferation, survival, and motility.[90] The MAPK-ERK kinase (MEK) is a critical signaling protein for multiple oncogenic pathways including the EGFR family, VEGF, PDGF, and activated Ras. PD 184352 (CI-1040) was the first MEK inhibitor reported to modulate phosphorylation of MAPK in tumor-bearing animals with approximately 80% suppression on colon cancer tumor growth in mice.[90] Tumor models of colon, pancreatic, and breast cancer have been shown to be sensitive to CI-1040. MEK inhibition has also shown synergy with standard anticancer chemotherapies.[91]

The MAPK pathway is activated in ovarian cancer. CL100 is an endogenous dual-specificity phosphatase that inhibits MAPK. Downregulation of CL100 may play a role in the progression of human ovarian cancer by activating the MAPK pathway, as malignant ovarian epithelial cells displayed 10–25 times less activity of CL100 compared with that of normal ovarian epithelial cells.[92] Induced expression of CL100 in ovarian cancer cells suppressed intraperitoneal tumor growth in nude mice. The MEK inhibitor PD98059 sensitized ovarian cancer cell lines to cisplatin.[93]

AZD6244 (ARRY-142886) is a potent, selective, orally available, and non-ATP competitive small-molecule inhibitor of MEK-1/2.[94] Significant suppression of tumor growth in response to AZD6244 treatment was observed in several xenograft mouse models derived from a range of tumor types including melanoma, breast, pancreatic, lung, colon, and hepatocellular carcinomas.[95] In the Calu-6 lung cancer xenograft model, AZD6244 suppressed tumor growth, and studies using human colorectal xenograft models (SW620, Colo205) demonstrate that AZD6244 inhibits tumor growth by slowing cell proliferation and by induction of apoptosis.

Four phase II monotherapy studies are currently ongoing, one each in the indications of melanoma, pancreatic cancer, colorectal cancer, and NSCLC. Because all four phase II clinical studies have only recently started, no efficacy data and only limited safety data are currently available. The GOG is also about to activate GOG 239, a phase II study of AZD6244 in the treatment of recurrent low-grade serous carcinoma of the ovary.

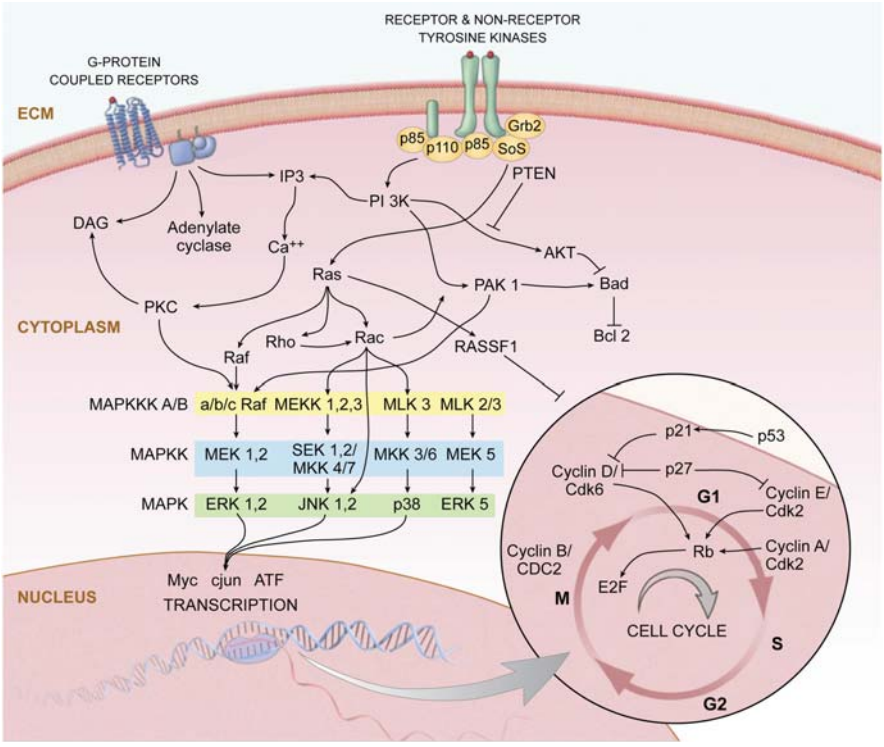

Fig. 3.1 Signal transduction, regulation of the cell cycle, and apoptotic pathways. The covalent modification of intracellular constituents is illustrated by receptor and nonreceptor protein tyrosine kinases. Input from tyrosine kinases results in increased generation of activated ras bound to GTP, which in turn associates with ras effectors: raf, Rho, Rac, and RassF1. Raf MAP kinase kinase kinase (MAPKKK) propagates the signal to microtubule-associated protein kinase kinase (MAPKK), which activates MAPK. MAPK phosphorylates a host of substrates, including cytoplasmic phospholipase A_2, cytoskeletal components, protein synthesis machinery, and, most importantly, transcription factors such as myc, c-Jun, and ATF. Parallel pathways exist that use different MAPKKKs, MAPKKs, and MAPKs. Different MAPKs, such as ERK, JNK, and p38, phosphorylate and activate downstream targets that ultimately drive the cell cycle. The upstream activators of the MAPKKK are not as well characterized as other portions of the cascade; as such only ras, PAK 1, and rac have been listed for simplicity. Although the MAP kinase cascades are parallel in nature, there is extensive cross-talk between these pathways. The generation of "secondary messengers" that act upon intracellular receptor sites is exemplified by G-protein–coupled receptors. G-proteins interact with adenylate cyclase and certain phospholipases. Consequent hydrolysis of membrane phosphatidylinositol 4,5-bis-phosphate yields inositol 1,4,5-tris-phosphate (IP3), which releases Ca^2, from internal stores, and diacylglycerol (DAG), which activates protein kinase C (PKC). Activation of PI3'K by tyrosine kinases increases IP3 levels, allowing cross-talk between growth factor and G-protein–coupled receptors. The ultimate effect of growth factors is to trigger the enzymatic cascade involving cyclins and cyclin-dependent kinases (CDKs) that play critical roles in stimulating cells to enter and transit through the cell cycle

Inhibitors of Cell Cycle Regulators

The progression of the cell from the G1 to the S phase is accompanied by phosphorylation and inactivation of the retinoblastoma gene product (Rb protein) by serine/threonine kinases known as cyclin-dependent kinases (CDKs).[96] The CDKs form complexes with proteins called cyclins. There are at least nine CDKs (CDK1–CDK9) and 15 cyclins (cyclin A through cyclin T).[96] These complexes are in turn inhibited by a combination of small proteins called CDK inhibitors (CKIs). The INK4 (inhibitor of CDK4) family consists of $p16^{ink4a}$, $p15^{ink4b}$, $p18^{ink4c}$, and $p19^{ink4d}$, and they specifically inhibit cyclin D–associated kinases.[97] The protein kinase inhibitor protein family of $p21^{waf1}$, $p27^{Kip1}$, and $p57^{kip2}$ inhibit the cyclin E/cdk2 and cyclin A/cdk2 complexes.[98] Loss of expression of CKIs confers a poor prognosis in a variety of cancers.[99] Loss of $p27^{Kip1}$ predicts poor prognosis in breast, lung, colon, or gastric cancer. Loss of $p16^{ink4a}$ is associated with poor prognosis in NSCLC and melanoma.[99] Strategies for therapeutic intervention in the modulation of CDK activity are divided into direct efforts that target the CDK subunit or indirect efforts that target the regulatory pathways that govern CDK activity.[97]

Small-molecular CDK inhibitors can be divided into eight families: purine derivatives, butyrolactone I, flavopiridols, staurosporins (UCN-01), toyocamycin, 9-hydroxyellipticine, polysulfates, and paullones.[97] Of the CDK inhibitors, flavopiridol has advanced the closest toward clinical application. Flavopiridol is a semisynthetic flavonoid isolated from a plant that is indigenous to India.[97] Flavopiridol inhibits cdk1, cdk2, and cdk4, decreases cyclin D1, causes selective induction of apoptotic cell death, and has anti-angiogenic properties.[100,101] Two phase I clinical trials with flavopiridol administered as a 72-hour infusion every 2 weeks, and one clinical trial with flavopiridol administered as a 1-hour infusion every 3 weeks, have been completed.[102,103] Minor response rates of 10% have occurred, but more importantly disease stabilization occurred in approximately 7% to 10% of patients who received flavopiridol.[102,103]

UCN-01, a staurosporine analogue, has activity against several protein kinase C isoenzymes.[97] UCN-01 is able to override the G2 checkpoint induced by DNA-damaging agents and override the DNA damage–induced S-phase checkpoint,[104] thus driving the cells toward apoptosis. UNC-01 also increases cytotoxicity in cells that contain mutated p53 genes and enhances the effect of a variety of chemotherapeutic agents to include cisplatin and camptothecan.[105] In ovarian cancer cell lines, UCN-01 enhances cisplatin cytotoxicity and apoptosis regardless of p53 status, but wild-type p53 was observed to increase the degree of sensitization.[105] A phase I trial of UCN-01 has recently been completed in humans.[97] Clinical features included an unusually long half-life that was 100 times longer than that observed in preclinical models. The recommended phase II dose of UCN-01 was 42.5 mg/m^2 given as a 72-hour infusion. A phase II study of topotecan and UCN-01 in patients with advanced ovarian cancer has also been conducted.[106] Twenty-nine patients are evaluable for toxicity and efficacy. Three patients (10%)

achieved a partial response.[106] The median time to progression was 3.3 months, and the median overall survival was 9.7 months. The most common grade 3–4 toxicities were neutropenia (79%), anemia (41%), thrombocytopenia (14%), hyperglycemia (10%), and pain (10%). The combination of UCN-01 and topotecan was generally well tolerated, however, this combination is not considered to have significant antitumor activity against advanced ovarian cancer.

Inhibitors of Poly(ADP-ribose) Polymerase

Poly(ADP-ribose) polymerase (PARP) comprises a family of enzymes that add ribose moieties to areas of damaged DNA in order to signal recruitment of a DNA repair complex.[107] To date, seven isoforms have been identified: PARP-1, PARP-2, PARP-3, PARP-4 (Vault-PARP), PARP-5 (tankyrases), PARP-7, and PARP-10. PARP is involved in base excision repair, a key pathway in the repair of DNA single-strand breaks.[107,108] PARP-1, the best characterized member, works as a DNA damage nick-sensor protein that uses beta-NAD+ to form polymers of ADP-ribose and has been implicated in DNA repair, maintenance of genomic integrity, and mammalian longevity.[107]

Carriers of germ-line heterozygous mutations in BRCA1 or BRCA2 are at highly elevated risk of developing breast, ovarian, and other cancers. Tumors arising as a result of a BRCA mutation generally show loss of the wild-type allele and retention of the mutated allele, suggesting that they are functionally deficient for BRCA1 or BRCA2. Both BRCA1 and BRCA2 proteins are important for the repair of double-strand DNA breaks by homologous recombination and the gene conversion pathway.[107,108] As a result, both BRCA1- and BRCA2-deficient cells have an elevated use of error-prone repair pathways, including the base-excision repair pathway initiated by PARP.[107,108]

PARP-1 activity is essential in BRCA mutant cells.[109] BRCA1 or BRCA2 dysfunction profoundly sensitizes cells to the inhibition of PARP enzymatic activity, resulting in chromosomal instability, cell cycle arrest, and subsequent apoptosis.[109,110] This may be because the inhibition of PARP leads to the propagation of single-strand breaks to double-strand breaks and the persistence of DNA lesions normally repaired by homologous recombination. These results illustrate how different pathways cooperate to repair damage and suggest that the targeted inhibition of particular DNA repair pathways may allow the design of specific and less toxic therapies for cancer.

There is a wealth of preclinical data showing that coadministration of a PARP-1 inhibitor with cytotoxic drugs that cause single- and double-strand DNA breaks potentiates the activity of these agents and causes persistent DNA single-strand breaks.[111–113] It is these intriguing data regarding BRCA dysfunction and sensitivity of cancer cells to PARP inhibition that have led to the investigation of an orally available PARP-1 inhibitor KU-00559436 (KuDOS; AstraZeneca, Astrazeneca Pharmaceuticals, P.O. Box 15437 DE 19850-5437) as a

single anticancer agent; a phase I study ongoing with this agent has already shown indications of activity in BRCA-defective patients with metastatic disease using a continuous oral dosing schedule.[112] This PARP inhibitor is also under investigation in combination with carboplatin as a DNA-damaging agent in the phase I setting for the assessment of toxicity in women with known BRCA-mutant breast or ovarian cancer (Annunziata and Kohn, personal communication).

A phase II study of AG014699, a potent tricyclic indole PARP inhibitor, in metastatic breast and ovarian cancer in proven carriers of a BRCA1 or BRCA2 mutation is in development (sponsored by Cancer Research UK). AG014699 has also completed both phase I and II studies in combination with temozolomide.[112,114] Inhibition of the target enzyme was shown in peripheral blood cells and tumor biopsies. Enhanced temozolomide-induced myelosuppression was observed when full-dose temozolomide was combined with a PARP inhibitory dose of AG014699; however, a 25% dose reduction of the temozolomide dose meant that the regimen was well tolerated, and this small phase II study reported a doubling of the response rate and median time to progression compared with that of temozolomide alone.[114,115] After these initial studies, there are several PARP inhibitors also scheduled to begin clinical trials as chemopotentiating or radiopotentiating agents. Novel agents INO-1001 (Inotek, now part of Genentech, Genentech, Inc. 1 DNA WaySouth San Francisco, CA 94080(650) 225-1000), ABT888 (Abbott, Abbott Laboratories, 100 Abbott Park Road, Abbott Park, Illinois 60064-3500), and GPI 21016 (MGI Pharma, 6611 Tributary St Baltimore, MD 21224) are in late preclinical development in this indication.

Conclusion

The molecular revolution has provided enormous potential for the treatment of many human diseases including cancer. The variety of cell surface receptors, signaling pathways, and nuclear proteins that stimulate cellular proliferation or inhibit cell death provide a rich environment for the development of clinically relevant molecular agents for the treatment of cancer. In the field of gynecologic oncology, we are now just beginning to investigate these new targeted agents. These new biologic therapies will usher in a new era of customized therapy that will certainly revolutionize the way we approach gynecologic cancer patients.

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Chapter 4
Biomarker Targets and Novel Therapeutics

Mary Clouser, Lisa M. Hess, and Setsuko K. Chambers

Introduction

Epithelial ovarian cancer is the leading cause of gynecologic cancer death among developed nations worldwide[1] and is the fifth leading cause of overall cancer mortality among women in the United States.[2] Despite the high mortality rates, ovarian cancer has a relatively low prevalence (0.075% of the U.S. female population). For women with regional and distant disease, the 5-year survival rates are 69% and 30%, respectively.[3]

The majority of women diagnosed with ovarian cancer (approximately 75%) present with advanced-stage disease.[4] Barriers to developing effective screening programs for ovarian cancer include its relatively low prevalence; lack of specific clinical symptoms; and that the cell of epithelial cancer origin is not clear with lack of a well-defined molecular precursor. Most ovarian cancer diagnoses (approximately 90%) are sporadic, and the remaining 10% are hereditary (e.g., primarily related to BRCA1/2 mutations). There are no known, specific, validated biomarkers to date that could be used for early detection among those at high risk for ovarian cancer.

Although a meta-analysis of 53 studies including those using modern chemotherapeutic agents demonstrated that greater primary surgical cytoreduction significantly increases median survival of ovarian cancer, it is unlikely that any further advances in surgical technology will significantly impact patient survival.[5] However, surgical improvements are needed to further reduce surgical complications, to improve patient quality of life, and to ensure that as many patients as possible are optimally debulked. Thus, the pursuit of new therapeutics is of utmost importance if advancements are to be made in the survival of ovarian cancer.

In the 1990s, the combination of intravenous platinum plus a taxane became the standard of care based on phase III studies done by the Gynecologic

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Oncology Group (GOG).[6,7] The majority of advances in cytotoxic drug development since then have been limited to patients in the best category, including those optimally debulked (less than 1 cm of residual disease), those who are candidates for consolidation therapy, or those who have platinum-sensitive disease. More recently, a transition is being made to the use of intraperitoneal cisplatin-based chemotherapy as the standard of care for the front-line treatment of patients with optimally debulked, advanced ovarian cancer. This transition is taking place due to a series of randomized trials of intraperitoneal cisplatin.[8–11] The most recent large phase III study conducted by the GOG showed a 16-month median survival advantage for women treated with the intraperitoneal regimen and a 25% decrease in risk of death (hazard ratio [HR], 0.75; 95% confidence interval [CI], 0.58–0.97).[9]

The natural history of epithelial ovarian cancer is such that after the primary surgery and chemotherapy, there is usually a remission period. The goal of primary therapy is to extend this remission period as long as possible and to maintain high patient quality of life. In an effort to extend this initial remission period, consolidation therapy is used by some clinicians but remains controversial due to the impact on patient quality of life and varied findings related to efficacy.[12] Unfortunately, the majority of these advanced ovarian cancer patients will experience recurrent disease, regardless of consolidation therapy, and will require additional chemotherapy regimens. Ultimately, these patients are very likely to develop drug-resistant disease. This remains an area in high need of additional therapies.

Targeted therapy has been successful in a number of other solid tumors, but these may not serve as valid models for the development of targeted therapies in ovarian cancer. For example, gastrointestinal stomal tumors (GISTs) were found to arise from a mutation in c-kit in up to 90% of all cases. Treatment with the c-kit tyrosine kinase inhibitor, imatinib, resulted in a 53% response rate in advanced GIST.[13] This has been a major success that has changed the treatment of these tumors but is unlikely to provide an example for therapeutic development in ovarian cancer due to the fact that multiple gene interactions govern the pathogenesis of ovarian cancer. In breast cancer, the combination of bevacizumab and paclitaxel (combination biologic and cytotoxic approach) improved progression-free survival compared with that of paclitaxel alone (11.8 vs. 5.9 months; HR, 0.60; $p < 0.001$) but was associated with significantly higher toxicity and no significant differences in overall survival (26.7 vs. 25.2 months; HR = 0.88; $p = 0.16$), precluding the development of the combination for U.S. Food and Drug Association (FDA) approval.[14] Combination therapy targeting multiple pathways may, nevertheless, serve as a model for how biologics may be useful to treat ovarian cancer.

Rationale for Molecularly Targeted Agents

Ovarian cancer is believed to be the result of an accumulation of genetic alterations, and therefore ongoing work in the area of treatment and prognostics has focused on targeting specific pathways related to the genes thought to be

involved in the carcinogenic process. Increasingly, evidence supports a two-pathway model of complex multiple genetic alterations in ovarian carcinogenesis.[15,16] This model differentiates between low- and high-grade pathways that are characterized by either a stepwise mutation process or by greater genetic instability that leads to rapid metastasis.[15] Early data show that there are different expression patterns between the low-grade and high-grade carcinomas, with low-grade tumors expressing higher estrogen and progesterone receptor and E-cadherin, whereas high-grade tumors were more likely to express matrix metalloproteinase-9 (MMP-9), B-cell leukemia/lymphoma 1 (BCL1), p53, and Ki-67.[16] This suggests that no single approach to targeted therapy or molecularly targeted prognostic factors may be valid across all ovarian carcinomas.

Genes that have been studied in ovarian cancer include oncogenes, including human epidermal growth factor receptor 2 (HER2/neu), c-myc, c-fms, and tumor suppressor genes, such as p53, BRCA1, and BRCA2. Both oncogenes and tumor suppressor genes have a role in normal cell growth regulation. Proto-oncogenes normally stimulate cell growth but when altered can promote the transformation of cells into cancer. Tumor suppressor genes normally inhibit cell division and/or promote cell death but when inactivated allow for the potential immortalization of cells and the growth of cancer. Multiple molecular markers have been studied in untreated ovarian cancer tissue samples, ascitic cells, and normal ovarian tissues in hope of gathering information on their correlation with each other and also with the apoptotic index.[17] Unfortunately, it would appear that multiple gene interactions govern the pathogenesis of ovarian cancer thus making single-gene–targeting therapies unlikely to succeed.[15–17]

There is accumulating evidence that angiogenesis plays a central role in ovarian cancer disease progression.[18,19] Angiogenesis is one of the cardinal processes leading to invasion and metastasis of solid tumors. Yoneda et al. studied the expression of angiogenesis-associated genes and progression of tumors generated by ovarian carcinoma cell lines in a nude mouse xenograft model.[19] They found that the formation of ascites was associated with the expression of vascular endothelial growth factor (VEGF) and other angiogenesis factors and that tumor growth rate was proportional to tumor vascularity.

The angiogenic-signaling pathway may be triggered by downregulation of p53 function. p53 functions to downregulate the angiogenic promoter VEGF and to upregulate the angiogenic inhibitor thrombospondin-1 (TSP-1). Loss of p53 function, extremely common among epithelial ovarian cancers, may therefore be associated with a pro-angiogenic state. In normal tissues, the amount of angiogenesis occurring is controlled by a balance of pro-angiogenic and anti-angiogenic factors; however, in tumor tissue, this balance is often disrupted. Pro-angiogenic proteins such as VEGF, basic fibroblast growth factor, and angiogenin are often more highly expressed than in normal tissue, whereas anti-angiogenic proteins such as angiostatins, endostatin, and TSP-1 are present at low levels or not at all.

Recent evidence has shown that dendritic cell precursors infiltrating tumors are transformed into endothelial-like cells in the tumor microenvironment under the influence of VEGF. These dendritic precursor cells were detected at

high frequency in ovarian cancers; growth of these cancers may be dependent on their presence.[20]

Epidermal growth factor receptor (EGFR) is overexpressed in 35% to 70% of epithelial ovarian cancers. EGFR is a member of the type 1 tyrosine kinase (TK) family of growth factor receptors, which play critical roles in cellular growth, differentiation, and survival. Activation of these receptors typically occurs via specific ligand binding, resulting in hetero- or homodimerization between receptor family members, with subsequent autophosphorylation of the tyrosine kinase domain. This activation triggers a cascade of intracellular signaling pathways involved in both cellular proliferation (the ras/raf/MAP kinase pathway) and survival (the PI3 kinase/AKT pathway). Members of this family, including EGFR and HER2/neu, have been directly implicated in cellular transformation.

There is correlative evidence in the ovarian cancer literature showing a relationship between EGFR positivity and a shorter progressive-free period as well as shorter overall survival for those with ovarian cancer.[21] Chemotherapy response has been shown to be significantly correlated with EGFR status as well. After 5 years, 63% of patients with negative versus 25% with positive EGFR were still alive indicating the possibility of impaired response of EGFR-positive carcinomas to chemotherapy containing platinum compounds.[21]

Similar to EGFR, other EGF receptors are overexpressed in ovarian cancer as well, such as HER2/neu (10% to perhaps 30% of epithelial ovarian cancers), for which the literature on prognosis is conflicting. HER2/neu encodes a cell surface glycoprotein similar in structure to EGFR.[22] Based on work by Vermeij et al., it would appear that the EGFR or HER2/neu directed molecular treatments could benefit approximately 25% of all patients with invasive epithelial ovarian cancer.[23] However, results are not consistent, with other studies showing no association with HER2/neu expression and stage, grade, cell type, and residual tumor and no significant survival effect.[24]

Platelet-derived growth factor receptor (PDGFR) has also been reported to be associated with shorter survival.[25] Therefore, the inhibition of EGFR, HER2, and PDGFR are among the targets that could play a significant role in preventing progression of ovarian cancer.

Continued clinical testing of novel noncytotoxic agents is critical for the development of effective salvage and primary treatment regimens. Ideally, these noncytotoxic agents should be directed toward mechanisms thought to be important in the process of ovarian tumor progression, such as angiogenesis and EGFR inhibition.

Molecularly Targeted Agents for Recurrent Disease

There are many new novel molecularly targeted agents being tested in recurrent ovarian cancer. Drugs that act on VEGF/VGFR include bevacizumab, sunitinib, and sorafenib. Drugs that act as an EGFR TK inhibitor include erlotinib,

gefitinib, and cetuximab. Imatinib was developed to target c-kit and has also been also found to inhibit colony stimulating factor 1 (CSF-1)–induced proliferation of a cytokine-dependent cell line, suggesting it may also act through inhibition of c-fms signaling.[26,27] HER2/neu agents include trastuzumab and pertuzumab. Protein kinase C (PKC) and rapamycin (mTOR) have also been targeted in ovarian cancer in the form of enzastaurin and temsirolimus. The epithelial cell adhesion molecule (EpCAM) inhibitor, catumaxomab, has been tried in patients with refractory ascites or as consolidation therapy. There are also agents that act on multiple pathways including imatinib (discussed above), lapatinib, HKI-272, CI-1033, bortezomib, and others.

In terms of single-agent, molecularly targeted approaches, the experience with bevacizumab to date has shown the most promise (Table 4.1). In a patient population that was less heavily pretreated and in whom more than 50% were platinum sensitive, 40% of patients were progression-free at 6 months.[31] What is notable, however, is that the toxicity profile in platinum-resistant patients is dramatically different from that of standard cytotoxic chemotherapy.[30] Unfortunately, the experience with single-agent EGFR inhibitors has been largely disappointing (Table 4.1).

In addition to the molecularly targeted agents summarized in Table 4.1, a number of other agents or their combinations are in clinical trials. In order to target those factors known to be critical to ovarian cancer progression, the Arizona Cancer Center has completed accrual of a phase II open label study of the combination of erlotinib (150 mg/day) plus bevacizumab (10 mg/kg) in ovarian cancer patients who are refractory to standard drug regimens. Targeting VEGF and EGFR signal transduction pathways simultaneously, this novel strategy is hoped to further enhance the response seen in previous clinical trials of bevacizumab or erlotinib alone. Further, resistance to EGFR antibody may result from activation of the VEGF pathway, with redundancy of signaling pathways, advancing the rationale for combination therapy. To date, we confirm both the impressive responses as well as the dramatically different toxicity profiles by application of this dual molecularly targeted approach.

Agents are being developed that target more than one signaling pathway, including several described below. Lapatinib (GW572016) is an orally active small molecule that inhibits multiple receptor dimers including downstream signaling pathways regulated by erbB1 (EGFR) and erbB2 (HER2/neu) TKs.[38] Studies of lapatinib done in solid tumors as a single agent or in combination showed evidence of safety and activity.[39,40] To date, only one phase I combination (lapatinib plus carboplatin) trial has been done in recurrent, platinum-sensitive epithelial ovarian or primary peritoneal carcinoma cancer patients.[41] At the time the study abstract was published, two subjects were evaluable for response, and both exhibited a complete response with no evidence of disease.[41]

HKI-272 is a low-molecular-weight, irreversible, pan-erbB receptor TK inhibitor.[42] It has been shown to inhibit the growth of tumor cells that express EGFR and HER2/neu in both culture and xenograft studies.[42] A phase I study

Table 4.1 Several molecularly targeted agents under investigation for recurrent ovarian cancer

Target	Agents	Clinical trial regimen	Response rate in ovarian cancer
VEGF/ VGFR	Bevacizumab	10 mg/kg IV Q 2 wk + oral cyclophosphamide (50 mg/d)	24% partial response[28]; 13.3% complete response, 26.7% partial response, 20.0% with stable disease[29]
		Single agent, 15 mg/kg IV Q 3 wk	16% to 21% partial response[30,31]
EFGR-TK	Erlotinib	150 mg/day (refractory disease)	5.9% partial response, 47% stable disease[32]
	Gefitinib	500 mg/day	9% response rate among patients with EGFR + tumors[33]
	Cetuximab	400 mg/m^2 IV cycle 1, day 1, followed by 250 mg m^{-2} week^{-1} + carboplatin (AUC = 6) day 1 and Q 3 wk	11% complete response; 21.4% partial response rate among EGFR + /platinum sensitive patients[34]
C-kit	Imatinib	400 mg/day	No objective responses[35]
HER2/neu	Pertuzumab	840 mg IV followed by 420 mg Q 3 wk or 1050 mg Q 3 wk	4.3% response rate[36]
		840 mg day 1 followed by 420 mg Q 3 wk + gemcitabine 800 mg/m^2 on day 1, 8 Q 3 wk	Progression-free survival HR: 0.67 (95% CI: 0.43–1.02), $p = 0.06$ for pertuzumab + gemcitabine vs. gemcitabine alone[37]

Q, every; IV, intravenously; AUC, area under the curve.

in 73 solid tumor patients, 3 of whom had ovarian tumors, saw two responses in HER2–positive breast cancers.[42]

CI-1033 is a pan-erbB inhibitor and acts directly with the adenosine triphosphate binding site of the erbB receptor family (EGFR, HER2/neu, erbB3, and erbB4), inhibiting their activation and downstream signaling pathways.[43] A phase II study done in 105 ovarian cancer patients who had failed prior platinum-based therapy showed no response.[43] Stable disease was confirmed in 34% patients with a 1-year survival rate of 38.5%.[43] Analysis of archival baseline tumor samples showed that the highest frequencies of expression were in erbB3 and erbB4 and the lowest in erbB2 leading to the conclusion that there appears to be no association between baseline erbB expression and disease stability.[43]

The E1A gene product of human adenovirus type 5 downregulates HER2/neu. A phase I intraperitoneal administration study of the E1A-lipid complex in ovarian cancer patients found that intraperitoneal therapy is feasible with the most common toxicities being asthenia, abdominal pain, nausea/vomiting, and fever.[44] HER2/neu was found to be downregulated in the tumor cells of 2 of the 15 (18%) patients, but no correlation between dose and biological activity was found.[44]

BIBF 1120 is an inhibitor of VEGF, PDGF, and fibroblast growth factor (FGF) receptor kinases and also inhibits members of the Src family of tyrosine kinases.[45] One phase I study in patients with solid malignancies showed it was well tolerated with the most common toxicities being nausea, vomiting, diarrhea, abdominal pain, and fatigue.[45]

Matuzumab (EMD72000) is a humanized monoclonal antibody that binds the ligand-binding portion of EGFR with higher affinity than natural ligands, therefore this antibody should be able to cause antibody-dependent cellular cytotoxicity in human tumor cells expressing EGFR.[46] A phase II study that treated 37 women with platinum-resistant ovarian and primary peritoneal malignancies, the majority of who had more than four prior lines of chemotherapy, showed no evidence of significant clinical activity as monotherapy.[46] Seven patients (2.1%) were on therapy for more than 3 months with stable disease.[46]

Phosphatidylinositol 3′-kinase (PI3K) has been found to be involved in a wide range of cancer signaling pathways. Amplification and/or mutations of PI3K and its subunit *PIK3CA* are found in ovarian tumors, and amplification of *PIK3CA* has been associated with early tumor-related mortality.[47] PI3K triggers the activation of AKT, promoting the inhibition of apoptosis and cell cycle progression.[47] Transcription of *PIK3CA* may also play a key role in platinum resistance.[48] A microarray study investigating a number of PI3K genes in 89 ovarian cancer specimens found that only *PIK3R3* had significantly upregulated mRNA expression in ovarian cancers compared with normal ovarian tissue, suggesting that this gene may serve as a future therapeutic target.[49] Targeting this important pathway is a broad-spectrum PI3K inhibitor, SF-1126, undergoing phase I trials, including at the Arizona Cancer Center.

Bortezomib (PS-3410) is a small-molecule proteasome inhibitor derived from leucine and phenylalanine.[50] It has multiple activities including the degradation of several critical intracellular proteins, including p53 and p21, involved in cell cycle regulation, blocking cell growth and division.[50] In addition, it blocks nuclear factor kappa B (NF-κB) transcriptional activity leading to reduced levels of growth factors such as VEGF, interleukin-6, and cell adhesion molecules.[50] Preclinical studies have also hinted that the proteasome inhibitors may overcome ovarian cancer cells that have become platinum resistant making this a very interesting molecule.[51] At least two phase I studies have examined bortezomib in combination with carboplatin in ovarian cancer. In study of 21 women (18 evaluable for response) with platinum- and taxane-resistant ovarian cancer, all patients had stable disease or progressive disease.[50] The second study in 15 patients with no more than three prior chemotherapy regimens for recurrent disease had and overall response rate of 47% with two complete responses and five partial responses including one complete response in a patient with platinum-resistant disease.[52]

ECO-4601 is a farnesylated dibenzodiazepinone with broad micromolar in vitro cytotoxic activity and also in tumor xenograft models and selectively binds to the peripheral benzodiazepine receptor resulting in both apoptosis and the inhibition of the Ras-mitogen activated protein kinase (MAPK) pathway.[53] A phase I study of 15 solid tumor patients including two ovarian cancers observed stable disease in six of the seven evaluable patients, one of which was ovarian.[53]

In ovarian cancer, urokinase plasminogen activator (uPA) and/or plasminogen activator inhibitor 1 has been shown to be a strong prognostic indicator of both progression-free and overall survival.[54–56] uPA has also been shown to be elevated in malignant compared with benign ovarian tumors and in comparison with normal ovarian epithelium.[54,57] uPA binding to its receptor (uPAR) has been shown to result in a variety of carcinogenic processes, including invasion, adhesion, migration, proliferation, and induction of signaling events that result in cellular differentiation,[58] suggesting that it mediates a host of cellular activities. Furthermore, the activity of uPA appears to be dynamic and furthermore dependent on the particular cellular environments and states it encounters.[58] The actions of uPA appear to underlie promotion of invasiveness of ovarian cancer cells by CSF-1.[59] Taken together, the uPA/uPAR axis appears to be a logical therapeutic target in ovarian cancer. Earlier trials in refractory ovarian cancer that had targeted the matrix metalloproteinase (MMP) pathway, a pathway also important to invasiveness and adhesiveness of ovarian cancer cells, had not shown benefit.[60]

Å6 is a capped, 8-amino-acid peptide derived from human single-chain uPA.[61] This peptide interferes with binding of endogenous uPA to uPAR. A phase I trial in 16 patients with advanced gynecologic cancer showed five patients with stable tumor measurements for at least four cycles, with one staying on study for 12 months.[61] In addition, one patient had a confirmed cancer antigen 125 (CA-125) response with stable disease on computed tomography (CT) scan after 14 cycles.[61] Baseline biomarker levels were not found to be predictive of

response, and trends over time did not correlate with outcome.[61] Encouragingly, a phase II randomized trial of Å6 in patients with asymptomatic CA-125 progression of epithelial ovarian, fallopian tube, or primary peritoneal cancer revealed that Å6 therapy was associated with a statistically significant progression-free survival (log rank p value = 0.01) with a median progression-free survival of 100 days (95% CI, 64–168) compared with 49 days (95% CI, 29–67) in patients who took placebo.[62] Treatment was not associated with CA-125 response (Fishers's exact = 0.44).[62]

Immunotherapy

Although a complete review of immunotherapy research in ovarian cancer is beyond the scope of this chapter, it is important to note a few points. The role of the immune system is well documented in other cancers, such as cervical cancer and melanoma, and is increasingly being found to play a role in ovarian tumorigenesis. In general, tumor-related antigens fall into a number of distinct categories, such as differentiation antigens (e.g., tyrosinase, mesothelin), post-translational/modification (e.g., MUC1, cathepsin D), mutational antigens (e.g., CD4, β-catenin, caspase-8, p53), amplification antigens (e.g., HER2/neu, HSP90, HoxB7, folate receptor), slice variant antigens (e.g., NY-CO-37, ING1), viral antigens (e.g., HPV), and cancer-testis antigens (e.g., NY-ESO-1, LAGE-1).[63,64] NY-ESO-1 peptide vaccines have been investigated in phase I studies of ovarian cancer, as NY-ESO-1 has been shown to be expressed in more than 40% of ovarian carcinomas.[65] Intratumoral T-cells are associated with improved progression-free and overall survival from ovarian cancer.[66] This furthers the potential value of NY-ESO-1 in ovarian cancer, due to the strong T-cell response to NY-ESO-1–derived peptides.[63]

p53 has been targeted in ovarian cancer, as the clinical and prognostic significance of p53 mutations in patients with ovarian carcinoma has long been appreciated. p53 acts as a tumor suppressor by inducing both growth arrest and apoptosis. The tumor suppressor gene TP53 is found mutated in a high percentage of ovarian tumors and may act as a checkpoint control for recognizing damaged DNA and inducing repair or apoptosis.[67] Tumor cells lacking functional p53 can evade apoptosis induced by both p53 transcription targets and Fas ligand with intracellular sequestration of death receptors causing resistance to apoptosis and insensitivity to chemotherapeutic agents.[68] Intraperitoneal gene therapy of ovarian cancer patients using adenoviral vectors for delivery of wild-type p53 has been investigated in a variety of phase I and II trials but has failed to show therapeutic benefit.[69]

The finding of a p53-specific memory T-cell response in a case-control study of women with primary epithelial ovarian cancer and patients with benign ovarian tumors and healthy controls[70] suggests that in the cancer patients, sufficient amounts of cancer-derived p53 was presented to induce the formation

of such a T-cell response and supports the rationale of using p53 peptides in vaccination strategies aimed at the induction of p53-specific immunity.[70] The highest p53 responses occur after primary treatment, a time point at which most ovarian cancer patients have minimal residual disease making this the important target period for these therapies.[70]

Nearly 50% of ovarian cancer patients generate antibodies to cathepsin D and 40% generate antibodies to glucose-regulated protein 78 (GRP78), neither of which were detected in the serum from controls.[64] Mesothelin (a differentiation antigen expressed in the mesothelia of the peritoneum) has been detected in 50% to 100% of ovarian cancers. This antigen has been shown to bind to CA-125, and it is thought to play a role in the peritoneal spread of ovarian cancer.[71] A number of agents that target mesothelin are under early clinical development (e.g., SS1P/CAT-5001, MORAb-009 CRS-207).[71]

The CA-125 antigen is a marker for initial disease progression or regression and is a common clinical measure used to determine disease recurrence. Oregovomab is a murine monoclonal antibody used to stimulate immunity against CA-125.[72] A 5-year phase II trial follow-up assessment failed to find a statistically significant survival effect of maintenance therapy with oregovomab compared with placebo: 57.5 versus 48.6 months, respectively (adjusted HR, 0.72; 95% CI, 0.41–1.25).[73] Accrual is complete for the subsequent phase III trials, with survival data pending completion of long-term follow-up (OVA-Gy-17A and B trials).

Readers are referred to work of Odunsi and Sabbatini and that of others for a more complete review of immunotherapy and the potential prognostic and therapeutic role of tumor-related antibodies in ovarian cancer.[63,64,74,75]

In summary, unlike the successes seen in breast cancer, to date there have been no new molecularly targeted agents that have translated to a significant survival advantage in ovarian cancer. Targeting single receptors in a pathway (e.g., EGFR) has not been a successful strategy to date due to the redundancies in signaling pathways in ovarian cancer. Further, little correlation is observed in these molecularly targeted trials between expression of the target and clinical response. Future strategies in the treatment of ovarian cancer are likely to require hitting multiple targets if they will ultimately prove to be successful. Prescreening of the tumor for target expression prior to trial eligibility is unlikely to be appropriate, as expression of the target alone is not likely to be indicative of activation of its signaling pathways.

Molecular Targets as Prognostic Factors

There are a number of molecules described to be prognostic factors in ovarian cancer, some of which are already targeted in clinical trials, and others that may also prove to be appropriate for therapeutic targeting in the future. Several of these molecular targets are summarized in Table 4.2; some have been addressed in the prior section, and others are described in more detail below.

Table 4.2 Selected prognostic molecular targets implicated in ovarian cancer

Molecular target	Expression	Prognostic evidence
p53	Overexpressed in approximately 50% of ovarian tumors[83,84]	• Patients with p53 overexpression and the wild-type TP53 gene had shorter overall survival time ($p = 0.019$) and were more refractory to chemotherapy ($p = 0.027$)[85] • p53 immunopositivity a predictor of poor prognosis[86] • Associated with advanced stage ($p = 0.04$), higher grade ($p = 0.0003$), serous histology ($p = 0.008$), and patient age >61 years ($p = 0.013$) but not survival[84] • Aneuploidy, but not survival[83]
EphA2	Overexpressed in 75% of ovarian cancers and in 91% of cancers with p53 mutations[76]	• EphA2(+)/p53 null genotype has been shown to be associated with reduced survival and increased metastasis and occurrence of ascites in ovarian cancer[87] • EphA2 overexpression and p53 null status associated with decreased survival and increased incidence of ascites and distant metastasis[76]
HER2/neu	Overexpressed in 8% to 66% of ovarian cancers[22,88,89]	• Overexpression is involved in cell proliferation, differentiation, migration, and apoptosis[88] • Patients with overexpression have significantly worse survival than that of those with normal HER2/neu expression[22] • High expression also less likely to have a complete response to primary therapy or have a negative second-look laparotomy when serum CA-125 levels were normal preoperatively[22] • Positive immunostaining associated with poor overall survival ($p = 0.03$)[90] • Overexpression correlated with overall survival (HR = 2.59, $p = 0.005$)[89]

Table 4.2 (continued)

Molecular target	Expression	Prognostic evidence
c-fms/CSF-1	Overexpression in 64% to 100% of ovarian cancers[91,92]	• Overexpression of CSF-1 in ovarian cancer cells promotes invasiveness and metastasis in vivo[93] and is associated with decreased progression-free and overall survival[80] • High levels correlate with advanced disease and poor prognosis[92,94]
uPA	17- to 38-fold higher levels in ovarian carcinoma compared with normal ovarian tissue[95]	• Associated with malignant progression (intra-abdominal spread and reimplantation), and lower progression free and overall survival[96]
PIK3CA	Amplified in 26% of patients with poorly differentiated and 16% with moderately differentiated carcinomas; amplified in 43% of stage I but not in stage IV cancers[47]	• Could be an early event in development of high-grade disease • PIK3CA amplification associated with reduced median survival (1.29 for high vs. 6.72 years for no amplification)[47]
Tumor suppressor genes		
OVCA1	Deleted in 80% or more of ovarian cancers[78,97–99]	• Potential use for detecting malignancy; detectable in normal epithelium but not evident or reduced in 92% of ovarian cancers[78,99]
NOEY2 (AHRI)	Loss of heterozygosity detected in 41% of ovarian and breast cancers[100]; downregulated in 63% of invasive ovarian cancer specimens and undetectable in 47%[101]	• Inversely correlates with progression-free survival; reexpression increases the sensitivity of ovarian cancer cells to cisplatin and paclitaxel and also to inhibit proliferation, motility, and invasiveness[101] • Reexpression inhibits ovarian cancer growth[102]
Lot1	Not detectable in 39% of ovarian cancers and variable in the remaining tumor specimens studied[103]	• Unknown
DOC-2	Downregulated in ovarian cancer, particularly serous tumors[104]	• Transfected cells reduced growth rate and ability to form tumors in nude mice[104]
SPARC	Detected in the stroma of 63% of ovarian carcinomas and 29% of normal ovaries; limited detection in normal ovaries of premenopausal patients[105]	• High levels of SPARC mRNA and protein detected in stroma of malignant ovaries; diffuse pattern seen in samples of low malignant potential[106] • SPARC confers growth inhibition in vitro and in vivo[106]

Table 4.2 (continued)

Molecular target	Expression	Prognostic evidence
PTEN	Mutations in 4% to 21% of endometrioid type cancers[107]; Loss of heterozygosity in endometrioid (43%) and serous (28%) tumors but infrequent in the other histologic types[108]	• PTEN expression diminished the risk of death among TP53-positive patients (HR = 0.35, $p = 0.029$)[107] • Inactivation of PTEN may be an early event in ovarian tumorigenesis[108]; no correlation between FIGO stage and PTEN expression in adenocarcinomas[109] • Reduced expression significantly higher among the adenocarcinomas than among borderline tumors ($p < 0.001$)[109] • Tumors with reduced expression associated with higher proliferation than those with normal PTEN[109]

OVCA1, DPH1 homolog (S. cerevisiae); NOEY2 (AHRI), DIRAS Family, GTP- binding RAS- like 3; DOC-2, disabled homolog 2, mitogen-responsive phosphoprotein; SPARC, secreted protein acidic and rich in cysteine; PTEN, phophatase and tensin homolog; FIGO, International Federation of Gynecology and Obstetrics

The EpHA2 TK receptor is overexpressed and correlates with poor prognosis in ovarian cancer.[76] A particularly novel therapeutic approach is being investigated preclinically by targeting EpHA2 via small interfering ribonucleic acids (siRNAs) incorporated in liposomes, delivered intravenously or intraperitoneally in mouse models.[77]

The macrophage colony-stimulating factor (CSF-1) receptor, c-fms, is activated in many ovarian cancers but is not activated in normal ovarian epithelium.[59,78,79] CSF-1 is also found in the plasma of patients with ovarian cancer with normal CA-125 levels (and is elevated in 70% of all ovarian cancer patients) and may be a complementary marker for these patients.[79] The co-expression of CSF-1 with c-fms in the metastasis of ovarian cancer patients leads to a 2.3-fold increased risk for recurrence[80]; such co-expression suggests that CSF may regulate cellular growth/ invasion by autocrine processes.[81] A number of agents have been developed that target c-fms (e.g., vatalanib, ABT869, CYT645, Ki20227), but to date, only imatinib, developed to target c-kit, has been tested in ovarian cancer and has not demonstrated significant activity among recurrent ovarian cancer patients.[35,82]

Several tumor suppressor genes (Table 4.2) have been identified to be important for development of ovarian cancer. In the future, in women at risk for development of the disease, these can serve as candidates for gene therapy reinstitution of normal function. Further, identification of regulatory molecules that downregulate their expression may serve as therapeutic targets in this high-risk population.

Conclusion

Since the introduction of platinum-based chemotherapy, no treatment has been shown to improve survival in patients with advanced ovarian cancer until the incorporation of taxanes into the primary treatment for this disease. Several large randomized trials have suggested that the combination of platinum with a taxane is an active first-line therapy for women with advanced ovarian cancer. Although a majority of women with advanced ovarian cancer will demonstrate an objective or subjective response to these drug combinations, the responses are generally of limited duration. Second-line chemotherapy for ovarian cancer has, in general, been a disappointment in the setting of platinum- and taxane-resistant disease with the best single agents yielding approximately 20% response rates. Therefore, there remains a need for an improved therapeutic approach in the management of ovarian cancer.

However, a molecularly targeted approach in ovarian cancer is challenged by the differing pathways of carcinogenesis, the redundant signaling pathways present in ovarian carcinogenesis, and the lack of a single pathway that can be targeted for therapeutic intervention or prognostic evidence. It appears from the experience to date that tumor expression of a molecular target alone is not a

good predictor of clinical response to an agent targeted to that molecule and its downstream signaling pathways. Further, cross-talk between pathways, adaptability of the cancer cell, incomplete inhibition of the target due to inability to efficiently deliver the agent, and/or excessive toxicity due to lack of selectivity of the agent confound success in this era of molecularly targeted therapies. It is highly likely that a better understanding of the multiple pathways in ovarian tumorigenesis and combinations of key targeted therapies will be needed to significantly impact the survival of ovarian cancer. Trials of combinations of molecularly targeted agents or those agents combined with cytotoxic chemotherapies are in progress or under development as described here. The optimal timing, dose, and schedules of these combinations will be the key to preventing unpredictable and excessive toxicities yet maximizing durable therapeutic effect.

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Chapter 5
Tumor Suppressor Genes

Zhen Lu and Robert C. Bast, Jr.

Introduction

A tumor suppressor gene inhibits one or more function(s) required for malignant transformation, including self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, and tissue invasion and metastasis or sustained angiogenesis.[1] Some tumor suppressor genes are lost in the germ line, whereas the function of other tumor suppressor genes is lost through somatic change. At present, loss of function has been described for some 16 putative tumor suppressor genes in epithelial ovarian cancer (Table 5.1). During oncogenesis, loss of tumor suppressor gene function can occur through deletion, mutation, or epigenetic silencing. Loss of heterozygosity has been observed in 11 putative tumor suppressors, inactivating mutations in four of these genes (*p53, PTEN, BRCA1, and BRCA2*), and promoter methylation and silencing in eight. In each case, only a fraction of ovarian cancers from different patients lose the function of a particular suppressor protein.

Epithelial ovarian carcinomas have been thought to arise from the ovarian surface epithelium or from cysts immediately beneath the ovarian surface. Recent evidence suggests that cancers with similar histology can arise from endometriosis, the fallopian tube, or directly from the peritoneal surface. More than 90% of epithelial ovarian cancers are clonal diseases that arise from the progeny of single cells.[2] Strong hereditary factors (BRCA1, BRCA2, and HNPCC) predispose to ovarian cancers in no more than 10% of cases. More than 90% of ovarian cancers arise from somatic genetic changes. With a few possible exceptions, chemical carcinogens that drive somatic genetic changes

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Table 5.1 Putative tumor suppressor genes in epithelial ovarian cancer (modified from Bast and Mills[149])

Gene	Chromosome	Mechanisms of downregulation	Function
ARHI (DIRAS3)	1p31	Imprinting; LOH; promoter methylation; transcription downregulated by E2F1 and E2F4	26-kDa GTPase; inhibits proliferation and motility; induces autophagy and dormancy; upregulates p21 and p27; inhibits cyclin D1, PI3K, Ras-MAP, Stat3
RASSF1A	3p21	Promoter hypermethylation	Inhibits proliferation and tumorigenicity; interacts with Ras downregulating cyclin D1
DLEC1	3p22.3	Promoter hypermethylation	166-kDa cytoplasmic protein that inhibits anchorage-dependent growth
SPARC	5q31	Promoter hypermethylation	32-kDa Ca^{++} binding protein; regulates tumorigenesis and proliferation, also adhesion and metastasis
DAB-2 (DOC2)	5q13	LOH	105-kDa a mitogen-rsponsive phosphoprotein which inhibits anchorage-independent growth and tumorigenicity
LOT-1 (ZAC1)	6q25	Imprinting; LOH; transcription downregulated by EGF, TPA	55-kDa nuclear zinc finger protein inhibits proliferation and tumorigenicity
RPS6KA2	6q27	Monoallelic expression in ovary; LOH	90-kDa ribosomal S6 serine threonine kinase that inhibits growth and arrests cells in G1 phase
PTEN (MMAC-1)	10q23	Promoter methylation; LOH; mutation; aberrant expression of microRNA	PI3 phosphatase; decreases proliferation; induces apoptosis; inhibits angiogenesis and invasion by regulating COX-2; inhibits the production of VEGF

Table 5.1 (continued)

Gene	Chromosome	Mechanisms of downregulation	Function
OPCML	11q25	Promoter methylation; LOH; mutation	GPI-anchored IgLON family member; suppresses oncogenic Ras activity; inhibits proliferation and tumorigenicity
BRCA2	13q12-13	Mutation; LOH	Binds RAD51 in repair of DNA double-strand breaks (DSBs)
ARLTS1	13q14	Promoter methylation	ADP ribosylation factor induces apoptosis
WWOX	16q23	LOH; mutation	Decreases anchorage-independent growth and tumorigenicity
P53	17p13.1	Mutation	53-kDa nuclear protein induces autophagy and cell cycle arrest; promotes DNA stability; induces apoptosis
OVCA1	17p13.3	LOH	50-kDa protein; decreases proliferation and clonogenicity; decreases cyclin D1
BRCA1	17q21	Mutation; LOH	Participates directly in repair of DNA DSBs through homologous recombination; regulates the S-phase checkpoints and the G2-M transition
PEG3	19q13	Imprinting; LOH; promoter methylation;	Induces p53-dependent apoptosis

have not been identified. The pattern of p53 mutations in ovarian cancers is consistent with spontaneous mutation associated with cell proliferation.[3]

Prior to menopause, spontaneous mutations can occur during proliferation of ovarian surface epithelial cells to repair ovulatory defects. After menopause, epithelial proliferation can occur in response to follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrone, or androgen. If proliferation is the critical mechanism driving ovarian oncogenesis, genetic events requiring only a "single hit" may be favored. Oncogenes can be activated with alteration of a single allele with mutation or amplification. In general, however, both alleles of a tumor suppressor gene must be inactivated to ensure loss of function, consistent with the "two-hit hypothesis" originally proposed to explain the

behavior of the Rb tumor suppressor gene in patients with familial or sporadic retinoblastoma. If only one allele of a tumor suppressor gene is lost or damaged, the second allele can still produce the correct protein. Although levels of the normal suppressor protein may be reduced, they are often still adequate to check tumor progression.

There are, however, notable exceptions to the two-hit rule for tumor suppressors. Certain mutations in the p53 gene product can function as a "dominant negative," where the mutated p53 protein can complex with the wild-type p53 protein from the nonmutated allele.[4] Another group of genes that can be lost with a single hit are imprinted tumor suppressor genes. Approximately 70 human genes are known to be imprinted. Although the nucleotide sequence remains unchanged, either the maternal or the paternal allele is silenced by epigenetic change, generally involving promoter methylation. Because one allele has been permanently silenced in all normal tissues, function of the remaining allele might be lost with only a single genetic or epigenetic event. Three of the putative tumor suppressor genes whose function is lost in ovarian cancer are known to be imprinted (*ARHI, LOT1, and PEG3*).

Putative tumor suppressor genes are distributed throughout the genome of ovarian cancer cells. Each of these genes is described below according to its chromosomal assignment.

Chromosome 1

ARHI is a maternally imprinted tumor suppressor gene that maps to a site of loss of heterozygosity (LOH) on chromosome 1p31 and encodes a 26-kDa GTPase with 50% to 60% homology to Ras and Rap.[5] ARHI is downregulated in >60% of invasive ovarian and breast cancers and cannot be detected in approximately one half. Loss of ARHI expression from the single functioning maternal allele can occur through several mechanisms including LOH, methylation and silencing of the functional allele, transcriptional regulation with E2F1 and E2F4, and shortened mRNA half-life.[5–10]

When ARHI expression was measured in normal, benign, and malignant ovarian tissues using immunohistochemistry (IHC) and in situ hybridization (ISH), strong ARHI expression was found in normal ovarian surface epithelial cells, cysts, and follicles.[11] Within individual cells, ARHI protein was detected predominately in the cytoplasm but occasionally in the nucleus. Reduced ARHI expression was observed in tumors of low malignant potential as well as in invasive cancers. ARHI expression was downregulated in 63% of 407 invasive ovarian cancer specimens and could not be detected in 47% of the samples.[11,12] Conversely, 37% of ovarian cancers expressed ARHI at levels comparable with those observed in normal ovarian epithelial cells. ARHI protein expression varied between different histotypes, with more frequent expression in clear cell and endometrioid cancers than in serous, mucinous, or transitional cancers. ARHI

expression did not correlate with grade, stage, or overall survival but was associated with prolonged disease-free survival.[11]

Reexpression of ARHI at supraphysiologic levels (1) inhibits growth of ovarian and breast cancer cells and xenografts, (2) decreases invasiveness, and (3) induces caspase-independent, calpain-dependent apoptosis.[13] Reexpression of ARHI at physiologic levels still inhibits clonogenic growth and motility of ovarian cancer cells but is associated with autophagy rather than apoptosis. ARHI participates directly in the formation of autophagosomes, and knockdown of ARHI inhibits both spontaneous and rapamycin-induced autophagy. ARHI inhibits signaling through Ras/mitogen-activated protein (MAP) and phosphoinositide 3 (PI3) kinase, upregulating tuberous sclerosis complex 1/2 (TSC1/2) and downregulating mammalian target of rapamycin (mTOR), a known stimulus for inducing autophagy in multiple systems.[14] ARHI-expressing autophagic ovarian cancer cells die within 6 days in culture. In xenografts, reexpression of ARHI also induces autophagy, but cancer cells survive, remain dormant for weeks, and grow promptly when ARHI levels are reduced. Growth factors and cytokines found at the xenograft site—Vascular endothelial growth factor (VEGE), IL-8, and insulin-like growth factor 1 (IGF-1)—can rescue ovarian cancer cells from autophagic death in culture and can partially reverse ARHI-mediated inhibition of signaling through PI3 kinase. These findings suggest that reexpression of ARHI can induce autophagic cell death or contribute to dormancy depending upon the tumor microenvironment.

In addition to regulating autophagy, ARHI downregulates cyclin D1, induces the cyclin-dependent kinases (CDK) inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, and prevents translocation of Stat3 to the nucleus.[15] Physiologic expression of ARHI not only inhibits proliferation through cell cycle arrest but also inhibits motility and invasion of ovarian cancer cells.

Chromosome 3

RASSF1A, a new ras effector, was characterized by Dammann et al.[16] The gene is located at chromosome 3p21.3, a region that frequently shows allelic loss in many cancers.[17] RASSF1 encodes for more than seven isoforms including RASSF1A, RASSF1B, and RASSF1C, derived from alternative messenger RNA (mRNA) splicing and promoter usage.[18] Methylation of the RASSF1A gene promoter has been observed in many human tumors including ovarian cancer and is an important mechanism for inactivation of the RASSF1A.[19–24]

In endometrial cancers, RASSF1A hypermethylation has been reported to be associated with advanced-stage disease,[25] and association with risk of recurrent cancer and poor survival is controversial.[25,26] The studies confirmed that RASSF1A hypermethylation was significantly associated with advanced staging of the cancers but did not correlate with survival. In addition, the frequency of RASSF1A hypermethylation was distinctly higher in type I endometrial carcinomas (61%) when compared with type II cancers (27%) suggesting that the two types

of cancers may adopt different genetic and epigenetic pathways, and this feature may be helpful in distinguishing the two subtypes of endometrial cancers.[24]

RASSF1A is thought to be responsible for the Ras-dependent growth inhibition through its proapoptotic function.[27] Shivakumar and co-workers[28] showed that RASSF1A can induce cell cycle arrest by inhibiting accumulation of cyclin D1, thus preventing G1/S-phase cell cycle progression. Loss of RASSF1A expression by methylation may shift the balance toward a growth-promoting effect without the necessity of activating Ras mutations.

DLEC1 is located at chromosome 3p22-p21.3,[29] and the gene contains 37 exons and spans approximately 59 kb. The predicted DLEC1 protein contains 1755 amino acids. However, its exact biologic function is still unclear because the predicted amino acid sequence of DLEC1 has no significant homology to any of the known proteins or domains.[30]

The potential involvement of DLEC1 in epithelial ovarian cancer remains unknown. DLEC1 downregulation was found in ovarian cancer cell lines and primary ovarian tumors. Overexpression of DLEC1 in two ovarian cancer cell lines resulted in 41% to 52% inhibition of colony formation. Whereas methylation was not detected in normal ovarian epithelium, hypermethylation of DLEC1 was detected in ovarian cancer cell lines with reduced DLEC1 transcripts. Treatment with a demethylating agent and a histone deacetylase enhanced DLEC1 expression in 90% and 80% of ovarian cancer cell lines, respectively. No chromosomal loss of chromosome 3p22.3 in any ovarian cancer cell line or tissue was found.[31]

Chromosome 5

SPARC (secreted protein acidic and rich in cysteine) is a secreted calcium-binding, acidic matrix glycoprotein that maps to chromosome 5q31.[32,33] A significant downregulation of SPARC mRNA expression was observed in ovarian tumor tissues. Lower levels and a more diffuse pattern of SPARC mRNA expression were associated with low malignant potential specimens.[33] Despite the lack of detectable SPARC mRNA, SPARC immunoreactivity was consistently observed within the cytoplasm of cancer cells. Variable SPARC immunostaining was observed in normal surface epithelial cells.[34] In contrast, high-level expression of SPARC mRNA and protein was detected in stroma of ovaries containing malignant tumor cells, particularly at the tumor-stromal interface of the invading tumors.[35] The downregulation of SPARC mRNA expression can be accounted for by aberrant hypermethylation of its CpG-rich region.[33]

Treatment with SPARC inhibits the proliferation of both normal and cancer cells but induces apoptosis only in cancer cells.[36] Putative SPARC receptors are present on ovarian epithelial cells.[36] Higher levels of SPARC receptors are found on human ovarian surface epithelial cells than on cancer cells, consistent with its tumor suppressor function. Decrease in ligand-receptor interaction by

the downregulation of SPARC and/or its receptor could contribute to ovarian carcinogenesis. SPARC not only can regulate proliferation but can also inhibit adhesion and metastasis. Loss of SPARC enhances ovarian carcinoma cell adhesion, a key step in peritoneal implantation.[37] SPARC appears to inhibit adhesion by regulating levels of lysophosphatidic acid (LPA) receptors and by attenuating extracellular signal-regulated kinase (ERK) 1/2 and protein kinase B/Akt signaling.[38] Evidence from in vivo studies also suggests that stromal expression of SPARC can normalize the microenvironment of ovarian cancer cells by downregulating the VEGF-integrin-matrix metalloproterinases (MMP) axis, as well as decreasing the levels and activity of bioactive lipids.[39]

Dab2/DOC2 (for "disabled-2/differentially") is located at chromosome 5p13[40,41] and is frequently inactivated by homozygous gene deletion in tumors.[42]

Expression of Dab2 is absent in ovarian carcinoma cell lines and in 58% to 85% of ovarian cancers examined but was present in normal ovarian epithelial cell lines and tissues.[43] Loss of Dab2 expression appears to be an early event in ovarian oncogenesis.[44] There is at least a fivefold decrease in expression of Dab2/DOC2 in serous epithelial ovarian cancer cells compared with that in normal ovarian epithelium.[45]

Dab2 functions in the mitogenic signal transduction pathway,[42] and furthermore, when DOC-2 was transfected into the ovarian carcinoma cell line SKOv3, the stable transfectants showed a significantly reduced rate of growth and a decreased ability to form tumors in nude mice.[41] It has been postulated that the functional role of DOC-2/DAB2 is to modulate signaling mediated by peptide growth factor receptor tyrosine kinases. Using sequence-specific peptides, it has been demonstrated that in the presence of growth factors, the second proline-rich domain of DOC-2/DAB2 binds to Grb2, interrupting the interaction between SOS and Grb2, consequently suppressing the downstream phosphorylation of ERK. These findings suggest that DOC-2/DAB2 has multiple effects on the RAS-mediated signal cascades in malignant cells.[46]

Chromosome 6

LOT1, the human homologue of the rat Lot1 gene, is localized at 6q25 and encodes a zinc-finger nuclear transcription factor that was identified through analysis of differential gene expression in normal and malignant rat ovarian surface epithelial cells. Decreased or lost expression of LOT1 occurs in human and rat ovarian carcinoma cells. Loss of LOT1 expression can occur through several mechanisms including LOH, imprinting, and transcriptional downregulation in the presence of epidermal growth factor (EGF) and 12-0-Tetradecanoylphorbol 13-acetate (TPA).[47–50]

Both human and rat ovarian carcinoma cell lines exhibit loss or decreased expression of this gene. Furthermore, transfection of LOT1 into ovarian cancer cell lines suppresses cancer cell growth.[47]

RPS6KA2, the p90 ribosomalS6 kinase-3 gene, maps to 6q25 and is monoallelically expressed in normal ovary.[51] Thus, loss of the single expressed allele may be sufficient to eliminate RPS6KA2 function. Although the loss of a single expressed allele of RPS6KA2 is sufficient to cause loss of expression in cancer cells, sequencing analysis of families harboring polymorphisms showed no evidence for imprinting.

Reexpression of RPS6KA2 in cell lines reduced proliferation, suppressed colony growth, and arrested cells in G1 phase of the cell cycle with a significant increase in apoptosis.[51] The kinase activity of RPS6KA2 is essential for its antiproliferative effect, but the biochemical mechanism is as yet unknown.

Chromosome 10

Phosphatase and tensin homolog **(PTEN)**, found on chromosome 10q23, is a dual protein/lipid phosphatase. Phosphatidyl-inositol 3,4,5 triphosphate (PIP3), its main substrate, is the product of phosphoinositide 3 kinase (PI3K). Increased PIP3 recruits AKT to the membrane where It can be activated by other kinases. Thus, loss of PTEN function can enhance signaling through the PI3 kinase pathway. Loss of PTEN function can occur through several mechanisms including mutation, deletion, promoter methylation,[52,53] or aberrant expression of microRNAs (miR-214, miR-199a, miR-200a, and miR-100).[54]

Mutation of PTEN is found in approximately 15% of ovarian endometrioid carcinomas that are mostly of low grade.[55,56] PTEN mutations are uncommon in other tumor types, consistent with a separate pathway of development for endometrioid carcinomas. Loss of heterozygosity at 10q23 was found not only in endometrioid and clear cell ovarian cancers but also in adjacent deposits of endometriosis.[57] PTEN mutations have also been detected in 21% of endometriotic cysts that are not associated with carcinoma. These findings support endometriosis as a precursor for both endometrioid and clear-cell carcinomas. Sporadically, PTEN alterations can be observed in other histologic types of ovarian cancer.[58,59] Downregulation of PTEN protein is frequently detected in serous and mucinous epithelial ovarian tumors[60] and can be associated with elevated levels of miR-214. Significantly, miR-214 negatively regulates PTEN by binding to its 3′ untranslated region (3′-UTR) leading to inhibition of PTEN translation and activation of the Akt pathway.[61]

PTEN mutation and enhanced Akt activity is often found in primary endometrial cancers in which cyclooxygenase 2 (COX-2) is expressed at high levels, associated with an aggressive phenotype. Akt can regulate COX-2 gene and protein expression in these lesions.[62] When PTEN is mutated, Akt signals via the NF-κB/IκB pathway to induce COX-2 expression. COX-2, in turn, can inhibit apoptosis, increase angiogenesis, and promote invasiveness.[62] As COX-2 can be elevated in ovarian cancers, a similar interaction could be sought in ovarian neoplasms.

Recent studies have reported that reexpression of PTEN inhibits the production of VEGF, an angiogenesis and vascular permeability factor.[63–66] PTEN overexpression markedly inhibited ascitic fluid production and peritoneal dissemination, as well as tumor growth in ovarian cancer (SHIN-3) xenografts.[67]

Chromosome 11

OPCML (also called OBCAM) is a member of the IgLON family of immunoglobulin (Ig) domain-containing glycosylphosphatidylinositol (GPI)-anchored cell adhesion molecules and is located at 11q25. OPCML is frequently somatically inactivated in epithelial ovarian cancer by allele loss and by CpG island methylation.[68–70] OPCML mRNA is expressed in 19% of ovarian epithelial carcinoma compared with 85% of normal ovarian tissues and 76% of benign ovarian tumors.[71] A somatic missense mutation of OPCML detected in a clinical specimen of epithelial ovarian cancer has also shown clear evidence of loss of function.[68]

Because activation of the Ras signaling pathway occurs frequently in human ovarian cancers, the effect of oncogenic Ras on the expression status of OPCML was explored. Studies revealed that Ras(V12)-mediated oncogenic transformation was correlated with loss of OPCML expression. Furthermore, the OPCML promoter was found to be hypermethylated in Ras-transformed human ovarian epithelial cells, and treatment with the DNA methyltransferase inhibitor 5′-aza-2′-deoxycytidine increased the activity of the OPCML promoter and restored OPCML expression in ovarian cancer cells. In addition, suppression of oncogenic Ras activity by stable siRNA specific for HRas(V12) led to the demethylation and reexpression of OPCML in ovarian cancer cells.[72] These studies demonstrate that oncogenic Ras activity may be responsible for the observed OPCML promoter hypermethylation and epigenetic gene silencing of OPCML and suggests a possible link between RAS signaling pathway and inactivation of OPCML in ovarian cancer.

Chromosome 13

BRCA2, located at 13q12-q13,[73,74] is a well-established tumor suppressor gene that contributes to the pathogenesis of a fraction of familial ovarian and breast cancers. Germ-line BRCA2 mutations are present in 3% to 6% of all epithelial ovarian cancers.[75,76] In addition, decreased BRCA2 expression may be associated with loss of heterozygosity.[77,78]

BRCA2, like BRCA1, has been implicated in homologous recombination, a process that repairs DNA double-strand breaks in an error-free manner, through interaction with the RAD51 protein.[79] Cells lacking BRCA2 repair DNA by the error-prone mechanism of nonhomologous end-joining (NHEJ),

leading to chromosomal rearrangements and genomic instability.[79] Many types of BRCA2-deficient cells exhibit increased sensitivity to DNA-damaging agents.[80,81]

ARLTS1 is located on chromosome 13q14.3. It is composed of two exons with the second exon encompassing the entire open reading frame. This open reading frame encodes a protein with 196 amino acids with a molecular mass of 21 kDa. ARLTS1 has significant homology with members of the Ras superfamily, particularly the ARF (ADP-ribosylation factor) and ARL (ARF-like) members, leading to its designation as ADP-ribosylation factor-like tumor suppressor gene 1.[82] ARLTS1 is downregulated by promoter hypermethylation in ovarian cancer.[83]

ARLTS1 is frequently downregulated in ovarian primary tumors and cell lines, and restoration of its expression by adenoviral ARLTS1 or by the demethylating agent 5-AZA-2-deoxycytidine (5-AZA) effectively induced apoptosis in vitro and suppressed ovarian cancer tumorigenicity in nude mice. In addition, ARLTS1 Trp149Stop polymorphism greatly reduced the protein apoptotic function in ovarian cancer.[83]

Chromosome 16

WWOX is a candidate tumor suppressor gene that exhibits LOH or homozygous deletion in several tumor types. The protein, encoded by an alternatively spliced transcript, variant 4, which lacks exons 6—8, may interfere with normal WWOX function in a dominant negative fashion in ovarian cancer.[84,85]

Immunohistochemical staining showed very low levels of WWOX expression in 30% of ovarian tumors. The remaining ovarian carcinomas (70%) stained moderately to strongly positive for this protein. Reduced expression of WWOX was found in mucinous (70%) and clear cell (42%) histotypes. Reduced WWOX expression demonstrated a significant association with clinical stage IV, lack of progesterone receptor (PR) expression, and shorter overall survival.[86] WWOX expression strongly inhibits anchorage-independent growth. Additionally, WWOX induces a dramatic inhibition of xenograft growth in vivo.[84]

Chromosome 17

p53, which maps to 17p13.1, is the most common genetic alteration discovered to date in ovarian cancer. It is often accompanied by an overexpression of the mutant form of p53 protein.[87] LOH on chromosome 17p13.17p13.3 is one of the most frequent changes in ovarian cancers.[88–93] Loss of functional p53 often involves inactivation of one allele by point mutation and the other by chromosomal deletion.[94]

Immunocytochemically detectable p53 expression is correlated with subcellular localization of aberrant p53 and particular types of mutations. Missense mutations showed strong nuclear or nuclear plus cytoplasmic staining, whereas deletion of the major nuclear localization signal showed exclusive staining of the cytoplasm.[95] In ovarian cancer, inactivated p53 can accumulate in both major cell compartments, depending on the type of the underlying mutation. The most common alteration of p53 in ovarian cancer results in its inactivation and the overexpression of a nonfunctional protein in the nucleus of the cell.[96–99] p53 overexpression is seen in approximately 4% of borderline tumors,[100] 10% to 15% of early, and 40% to 50% of advanced cancers.[101] The frequency of overexpression of a mutant p53 is also found to be significantly higher in advanced stage III/IV disease (40% to 60%) compared with stage I cases (10% to 20%).[98] Therefore, the higher frequency of p53 overexpression in advanced-stage cases may indicate that this is a late event in ovarian carcinogenesis. Alternatively, it is possible that loss of p53 may give an aggressive phenotype associated with a more rapid dissemination of the disease. Although p53 mutations are found in all histologic types, it is most common in high-grade serous carcinoma.[102]

The p53 protein contains four functional domains: a transcriptional activation domain, a tetramerization domain, and two DNA-binding domains. In addition to possessing transcriptional activation properties, transcriptional repression has been described, although these promoter sites are less well characterized[103–107]. Either loss of wild- type p53 function, gain of oncogenic function, or the ability to activate p53 inappropriately severely decreases the capacity for controlled cellular proliferation and growth. Numerous stimuli can activate p53, including UV irradiation-induced DNA damage, inappropriate proto-oncogene activation, mitogenic signaling, and hypoxia. Depending on the cellular context, one of several responses is implemented, such as cell cycle arrest, senescence, differentiation, or induction of the apoptotic cascade.

The majority of genetic alterations in p53 are missense mutations of single residues, largely occurring in the DNA-binding domain.[108] Mutant p53 protein has the ability to form a tetramer with wild-type p53, acting as a dominant negative to repress normal physiologic processes of p53, possible by inducing an inactive conformation of the DNA-binding domain and reducing the ability to transactivate/repress target genes.[109–112] Normally, p53 exists in a negative feedback loop with human double-minute 2 oncoprotein (HDM2), which tightly controls both p53 and HDM2 levels in the cell. Loss of transcriptional activity, however, may result in decreased HDM2, stabilizing the p53 mutant and increasing the p53 mutant protein.[113] A recent study has shown that p53 activation can also induce autophagy by the activation of AMP-activated kinase (AMPK), which, in turn, inhibits mammalian target of rapamycin (mTOR) through the TSC1/TSC2 complex.[114] Recent research has also demonstrated that p53 controls microRNA expression, directly transactivating miR-34a, miR-34b, and miR-34c through the presence of a p53 response element 30 kb upstream of the miRNA mature sequence in several cell types.[115–118]

OVCA1 is a candidate tumor suppressor gene and is located at a highly conserved region on chromosome 17p13.3 that shows frequent loss of heterozygosity in breast and ovarian carcinomas.[84]

Subcellular localization studies indicate that OVCA1 is localized to punctate bodies scattered throughout the cell but is primarily clustered around the nucleus. Overexpressing OVCA1 resulted in 50% to 60% reduction in colony number in colonogenic assay. The clones that expressed exogenous OVCA1 were found to have dramatically reduced rates of proliferation. Reduced growth rates correlated with an increased proportion of the cells in the G1 fraction of the cell cycle and decreased levels of cyclin D1. The low levels of cyclin D1 appeared to be caused by an accelerated rate of cyclin D1 degradation. Overexpression of cyclin D1 was able to override OVCA1's suppression of clonal outgrowth.[119] These results suggest that slight alterations in the level of OVCA1 may result in cell cycle deregulation and promote tumorigenesis.

BRCA1, located at 17q21[120–123] encodes a second tumor suppressor that has been implicated in familial ovarian and breast cancer. Germ-line BRCA1 mutations are present in 6% to 8% of all epithelial ovarian cancers.[124,125] Less commonly, epithelial ovarian cancers contain somatic mutations in this gene.[126] In addition, aberrant expression of BRCA1 may occur through loss of heterozygosity. LOH has been correlated with clinical parameters such as tumor histology and stage. A study revealed an incidence of 35% of LOH at at least one locus, with an increased prevalence in advanced stage (44%) versus early-stage (14%) tumors as well as with serous histology (39%).[127] In addition, the loss of BRCA1 expression can also occur through promoter hypermethylation[128–132] and haploinsufficiency leading to an overall decrease in BRCA1 function as a result of inactivation of one BRCA1 allele.[133]

Immunohistochemical analysis of BRCA1 in sporadic epithelial ovarian cancers revealed a significant reduction in the BRCA1 protein in 75% of benign cystadenomas, 100% of borderline epithelial ovarian tumors, and 34% of serous epithelial ovarian cancers.[134] There was also a sharp decline in BRCA1 expression with increasing tumor grade. Russell et al.[135] demonstrated reduced or absent protein expression in 90% of serous epithelial ovarian cancers, with a 44% incidence of LOH at the BRCA1 locus. However, there was no significant correlation between LOH status and levels of RNA and protein expression. This group also reported that 80% of BRCA1-positive tumors were stage I or II compared with 21% and 38% of tumors classified as having intermediate or low levels of BRCA1 staining, respectively,[135] implying that BRCA1 loss is a late event in tumor progression.

BRCA1 is a target for ATM/ATR phosphorylation and functions as a mediator protein in the DNA damage checkpoints.[136] Loss of function of the BRCA1 gene is associated with defects in the S-phase checkpoint and G2-M transition.[137,138] In response to DNA damage, the role of BRCA1 in the regulation of the G2-M checkpoint is linked to ATM-mediated phosphorylation and activation of the CHK1 signaling cascade,[139,140] and cells with deficient BRCA1 are unable to undergo G2-M arrest.[141] BRCA1 is required for

DNA double-strand break repair by homologous recombination. BRCA1 associates with protein complexes that are involved in the repair of DNA double-strand breaks usually through the error-free mechanism of homologous recombination (HR).[142] Cells without normal BRCA1 activity, which are reduced to using error-prone pathways, accumulate genetic alterations as a result of failure to arrest and repair DNA damage or self-destruct, thereby leading to genomic instability and neoplastic progression. BRCA1 is important for maintaining the genomic stability. Cells lacking BRCA1 showed the accumulation of chromosomal abnormalities including chromosomal breaks, severe aneuploidy, and centrosome amplification.[143]

Chromosome 19

PEG3, also known as Pw1, is one of several genes identified in an imprinted region mapped to human chromosome 19q13.4.[144,145] This 19-kb gene encodes a multifunctional protein with primarily nuclear localization.[146] PEG3 silencing is associated with DNA hypermethylation, but not gene deletion. PEG3 was downregulated in 75% of 40 ovarian cancers. The gene was hypermethylated in 11 of 42 (26%) ovarian cancers, and its expression was downregulated in 10 of those 11 cancers. LOH at PEG3 was detected in 5 of 25 (20%) informative cases.

Loss of PEG3 expression is frequently observed in ovarian cancer, and reexpression of PEG3 markedly inhibited ovarian cancer growth.[147] The role of PEG3 in p53-mediated apoptosis was explored. It has been shown that Peg3/Pw1 is upregulated in the p53-mediated cell death process and induces Bax translocation independent of apoptosis.[148] Results from these studies suggest not only that Bax translocation is an important regulatory step in p53-mediated apoptosis, but also that PEG3 may function as a downstream modulator of p53 to regulate Bax redistribution. These events lead to the cell's propensity to favor apoptosis over growth arrest after p53 induction.

Conclusion

Of the 16 tumor suppressor genes reviewed, loss of heterozygosity or inactivating mutations has been observed in 11. Five putative tumor suppressor genes have been silenced by somatic promoter methylation without genetic alterations. Further studies will be required to determine whether the loss of these epigenetically silenced genes is associated with a poor prognosis in the clinic or an increase in tumorigenesis in murine knockout models. Loss of *ARHI* function is associated with genetic alterations in 30% to 40% of ovarian cancers, and downregulation of ARHI has been linked to a significantly shorter disease-free survival. Knockout of *ARHI* in murine systems has not been possible.

Cattle and swine express *ARHI* orthologs, but mice do not, precluding convenient preparation of knockout animals. *ARHI* is one of several genes that are expressed by man, but not by mice, including *COL21A1, STK17A*, and *GPR145*. *ARHI* maps to an apparent evolutionary breakpoint in the rodent lineage where chromosomes have been rearranged relative to the human genome since the two species shared a common ancestor.[149]

During oncogenesis, loss of tumor suppressor gene function can occur through deletion, mutation, or epigenetic silencing, and the frequency of loss for different genes in ovarian cancers varies substantially. The function of several putative tumor suppressor genes is lost in a majority of ovarian cancers, including p53 (50% to 70%), ARHI (64%), DLEC1 (73%), SPARC (70% to 90%), DOC2 (58% to 85%), RPS6KA2 (64%), OPCML (56% to 83%), ARLTS1 (62%), and PEG3 (75%). Other tumor suppressor genes are dysregulated less frequently, including RASSF1A (46%), LOT-1 (39%), OVCA1 (37%), PTEN (35%), WWOX (30% to 49%), BRCA1 (6% to 8%), and BRCA2 (3% to 6%). Taken as a group, the many presumed functions of tumor suppressor genes control critical points in a wide range of cellular signaling pathways that regulate proliferation, differentiation, apoptosis, and response to genetic damage. Several tumor suppressor genes affect proliferation, whereas others, such as ARHI, affect both proliferation and motility. Of the signaling pathways that are dysregulated in ovarian cancer, Ras/MAP is affected by ARHI and DOC2, whereas PI3K is inhibited by PTEN and ARHI. Stat3 is also functionally inhibited by ARHI. Further study of the interactions between tumor suppressors and activated oncogenes will enable us to understand the genetic basis of ovarian cancer and to manage patients with ovarian cancer more precisely and effectively in the clinic.

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Chapter 6
Epigenetics and Ovarian Cancer

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Cancer Epigenetics: Introduction

Epigenetics is a broad term that refers to all stably heritable alterations in gene expression that occur without changes in DNA base sequence. Epigenetic phenomena include deoxycytosine methylation, histone protein modifications, nucleosome position effects on DNA, and gene regulation by noncoding RNA molecules (Fig. 6.1); the overall epigenetic state corresponding with a specific cell phenotype is referred to as an "epigenome." The Human Genome Project, completed in 2003, has provided a wealth of data regarding the relationship of DNA sequence to human health, and one interesting outcome of that project was the observation that humans possess far fewer genes than previously predicted.[1] That vast underestimation of human genes suggested a much greater role for phenotype-specific gene regulation by other mechanisms, including epigenetic modifications. Consequently, a Human Epigenome Project, an international public/private consortium, has now been initiated to provide a set of normal, reference epigenomes to permit study of the role of epigenetics in biological processes such as differentiation, proliferation, and various disease states, including cancer.[2]

To date, the best-studied epigenetic process is methylation of deoxycytosine, usually located within the dinucleotide CpG, and catalyzed by enzymes known as DNA methyltransferases (DNMTs).[3] DNA methylation is strongly correlated with gene silencing, and in normal cells, heterochromatin and the inactive female X chromosome are extensively methylated.[4] This modification also occurs within repeat elements, where it is believed to silence potentially harmful transposable elements.[5] Aside from heterochromatin and repeats, distinct regions, usually within 5′ untranslated regions and the first exons of genes, are normally protected from this modification.[3] It is now well established, however, that these localized "CpG islands" become increasingly hypermethylated during tumor advancement

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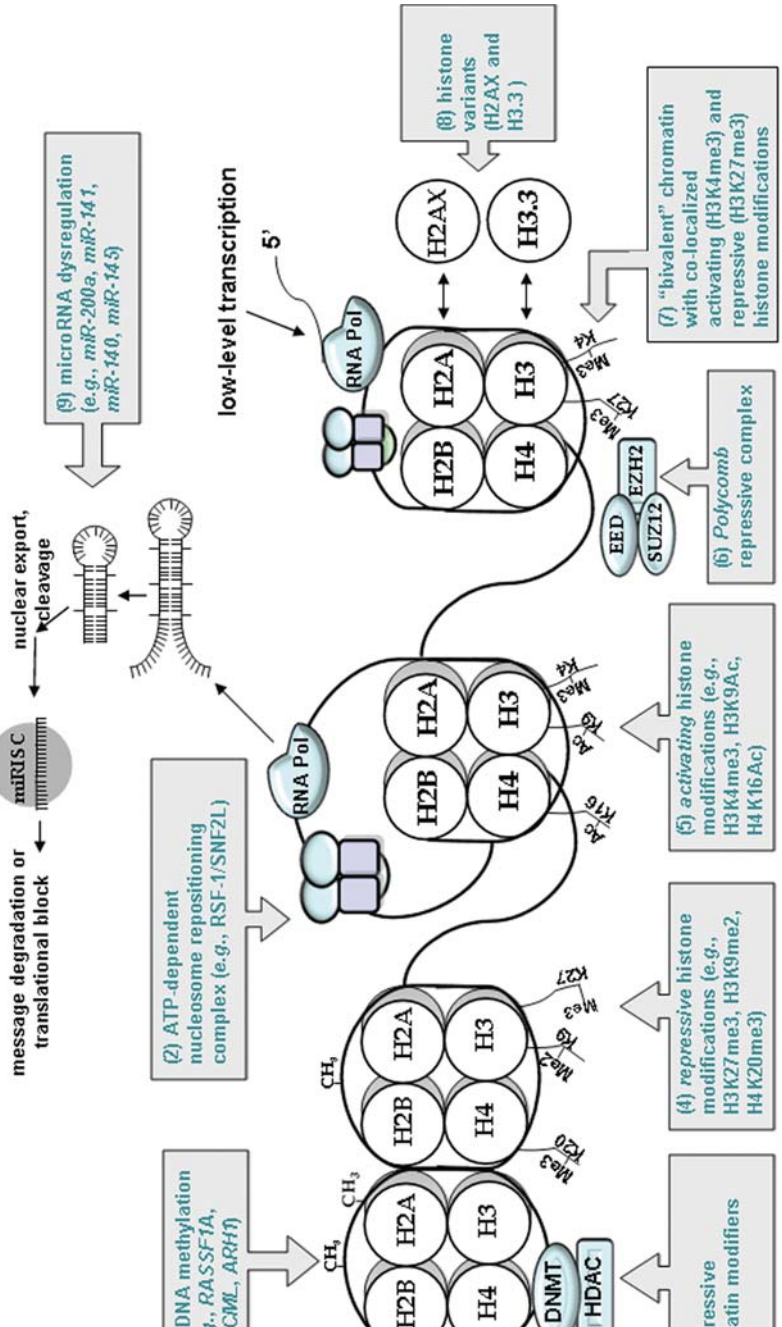

Fig. 6.1 Epigenetic modifications in ovarian cancer (specific examples shown in parentheses). *HDAC* , histone deacetylase; *DNMT* , DNA methyltransferase; *EZH2* , enhancer of Zeste-2; *EED* , embryonic ectoderm development protein; *SUZ12* , suppressor of Zeste-12; *RNA Pol* , RNA polymerase II; *miRISC* , microRNA-induced silencing complex

from benign to invasive disease, with an inverse progressive decrease in *global* DNA methylation levels.[6]

In addition to methylation of DNA, modifications to its associated histone proteins represent another epigenetic level of gene regulation, although it is now accepted that both DNA methylation and histone alterations jointly cooperate to govern specific gene expression patterns.[7] Acetylation of histones (catalyzed by *histone acetyltransferases*) is associated with chromatin relaxation and gene activation, whereas histone deacetylation (catalyzed by *histone deacetylases*) is well correlated with chromatin compaction and gene silencing.[8] Whereas DNA methylation is associated with gene repression, histone methylation can be either activating or repressive in a site-specific manner. For example, di- and tri-methylation of histone H3 lysine 4 (H3K4me2 and H3K4me3) is associated with active transcription, whereas trimethylation of H3K27 (H3K27me3) is strongly linked with gene repression.[9] Such histone modifications, and their specific associations with gene activation/repression have led to a "histone code" hypothesis in which various adapter proteins bind to designated histone "marks" and translate the code to effectuate the appropriate transcriptional responses.[10] Similar to DNA methylation, distinct aberrations in histone patterns occur in neoplasia, including a global loss of H4K20 acetylation and H4K16 trimethylation, in addition to extensive rearrangements of other activating and repressive histone marks.[11] The H3K4 methyltransferase hASH2 (a human homolog of a *Drosophila trithorax* protein) has been recently identified as a transforming oncogene in mouse embryonic fibroblasts,[12] and the H3K27 methyltransferase EZH2, and its associated polycomb proteins, have also been demonstrated as overexpressed in numerous malignancies.[13]

In one global analysis of histone marks in embryonic stem (ES) cells, a number of gene promoters were found to (seemingly paradoxically) simultaneously possess both H3K4me2 activating and H3K27me3 repressive marks; this "bivalent" epigenetic state was associated with low levels of transcriptional activity and was restricted to various development and lineage-specific genes.[14] In 2007, Ohm et al. discovered that a number of DNA-methylated genes in adult tumors similarly possess a bivalent state in ES cells, which subsequently lose the H3K4me2 activating mark (while also gaining additional repressive histone marks) in embryonic carcinoma cells.[15] That finding has now led to a carcinogenesis model in which aberrant gene repression is initiated by H3K27 trimethylation, catalyzed by EZH2, and ultimately leading to permanent gene silencing by DNA methylation in adult tumors.[16,17] Thus, although numerous genetic mutations have now been established as contributory to tumorigenesis, it is now becoming apparent that epigenetics plays an equal (or perhaps even greater) role in the establishment of the recently hypothesized "cancer stem cell" pluripotent phenotype.[16–19]

As the nucleosome, the basic chromatin-packaging unit of 147 bp of DNA wrapped around a histone octamer, precludes DNA access by transcription factors and polymerases, nucleosome positioning represents another epigenetic modulator of gene expression. To alter DNA/nucleosome packaging, ATP-dependent

chromatin-remodeling protein complexes elicit octamer *transfer* (in *trans*) or *sliding* (in *cis*) and include the SWI/SNF family, the imitation SWI (ISWI) group, and the Mi-2 family of complexes,[20] and can, depending on the specific constituent enzymes, be either transcriptionally repressive or activating. Similar to DNA methylation and histone modifications, cancer cells frequently possess altered composition of nucleosome-destabilizing complexes, including dysregulation of components of both activating (SWI/SNF) and repressive (NuRD) multisubunit chromatin-remodeling assemblies.[21,22]

The most recently discovered epigenetic phenomenon is gene expression regulation by noncoding RNA molecules, including 21- to 23-nucleotide single-stranded RNAs known as *microRNAs*. MicroRNAs are often transcribed as polycistrons that are then processed to 60- to 70-nucleotide hairpin structures ("pre-miRNAs") that are then exported from the nucleus. In the cytoplasm, pre-miRNAs are further processed by an endonuclease, Dicer, to the single-stranded mature molecule that is incorporated into a multisubunit riboprotein known as the RNA-induced silencing complex (RISC).[23] Within the RISC, another group of small RNAs associate directly with a subunit of the degradation complex known as the Piwi family of proteins.[24] These "Piwi-interacting RNAs" (piRNAs) are believed to interact with other novel small RNAs, target the RISC complex to parasitic DNA sequences (such as transposons), and contribute to germ-line and stem cell maintenance.[25,26] Although microRNAs are believed to play a significant role in animal development and normal pluripotent stem cell regulation,[27,28] a large number of these small RNA molecules have also been shown as dysregulated in various tumors, where they may act as either tumor suppressors or oncogenes.[29] Several now well-known tumor-suppressing microRNAs include let-7, which targets the *RAS* oncogene, and mir-15/16, targeting the apoptosis inhibitor BCL2, while oncogenic miRNAs include miR-372/373, inhibitory to the p53 tumor-suppressing pathway, and the miR17-92 cluster, which cooperates with the Myc oncoprotein to elicit cell proliferation.[6]

Epigenetic Aberrations in Ovarian Cancer

The two most significant obstacles to the effective treatment of ovarian cancer are the lack of early diagnostic markers and the development of drug resistance after therapy of advanced disease. A dire need for improved early-detection strategies is based on the grim statistic that more than 70% of patients are initially diagnosed with stage III/IV tumors due to a lack of distinct symptoms and biomarkers for early malignancy.[3,30,31] Moreover, although surgical debulking followed by chemotherapy regimens (typically paclitaxel and carboplatin) results in complete remission in 80% of advanced-stage ovarian cancer, chemoresistance almost inevitably ensues (after a median period of 18 months), after which the disease is essentially untreatable.[32]

Similar to all cancers studied to date, ovarian tumors possess numerous epigenetic aberrations, including the characteristic global DNA hypomethylation and CpG island hypermethylation described above.[33] Specific examples of hypomethylation include chromosome-1 satellite-2 and Long interspersed nuclear element-1 (LINE-1) repetitive elements.[33,34] Using an RNA interference approach, our group demonstrated that knockdown of two DNA methyltransferase enzymes in ovarian cancer cells resulted in extensive loss of CpG hypermethylation and substantially reduced cell survival.[35] A number of genes have now been characterized as silenced by promoter methylation in ovarian cancer cells and tumors (for more extensive review, see Refs. Balch et al.[8] and Barton et al.[36]), including the imprinted genes *ARH1* and *PEG3*,[37] proapoptotic genes such as *LOT1*, *DAPK*, *TMS1/ASC,* and *Apaf-1*,[8,38] and cell adhesion genes including *OPCML*, *ICAM-1*, and *CDH1*.[9–41] One of the most commonly methylated genes in ovarian cancer is the *RAS association domain family isoform 1A* (*RASSF1A*), encoding a microtubule-stabilizing protein that is also an inhibitor of the anaphase-promoting complex, a ubiquitin ligase that targets mitotic cyclins for degradation.[42] A newly identified gene, *HSulf-1*, encoding an arylsulfatase that acts on cell surface heparin sulfate proteoglycans and inhibits growth factor signaling and angiogenesis,[43] was found methylated in more than 50% of ovarian tumors and cell lines.[44] Methylation of the well-known tumor suppressor *PTEN*, which negatively regulates growth signaling through the oncoprotein Akt, has also been observed in ovarian cancer,[45] as has methylation of the p53-stabilizing gene *14-3-3 sigma*.[46] Another interesting gene associated with ovarian tumor chemoresistance is *MCJ* (*methylation controlled DNAJ*), recently discovered to encode an inhibitor of c-jun–mediated upregulation of the multidrug-resistance transporter ABCB2.[47,48] Interestingly, in addition to hypermethylated genes, a number of genes have also been demonstrated as *hypomethylated* and overexpressed in ovarian cancer, including *SNCG*, an oncogene of unknown function; *claudin-4*, encoding a tight junction protein; *maspin*, encoding a serine protease inhibitor; and *TRAG-3*, a gene implicated in Taxolresistance.[49–52]

In addition to redistributions of DNA methylation patterns, another epigenetic aberration in ovarian cancer is atypical modifications of histones and dysregulated expression of histone-modifying enzymes; histone modifications also contribute significantly to normal ovarian functions, including estrogen synthesis, folliculogenesis, and luteal phase activity.[53] In malignancy, however, ovarian tumors were found to possess significantly altered expression of chromatin-modifying proteins,[54] and another study demonstrated several specific histone modifications (but not promoter DNA methylation) as responsible for silencing of the differentiation genes *GATA4* and *GATA6* in ovarian cancer cells.[55] Two other genes upregulated by histone acetylation include the well-known tumor suppressor *Rb* and the cyclin-dependent kinase inhibitor *CDKN1*, whereas interestingly, the *Id1* (*inhibitor of differentiation-1*) gene was found *downregulated* by a histone deacetylase (HDAC) inhibitor.[56] Using a dominant-negative histone overexpression approach, our group established a role for H3K27 in ovarian cancer chemoresistance,[57] and another group demonstrated that loss of H3K27 trimethylation is associated with poor prognosis in ovarian and other malignancies.[58]

As mentioned above, another epigenetic phenomenon is repositioning of nucleosomes (histone octamers) during gene activation or repression. In ovarian cancer, overexpression of Rsf-1, a component of the *remodeling and spacing factor* (RSF) chromatin-remodeling complex, has been strongly correlated with advanced-stage and high-grade malignancies.[59,60] Another protein overexpressed in ovarian cancer, MTA-1 (metastasis-associated gene-1), is a component of the *nuclear remodeling and deacetylation* (NuRD) gene-repressive complex.[61,62] Similarly, the protein product of *BRCA1*, a gene having germ-line mutations linked to greatly increased ovarian cancer risk, was demonstrated to interact with a component of the ATP-dependent nucleosome-destabilizing complex SWI/SNF, likewise suggesting altered chromatin modeling in the pathogenesis of this malignancy.[63] In another study of the SWI/SNF component Brm (Brahma), a protein frequently downregulated in ovarian cancer, Brm expression was found to be restored by HDAC inhibitors, suggesting a link between repressive histone modifications and altered chromatin remodeling in carcinogenesis.[64]

The most recently discovered epigenetic phenomenon, gene regulation by non–protein-encoding microRNAs, has also now been established as altered in ovarian cancer. Specifically, in a panel of 69 ovarian tumors, the most significantly upregulated microRNAs were 200a, 200b, and 141; one common target of these is the tumor suppressor BAP-1 (BRCA-1-associated protein), and a microRNA found downregulated, miR-140, targets oncoproteins such as c-SRK, MMP13, and FGF2.[65] Similar to other malignancies, the tumor suppressor phosphatase and tensin homolog (PTEN)–targeting microRNA miR-214 was found upregulated in ovarian cancer; additionally, expression of miR-214 and two other microRNAs, miR-199a and miR-200a, was found to correlate positively with advanced-stage and high-grade tumors.[66] Interestingly, a group of six miRNAs clustered on chromosome 19, and seven clustered on chromosome 14, were upregulated by the DNA methyltransferase inhibitor decitabine (described later), demonstrating that microRNA genes can be regulated by DNA methylation.[67]

Epigenetics in Ovarian Cancer Management

Methylation Biomarkers for Screening/Prognosis

As redistributed DNA methylation (genomic hypomethylation with localized CpG island hypermethylation) is a hallmark of ovarian tumors, specific hypomethylated sequences and individual or panels of hypermethylated genes represent possible biomarkers for disease detection and prognosis. Hypomethylated sequences that have been put forth as ovarian cancer biomarkers include LINE-1 repetitive elements and chromosome-1 satellite-2 sequences.[33,34] In a study of CpG island hypermethylation, using a microarray-based genome-wide approach, our group identified a panel of 112 methylated loci 95% predictive of short (<12 months) progression-free survival (PFS) in 40 stage III/IV ovarian cancer

patients.[68] Similarly, an examination of peritoneal fluid from 57 ovarian cancer patients revealed that methylation levels of a 15-gene panel likewise could distinguish short versus long overall patient survival,[69] and another study by our group demonstrated an association of PFS with hypermethylation of 28S and 18S ribosomal DNA genes.[70] Other groups have also identified differential methylation patterns in ovarian cancer cells and tumors, using simultaneous PCR-based methylation assessment of multiple candidate genes.[71–73] Using another approach, gene expression profiling, it was found that transcription levels of 12 chromatin-modifying genes could successfully define a distinct "signature" for ovarian tumors.[54] Individual methylated genes with possible prognostic value in ovarian cancer include *HOXA11*, linked with postsurgical residual tumor and overall poor prognosis[74]; *MCJ*, strongly associated with chemoresistance; and *14-3-3-sigma*, significantly correlated with elevated serum CA-125 levels and high-grade malignancy.[46,48]

In addition to DNA-methylated genes, microRNAs could also conceivably be used as biomarkers for ovarian cancer prognosis. For example, it was found that overall microRNA profiles could clearly distinguish normal from malignant ovarian tissues and that specific upregulated or downregulated clusters could further specify clear cell, endometrioid, and serous subtypes.[65] In another study, 10 downregulated miRNAs were found to correlate positively with advanced-stage malignancy and 13 with high-grade ovarian tumors,[67] and a separate report noted an association of miR-214 (an inhibitor of the tumor suppressor PTEN) with chemoresistance in ovarian cancer cell lines and tumors.[66]

In addition to tumor-methylated genes, a number of methylated genes have also been identified in patient serum and plasma, including *hMLH1*, *BRCA1*, and *RASSF1A*, offering the possibility of minimally invasive biomarker assessment.[75,76] Specifically, *hMLH1* methylation was found to correlate with patient tumor relapse in 25% of patients, after carboplatin/Taxol therapy, and also associated with microsatellite instability.[75] Though much improved and now increasingly reproducible, gene expression signatures for ovarian cancer have, to date, been limited to invasive removal of tumors/biopsies and thus may not represent a practical approach for early detection. Similarly, though also now improved, serum proteome profiles have suffered validation setbacks due to bias and a lack of reproducibility.[77] Consequently, methylated DNA sequences yet represent a feasible, minimally invasive approach for ovarian cancer screening, possibly in conjunction with other indicators such as cancer antigen 125 (CA-125) levels and transvaginal sonographs.[78]

Epigenetic Therapies

DNA Methylation Inhibitors

Several drugs have been discovered that inhibit DNA methyltransferase activity, resulting in genomic hypomethylation. Most of these are cytosine analogues that covalently and irreversibly bind to the active site of these enzymes,

ultimately resulting in their cellular depletion.[79] The hypomethylating activity of these inhibitors is replication-dependent, requiring several cell divisions to complete the demethylation of each DNA strand.[80] The first of these to be characterized was the ribonucleotide 5-azacytidine (trade name Vidaza; Pharmion Corp., Boulder, CO), now approved by the U.S. Food and Drug Administration (FDA) for the treatment of myelodysplastic syndrome (MDS).[81]

In addition to azacytidine, its deoxyribose analogue, 5-aza-2′-deoxycytidine (5-aza-dC; decitabine; Dacogen; MGI Pharma, Bloomington, MN)[82] is a potent methylation inhibitor. Decitabine is effective at submicromolar concentrations and has been shown to effect demethylation in numerous ovarian cancer cell lines, with reversal of silencing of several tumor suppressor genes.[8,83] Decitabine-mediated DNA methylation was also associated with the reversal of repressive histone methylation at H3K9 and H3K27.[57,84] In addition to decitabine, our group has also examined another DNA methylation inhibitor, zebularine, demonstrating that this agent possesses potent antiproliferative activity against a number of ovarian cancer cell lines.[85] Although decitabine remains a promising therapy for ovarian and other malignancies, its potency is somewhat limited by inactivation by intracellular deaminases. To achieve a more stable compound, a decitabine-guanine dinucleotide, S-110, was synthesized and found to be slightly less toxic than the mononucleotide, with substantially less deamination.[86] In that pilot study, S-110 was also found to reactivate the methylated tumor suppressor *p16* in bladder cancer cells, apparently using a DNA methylation inhibition mechanism identical to that of decitabine, after cleavage of the dinucleotide by intracellular phosphodiesterases.[86]

A number of non-nucleotide agents, including the antiarrhythmic procainamide, epigallocatechin-3-gallate (EGCG; a component of tea), procaine (a local anesthetic), and the antihypertensive hydralazine, were also found to possess DNA-demethylating activity, albeit with a much lower potency than that of decitabine.[87–89] As these drugs and natural compounds are already approved for human consumption, examination of their use in clinical trials might be greatly facilitated.

Histone Deacetylase Inhibitors

As histone deacetylation is another transcriptional silencing mechanism, the use of histone deacetylase inhibitors (HDACIs) is another strategy to relieve epigenetic gene repression. Numerous HDACIs, which antagonize the action of histone deacetylases by chelation of an essential zinc ion cofactor,[90] are now under extensive investigation, based on their abilities to selectively induce differentiation and apoptosis in tumor, but not normal, cells.[90] Interestingly, these compounds can induce specific genes while repressing others and also inhibit other protein (i.e., nonhistone) deacetylases, including those that act on p53[91] and tubulin.[92]

To date, the hydroxamate structural class of HDACIs has demonstrated the greatest promise in preclinical and clinical studies. One such hydroxamate,

suberoylanilide hydroxamic acid (SAHA; vorinostat; Zolinza; Merck & Co, Whitehouse Station, NJ), has recently been approved for the therapy of cutaneous T-cell lymphoma and is currently being evaluated in 80 separate clinical trials for various malignancies (clinicaltrials.gov). In ovarian cancer cells and xenografts, vorinostat has demonstrated potent antigrowth activity and minimal toxicity,[93] although a phase II trial for advanced ovarian cancer demonstrated low toxicity but minimal patient response.[94] Another hydroxamate, PXD101 (belinostat; CuraGen Corp., Branford, CT), has also shown potent activity against human ovarian cancer xenograft models[95] and is now being studied in a phase I trial in the United Kingdom (www.beatson.org.uk). A third hydroxamate, scriptaid, was also recently demonstrated as highly antiproliferative against a number of ovarian cancer cell lines,[96] and another novel HDACI, R306465, was demonstrated to inhibit xenograft tumor growth of A2780 ovarian cancer cells and to activate the cell cycle regulator *p21* in vivo; interestingly, R306465 possessed isotype-preferential activity toward class I (but not class II) histone deacetylases.[97]

Another potentially clinically useful HDACI is the short-chain fatty acid valproate (VPA), which has been previously used as an anticonvulsant.[90] In two preclinical studies, VPA was found to be strongly antiproliferative against the human ovarian cancer cell line SK-OV-3 in both cultured cells and in xenograft tumors.[93]

Novel Epigenetic Therapies and Combination Strategies

While HDAC and methyltransferase inhibitors have demonstrated ovarian cancer activity as single agents, it is strongly believed that combinations of these two inhibitor types, in addition to conventional therapies, will be most effective.[98] Based on the fact that pluripotent/multipotent progenitors possess increased epigenetic repression,[99,100] it has been hypothesized that epigenetic inhibitors could (possibly in combination with conventional chemotherapies) elicit differentiation of the cancer stem cells believed to be solely responsible for tumorigenesis.[98] Also in accordance with the cancer stem cell theory, epigenetic therapies might also allow tumor resensitization to conventional agents by reversing repressive chromatin states associated with the pluripotent phenotype of tumor progenitors, which are believed to be recalcitrant to traditional therapies directed toward their rapidly dividing descendants. Consequently, a number of combinatorial strategies have demonstrated promise in preclinical and clinical studies for ovarian cancer. In two preclinical studies, it was found that vorinostat was effective alone against paclitaxel-resistant ovarian cancer cells but was more effective in combination with this taxane.[101,102] Another study, both in vitro and in vivo, combined belinostat with carboplatin, demonstrating substantial benefit, over either alone, in three-dimensional cultures and mouse xenografts of platinum-resistant ovarian cancer cells,[103] whereas a similar approached used the HDACI VPA to resensitize platinum-resistant cells to cisplatin.[104] In an assessment of the methyltransferase inhibitor zebularine, our

group demonstrated that agent to completely reverse cisplatin resistance in recalcitrant ovarian cancer cells,[85] and others have reported similar resensitizing effects using decitabine in cisplatin-resistant cells and xenografts.[105,106] A comparable approach is currently being examined by our group in a phase I/II trial of decitabine paired with carboplatin, with a similar study under way in the United Kingdom (clinicaltrials.gov). Another phase I/II study, combining Vidaza with VPA and carboplatin, is currently in progress at the M.D. Anderson Cancer Center (Houston, TX) (www.mdanderson.org). In a study of the non-nucleoside hydralazine, in combination with VPA and standard chemotherapy, some degree of response was observed in 7 of 7 ovarian cancer patients, with most patients showing primarily mild hematologic toxicities.[107]

In addition to DNA methylation and histone deacetylase inhibitors, various recently developed agents have been designed to target other components of the repressive epigenetic machinery, including inhibitors of histone acetyltransferases and methylcytosine-binding proteins.[108,109] Another epigenetic repressor, the polycomb protein EZH2 (trimethyltransferase for H3K27), is highly expressed in various aggressive malignancies[108,109] and has been demonstrated as largely responsible for gene repression and maintenance of embryonic stem cells.[110,111] Consequently, an *S*-adenosyl-homocysteine inhibitor, 3-deazaadenosine (DZNep), has now been developed and demonstrated to substantially reduce H3K27 levels, activate a large number of apoptosis and differentiation-related genes, and elicit apoptosis in MCF-7 breast cancer cells.[112] Such novel inhibitors may allow for combinatorial regimens targeted toward repressive epigenetic events directly responsible for tumor initiation and progression.

Conclusion

Epigenetic alterations have now been established as intimately involved in the initiation, development, and progression of ovarian malignancies, including the eventual evolution to a drug-resistant phenotype. As a large number of these alterations have now been demonstrated to be highly specific to this gynecologic malignancy, such modifications represent highly promising biomarkers for early detection, therapy response, and prognosis. Further, as transcriptionally repressive epigenetic changes, such as DNA methylation, occur in several genes involved in apoptotic and differentiation pathways, reactivation of those genes might allow for resensitization of tumor cells to drugs that rely on such pathways. An increasing body of evidence now supports a role for possible tumor progenitors, known as "cancer-initiating cells," in the propagation of fully drug-resistant tumors. As normal multipotent stem cells possess a high degree of epigenetic repression, it is possible that therapies that reverse such repression could be highly effective against drug-resistant disease. Toward this end, our group has now identified candidate ovarian cancer-initiating cells from human ovarian stage III serous adenocarcinomas, based both on the ability of those

cells to form anchorage-independent spheres and to serially propagate in consecutively engrafted animals.[113] Based on five distinct attributes,[114,115] those tumor progenitors fulfilled all currently accepted criteria for the existence of a population of ovarian cancer-initiating cells, which are now being subjected to comprehensive genome-wide analyses to identify specific epigenomes responsible for tumor initiation and growth. In summary, it is increasingly evident epigenetics plays a prominent role in ovarian cancer, and a greater understanding of this phenomenon will likely lead to much improved diagnostic and therapeutic interventions.

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Chapter 7
Aberrant Epithelial Differentiation in Ovarian Cancer

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Introduction

It is well recognized that cancer cells often appear in an inappropriately differentiated stage, and the term "dedifferentiation" is often used.[1–4] Dedifferentiation is a prominent feature of cancer cells; however, the causative mechanism is not well understood. Even the meaning of "dedifferentiation" is not well defined and is often used to refer to various aspects of cell changes associated with neoplastic transformation. The phenotype of ovarian cancer described as "undifferentiated" or "dedifferentiated" often refers to the apparent changes of the cancer cells toward a less epithelial-like morphology. However, such a definition is subjective and lacks molecular determinants.

To provide a molecular interpretation of "dedifferentiation" of cancer cells, one may consider the concept of cell lineage differentiation prominent in developmental biology, in which a primordial cell undergoes change in its global gene expression profile toward a specific mature cell type. By analogy, dedifferentiation of cancer cells may be caused by the loss of one or more critical genes that are key in inducing the differentiation of a primordial cell type to derive the ovarian surface epithelial cells (Fig. 7.1). "Dedifferentiation" may seem to mean a reversion of the process of differentiation; however, cancer cells are not just epithelial cells of the reversed differentiation but are cells with an aberrantly differentiated state. The dedifferentiation of cancer cells thus is defined as a loss of key gene(s) involved in the process of differentiation to derive ovarian surface epithelial cells, resulting in an apparent differentiation stage that is not identical to a normal cell lineage. Likely, the one or more critical gene(s) is a transcription factor(s)/regulator(s), which can activate or suppress the transcription of a large panel of genes that enable the cells to take

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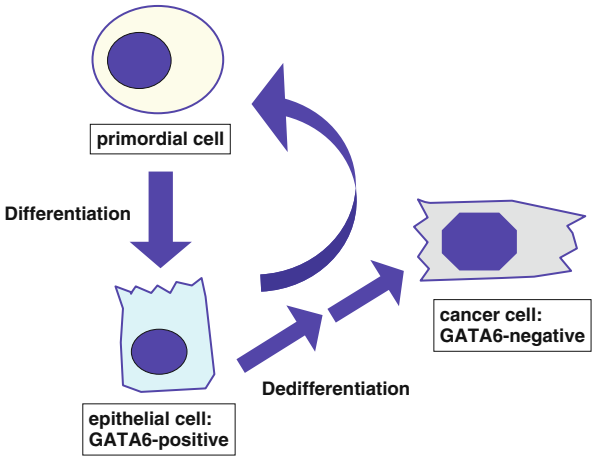

Fig. 7.1 Definition of "dedifferentiation" in cancer. "Dedifferentiation" of cancer cells is defined as the loss of a gene(s) such as GATA6 that is critical for the derivation of the epithelial cells from a primordial cell type. However, dedifferentiation of cancer cells is not the reversion of differentiation back to a normal, more primitive cell type; rather, dedifferentiation leaves the cancer cells in an aberrant stage that is different from a normal cell lineage[5,6]

on a new identity. Recent works suggest the loss of the transcription factor GATA6 in ovarian cancer may be the underlying mechanism of "dedifferentiation" of ovarian cancer cells. In this chapter, we summarize recent studies on the cause and consequences of the loss of GATA6 in ovarian cancer and discuss the relevance of dedifferentiation in ovarian tumorigenesis.

GATA Transcription Factors in Cell Lineage Differentiation: The Extraembryonic Endoderm Lineages

The expression or the nuclear functions of GATA6 transcription factor are lost in a majority of epithelial ovarian cancer cells.[5,6] However, whether GATA6 is important for the formation of the ovarian surface epithelium is unclear, as little is known about the derivation of the ovarian surface epithelial cells. Nevertheless, the role of GATA6 in the formation of extraembryonic endoderm may provide an excellent analogy for the role of GATA6 in ovarian surface epithelial cells (Fig. 7.2). Similar to the ovarian surface epithelium, the primitive endoderm is a simple epithelium consisting of a single layer of cells that covers the inner cell mass.[7] The primitive endoderm cells are derived from the differentiation of the pluripotent cells of the inner cell mass at approximately embryonic day 4.5 (E4.5), around the time of implantation of the blastocysts onto the uterine surface. GATA6 is necessary for the differentiation and formation of the primitive endoderm cells derived from the inner cell mass (Fig. 7.2). Subsequently, some primitive endoderm cells mature to become visceral endoderm cells, and others migrate to cover the

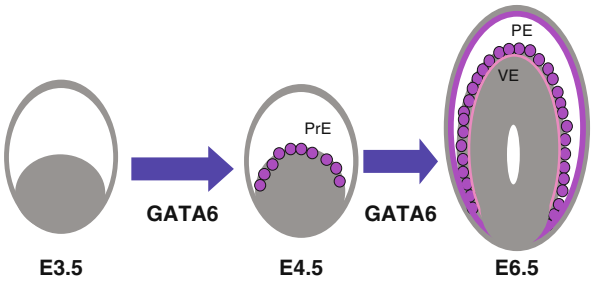

Fig. 7.2 The role of GATA6 in the development of extraembryonic endoderm. Around the time of implantation of mouse blastocysts at E3.5, an early cell lineage, the primitive endoderm is derived from the pluripotent cells of the inner cell mass (ICM), and the primitive endoderm cells form an epithelium covering the ICM. GATA6 is required for the differentiation and formation of primitive endoderm (PrE) epithelium. GATA6 deficiency results in the failure to form both visceral endoderm (VE) and parietal endoderm (PE), which are extraembryonic endoderm and are derived from PrE at a later stage. Thus, GATA6 is an essential and a determining factor in the differentiation of extraembryonic endoderm cell lineages

surface of the blastocysts and develop into parietal endoderm.[7,8] Both visceral and parietal endoderms are known as extraembryonic endoderm, precursors for the extraembryonic tissues of the embryos. The development of extraembryonic endoderm can be modeled in embryoid bodies in vitro.[9] After aggregation, embryonic stem cells differentiate and form an extraembryonic endoderm-like cell layer on the surface. The resemblance of embryoid bodies to early embryos can be seen in a side-by-side comparison of a section of an E5.5 mouse embryo embedded in uteri to a section of an embryoid body (Fig. 7.3). The endoderm cells stain positive for GATA4, an extraembryonic endoderm marker (Fig. 7.3). However, aggregation

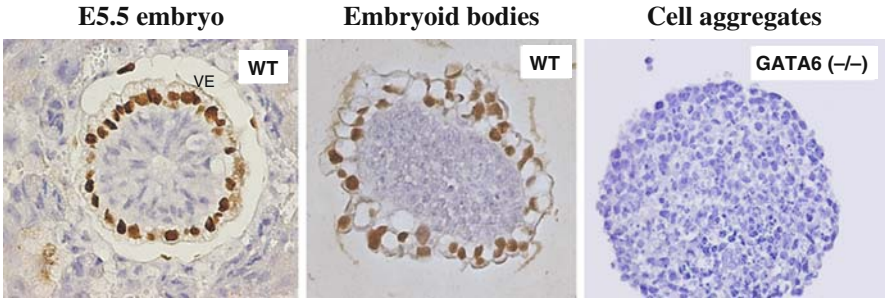

Fig. 7.3 Role of GATA6 in the differentiation and formation of visceral endoderm in embryoid body models. The requirement of GATA6 in the formation of visceral endoderm is shown in the formation of embryoid bodies from aggregation of embryonic stem cells. When mouse embryonic stem cells are allowed to aggregate, a visceral endoderm-like extraembryonic endoderm outer layer forms, as shown in immunostaining of a section with GATA4, which is strongly expressed in extraembryonic endoderm cells. The embryoid body resembles a section of an E5.5 mouse embryo implanted into a uterus. Aggregation of GATA6-null embryonic stem cells fails to form an extraembryonic endoderm, suggesting the requirement of GATA6 in the differentiation and formation of extraembryonic outer layer

of GATA6-null embryonic stem cells does not lead to the formation of extraembryonic endoderm in embryoid bodies (Fig. 7.3).[9] Nevertheless, differentiation of the embryonic stem cells does occur in GATA6-null embryonic stem cells, as indicated by the loss of the pluripotent marker Oct-3/4.[9] Thus, in the absence of GATA6, embryonic stem cells undergo aberrant differentiation into an abnormal cell type.

The GATA transcription factors bind a consensus A/T-G-A-T-A-A/G sequence in promoters and are conserved in insects and vertebrates, from fly to humans. All GATA family members contain DNA binding motifs, a transcription activation domain, and a nuclear localization sequence (Fig. 7.4). The GATA factors have been well studied in a developmental biology context: they function in cell lineage specification during embryonic development and organ formation.[10] In mammals, there are six GATA family members: GATA1, 2, and 3 are involved mainly in the development of the hematopoietic systems[11,12]; GATA4, 5, and 6 are expressed in a wide range of tissues and function in the formation of most, if not all, organs during embryonic development.[10] GATA4 and GATA6 are first expressed during the formation of extraembryonic endoderm differentiated from the pluripotent embryonic stem cells of the inner cell mass during early embryonic development.[9,13,14] Using an embryoid body model for in vitro analysis, GATA4 and GATA6 were shown to be two of the most upstream factors during the extraembryonic endoderm differentiation of pluripotent embryonic stem cells.[9,15] Thus, in embryonic stem cells, GATA6 is both necessary and sufficient and is a key gene in the differentiation of extraembryonic endoderm lineages.

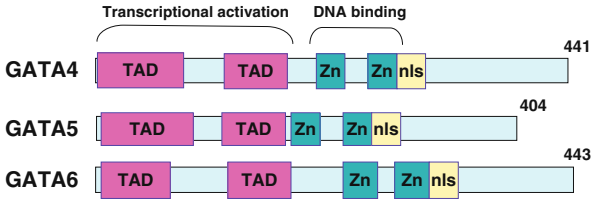

Fig. 7.4 GATA transcription factors. GATA4, 5, and 6 genes contain two zinc-finger DNA binding domains (Zn), 2 transcription activating domains (TAD), and a nuclear localization signal sequence (nls). GATA transcription factors bind to specific sequences in promoters and regulate gene transcription. Mouse GATA4, GATA5, and GATA6 have 441, 404 and 443 amino acid respectively

The importance of GATA6 and other family members in the differentiation and cell lineage formation in many other tissues is recognized. GATA4 and GATA6 are expressed in the heart,[16] liver,[17] lung,[18] gastric epithelium,[19] intestine and colon,[20] testis,[21] and ovary[22,23] and likely play critical roles in the development of these organs. GATA factors are not tissue-specific but rather function in the specification and differentiation of cell lineages within an organ,

such as the differentiation of an epithelial cell lineage from stromal cells. GATA6 is expressed in ovarian surface epithelial cells,[9,10] and presumably, GATA6 is important for the formation and maintenance of the differentiated state of ovarian surface epithelial cells. Thus, loss of GATA6 in ovarian cancer cells may be the underlying mechanism of dedifferentiation.

Loss of GATA6 Function in Ovarian Cancer

GATA factors are often expressed in the differentiated cells after completion of development. However, in adult cells, the functions of these transcription factors have not been as extensively investigated. One can speculate that the expression of GATA factors in the differentiated cells functions to maintain the differentiated states of the cells in postdevelopment tissues. Thus, GATA transcription factors play a critical role in lineage determination during development and function in maintenance of cell differentiation in adult tissues.[10]

GATA6 is expressed in many epithelial cell types, and GATA6 likely plays a role in the initial differentiation of the cells.[10] The critical role of GATA6 and other family members in the differentiation and cell lineage formation has been investigated in the development of colon,[20] lung,[18] mammary glands,[24,25] and liver.[17] Both GATA4 and GATA6 are expressed in the epithelial cells of morphologically normal human ovarian surface epithelium.[5,6] By immunostaining, all of the surface epithelial cells are intensely positive for GATA6 staining in the nucleus. Western blot analysis and immunofluorescence microscopy also confirmed the expression of GATA6 in isolated primary surface epithelial cells from human and rat ovaries.[5]

The investigation of GATA6 expression in ovarian cancer was first prompted by the earlier finding of the loss of expression of Dab2,[26] which is a transcription target of GATA6.[27] The loss of Dab2 was found to be an early event in ovarian tumorigenicity, correlating closely with dysplastic morphologic transformation of ovarian surface epithelia.[26,28,29] It was suggested that the distortion of an ovarian surface epithelium needs two factors: the loss of Dab2 expression and the loss of a collagen IV and laminin that compose the basement membrane.[28] Interestingly, both Dab2 and collagen IV are regulated by GATA6 during the differentiation of embryonic stem cells to epithelial-like extraembryonic endoderm cells.[27] Indeed, the finding that GATA6 expression is lost in ovarian cancer fits with the hypothesis that GATA factors might function in the maintenance of ovarian surface epithelial differentiation by regulating expression of Dab2 and collagen IV. In immunostaining of ovarian cancer, GATA6 was found completely lost in 30% of ovarian cancer in the initial report.[5] With the subsequent accumulation of additional samples and analyses, the current estimate of the percentage of GATA6-negative cancer is higher, about 50%. The GATA6 staining patterns of most of the rest of ovarian cancer cases are abnormal, either very weak, cytoplasmic instead of nuclear

staining, or only present in a fraction of cancer cells. The truly GATA6-positive ovarian cancer, with strong nuclear staining and good quality tissues, is less than 10%. Thus, it is certain that about 90% of ovarian cancer exhibits a loss of GATA6 function, either by the absence or the mislocalization of the GATA6 protein.

The loss of GATA6 was also demonstrated in cultured cells by both Western blots to examine the protein or Northern blot to measure mRNA.[5,6] Primary ovarian surface epithelial cells show strong GATA6 expression as determined by Western blot, Northern blot, and immunofluorescence microscopy. In contrast, GATA6 mRNA and protein are absent in the majority of ovarian cancer cell lines, suggesting that the loss of GATA6 in ovarian cancer is at the transcription level.[6] The ovarian clear cell carcinoma line, ES2, in which both GATA6 and Dab2 are expressed strongly, is one of the few exceptions among ovarian cancer cell lines.[5] Even in some of the nontumorigenic HIO lines, GATA6 is already absent, correlating with the loss of expression of Dab2, collagen IV, and laminin. These HIO lines are primary ovarian surface epithelial cells transfected with the SV-40 T-antigen to prolong life span in culture.[5] Thus, it appears that loss of GATA6 is an early event and not a consequence of neoplastic transformation, and loss of GATA6 is common in ovarian cancer.

In some ovarian cancer classified as GATA6-positive, heterogeneity in the expression of GATA6 among cancer cells of the same tumors is a general feature. Often, 10% to 20% of the tumor cells that are GATA6-positive in the nucleus are scattered among GATA6-negative tumor cells. It is not known if these GATA6-positive cells are derived from cancer cells by regaining their GATA6 expression or if these GATA6-positive tumor cells have never lost the expression. Thus, variable GATA factor expression exists in morphologically indistinguishable tumors cells. This heterogeneity in GATA factor expression may contribute to the heterogeneity among cancer cells within a tumor mass.

Beside loss of expression, the inactivation of GATA6 function may be the result of mislocalization of GATA6 protein in the cells. In some of the ovarian cancers in which GATA6 expression was classified as positive, the staining appears to be cytoplasmic rather than nuclear.[5] Analysis of established ovarian cancer cell lines by immunofluorescence microscopy has confirmed that in some cell lines such as SKOV-3 cells, GATA6 is cytoplasmic, whereas it is nuclear in others such as ES2. In primary ovarian surface epithelial cells, GATA6 is nuclear. Because GATA6 is a transcription factor, its function is believed to reside in the nucleus, where GATA6 interacts with specific promoters to modulate transcription. Thus, mislocalization of GATA6 predicts a loss of its cellular function. By combining the number of tumors that have lost or have nuclear exclusion GATA6 protein, the majority (90%) of ovarian cancer lose the function of GATA6.

The loss of GATA6 expression correlates closely with neoplastic transformation of the ovarian surface epithelium, as observed in contiguous ovarian surface epithelium connecting morphologically normal to malignant cells (Fig. 7.5). In previous studies,[25,28] the ovarian surface epithelium immediately

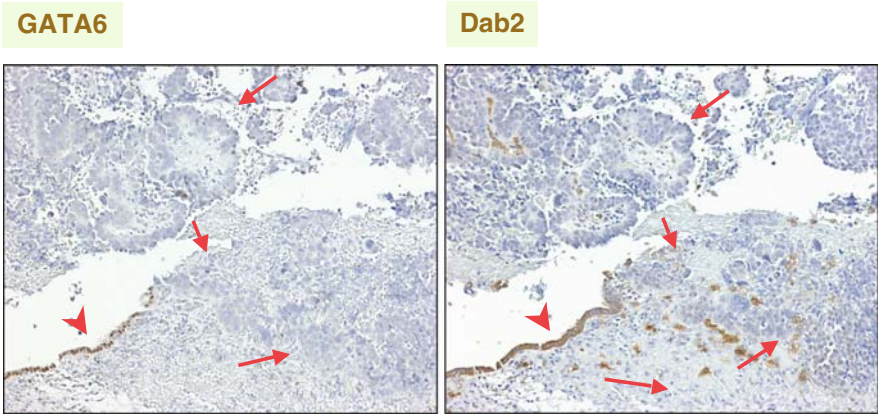

Fig. 7.5 Loss of GATA6 correlates closely with neoplastic transformation of ovarian surface epithelial cells. An example of human ovarian cancer stained with GATA6 and Dab2 in adjacent tumor sections. This slide shows an ovarian surface epithelium that consists of a morphologic normal surface epithelium (arrowhead) contiguously linking malignant cells (arrow) either invaded into stroma or expanded into areas around the ovary. The epithelial cells in the morphologic normal epithelium are positive for GATA6 and Dab2, and the malignant cells are negative for GATA6 and Dab2. Some scattered cells in the stroma are Dab2 positive but negative for GATA6. These cells are macrophages in which Dab2 expression is not GATA6-dependent

adjacent to tumor areas was considered to be preneoplastic. These preneoplastic lesions often lack an intact basement membrane as indicated by the absence of collagen IV and laminin staining, as well as the loss of Dab2 expression. In the morphologically normal epithelia immediately adjacent to tumor areas, both GATA6 and Dab2 are positive. However, the contiguously connected neoplastic cells are negative for both GATA6 and Dab2 staining. The neoplastic cells in the transformed epithelium that is immediately connected to the morphologically normal ovarian surface epithelial cells are likely most similar to the normal cells and thus are considered early tumor cells. Such analysis suggests that the loss of GATA6 is an early event in ovarian epithelial transformation.

Loss of GATA4, GATA5, and GATA6 factors has been implicated in colon, lung, and gastric cancer development.[30,31] Loss of GATA3 is reported to be involved in the loss of differentiation and development of mammary tumors.[32] GATA6 was identified as a tumor suppressor of gliomas.[33,34] In ovarian cancer, GATA6 expression is lost or the protein is mislocalized: cytoplasmic instead of nuclear. The loss of GATA6 is an early and general event and correlates closely with neoplastic transformation of ovarian surface epithelia.[5,6] It is suggested that loss of the differentiation–determining GATA6 accounts for the dedifferentiation of epithelial characteristics in tumors as GATA6 inactivation is associated with the loss of proteins required for epithelial organization, such as Dab2 and laminin.

Mechanism for the Loss of GATA Factors in Ovarian Cancer

The majority of ovarian cancer is negative for GATA6 expression; however, GATA6 gene deletion or mutation is not common, and additional mechanisms must explain the loss of GATA6 function in ovarian cancer. In a subset of ovarian cancer, GATA6 protein is present but does not reside in the nucleus where GATA6 protein fulfills its function in transcriptional regulation.[5] Thus, one mechanism for GATA6 inactivation is mislocalization. GATA6 has a classic nuclear localization motif, such that aberrant changes in the nuclear importing machinery may affect GATA6 nuclear entry. Alternatively, gain of a cytoplasmic protein that sequesters GATA6, or loss of a nuclear protein that can help to station GATA6 in the nucleus, may be an additional possibility. GATA factors are known to interact with coreceptors, such as Nkx type transcription factors or FOG (friend of GATA).[10] It is possible that the absence of a GATA6 cofactor may affect GATA6 nuclear localization and function in transcriptional regulation. These potential mechanisms for the mislocalization of GATA6 remain to be investigated.

Epigenetic silencing of GATA6 is likely a mechanism for the loss of GATA6 expression in ovarian cancer, as that was established in ovarian cancer cell lines. Chromatin remodeling and chromatin structure are thought to be responsible for establishing and maintaining states of differential gene expression and thus cell functional differentiation during embryonic development.[35] The various histone modifications that can determine transcriptional activity are known as the "histone codes."[36] Multiple acetylations at both histone H3 and H4 subunits lead to a relatively open chromatin structure and are associated with active transcription; lack of histone acetylation (hypoacetylation) produces a more condensed chromatin structure and correlates with transcription silencing.[37] The modulation of the levels of acetylation at histone H3 and H4 are catalyzed by enzymatic activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Additionally, the unacetylated histone lysine residues can be further modified by histone methyltransferases (HMTs) to further strengthen the transcriptional status. Histone H3 di- or trimethylation at lysine 4 also associates with active transcription.[38,39] In contrast, histone H3 di- and trimethylation at lysine 9 are associated with transcription silencing. The histone H3 lysine methylation recruits the chromodomain-containing proteins HP1 to the locus and leads to the assembling of heterochromatin, a transcriptional inactive state.[40]

In cancer, genes can be silenced after changes in DNA and histone epigenetic modification. Epigenetic modulation of chromatin through DNA methylation on CpG sites, histone covalent methylation or acetylation, and RNA-associated interference are thought to be common mechanisms accounting for the alterations of gene expression in cancer.[41–43] Aberrant methylation of CpG islands, CpG-rich sequences around promoter regions, is generally believed to be the cause of gene silencing.[42,43] The methylated nucleotide of the CpG islands then recruits histone-modifying enzymes that modify histone of the chromatin in those regions, resulting

in changes in transcriptional states.[42] The promoter region of the GATA6 gene contains CpG-rich sequences. However, in GATA6-negative ovarian cancer cells, the GATA6 promoter was found unmethylated.[6] Treatment with 5-azadeoxycytidine, a DNA methyltransferase inhibitor, did not reactivate GATA6 expression, consistent with the finding that the GATA6 promoter is unmethylated in ovarian cancer cells. Thus, methylation of promoter CpG island appears not to be a common mechanism for the silencing of GATA6 in ovarian cancer.

Histone modification and transcription silencing can occur before DNA methylation, as has been reported for transgenes[44] and in the case of p16INK4a.[45] However, without CpG methylation, the patterns of histone modification at the GATA6 promoter region of GATA6-positive normal cells and GATA6-negative cancer cells differ significantly, as determined by chromatin immuno-precipitation (ChIP) assays.[6] The loss of GATA6 expression associates with histone H3 and H4 hypoacetylation and loss of histone H3/lysine 4 trimethylation at the promoter, compared with GATA6-positive ovarian surface epithelial or cancer cells. Either an increase in HDAC or a decrease in HAT activity specifically associated with GATA6 gene loci, but not a change in the global activities of the histone-modifying enzymes, may account for the GATA6 promoter histone H3 and H4 hypoacetylation.[6] However, the presence of high levels of histone H3/lysine 4 dimethylation, the absence of histone H3/lysine 9 dimethylation, and the lack of HP1 association with the loci suggest that the GATA6 gene exhibits euchromatic, but not heterochromatic, structures despite the silencing of GATA6 expression. Consistently, inhibition of HDACs by the inhibitor trichostatin A is sufficient to restore the acetylation of histone and reexpression of the GATA6 gene in ovarian cancer cells. Inhibition of histone deacetylation increases the DNase I sensitivity of the GATA6 promoter. DNase I sensitivity indicates the accessibility of the DNA strands, correlating with the openness of the chromatin conformation. A recent report[46] shows that GATA4 and GATA5 promoters are methylated in a fraction of ovarian cancer cell lines examined. Thus, the exact mechanism for the silencing of GATA factors may vary among cell lines. Nevertheless, the opened or condensed chromatin conformations as a result of epigenetic marking determine the expression of GATA6 and thus dedifferentiation in ovarian cancer cells.

Thus, in ovarian cancer cells, the alteration of chromatin conformation as a result of histone modification, including the hypoacetylation of histone H3 and H4 and the associated reduction in di- and trimethylation of histone H3 lysine 4, is a causative mechanism for the loss of GATA6 expression. No consistent global changes in histone-modifying enzymatic activities were identified to explain the changes in histone modification. One possibility is that the alteration of histone modification at the GATA6 loci is a result of a random error and infidelity of the epigenetic marking machineries. However, the direction of the drift may favor the silencing of GATA6, assuming the loss of GATA6 allows the cells to escape the constraint of the tissue environment and proliferate. Ultimately, cells with modified chromatin and silencing of GATA6 expression may emerge.

Therefore, before factors that can regulate and promote dedifferentiation are identified, a hypothesis is put forward that dedifferentiation may be the result of errors and infidelity in the maintenance of histone codes and the subsequent selection of dedifferentiated cells, which escape growth regulation imposed on the differentiated cells of the tissue environment.

Consequence of GATA6 Inactivation and Epithelial Dedifferentiation

In ovarian cancer, the loss of GATA6 correlates closely with the neoplastic morphologic transformation of ovarian surface epithelia (Fig. 7.5).[5,6] The identification of potential links of the loss of GATA6 to the possible dedifferentiated cell properties and neoplastic phenotypes is an important task. Presumably, the effectors are the transcriptional targets of GATA6, and several GATA6 transcriptional targets with tissue specific functions are known.[10] For example, in the comparison of endoderm differentiation of wild-type and GATA6 null embryonic stem cells, several GATA6 transcriptional targets, including Dab2 and collagen IV, have been identified.[27]

In ovarian surface epithelial cells and derived carcinomas, GATA6 and Dab2 expressions are closely correlated (Fig. 7.5).[5,6] The consequential loss of Dab2 and laminin after GATA6 suppression by small interfering RNA (siRNA) was demonstrated in ovarian epithelial cells.[5] A transcriptional relationship between GATA6 and Dab2 has been established; however, whether GATA6 directly regulates the transcriptional expression of laminin and collagen IV has not yet been demonstrated.

The GATA6 transcription target Dab2 is a tumor suppressor, and loss of Dab2 is an early step in ovarian tumorigenicity.[26] Dab2 is an adaptor protein in cellular vesicular trafficking,[47] and Dab2 associates with endocytic cargos through its binding to a N-P-X-Y motif often found in the cytoplasmic tail of glycoproteins such as low-density lipoprotein (LDL) receptor family members, as well as to adaptor protein 1 (AP-1) and the vesicular coat protein clathrin. The C-terminal part of Dab2 binds the motor protein myosin VI. As a result, Dab2 mediates directional transport of cargos along actin filaments. The directional trafficking of the cargos is a critical component of epithelial polarity, and the loss of apical polarity of the endoderm cells in Dab2 null embryos is thought to be the basis for the disruption of epithelial structure of the extra-embryonic endoderm.[48] The early lethality and embryonic phenotype of Dab2 knockout resembles the close correlation between Dab2 loss and morphologic neoplastic transformation of ovarian surface epithelia. Thus, loss of Dab2 likely accounts for a major factor in the loss of structural organization of ovarian surface epithelia in cancer.[29]

When ovarian surface epithelial cells in culture are treated with specific siRNA to downregulate GATA6 expression, Dab2 and laminin expression

are lost as a consequence of GATA6 suppression, though the expression of collagen IV was not determined in the study.[5] In ovarian cancer tissues, the expression of GATA6 and collagen IV is not well correlated as determined by immunostaining. In some ovarian cancer cell lines, either GATA4 or GATA6 is absent, though collagen IV is expressed.[49] It is possible that tumor cells lose the expression of a GATA factor and collagen IV initially, and collagen IV expression is a gain-of-function that occurs in later stages of tumor development. This would be consistent with the observation that ovarian cancer cells often lose extracellular collagen IV and laminin initially, and the restoration of collagen IV and laminin expression correlates with tumor cell spreading in later stages.[49]

The majority of ovarian malignancies are derived from ovarian surface epithelial cells, which are flat or cuboidal cells, form a single-cell layer of epithelium, and are organized by a sheet of basement membrane.[50] Normal ovarian surface epithelial cells are polarized, and Dab2 is essential to maintain the epithelial polarity, which is also important for epithelial organization.[8] The basement membrane consisting of a layer of collagen IV and laminin, which are produced and deposited by the epithelial cells, is also important for the organization of epithelium.[28] The expression of Dab2 that is involved in establishment and maintenance of epithelial polarity and collagen IV and laminin that compose the epithelial basement membrane are markers or characteristics of epithelium, and the losses of Dab2, laminin, and collagen IV as results of GATA6 suppression indicate the loss of epithelial characteristics or dedifferentiation. Suppression of GATA6 and dedifferentiation would result in basement membrane independence and loss of apical polarity of epithelial cells. Based on the idea that GATA6 determines cell lineage differentiation in development, the loss of GATA6 functions and the potential transcription targets such as the epithelial specific markers Dab2, collagen IV, and laminin was postulated to be the underlying mechanism for epithelial dedifferentiation in ovarian cancer[5](Fig. 7.6). Indeed, losses of Dab2 and basement membrane are two events closely associated with neoplastic transformation of ovarian surface epithelia[28] and are proposed to be the required factors for disorganization and morphologic distortion of ovarian surface epithelia in tumorigenesis. Additionally, it is likely GATA6 regulates the expression of additional genes involved in other aspects of epithelial function and regulation, and loss of GATA6 has additional as yet unidentified consequences or targets in the neoplastic transformation of ovarian surface epithelial cells.

A scenario can be postulated that impairment in executing epigenetic inherent markers such as the histone acetylation status of GATA6 promoter leads to a gradual drift in epigenetic coding and ultimately the loss of GATA6 expression in some cells. The absence of GATA6 leads to the loss of expression of its transcription targets including Dab2, collagen IV, and laminin and presents the cells in a state of dedifferentiation. The dedifferentiated cells no longer obey the growth regulation imposed by epithelial tissue structure, and the dedifferentiated state of the cells will collaborate with acquired oncogenic mutations to initiate the development of ovarian tumors.

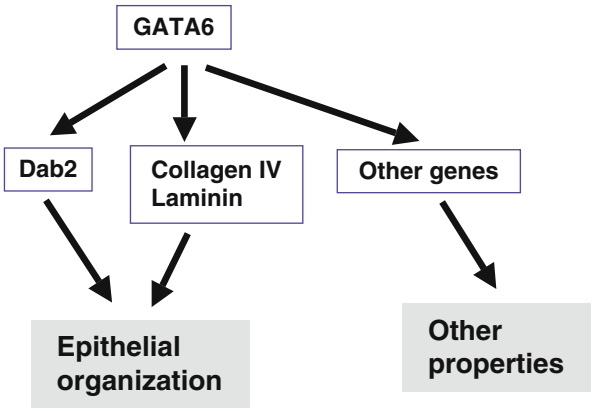

Fig. 7.6 Role of GATA6 in epithelial organization by the regulation of gene expression in ovarian surface epithelial cells. The scheme illustrates the role of GATA6 in the expression of Dab2, collagen IV, laminin, and additional unidentified genes. Dab2 is required for maintaining apical polarity of the ovarian surface epithelial cells. Laminin and collagen IV compose a basement membrane that is important for the organization of an epithelium. The loss of Dab2-medited epithelial apical polarity and a basement membrane are two critical events in the morphologic transformation and disorganization of an epithelium

Aberrant Differentiation of Cancer Cells and the Concept of Cancer Stem Cells

The stem cell concept explains the process of embryonic development.[51] In mammals, the entire embryo is derived from pluripotent cells of the inner cell mass.[8] These stem cells of the early embryos, known as embryonic stem cells, can be isolated, cultured, and expanded in vitro and can be used to derive genetically engineered animals. In the embryos, these pluripotent embryonic stem cells progressively differentiate into multipotent, unipotent, and differentiated cells of various tissue types. Multipotent and unipotent stem cells have also been identified in some postdevelopmental tissues. These tissue stem cells may differentiate into specific cell types in the tissues and provide the ability for tissue renewal and repair functions.

The concept of cancer stem cells, or initiating cells, has recently been revisited and started an excitement in the cancer research field.[52,53] The cancer stem cell theory is analogous to the stem cell concept in developmental biology. The hypothesis of the cancer stem cell theory holds that only a subpopulation within the cells of the tumor mass has the property of "stem cells," which may proliferate, self renew, and change/differentiate to give rise to the heterogeneous populations of neoplastic cells composing the tumor mass. These cancer stem cells are thought to underlie the ability for cancer to metastasize, to become drug-resistant, and to reoccur. Obviously, the concept of cancer stem cells makes drastic impact on the thinking on cancer etiology, mutation theory, and therapeutic strategy.

Several reports have claimed the isolation of ovarian cancer stem cells[54,55] or the "side population," a more conservative reference to the cancer stem cells.[56] The existence and the definite identification of ovarian cancer stem cells are urgent issues, and these will likely be resolved soon as many labs are actively pursuing these questions. The origin of the cancer stem cells is a critically important question that will impact on the understanding of the development of ovarian cancer. In theory, ovarian cancer stem cells may derive either from the mutations of the primordial cells (referred to as tissue stem cells) or from the dedifferentiation of ovarian surface epithelial cells (Fig. 7.7). During organogenesis in embryonic development, presumably, GATA6-positive ovarian surface epithelial cells are derived from a primordial cell type that is GATA6-negative. In adult ovaries, the existence of tissue stem cells, or primordial cells, that can generate ovarian surface epithelial cells has not been established. However, ovarian surface epithelial cells need to be constantly replenished over the reproductive period. During ovulation, the ovarian surface is ruptured at the area where the ovum is released. It is thought that the normal ovarian surface epithelial cells, not a special population of stem/primordial cells, surrounding the wound proliferate and heal the opening.[57] Yet another possibility is that during oncogenic transformation, the GATA6-positive ovarian surface epithelial cells undergo dedifferentiation, and GATA6 expression is lost. Possibly, some fraction of these dedifferentiated cancer cells may revert to an aberrant differentiated state that resembles the stem or primordial cells that exist during embryonic development. These dedifferentiated cancer cells may then further

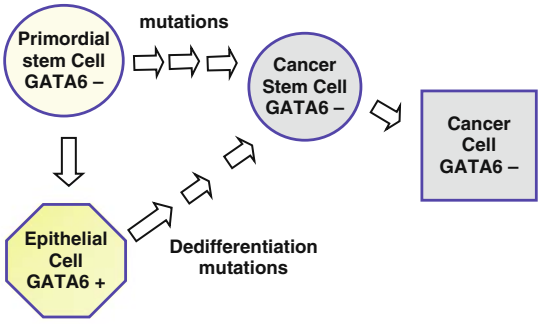

Fig. 7.7 Relationship of dedifferentiation to ovarian cancer stem cells. An illustration of relationships between precursor cells, cancer cells, and cancer stem cells is shown. As an analogy to the derivation of extraembryonic endoderm cells from pluripotent cells of the inner cell mass, the GATA6-positive ovarian surface epithelial cells are presumably derived from a GATA6-negative primordial cell lineage. Cancer stem cells might either originate from the accumulated mutations of this primordial cell lineage or might form as a result of dedifferentiation and progressive mutations of the ovarian surface epithelial cells. These cancer stem cells would be able to "differentiate," or change to produce a heterogeneous population of cells composing the tumor masses

change/differentiate to generate a heterozygous population of ovarian cancer cells and mediate the disseminations and spreading of ovarian cancer.

Conclusion

Rather than a subjective observation of cell morphology, we define "dedifferentiation" in cancer as a loss of critical gene(s) required for the differentiation/derivation of the cells from a primordial cell. The inactivation of this critical differentiation gene(s), often a transcription factor, results in the drastic alteration of gene expression profile, which makes the cancer cells show behaviors distinct from the normal precursor cells.

Recent findings lead to a suggestion that the loss of the GATA6 transcription factor may underlie the mechanism of "dedifferentiation" of ovarian cancer cells. GATA6 appears to be inactivated in ovarian cancer cells by alterations in histone modification and changes in chromatin conformation that result in a transcriptional inactive state. The observed consequences of the loss of GATA6 in ovarian surface epithelial cells further suggest that dedifferentiation is not an incidental bystander of neoplastic transformation but a critical co-conspirator and an active participant. In brain tumors, GATA6 was found to be a tumor suppressor gene.[33,34] It is then reasoned that dedifferentiation is an important factor in neoplastic transformation, and dedifferentiation, in synergy with mutations of critical genes, enable the cancer cells to display their malignant phenotypes (Fig. 7.8). Lastly, further understanding of dedifferentiation may lead to improved comprehension of cancer etiology and provide rationale and strategy for differentiation therapy and epigenetic targeting in the prevention and treatment of cancer.

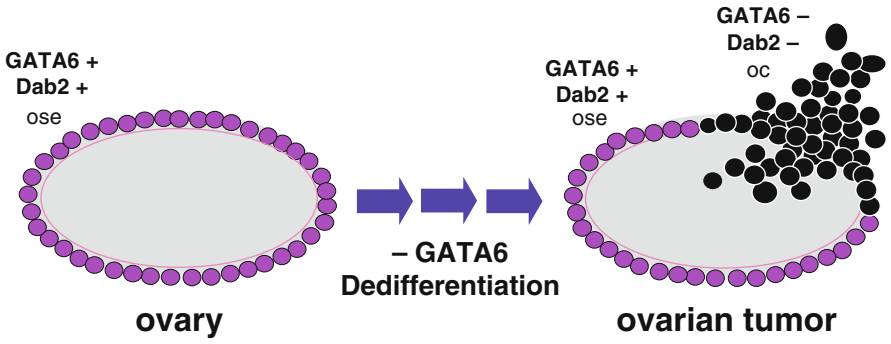

Fig. 7.8 Hypothesis that dedifferentiation as defined by the loss of GATA6 contributes to morphologic transformation and is an important step in ovarian epithelial tumorigenesis. Dedifferentiation, or the loss of GATA6 that is key in maintaining differentiation status, is a step in ovarian tumorigenesis. Loss of GATA6 leads to the loss of Dab2, a transcription target. Loss of Dab2 is thought to contribute to morphologic transformation of ovarian surface epithelium

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Chapter 8
The Human Kallikrein Gene Family: New Biomarkers for Ovarian Cancer

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The Human Kallikrein Gene Family

Structure and Genomic Organization

The term "kallikrein" (derived from the Greek *kallikreas*, for pancreas) was introduced in the 1930s to describe proteolytic enzymes that can release small vasoactive peptides from high-molecular-weight precursors. There are two categories of human kallikreins; the plasma and the tissue kallikreins. The plasma kallikrein is encoded by a single gene on chromosome 4. This enzyme (a serine protease) releases the vasoactive peptide bradykinin from a high-molecular-weight precursor synthesized in the liver.[1] The human tissue kallikrein family is localized on chromosome 19 and also encodes for serine protease enzymes.[2–4]

Recently, a new classification emerged for tissue kallikreins that is not based on the functional definition but rather on structural criteria and map location. Based on the newer definition, the number of genes that are included in this family increased to 15, a number that is comparable with that of homologous families found in rat and mouse.[5,6] Because all kallikreins (except KLK1) do not have classic "kallikrein" activity, they are better defined as "kallikrein-related peptidases." A list of the official names of all kallikrein genes and proteins is included in Table 8.1, and a schematic diagram showing the human tissue kallikrein gene locus on chromosome 19q13.4 is shown in Fig. 8.1. All kallikrein genes map within an approximately 300-kb region, and the lengths of the genes, the distances between them, as well as the direction of transcription have now been accurately defined.[7,8] The kallikrein family is bounded from the telomeric side by the Siglec family of genes[9] and

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Cancer Treatment and Research 149, DOI 10.1007/978-0-387-98094-2_8,

Table 8.1 Official and other gene and protein names for members of the human kallikrein gene family

Official Gene Symbol	Other Names/Symbols	GenBank Accession Number	UniGene Cluster	OMIM ID	SwissProt ID
KLK1	Pancreatic/renal kallikrein, hPRK	M25629 M33105	Hs.123107	147910	Q07276
KLK2	Kallikrein-related peptidase 2 Human glandular kallikrein 1, hGK-1	M18157	Hs.181350	147960	P20151
KLK3	Kallikrein-related peptidase 3 Prostate-specific antigen, PSA, APS	X14810 M24543 M27274	Hs.171995	176820	P07288
KLK4	Kallikrein-related peptidase 4 Prostase, KLK-L1, EMSP1, PRSS17, ARM1	AF113141 AF135023 AF148532	Hs.218366	603767	Q9Y5K2
KLK5	Kallikrein-related peptidase 5 KLK-L2, HSCTE	AF135028 AF168768	Hs.50915	605643	Q9Y337
KLK6	Kallikrein-related peptidase 6 Zyme, Protease M, Neurosin, PRSS9	AF013988 AF149289 U62801 D78203	Hs.79361	602652	Q92876
KLK7	Kallikrein-related peptidase 7 HSCCE, PRSS6	L33404 AF166330	Hs.151254	604438	P49862
KLK8	Kallikrein-related peptidase 8 Neuropsin; Ovasin; TADG-14, PRSS19, HNP	AB009849 AF095743 AB010780 AF055982	Hs.104570	605644	O60259
KLK9	Kallikrein-related peptidase 9 KLK-L3	AF135026	Hs.448942	605504	Q9UKQ9
KLK10	Kallikrein-related peptidase 10 NES1, PSSSL1	AF055481 NM_002776	Hs.69423	602673	O43240
KLK11	Kallikrein-related peptidase 11 TLSP/Hippostasin, PRSS20	AB012917	Hs.57771	604434	Q9UBX7
KLK12	Kallikrein-related peptidase 12 KLK-L5	AF135025	Hs.159679	605539	Q9UKR0
KLK13	Kallikrein-related peptidase 13 KLK-L4	AF135024	Hs.165296	605505	Q9UKR3
KLK14	Kallikrein-related peptidase 14 KLK-L6 protein	AF161221	Hs.283925	606135	Q9P0G3
KLK15	Kallikrein-related peptidase 15 Prostinogen, HSRNASPH	AF303046	Hs.250770	610601	Q9H2R5

OMIM, Online Mendelian Inheritance in Man.

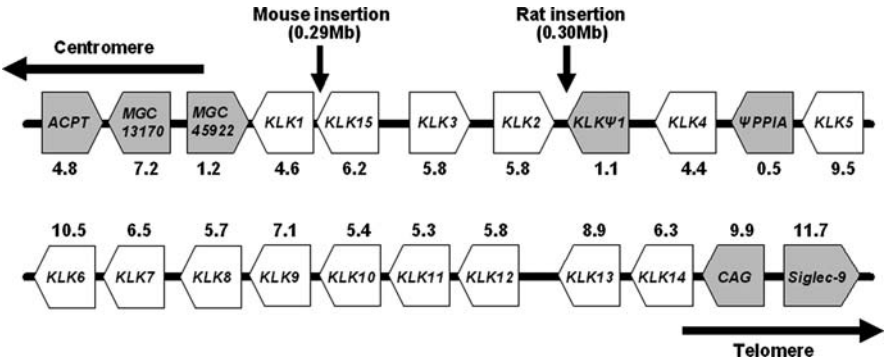

Fig. 8.1 Schematic presentation of the human kallikrein locus on chromosome 19q13.4. Gene locations are indicated by arrowheads that show the direction of transcription. Gene lengths are shown in kilobases. Position of the location of expanded regions in the mouse and rat genome that contain closely related *KLK1* paralogs is indicated. Non-kallikrein genes are shaded in gray

centromerically by the testicular acid phosphatase gene (ACPT).[10] New, uniform nomenclatures are now established for the human tissue kallikreins and their rodent orthologs.[11,12] There are many common structural features of the human kallikrein genes and proteins.[13] All genes are formed of five coding exons, and most of them have one or more extra 5′ untranslated exons. The first coding exon always contains a 5′ untranslated region, followed by the methionine start codon, located ~50 bp away from the end of the exon. The stop codon is always located ~156 bp from the beginning of the last coding exon. Moreover, exon sizes are nearly identical, and the positions of the residues of the catalytic triad of serine proteases are conserved. All kallikrein proteins are synthesized as a pre/pro peptides with a signal peptide of about 17–20 amino acids at the amino terminus, followed by an activation peptide of about 4–9 amino acids (with the exception of KLK5), followed by the mature (enzymatically active) protein. Finally, all proteins contain 10–12 cysteine residues that will form 5–6 disulfide bonds. The position of the cysteine residues is also fully conserved.

Tissue Expression and Hormonal Regulation

Many kallikreins are transcribed predominately in few tissues, as indicated by Northern blotting. By using the more sensitive RT-PCR technique, kallikreins are found to be expressed at lower amounts in several other tissues. The tissue expression of kallikrein mRNAs and proteins is summarized elsewhere.[14] Kallikrein abundance can be categorized as highly restricted (KLK2 and KLK3 in prostate), restricted (KLK5 in skin, salivary gland, breast, and esophagus; KLK6 in brain and central nervous system; KLK7 in esophagus, heart, liver, and skin; KLK8 in breast, esophagus, skin, and tonsil; KLK13 in

esophagus and tonsil), or wide (KLK1, 4, 9–12, 14, and 15). Interestingly, many kallikreins are expressed in endocrine-related organs, including the prostate, testis, ovary, and breast.[15]

In the ovary, there is abundant expression of the mRNA of KLK6–8 and KLK10, followed by lower levels of KLK1, 9, 11, 14, and 15. At the protein level, KLK1, 6, 7, 10–11 show highest expression levels, followed by KLK8 and KLK14.[14] Given the coexpression of many kallikreins in the same tissue, it is possible that these kallikreins may act in concert in cascade pathways, reminiscent of the coagulation and apoptotic processes.

Several reports confirmed that many kallikreins are under steroid hormone regulation.[16–18] An interesting observation is the different patterns of hormonal regulation in different tissues (e.g., KLK4 is upregulated by androgen in prostate and breast cancer cell lines and by estrogen in endometrial cancer cell lines).

Kallikreins in Normal Physiology

From a functional point of view, kallikreins are serine proteases (SPs). SPs are peptidases with a uniquely activated serine residue in the substrate-binding pocket. They are involved in many vital functions such as digestion, blood clotting, fibrinolysis, fertilization, and complement activation and are related to many diseases including cancer, arthritis, and emphysema.[19]

Accumulating evidence indicates that kallikreins might have diverse functions in different tissues and developmental stages. KLK1 has a known role in blood pressure regulation by cleaving low-molecular-weight kininogen to produce vasoactive kinin peptides. Intact kinin binds to bradykinin B_2 receptor in target tissues and exerts a broad spectrum of biological effects including blood pressure reduction via vasodilation, smooth muscle relaxation or contraction, pain induction, and mediation of the inflammatory response.[19] Low renal synthesis and urinary excretion of tissue kallikreins have been linked to hypertension in animals and humans.[20] Apart from its kininogenase activity, KLK1 has been implicated in the processing of growth factors and peptide hormones in light of its presence in pituitary, pancreas, and other tissues. As summarized by Bhoola et al.,[19] KLK1 has been shown to cleave proinsulin, low-density lipoprotein, prorenin, angiotensinogen, vasoactive intestinal peptide, procollagenase, and the precursor of atrial natriuretic factor.

KLK3 (also known as prostate specific antigen; PSA) has been shown to rapidly hydrolyze semenogelin I and semenogelin II, as well as fibronectin, resulting in liquefaction of the seminal clot after ejaculation.[21] Several other potential substrates for KLK3 have been identified, including IGFBP-3, TGF-β, parathyroid hormone-related peptide, and plasminogen.[22] KLK2 is found to be able to cleave semenogelin I and semenogelin II but at different cleavage sites and with lower efficiency than that of KLK3.[23] The mouse and porcine orthologs of KLK4 were originally designated "enamel matrix serine proteases" because of their predicted role in normal teeth development.[24] Recent evidence

shows that a splice variant of kallikrein 4 is a predominately nuclear protein that might have a role in controlling gene expression.[25]

A few kallikreins, especially KLK5 and KLK7, are expressed in the stratum corneum of the skin and are known to be involved in desquamation of corneocytes.[26] Another group of kallikreins, KLK6, KLK8, and KLK11, are highly expressed in the central nervous system where they are thought to play a role in neural plasticity.[27] Another possible mechanism for kallikrein action is the activation of proteinase-activated receptors (PARs). Activation of these receptors elicits different responses in several tissues. In addition, they switch-on cell signaling pathways (e.g., the MAP kinase pathway), leading to cell growth and division.[28]

Regulation of Kallikrein Activity

Kallikrein activity is controlled at both the mRNA and protein levels. Besides KLK3 and KLK2, and more recently KLK10, no other kallikrein gene promoter has been functionally tested. TATA box variants are found in the three classic kallikreins (KLK1–3).[29] Also, androgen response elements have been identified and experimentally verified.[30] No obvious TATA boxes are found in the promoter of other kallikreins. At the protein level, there are different mechanisms for controlling serine protease activity by which unwanted activation is avoided and precise spatial and temporal regulation of the proteolytic activity is achieved. One important mechanism is by producing kallikreins in an inactive "proenzyme" (or zymogen) form, which is activated as necessary. The N-terminal extension of the mature enzyme, or the "prosegment," sterically blocks the active site and thus prevents binding of substrates. The activation of the zymogen can occur intracellularly (i.e., in the trans-Golgi apparatus or in the secretory granules) or extracellularly after secretion, and it can be autolytic or dependent on the activity of another enzyme. Autoactivation is a common phenomenon among kallikreins. KLK2, but not KLK3, is capable of autoactivation.[31] KLK4 is also autoactivated during the refolding process, and there is evidence that KLK6 is also capable of autoactivation.[32]

Proteolytic activation is irreversible. Hence, other means of switching off the activity of these enzymes are needed. Once activated, serine proteases are controlled by ubiquitous endogenous inhibitors.[33] Some molecular complexes of kallikreins with protease inhibitors have clinical applicability because they can improve the diagnostic sensitivity or specificity of cancer biomarkers such as PSA.[34]

The coexpression of many kallikreins in the same tissues and the parallel differential regulation of groups of kallikreins in pathologic conditions raise the possibility of the existence of a common mechanism that controls expression of groups of kallikrein genes in a cluster, as a "locus control region." Added to this are the relatively short distances between adjacent kallikreins (which could be as short as the 1.5 kb between *KLK1* and *KLK15*) and the absence of classic promoter sequences, as shown by prediction analysis, in all kallikreins except *KLK1–3*.

Kallikreins can be also targeted by microRNAs (miRNAs). The first bioinformatic prediction of the potential interaction between miRNAs and kallikreins with experimental verification has been recently published.[35] miRNAs represent an important tool of posttranscriptional regulation of kallikrein activity that can explain aberrancies between the mRNA and protein expression levels.[36]

Kallikrein Expression in Cancer

Kallikreins as Cancer Biomarkers

Accumulating evidence indicates that many kallikreins are differentially expressed in various malignancies. KLK6 (zyme/protease M) was originally isolated by differential display from an ovarian cancer library,[37] and KLK10 was cloned by subtractive hybridization from a breast cancer library[38] and later proved to act as a tumor suppressor gene.[39]

A number of kallikreins were shown to be putative prognostic and/or predictive cancer markers. In breast cancer, the expression of *KLK5* and *KLK14* is indicative of poor patient prognosis,[40] whereas higher levels of *KLK9*, *KLK13,* and *KLK15* mRNA and the KLK3 protein forecast a favorable disease outcome.[41] The apparent relationship between kallikreins and testicular cancer has been published,[42] and the differential expression of *KLK10*, *KLK14,* and *KLK13* splice variants in testicular cancer tissues have also been reported.[43]

A microarray study has identified at least one kallikrein (*KLK11*) is overexpressed in lung carcinoma.[44] Recently, *in silico* analysis provided evidence that some kallikreins are differentially regulated in pancreatic cancer.[45] This was confirmed by microarray analysis.[46] Recent evidence also indicates overexpression of three kallikreins (*KLK7*, *KLK8*, and *KLK10*) in colon cancer.[45] Another report showed downregulation of the KLK10 gene in acute lymphoblastic leukemia.[47]

The potential clinical utility of kallikreins as cancer biomarkers has been proved by many reports. Prostate-specific antigen (KLK3) and, more recently, human glandular kallikrein (KLK2) are useful biomarkers for prostate cancer.[48] KLK11 is also shown to be a potential marker for ovarian and prostate cancer.[49] Recent reports demonstrate that kallikrein mRNA and proteins can be useful serum biomarkers for diagnosis, monitoring, and prognosis of different cancers.[15] In addition to their diagnostic/prognostic utilities, kallikreins have potential for being used for therapeutic applications. A synthetic KLK1 inhibitor is found to suppress cancer cell invasiveness in human breast cancer cell lines.[50]

An interesting observation is that many kallikreins were found to be dysregulated in malignancies of different tissues, for example, KLK5 in ovarian and breast cancer[51] and the downregulation of *KLK14* in multiple malignancies.[52] This lack of "tissue specificity" points to the possibility that kallikreins might be involved in a "common" pathway or biological process that is involved in cancer initiation and/or progression.

Differential Expression of Kallikreins in Ovarian Cancer

The dysregulation of kallikreins in ovarian cancer is well documented. KLK6 was isolated by differential display from an ovarian cancer library.[37] Kallikreins were identified among the top differentially expressed genes in ovarian cancer in a global analysis.[53] Recently, an *in silico* analysis of kallikrein gene expression in ovarian cancer was performed by using the databases of the Human Genome Anatomy Project. This study showed that at least seven kallikreins are upregulated in ovarian cancer compared with that in normal ovarian tissues. This was also confirmed at the protein level.[54] A review showing the prognostic value of many members of the human kallikrein family in ovarian cancer has been also published.[18]

Subcellular Localization of Kallikreins in Ovarian Cancer

Immunohistochemistry (IHC) enables kallikrein protein distribution in different cell types, independently from its quantity in the tissue. In addition, it provides a semiquantitative analysis of expression levels. Because kallikreins are secreted proteins, it was not unexpected that immunostaining of kallikreins was mainly cytoplasmic and in some tissues displayed a characteristic pattern that was membranous, droplet-like, supranuclear, subnuclear, or luminal. KLK4 appears to be a notable exception. Recently, Xi et al. suggested that one variant of KLK4 is a predominately nuclear protein that is overexpressed in prostate cancer.[25] Many kallikreins were analyzed by IHC and showed upregulation in ovarian cancer compared with that in normal ovarian tissues. KLK4 is localized to the cytoplasm of ovarian cancer, but not normal cells, with focal membranous staining.[55] KLK10 and KLK14 are found to have an intracytoplasmic pattern of staining in the epithelial cells (and occasional stromal cells) of serous ovarian cancers.[56,57] Underwood et al., using peptide antibody against the KLK8 protein, showed cytoplasmic granular staining (that might represent a secretion pathway) in tumor cells of different histologic types. In endometrioid carcinoma, the staining was most prominent in the glandular lumens.[58] KLK9 shows moderate cytoplasmic staining, with no nuclear or stromal staining pattern in ovarian cancer cells.[59]

Kallikrein Splice Variants in Ovarian Cancer

The mechanism by which a single gene gives rise to more than one mRNA transcript is referred to as differential splicing. This system is often tightly regulated in a cell type–specific or developmental stage–specific manner and increases genome complexity by generating different proteins from the same mRNA. The presence of more than one mRNA form for the same gene is common among kallikreins. These variant mRNAs may result from alternative splicing, a retained intronic segment, or use of an alternative transcription

initiation site. To date, there are at least 82 documented splice variants of the 15 kallikrein genes.[60] A better understanding of alternative splicing can lead to the use of gene variants as drug targets, therapeutic agents, or diagnostic markers.[60] Slawin et al. reported a prognostic significance of a splice variant–specific RT-PCR assay for *KLK2* in detecting prostate cancer metastasis.[61] Nakamura et al. reported differential expression of the brain and prostate types of *KLK11* between benign, hyperplastic, and malignant prostate cancer cell lines.[62] Some of the alternatively spliced forms were also found to be tissue specific.

Several kallikrein splice variants were identified in ovarian cancer. Dong et al.[55] identified three alternative splice forms of KLK4 expressed in ovarian cancer tumor tissues and cell lines, but not in normal ovaries: one with intronic insertion from intron 3; the second has intronic insertion from intron 2 and exon 4 deletion, and the third has deleted exon 4. A novel *KLK5* mRNA transcript with a short 5′ untranslated region and a *KLK7* splice variant with a long 3′ untranslated region are highly expressed in ovarian cancer cell lines but are expressed in very low levels in normal ovarian epithelial cells.[63] Another splice variant was identified, *KLK5-SV2*, which is overexpressed in ovarian cancer tissues and cell lines.[64] A recent report showed that *KLK6-splice variant 1* is expressed at much higher levels in ovarian cancer compared with the "classic" variant.[65]

Mechanisms of Kallikrein Involvement in Ovarian Cancer

The mechanism by which kallikreins might be involved in the pathogenesis and/or progression of ovarian cancer is not yet fully understood. Preliminary reports indicate a possible role of kallikreins in controlling vital processes, like apoptosis, angiogenesis, and tumor metastasis by cleavage of specific substrates, including growth factors, hormone receptors, or connective tissue. The involvement in growth and apoptotic activities was reported for KLK3 (PSA), which can digest insulin-like growth factor–binding protein (IGFBP-3)[66] and parathyroid hormone–related protein (PTHrP).

As proteolytic enzymes, kallikreins can be involved in tumor progression because of their role in extracellular matrix degradation. Many studies have shown that a variety of proteolytic enzymes are overproduced either by the cancer cells themselves or by the surrounding stromal cells, with an associated unfavorable clinical prognosis. Experimental evidence indicates that KLK2 and KLK4 can activate the proform of another serine protease, the urokinase-type plasminogen activator (uPA).[67] Urokinase activates plasmin from its inactive form (plasminogen), which is ubiquitously located in the extracellular space leading to degradation of the extracellular matrix proteins. Plasmin can also activate precursor forms of collagenases, thus promoting the degeneration of collagen in the basement membrane surrounding the capillaries and lymph nodes. Another kallikrein, KLK7, can degrade the alpha chain of human fibrinogen.[68]

Modulation of angiogenic activity is another possible mechanism for kallikrein involvement in cancer. The kinin family of vasoactive peptides, liberated by

KLK1 action, is believed to regulate the angiogenic process.[69] It was recently reported that immunolabeling of KLK1 is intense in the angiogenic endothelial cells derived from mature corpora lutea.[69] Also, KLK3 is reported to have anti-angiogenic activities.[70]

A recent study has shown that expression of kallikreins increases the malignant behavior of ovarian cancer cells.[71] Transfecting cancer cells with kallikreins led to significantly increased invasive behavior, and when these cells were inoculated into the peritoneum of nude mice, they resulted in a remarkable increase in tumor burden.

Ovarian cancer is a "hormone-related" malignancy. Sex hormones are known to affect its initiation and/or progression.[72] Oral contraceptive pills decrease the risk of ovarian cancer,[73] and the growth of ovarian carcinoma cell lines is sensitive to estrogen.[74] Progesterone promotes cell differentiation and apoptosis, and it has been shown to inhibit DNA synthesis and cell division.[75] Also, studies have shown a prognostic value of the progesterone receptor in ovarian cancer.[76] Moreover, appreciable evidence implicates androgens in the pathogenesis of ovarian cancer[77] and supports the existence of a physiologic interaction between androgens and the ovarian surface epithelium, as well as the possible role of this interaction in ovarian neoplasia.[78] Androgens have also been shown to stimulate growth of rodent ovarian epithelial cells in vivo, leading to benign ovarian neoplasms.[79] Ovarian cancer patients have higher levels of circulating androgens than do women without cancer.[80] Additionally, the majority of ovarian cancers express androgen receptor (AR),[81,82] and ovarian cancer cell growth is inhibited in vitro by antiandrogens.[83] Recent observations show a correlation between AR and susceptibility to ovarian cancer.[82] Given the fact that most kallikreins are regulated by sex hormones,[17] kallikreins could represent downstream targets by which steroids are involved in the malignant process. This, however, could not be verified in a recent study.[65] The elevation of serum concentration of kallikreins in cancer could also be due to the increased vasculature (angiogenesis) of cancerous tissues and the destruction of the glandular architecture of the tissues involved, with subsequent leakage of these proteins into the general circulation.

Clinical Utility of Kallikreins in Ovarian Cancer

Kallikreins as Diagnostic Markers

The clinical utility of kallikreins in ovarian cancer spans both the diagnostic and prognostic applications. For diagnostic purposes, many kallikreins have been shown to be elevated, at both the mRNA and protein levels, in ovarian cancer compared with patients with normal ovaries. Kallikreins can be measured in serum, tissue, or ascites fluid.[84] Reports on the diagnostic value of kallikreins in ovarian cancer are summarized in Table 8.2. Among all kallikreins, KLK6 and KLK10 show the best promise as serum biomarkers for ovarian cancer, specially the serous type.

Table 8.2 Kallikreins as diagnostic markers for ovarian cancer

Type of Analysis	Kallikrein	Clinical Significance	References
Protein in serum	KLK6	• Serum levels elevated in 66% to 68% of cancer patients • The diagnostic sensitivities at 90% and 95% specificity are 52% and 47%, respectively	90, 92
	KLK10	• Serum levels elevated in cancer compared with normal and benign disease (54% sensitivity at 90% specificity) • Elevated in 35% of CA-125–negative cancers (at 90% specificity) • Significantly associated with serous type	89
Protein in serum and ovarian tissue	KLK10	• A member of a multianalyte test for ovarian cancer diagnosis	88
	KLK14	• Elevated serum levels in 65% of ovarian cancer patients versus normal • Higher levels in 40% of ovarian cancer tissues compared with normal	57
Protein in ovarian tissue extract	KLK5	• Elevated in 55% of ovarian cancers compared with normal	97
mRNA from ovarian tissue	KLK4	• Elevated in 100% of serous carcinoma of late stage	55
	KLK6	• Significantly elevated in low-malignant-potential tumors and ovarian cancer	98
	KLK5, KLK7	• Significantly elevated in ovarian cancer, especially serous type	63
	KLK7	• Elevated in 67% of low-malignant-potential tumors and 78% of carcinomas	99
	KLK8	• Overexpressed in 67% of ovarian cancers and 40% of low-malignant-potential tumors compared with normal	58
	KLK10	• Significantly elevated in 91% of serous cancers, 73% of nonserous cancers, and 73% of primary peritoneal carcinoma compared with normal	100
	KLK14	• Downregulated in ovarian cancer • Stepwise decrease in normal > benign > cancer	52, 101
IHC of ovarian cancer	KLK6 and KLK10	• Expressed in 100% of CA-125–negative cancers	87
Protein from ascites fluid	KLK5–8, 10, 11, 13, 14	• Ovarian cancer ascites contained higher levels compared with benign effusions and ascites from other cancer types	86
Microarray	KLK5–8	• Among the top upregulated genes in ovarian cancer compared with normal tissue and other diseases	102

Table 8.2 (continued)

	KLK6–8, 10, 11	• Significantly upregulated in ovarian cancer	103
	KLK6–8, 10, 11	• Overexpressed in ovarian cancer	104
Bioinformatics analysis	KLK5–8, 10,11, 14	• Parallel overexpression in ovarian cancer compared with normal	54
	KLK6	• Elevated 25-fold in ovarian cancer	53, 63

Recent reports indicate the potential diagnostic utility of kallikreins in ascites fluid. Among kallikreins, KLK6–10 showed the highest statistical power in distinguishing ovarian cancer ascites from that of benign causes and other cancer groups. It was shown that kallikreins could identify false-negative cases of cytology.[85,86] Combinations of kallikreins achieved areas under the receiver operating characteristics (ROC) curve of 0.994 and 0.961 in separating ovarian cancer from benign effusion and from other cancer groups, respectively.[86]

The diagnostic utility of kallikreins can also extend to their use as immunohistochemical markers. A recent study has shown that in ovarian cancers that lacked CA125 expression by IHC, all specimens (100%) expressed KLK10 and KLK6.[87] Kallikrein mRNAs are also detected in circulating tumor cells in the blood and ascites fluid of ovarian cancer patients, but this application lacked sensitivity and specificity for detecting disseminated disease.[85]

Although the sensitivity and specificity of individual kallikrein proteins are not superior to that of standard markers, like CA-125, the use of kallikreins as a part of a multianalyte test significantly improves the diagnostic sensitivity and specificity.[88] In patients with early-stage cancer (stage I/II), the combination of CA-125 and KLK10 results in 21% increase in sensitivity compared with that of CA-125 alone.[89] The combination of KLK6 and CA-125 can also lead to improved sensitivity of detection of early-stage disease. When combined with CA-125, at 90% specificity, sensitivity increases to 72% (for all patients) and to 42% in stage I or II disease.[90] A recent review included kallikreins among the most promising new markers that are now being investigated to complement CA-125 for ovarian cancer diagnosis/prognosis.[91]

Another interesting application is distinguishing benign and borderline ovarian tumors. Preliminary reports indicate that KLK10 and KLK6 can have better ability than CA-125 in distinguishing ovarian cancer from benign ovarian tumors.[89,90]

Prognostic Applications

Clinical utility of kallikreins in ovarian cancer extends beyond diagnosis. Table 8.3 summarizes published data about the prognostic utility of different kallikreins in ovarian cancer. These data show that a group of kallikreins,

Table 8.3 Prognostic utility of kallikrein genes/proteins in ovarian cancer

Kallikrein Gene/Protein	Sample Type	Analysis Method	Prognostic Value	References
KLK4	mRNA	RT-PCR	*Unfavorable* prognosis: • overexpressed in late-stage, higher-grade tumors and no response to chemotherapy • associated with shorter DFS and OS • independent indicator of poor prognosis in patients with low-grade tumors	105
	Protein from effusion cells and solid tumor	IHC and immunoblotting	*Favourable* prognosis: • lower in grade IV compared with grade III • associated with longer OS	106
KLK5	mRNA	RT-PCR	*Unfavorable* prognosis: • overexpressed in late-stage and higher-grade tumors • associated with shorter DFS and OS • independent indicator of poor prognosis in patients with low-grade tumors	107
	mRNA and protein	SQ-RT-PCR, Southern, Northern, and Western blots and immunohistochemistry	*Unfavorable* prognosis: • overexpressed in ovarian tumor tissues and cell lines mainly of late stage and serous histotype	63
	Ovarian cancer cytosols	Immunoassay	*Unfavorable* prognosis: • overexpressed in patients with late-stage and higher-grade tumors • associated with shorter DFS and OS	108

Table 8.3 (continued)

Kallikrein Gene/Protein	Sample Type	Analysis Method	Prognostic Value	References
			• independent indicator of poor prognosis in patients with high-grade tumors and optimal debulking success	
KLK6	Ovarian cancer cytosols	Immunoassay	*Unfavorable* prognosis: • overexpressed in late-stage and serous tumors • associated with shorter DFS and OS • independent indicator of poor prognosis in low-grade tumors and optimal debulking success	109
	Serum from normal women, women with benign disease, and women with ovarian cancer	Immunoassay	*Unfavorable* prognosis: • higher serum levels in late-stage, higher-grade, serous tumors, suboptimal debulking, and a poor response to chemotherapy • indicator of decreased DFS and OS	90
KLK7	Ovarian cancer cytosols	Immunoassay	*Unfavorable* prognosis: • overexpressed in advanced stage, higher grade, suboptimal debulking, and serous or undifferentiated histotypes • associated with significantly shorter DFS but not OS • not an independent prognosticator for ovarian cancer	110
	mRNA from normal, benign, and cancerous ovarian tissues and late-stage	SQ-RT-PCR, Southern, Northern, and Western blots and immunohistochemistry	*Unfavorable* prognosis: • overexpressed in ovarian tumor tissues and cell lines mainly of late-stage and serous histotype	63

Table 8.3 (continued)

Kallikrein Gene/Protein	Sample Type	Analysis Method	Prognostic Value	References
	serous ovarian cancer cell lines			
	mRNA from cancerous ovarian tissue	Q-RT-PCR	*Unfavorable* prognosis: • overexpressed in higher-grade tumors • associated with shorter DFS • independent indicator of poor prognosis in patients with low-grade tumors and optimal debulking success	111
KLK8	mRNA from ovarian cancer tissues	RT-PCR	*Favorable* prognosis: • overexpressed in lower-grade tumors • associated with longer DFS and OS • independent indicator of longer DFS	112
	Protein from ovarian cancer cytosols	Immunoassay	*Favorable* prognosis: • overexpressed in lower grade, no residual tumor, and optimal debulking success • associated with significantly longer DFS and OS • an independent marker of favorable prognosis	113
	mRNA from ovarian cancer tissues	RT-PCR	*Favorable* prognosis: • overexpressed in younger age, lower-grade, and early-stage tumors	114
KLK9	mRNA from ovarian cancer tissues	Q-RT-PCR	*Favorable* prognosis: • overexpressed in early stage and optimal debulking success • associated with longer DFS and OS	59

Table 8.3 (continued)

Kallikrein Gene/Protein	Sample Type	Analysis Method	Prognostic Value	References
			• independent indicator of prolonged DFS in patients with low-grade tumors and optimal debulking success	
KLK10	Normal, benign, and cancerous ovarian cytosols	Immunoassay	*Unfavorable* prognosis: • overexpressed in cancer patients with late-stage, serous tumors and suboptimal debulking success • associated with shorter DFS and OS • independent indicator of DFS and OS in patients with late-stage tumors	56
	Serum from normal women, women with benign disease, and women with ovarian cancer	Immunoassay	*Unfavorable* prognosis: • higher serum levels in late-stage, advanced-grade, large residual tumor, suboptimal debulking, and poor response to chemotherapy • indicator of decreased DFS and OS • independent indicator of OS	89
KLK11	Proteins of ovarian tumor extracts	Immunoassay	*Favorable* prognosis: • overexpressed in early-stage and low-grade tumors • associated with longer DFS and OS • independent indicator of DFS	115
	Ovarian cancer cytosols	Immunoassay	*Favorable* prognosis: • overexpressed in patients with early-stage disease, and good response to chemotherapy • associated with longer DFS and OS	116

Table 8.3 (continued)

Kallikrein Gene/Protein	Sample Type	Analysis Method	Prognostic Value	References
			• independent indicator of OS • independent indicator of DFS and OS in patients with low-grade tumors	
KLK13	Ovarian cancer cytosols	Immunoassay	*Favorable* prognosis: • overexpressed in early stage, no residual tumor after surgery, and optimal debulking success • independent indicator of longer DFS and OS	117
KLK14	mRNA from normal, benign, and cancerous ovarian tissues	Q-RT-PCR	*Favorable* prognosis: • overexpressed in early stage, optimal debulking success, and good response to chemotherapy • independent indicator of longer DFS and OS	101
KLK15	mRNA from benign and cancerous ovarian tissues	Q-RT-PCR	*Unfavorable* prognosis: • independent indicator of decreased DFS and OS	118

IHC, immunohistochemistry; RT-PCR, reverse transcriptase–polymerase chain reaction; DFS, disease-free survival; OS, overall survival; SQ-RT-PCR, semiquantitative RT-PCR; Q-RT-PCR, quantitative RT-PCR.

namely *KLK4–7*, *KLK8*, *KLK10,* and *KLK15*, are markers of poor prognosis in ovarian cancer. That is, higher kallikrein mRNA and/or protein levels correlate with more aggressive forms of the disease and a decreased disease-free survival (DFS) and overall survival (OS) of patients. The remaining subset of kallikreins, namely *KLK8, KLK9*, *KLK11*, and *KLK13–14*, seem to be markers of favorable prognosis, with higher levels of their mRNA or proteins associated with earlier-stage disease and increased DFS and OS.

Data from a recent report suggested that KLK6 might have value for patient monitoring in ovarian cancer.[92] A recent pilot study shows that some kallikrein proteins could be included with other clinical variables to develop a multiparametric "score" that can predict the surgical outcome and thus help in preoperative risk stratification and identifying candidates for alternative or adjuvant therapeutic strategies.[93] Another study showed evidence that a group of kallikreins and multiparametric combinations with other biomarkers and clinical variables can significantly assist with ovarian cancer classification, prognosis, and response to platinum-based chemotherapy.[94]

Kallikrein expression in ovarian cancer may also be clinically useful in determining the prognosis in subgroups of patients. Subclassification of large heterogeneous groups into smaller subgroups is becoming an important tool for individualizing treatment options in ovarian cancer patients and thus avoiding unnecessary treatments with high costs and unwanted side effects.

Therapeutic Applications

It is possible that some kallikreins may become valuable therapeutic targets when the biological pathways that are involved are delineated. For example, the enzymatic activity of these serine proteases may initiate or terminate biological events (e.g., tumor invasion, angiogenesis, activation or inhibition of hormones, growth factors, other enzymes, receptors, or cytokines). Once known, these events could be manipulated, for therapeutic purposes, by specific enzyme inhibitors or activators. Another potential therapeutic approach is the cell-specific activation of therapeutic agents.[95] Preliminary reports show potential success by using the KLK3 (PSA) promoter to express molecules in a tissue-specific fashion.[96] A third possible therapeutic approach involves immunotherapy and/or development of cancer vaccines. With our increasing knowledge of the hormonal regulation of kallikreins, hormonal activation (or repression) of kallikrein activity could be investigated in the future.

Conclusion

Accumulating evidence, at both the mRNA and protein levels, indicates that many kallikreins are differentially expressed in ovarian cancer. The mechanism by which kallikreins are involved in the pathogenesis and/or progression of

cancer is not fully understood and is possibly through controlling vital processes, like apoptosis, angiogenesis, and tumor metastasis, by cleavage of specific substrates. Kallikreins can be measured in serum, tissue, and ascites fluid of ovarian cancer patients. Reports show that kallikreins can be useful serum biomarkers for diagnostic, monitoring, and prognostic purposes. They can be also useful immunohistochemical markers. In addition, kallikreins have potential for being used for therapeutic applications. KLK6 and KLK10 show the best promise as serum biomarkers for ovarian cancer. Although kallikreins might not have superior sensitivity and specificity to that of existing markers, the use of kallikreins as a part of a multianalyte test significantly improves the diagnostic and prognostic accuracy. Further large-scale studies are needed to evaluate the applicability of this approach.

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Chapter 9
Soluble Epidermal Growth Factor Receptor: A Biomarker of Epithelial Ovarian Cancer

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Definition: The Epidermal Growth Factor Receptor and Soluble Epidermal Growth Factor Receptor Isoforms

The epidermal growth factor receptor (EGFR) is the prototypic member of the ErbB receptor tyrosine kinase family, which includes ErbB1 (EGFR), ErbB2 (HER2, Neu), ErbB3 (HER3), and ErbB4 (HER4).[1–3] Biochemical and structural studies show that the mature form of EGFR is a 170-kDa plasma membrane protein that is composed of extracellular, transmembrane-spanning, and intracellular domains.[4] The extracellular domain is divided into four subdomains: subdomains I and III function in growth factor binding, and subdomains II and IV confer secondary and tertiary structure to the extracellular domain through cysteine residues. The intracellular domain also is divided into subdomains: a tyrosine kinase subdomain and a carboxy-terminal regulatory subdomain that includes several tyrosine autophosphorylation sites. In general, growth factor binding to EGFR results in receptor dimerization with any member of the ErbB receptor family, transphosphorylation of tyrosine residues, docking of numerous cytoplasmic proteins containing either SH2 or PTB motifs, and activation of protein phosphorylation cascades (e.g., Ras-Raf-MAP kinase or PI-3 kinase). Activation of these cascades, in turn, effect gene expression, cellular proliferation and survival, and changes in cell shape, adhesion, and motility.[5,6] Consequently, EGFR plays an important role in normal cellular proliferation, survival, and differentiation.[7,8]

In addition to the full-length EGFR, cells synthesize "soluble" isoforms of EGFR (soluble epidermal growth factor receptor; sEGFR) by alternative mRNA processing. These sEGFR isoforms contain only elements of the extracellular domain and, therefore, are secreted. Alternative *EGFR* transcripts

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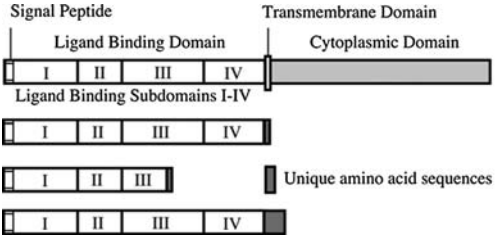
encoding sEGFR isoforms have been isolated from normal rat,[9] mouse,[10–12] chicken,[13] and human tissues,[12,14–16] as well as from malignant human cells.[17–21] Because alternatively spliced mRNAs use alternative polyadenylation signals and stop codons, amino acids distinct from full-length EGFR are commonly present at the carboxy-terminus of sEGFR isoforms (Fig. 9.1). The human vulvar carcinoma cell line A431, for example, synthesizes a mutant sEGFR isoform from a 2.8-kb mRNA transcript that resulted from a gene amplification and translocation event.[17–22] This mutant sEGFR isoform harbors 17 unique amino acids at its carboxy-terminus that are unrelated to full-length EGFR. Two naturally occurring alternative *EGFR* transcripts of 1.8 and 3.0 kb have been cloned and sequenced from normal human placenta.[12,14] The 1.8-kb transcript encodes a 60-kDa sEGFR protein that has two unique carboxy-terminal amino acids,[14] whereas the 3.0-kb transcript encodes a protein product that is homologous with the extracellular domain of EGFR through amino acid residue 603, but then adds 78 unique carboxy-terminal amino acids.[12] Heterologous expression studies demonstrate that this 3.0-kb transcript is translated into a 110-kDa sEGFR N-linked glycoprotein. Moreover, this 110-kDa sEGFR protein circulates in human blood and may have utility as a risk assessment, early detection, diagnostic, and prognostic biomarker of epithelial ovarian cancer.[23–27]



mRNA	Unique AA	Molecular Weight	Reference
10.5/5.8 kb	---	170 kDA	Ullrich et al., 1984
2.8 kb	17	115 kDA	Ullrich et al., 1984
1.8 kb	2	60 kDA	Reiter and Maihle, 1996
3.0 kb	78	110 kDA	Reiter et al., 2001

Fig. 9.1 A structural diagram comparing the full-length 170-kDa human EGFR with human sEGFR isoforms is shown. The full-length EGFR contains an extracellular domain with four distinct subdomains (I–IV), a transmembrane-spanning domain, and a cytoplasmic domain. sEGFR isoforms include the mutant p115 sEGFR from A431 cells and the wild-type placental p60 and p110 sEGFR proteins. The filled boxes correspond with unique carboxy-terminal amino acids not present in the full-length p170 EGFR. The chart to the right of the diagram indicates the mRNA transcript size, the number of unique amino acids, the molecular weight, and a reference for each transcript/protein

Soluble isoforms of plasma membrane receptors are widespread in animal systems and may play important physiologic roles in intercellular communication, cellular proliferation and differentiation, tissue morphogenesis, tissue regeneration and wound repair, inflammation, embryogenesis, and carcinogenesis.[3,28] Soluble receptor isoforms, for example, may function to: (i) activate signal transduction pathways through precursor molecules of membrane-bound

growth factors; (ii) transport growth factor ligands in body fluids; (iii) modulate growth factor degradation by specific proteolytic enzymes; (iv) competitively inhibit growth factor stimulation of their cognate holoreceptors; (v) affect the bioavailability of growth factors bound to the extracellular matrix; and (vi) regulate the tyrosine kinase activity of their cognate holoreceptors. In this regard, sEGFR molecules have been shown to bind to epidermal growth factor (EGF),[29–33] to inhibit the tyrosine kinase activity of the EGFR holoreceptor,[34,35] and to block ligand-dependent transformation and cellular proliferation in vitro.[9,20,36] Although sEGFR molecules can bind to EGF directly, the mechanism of signal abrogation appears to involve heterodimer formation between the sEGFR analogue and the membrane-bound EGFR holoreceptor, rather than EGF sequestration.[34,35] Consequently, sEGFR isoforms may function as dominant negative regulators of EGFR signal transduction pathways in animal systems.

Epidemiology of Epithelial Ovarian Cancer

Ovarian cancer is composed of a biologically diverse group of neoplasms of germ cell, stromal, and epithelial origin.[37,38] However, among women over the age of 35, 95% of ovarian tumors originate from the surface epithelium of the ovary. Globally, epithelial ovarian carcinoma (EOC) represents the seventh most common type of women's cancer after breast, cervix, colon and rectum, stomach, corpus uteri, and lung cancer.[39] Although the incidence of EOC varies worldwide, the highest incidence rates, with the exception of Japan, are found in industrialized countries. For women in the United States, EOC ranks fifth in incidence and is the leading cause of death for gynecologic malignancies: 22,430 new cases and 15,280 deaths from this disease were estimated for 2007.[40] Epithelial ovarian cancer, therefore, represents an important women's health problem of public concern.

Etiology of Epithelial Ovarian Cancer

Age, family history of EOC, positive germ-line *BRCA1* mutation status, and a personal history of breast cancer are well-known risk factors of EOC. The incidence of EOC is uncommon in women under 40 years of age, increases sharply between 40 and 80 years of age, and then decreases moderately.[41] Incidence rates for EOC increase from 2 to 3 cases per 100,000 women in the third decade of life to 59 cases per 100,000 women in the eighth decade. Combined data from 12 case-control studies performed in the United States estimate the risk of developing EOC before age 65 to be 0.8%.[42] However, the overall lifetime risk of developing ovarian cancer in the United States is between 1.4% and 1.8%[43]; therefore, approximately 1 woman in 60 will develop this

gynecologic cancer in her lifetime (American Cancer Society, *Cancer Facts & Figures 2007*; http://www.cancer.org/docroot/STT/stt_0.asp). In contrast, women with a positive family history of EOC have a 9.4% estimated lifetime risk of developing EOC.[42] Moreover, the risk of developing EOC increases to 1 in 2 (40% to 60% lifetime risk) for women with a family history of EOC who have a germ-line *BRCA1* mutation[44,45] and up to fourfold for women with a prior history of breast carcinoma.[38]

Pregnancy, oral contraceptive use, and lactation decrease the risk of developing EOC. Parity is an independent protective factor of EOC, whereby each birth is associated with a 16% reduction in a woman's risk of developing EOC regardless of age at first birth.[46] Oral contraceptives decrease a woman's risk for developing EOC by 30% to 60%, increasing approximately 11% with each year until reaching 6 years of use.[46,47] For women without a family history of EOC, plus three or more term pregnancies, and 4 or more years of oral contraceptive use, the lifetime risk of developing EOC is 0.6%. In contrast, nulliparous women who have not used oral contraceptives have a 3.4% lifetime risk of developing EOC. Finally, women who breast-feed their children have a decreased risk of developing EOC of approximately 1% per month of lactation.[48] In general, pregnancy, oral contraceptive use, and lactation are thought to be protective, because they suppress ovulation and wounding of the ovary's surface epithelium, thereby decreasing the probability of cellular transformation in this regenerative layer of epithelial cells.

EGFR plays an important role in follicle development within the ovary and in regulating the proliferation of ovarian surface epithelial cells.[49] It is, therefore, not surprising that EGFR and sEGFR isoforms may have clinical utility as tumor and serum biomarkers of EOC. Amplification of the *EGFR* proto-oncogene and overexpression of EGFR are common abnormalities of human ovarian carcinoma–derived cell lines and tumors; hence, EGFR is thought to play a critical role in the etiology of EOC.[50–53] Lower serum sEGFR concentrations in older, postmenopausal women and in EOC patients compared with that in healthy women of similar age are consistent with the following concepts: (i) sEGFR isoforms are dominant negative regulators of EGFR signal transduction, (ii) high serum sEGFR concentrations may represent endogenous protective factors of EOC, and (iii) low sEGFR concentrations in the circulation may increase a woman's risk of developing this "silent" cancer.[25,26]

Pathogenesis of Epithelial Ovarian Cancer with Respect to EGFR and sEGFR Concentrations

Although EOC is associated with industrialization, late age of occurrence suggests that this relationship may be biased by a higher standard of living and increased personal longevity. Unlike other cancers, there is little evidence that exposure to xenobiotic carcinogens or biological vectors plays an

important role in the etiology of EOC. Rather, molecular and biochemical studies indicate that genetic alterations in oncogenes and tumor suppressor genes may be more important in the etiology and pathogenesis of EOC than environmental exposures. In this regard, *EGFR*/*ERBB* family members are frequently amplified and/or overexpressed in human tumor cell lines and neoplasms,[54] including cancer of the ovary.[50,52] In addition, primary tumors expressing high levels of EGFR are often associated with increased production of TGF-α,[55] the most widely expressed EGF-family ligand. In the ovary, malignant tumor tissues have been shown to release more EGF and TGF- α than do benign tissues, and aneuploid carcinomas have been shown to release more EGF and TGF-α than do diploid carcinomas.[56] Elevated coexpression of EGFR and its ligands, therefore, may establish growth stimulatory autocrine and/or paracrine loops in ovarian carcinomas.[53] Clinically, amplification of *EGFR* and/or overexpression of EGFR are associated with a shorter time to disease recurrence, as well as decreased patient survival for EOC.[50] The EGFR/ErbB receptor tyrosine kinase family and its growth factor ligands thus play an important clinical role in the molecular pathogenesis of EOC.

Clinical Presentation of Epithelial Ovarian Cancer

History

Physicians rely on the patient's history of symptoms and physical examination; laboratory tests for tumor antigens such as CA-125, alpha-fetoprotein (endodermal sinus tumors), and Müllerian-inhibiting factor (granulosa cell tumors); and on various imaging modalities such as x-ray, sonography, computed tomography (CT) and magnetic resonance imaging (MRI) scans to detect, confirm, and/or monitor for the presence of epithelial ovarian tumors.[37,38,57,58] These methods of detection are limited in that they only identify the tumor when it is macroscopic and perhaps already metastatic. In addition, several of these detection methods are expensive (e.g., CT and MRI scans) or relatively nonspecific for epithelial ovarian cancer (e.g., CA-125). Therefore, simple, cost-effective, specific, and sensitive methods of screening are needed to detect and better diagnose EOC.

Symptoms

Women who have EOC usually are asymptomatic or have vague, nonspecific symptoms such as indigestion, bloating, and/or changed bowel habits.[59] Consequently, EOC typically goes undetected and untreated until in its advanced stages. In the unscreened population, 70% to 75% of women with EOC are diagnosed with stage III or IV disease,[60,61] which is associated with 5-year

survival rates of just 15% to 31% and disease-free survival rates of less than 10%.[40,57,62,63] In comparison, 5-year survival rates for stage I EOC patients are substantially better, in the range of 90% to 95%.[40,64] Despite new therapy options,[65,66] the age-adjusted mortality rate for patients with EOC has not changed substantially over the past 20 years.[40,67] Look has estimated that the mortality rate of EOC could be reduced by 50% if the number of patients diagnosed with early-stage EOC could be increased from 20% to 80% by a screening program.[39] Early detection, therefore, represents a potentially practical approach for controlling the burden of EOC in the human population.

Laboratory Findings of sEGFR and EGF Concentrations in Epithelial Ovarian Cancer

Sera from healthy men and women contain a p110 sEGFR isoform.[23,25,26] Furthermore, patients with stages I through IV EOC have significantly lower preoperative serum values of p110 sEGFR than that of healthy women.[24,26] In a first report, preoperative serum sEGFR concentrations were shown to have 100% sensitivity and 100% specificity to discern 21 stage III/IV EOC cases from 21 healthy women of similar ages using a cutoff threshold of 1500 fmol/ml.[24] After cytoreductive tumor debulking surgery, serum sEGFR concentrations increased to normal values for many EOC patients enrolled in a clinical trial of cyclophosphamide plus carboplatin combination chemotherapy. Compared with that in healthy women, serum EGF concentrations were significantly higher only in EOC cases immediately after but not prior to cytoreductive surgery.[24] Moreover, serum sEGFR and EGF concentrations were strongly associated in EOC patients prior to tumor debulking surgery but not in healthy women or in EOC patients after cytoreductive surgery. Taken together, these data suggest that epithelial ovarian tumors may concomitantly affect sEGFR and EGF concentrations in the systemic circulation and that serum sEGFR may be a useful diagnostic, prognostic, and/or theragnostic biomarker of EOC (Table 9.1).

Table 9.1 Major points

- Men and women express a 3.0-kb alternative mRNA transcript of the *EGFR* proto-oncogene that encodes a 110-kDa sEGFR serum protein.
- Patients with EOC have significantly lower preoperative serum p110 sEGFR concentrations than that of healthy women (nonsurgical volunteers).
- Serum sEGFR concentrations in EOC patients increase to normal values after cytoreductive tumor debulking surgery.
- Serum EGF concentrations are significantly higher in EOC patients after cytoreductive surgery but not preoperatively.

Table 9.1 (continued)

- Serum sEGFR and EGF concentrations are strongly associated in EOC patients preoperatively but not postoperatively.
- Serum sEGFR concentrations are inversely associated with serum concentrations of FSH and LH and with age in healthy women.
- Age- and menopause-specific cutoff thresholds are appropriate when using serum sEGFR concentrations to discern EOC patients from healthy women.
- Serum sEGFR concentrations are most useful for detecting EOC among younger, premenopausal women.
- Serum sEGFR concentrations are not associated with disease stage or tumor grade.
- Serum sEGFR concentrations are lower in EOC patients than in women with benign ovarian and non-ovarian gynecologic conditions.
- Age and serum sEGFR concentrations modify the association between CA-125 values and EOC versus benign gynecologic disease.
- Parallel testing with fixed sEGFR and CA-125 cutoff thresholds optimizes sensitivity to detect EOC.
- Serial testing with age- and sEGFR-dependent CA-125 cutoff thresholds optimizes test specificity and accuracy to discern women with benign ovarian and non-ovarian gynecologic conditions from patients with EOC.

A cross-sectional study of serum sEGFR concentrations among healthy women has shown that sEGFR concentrations are inversely associated with serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) concentrations and with age.[25] Consequently, sEGFR concentrations are lower in older, postmenopausal women compared with that in younger, premenopausal women. Accordingly, these data suggested that age- or menopause-specific cutoff thresholds for serum sEGFR might be more appropriate than fixed cutoff thresholds. Using a cutoff threshold of $\leq$624 fmol/ml, pretreatment serum sEGFR concentrations had 56% sensitivity and 95% specificity in discerning stages I through IV EOC cases from healthy women overall.[26] Sensitivity was lower for stage I/II (34%) compared with stage III/IV (61%) EOC cases. Using menopausal status-specific cutoff values that maintained 95% specificity across strata, sensitivity was found to be higher for premenopausal (74%) compared with postmenopausal (50%) women. Furthermore, test sensitivity for detecting stage I/II versus stage III/IV EOC among premenopausal women was 64% and 81%, respectively. Among postmenopausal women, test sensitivity was 28% for stage I/II versus 54% for stage III/IV EOC. Stratification of the cases and controls into groups between 20 and 40, 41 and 60, and 61 and 87 years of age followed by selection of cutoff values at the 95th percentile for each group of controls showed a sensitivity of 72.7%, 60.6%, and 33.3% for each age group, respectively. Accordingly, test sensitivity for detecting stage I/II or stage III/IV EOC in these age groups was shown to be 66.7% or 75.0%, 40.7% or 68.1%, and 28.6% or 34.0%, respectively. Taken together, these data indicate that (i) age- and menopause-specific cutoff thresholds are appropriate when using serum sEGFR concentrations to discern EOC patients from healthy women, and (ii) serum sEGFR concentrations may be especially useful in detecting EOC among younger, premenopausal women.

Demographic and clinicopathologic prognostic factors such as older age, advanced surgical stage, high tumor grade, large residual tumor volume after cytoreductive surgery, and lower performance status are associated with poor patient prognosis for EOC.[37,38] Consequently, postsurgical selection of chemotherapy for EOC is based on tumor histology and grade, surgical stage, and residual disease. In the future, therapeutic decisions also may be based on the expression of tumor and/or serum biomarkers. In this regard, preoperative serum p105 sNeu/sErbB2 concentrations have been shown to be (i) elevated in women with ovarian cancer in comparison with healthy women; (ii) unassociated with disease stage, tumor grade, histologic subtype, or serum CA-125 concentrations; and (iii) significantly associated with shorter overall survival for surgically treated EOC patients.[68–27] Moreover, like serum sNeu/sErbB2 concentrations, preoperative serum p110 sEGFR concentrations are not associated with disease stage or tumor grade after reciprocally adjusting for either tumor grade or disease stage alone or in combination with either age or menopausal status, respectively.[26] Although the relationship between serum p110 sEGFR concentration and tumor EGFR expression has yet to be explored, positive EGFR immunohistochemical status has been reported to be an independent prognosticator of EOC.[73] As such, circulating sEGFR isoforms also may serve as prognostic factors of EOC.

Differential Diagnosis of Benign Versus Malignant Gynecologic Disease

Recently, serum CA-125 values and sEGFR concentrations were compared in women with EOC and women with benign gynecologic conditions of ovarian and non-ovarian origin.[27] Serum sEGFR concentrations were significantly lower in patients with EOC than in women with benign gynecologic conditions, whereas serum CA-125 values were significantly higher in patients with EOC compared with that in women with benign gynecologic conditions. In addition, age and serum sEGFR concentrations modified the association between CA-125 values and EOC versus benign gynecologic disease. Hence, serial testing with both age- and sEGFR-dependent CA-125 cutoff thresholds optimized test specificity and accuracy to discern EOC patients from women with benign ovarian tumors and non-ovarian gynecologic conditions. Multivariate logistic regression and receiver operating characteristic (ROC) analyses demonstrated that age- and sEGFR-dependent CA-125 cutoff thresholds correctly discerned EOC cases from patients with benign ovarian neoplasms, benign non-ovarian gynecologic conditions, and both groups of women combined with 87.2% (95% CI: 83.6%, 90.7%), 90.1% (95% CI: 87.0%, 93.2%), and 87.4% (95% CI: 84.1%, 90.8%) accuracy across all cutoff thresholds, respectively. Sensitivity for detecting EOC where the multivariate logistic regression model converged to 100% specificity was 70%, 65%, and 60% for each of these respective

groups of women. In contrast, data analyses showed that parallel testing with fixed sEGFR and CA-125 cutoff thresholds optimized the overall sensitivity (85%) to detect EOC. Using fixed cutoff thresholds of $\leq$1000 fmol/ml sEGFR and $\geq$50 units/ml CA-125, these biomarkers detected 57% and 92% of the stage I/II and stage III/IV EOC cases, respectively. The combined use of serologic sEGFR and CA-125, therefore, may have improved utility for screening and diagnosing EOC and warrants further research.

Treatment and Expected Outcome with Respect to sEGFR Concentrations

Postoperative serum sEGFR and EGF concentrations have been quantified in a case series of 73 stage III/IV EOC patients enrolled in a phase III chemotherapy protocol to study the efficacy of cyclophosphamide plus conventional-dose versus double-dose carboplatin.[24] Serum sEGFR concentrations measured between days 1 and 34 after surgery were significantly lower in the 73 EOC patients compared with an age-matched group of 73 healthy women, whereas serum EGF concentrations were significantly higher in these EOC patients. For 33 EOC patients who provided longitudinal serum samples between 35 and 287 days after surgery, serum sEGFR concentrations were similar to those seen in healthy women. Comparison of the first versus the second blood draw for these 33 EOC patients showed that serum sEGFR concentrations increased for 73% (24 of 33) of the 33 patients who provided longitudinal blood draws. In contrast, serum EGF concentrations decreased for approximately 50% (18 of 33) of these 33 patients. Interestingly, no evidence was found for an association between sEGFR and EGF concentrations in the 73 EOC patients who provided postoperative serum samples between 1 and 34 days after cytoreductive surgery (Spearman's rank order correlation coefficient = 0.07923; $p = 0.5113$) or in the 73 normal age-matched women (Spearman's rank order correlation coefficient = 0.17033; $p = 0.1526$). Yet, preoperative serum sEGFR and EGF concentrations were strongly associated in EOC patients (Spearman's correlation coefficient = 0.61968; $p = 0.0027$). For two patients who provided five consecutive blood draws, sEGFR concentrations first increased temporally after cytoreductive surgery and then began to decrease (Fig. 9.2A), whereas EGF concentrations showed a consistent decreasing trend (Fig. 9.2B). Taken together, these preliminary data suggest that (i) epithelial ovarian tumors are not the primary source of serum sEGFR, (ii) serum sEGFR and EGF are in a state of equilibrium in healthy women, (iii) epithelial ovarian tumors affect circulating sEGFR and EGF concentrations, perhaps by altering their state of equilibrium, (iv) and sEGFR may be a serum transport protein of EGF. These data further suggest that serum sEGFR concentrations may be useful prognostic and theragnostic markers of EOC (i.e., sEGFR may be a useful predictor of disease

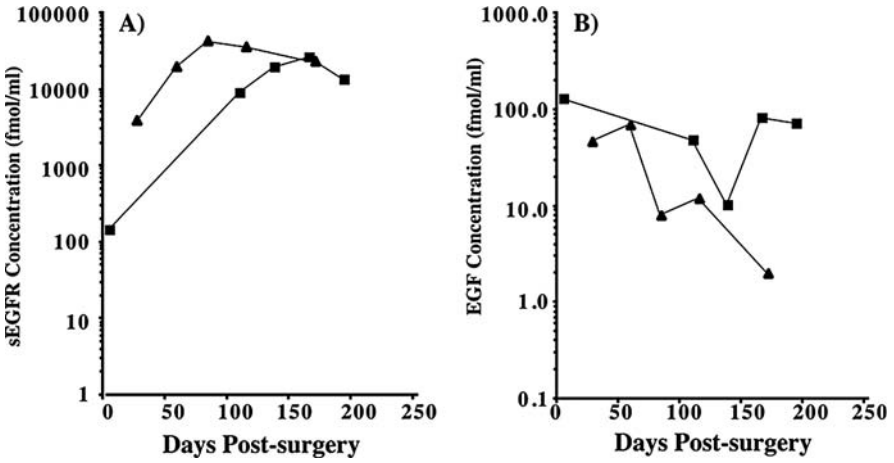


Fig. 9.2 Serum sEGFR and EGF concentrations after cytoreductive surgery are shown. Serum sEGFR and EGF concentrations were quantified after cytoreductive surgery in a cohort of 73 stage III/IV EOC patients enrolled in a phase III chemotherapy protocol to study the efficacy of cyclophosphamide plus conventional-dose versus double-dose carboplatin.[24] Measurement of serum sEGFR and EGF concentrations for two patients who provided five consecutive blood draws show that (A) sEGFR concentrations first increased and then decreased after cytoreductive surgery, whereas (B) EGF concentrations showed an overall decreasing trend. Each data point represents the median of the mean sEGFR concentration for one serum sample tested in duplicate from three separate assays and the mean EGF concentration for one serum sample tested in duplicate

outcome, responsiveness to therapy, and/or disease recurrence). Additional prospective studies are needed to address each of these potential applications for sEGFR as a clinically useful biomarker in ovarian cancer patients.

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Chapter 10
Activated Epidermal Growth Factor Receptor in Ovarian Cancer

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Background: The Epidermal Growth Factor Receptor

Growth factor receptors direct numerous cellular functions and behavior including cell proliferation and survival, apoptosis, differentiation, and migration. The receptor tyrosine kinase (RTK) family of growth factor receptors includes the epidermal growth factor (EGF) receptor subfamily (also known as the ErbB or type I RTKs).[1–5] The ErbB family includes four ErbB proteins: ErbB-1 (EGF receptor), ErbB2, ErbB3, and ErbB4. These structurally related, single membrane spanning receptors consist of an extracellular ligand-binding domain, a transmembrane domain, a juxtamembrane domain, the catalytic tyrosine kinase domain, and a C-terminal tail containing multiple tyrosine residues (Fig. 10.1). Ligand binding promotes EGF receptor homo- and heterodimerization with ErbB family members, activation of the intracellular tyrosine kinase domain, and phosphorylation of specific tyrosine residues of the receptor cytoplasmic domain. This leads to assembly of signaling complexes and stimulation of numerous downstream signaling cascades associated with cell growth and survival, increased angiogenesis, and metastasis in tumors.[1–10]

Numerous ligands interact with the ErbB receptor family.[4–6,11–13] EGF, transforming growth factor-α (TGF-α), and amphiregulin only bind to the EGF receptor. The ligands heparin-binding EGF-like growth factor (HB-EGF), betacellulin, epiregulin, and epigen bind both the EGF receptor and ErbB4. These EGF receptor ligands are synthesized as membrane-bound precursors then cleaved to release the mature form of the ligand. EGF receptor ligands can activate receptors on the cell of origin, on nearby cells, or on cells at more distant sites after systemic distribution. In some instances, receptor activation by the precursor (membrane-bound ligand) may occur as a consequence

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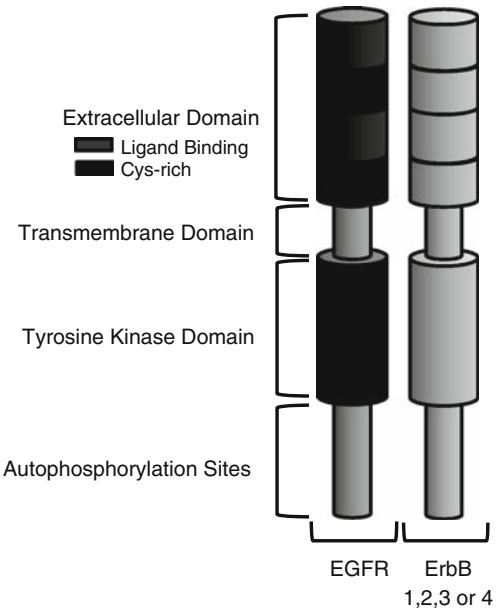

Fig. 10.1 Model of the EGF receptor. The extracellular N-terminal domain contains two subdomains that directly interact with ligand and two cysteine-rich subdomains. There is a single transmembrane domain that links the extracellular domain to the intracellular tyrosine kinase domain and the C-terminal tail, which contains the autophosphorylation sites. The EGF receptor dimerizes with other ErbB receptors

of cell:cell interaction.[12] Other ErbB receptors bind additional ligands: ErbB3 and ErbB4 both bind neuregulin-1 and neuregulin-2; neuroglycan C selectively interacts with ErbB3; and neuregulin-3, neuregulin-4, and tomoregulin are selective for ErbB4.[4,5,11–13] Activated ErbB receptors transactivate other ErbB family members leading to a robust signaling network with the biological consequences dependent on spatial and temporal expression of receptors and ligands.[3,9,13]

Based on the profound influence of ErbB receptor signaling on vital cellular functions, it is not surprising that dysregulation of the ErbB network is implicated in cancer. The discovery that the avian erythroblastosis retrovirus encoded a mutant homolog of the EGF receptor[14] established its oncogenic potential.[2,8,9,13–15] Numerous studies link EGF receptor activity to the development of tumors and tumor metastasis. Dysregulated EGF receptor activity is common in solid tumors due to receptor overexpression, activating mutations, or autocrine/paracrine stimulation by ligand and other mechanisms.[7–9,13,15–20] Aberrant expression and activity of the EGF receptor is generally understood to have a negative impact on the clinical outcome of cancer patients, which has led to focus on the EGF receptor as a therapeutic target. Recent reviews provide information regarding a systems-level approach to ErbB receptor signaling,[3,13,16] significance of ErbB receptors in the development and progression of cancer,[5–9,13] and ErbB receptors as targets for cancer therapeutics.[4,7,15–18]

This chapter will focus on the EGF receptor in ovarian cancer. We will summarize information regarding EGF receptor and ligand expression in ovarian cancer, identify consequences of EGF receptor activation, and discuss the

interplay between the EGF receptor and the ovarian tumor microenvironment. The EGF receptor impinges on multiple key hallmarks of cancer defined by Hanahan and Weinberg,[21] and the EGF receptor is associated with a gene expression pattern unique to invasive tumor cells,[22] illustrating the need to more fully understand the impact of EGF receptor activity in ovarian cancer.

Expression of the EGF Receptor and Ligands in Ovarian Cancer

The most common form of ovarian cancer arises from the ovarian surface epithelium (OSE). The OSE expresses EGF receptors in vivo, and EGF receptor activity is implicated in gonad development, growth and differentiation of the ovarian follicle, and postovulatory repair.[23–25] It has been proposed that EGF stimulation of the OSE contributes to its rapid postovulatory proliferation and to epithelial-mesenchymal transition (EMT) of OSE cells within the ruptured follicle. Malfunctions in postovulatory repair are believed to contribute to formation of epithelial inclusion cysts, which are the preferential sites of malignant transformation.[5,26,27] The normal OSE responds to EGF receptor–generated signals by displaying a phenotypic plasticity characterized by transition between epithelial and fibroblastic phenotypes, a characteristic usually limited to immature, regenerating, or neoplastic epithelia.[28] These attributes of the adult OSE suggest that this tissue is "primed" to respond to the EGF receptor during tumor development and progression.

In addition to its role in normal ovarian epithelium, there is abundant evidence of aberrant EGF receptor and/or ligand expression in ovarian cancer. A recent review[5] provides an excellent and comprehensive summary of immunohistochemical studies evaluating ErbB receptor and ErbB ligand expression in malignant ovarian tumors. Briefly, findings in the literature estimate EGF receptor is expressed in 10% to 70% of human epithelial ovarian cancer cases with an average of reported EGF receptor expression in 48% of ovarian tumors.[5] This broad range of EGF receptor expression detected in ovarian cancer may be due to the many variables that influence immunohistochemical studies, including those related to the processing of tissue samples, specific antibodies employed, detection methods, and scoring procedures. A smaller subset of studies has examined amplification of the EGF receptor gene in ovarian cancer. An advantage of this approach is the relative stability of DNA in archived samples, but because EGF receptor overexpression can occur in the absence of gene amplification, these studies may underestimate the frequency of elevated EGF receptor protein in tumors. Despite this caveat, recent findings report EGF receptor gene amplification in ~10% to 20% of ovarian cancer cases,[29–31] with low-level gains detected more frequently in 43% of tumors.[29] Thus, based on detection of protein or gene amplification, there is strong evidence for elevated EGF receptor expression in a significant fraction of ovarian cancer cases.

Additionally, there is evidence that increased EGF receptor expression is an early event in ovarian cancer development,[31–34] and a recent study provides evidence that early changes in EGF receptor expression may promote ovarian cancer.[35] Hyperplasia, hypertrophy, or mild dysplasia was detected in the ovaries of 100% of female mice expressing the EGF receptor under control of the mouse mammary tumor virus (MMTV) promoter. No changes in reproductive, hormone-responsive tissues of male transgenic animals were observed.[35] These findings suggest that the EGF receptor may contribute to early events in ovarian neoplasia.

Ligands for the EGF receptor including EGF, TGF-α, amphiregulin, and heparin-binding EGF (HB-EGF) have been identified in ovarian tumors[5] but appear to display different expression patterns. Both EGF and TGF-α are detected in the majority of epithelial ovarian tumors,[5,36,37] and levels are elevated in the urine and serum of ovarian cancer patients.[38–40] Overall, there are not strong associations between EGF or TGF-α expression in tumors and tumor subtype or disease stage.[36,37] HB-EGF mRNA expression is significantly increased in advanced ovarian cancer compared with that in normal ovaries.[41] There is some evidence for autocrine activation of the EGF receptor in ovarian tumor cell lines. In ovarian tumor cells expressing multiple EGF receptor ligands, small interfering RNA (siRNA) knockdown of HB-EGF inhibits EGF receptor tyrosine phosphorylation and extracellular signal-regulated kinase (ERK) activation.[42] Furthermore, ovarian tumor xenograft growth in nude mice is blocked by an inhibitor of HB-EGF or RNA interference.[43] In another study, the majority of ovarian carcinoma cell lines tested express TGF-α and amphiregulin, and antisense oligonucleotides to either ligand inhibit anchorage-independent growth.[44] Given the frequency of elevated EGF receptor expression in ovarian cancer, it is likely that in many cases the availability of one or more ligands due to autocrine, paracrine, or endocrine mechanisms leads to receptor activation and modulation of tumor cell behavior.

EGF Receptor Expression in Ovarian Cancer and Clinical Correlates

Overall, elevated EGF receptor is associated with less favorable disease outcomes in a number of human tumors.[6,9,13,19,20] Despite evidence for EGF receptor expression in ovarian tumors,[5] studies on the relationships between receptor and patient outcomes do not provide a uniform picture on the clinical consequences of elevated EGF receptor levels. Some studies report little or no relationship between EGF receptor expression and a variety of clinical endpoints such as disease stage, tumor grade, histologic subtype, response to treatment, or overall survival.[5] In contrast, other studies find significant associations between increased EGF receptor expression and advanced-stage disease, tumor grade, disease progression, and decreased overall survival.[5] Technical factors inherent in

immunohistochemical studies hamper efforts to identify relationships between EGF receptor expression levels and specific clinical end points. Notably, many studies classify tumors based simply on EGF receptor–positive staining rather than compare EGF receptor expression in tumors with that detected in normal ovarian tissue controls. As EGF receptor is expressed in normal ovarian epithelium, it is not surprising that certain studies report very high frequency (>50%) EGF receptor expression in ovarian tumors when positives are scored based on detectable staining. In one study that used EGF receptor expression level in metaplastic OSE and normal tubal epithelium as a reference control, EGF receptor overexpression was detected in 17% of the tumors, and overexpression significantly correlated with aggressive disease characteristics.[29] Another tissue microarray study focused on advanced-stage ovarian cancers from patients that had received comparable treatments.[45] An automated in situ quantitative measurement of protein analysis found that high tumor EGF receptor expression was associated with poor patient outcome as defined by overall survival and disease-free survival at 3 years.[45] In this study, EGF receptor expression status was identified as the most significant prognostic factor for disease-free and overall survival. Increased use of tumor tissue microarrays with appropriate control tissue and refinement of study parameters may ultimately resolve the current lack of consensus regarding the consequences of EGF receptor overexpression in ovarian cancer. Despite differences in individual study results, the overall conclusion that aberrant EGF receptor status is a factor in ovarian cancer outcome is supported by a meta-analysis study revealing a relationship between EGF receptor and decreased survival[46] and the abundant evidence linking EGF receptor to poor patient outcome in other cancers of epithelial origin.[6,9,13,19,20]

Other possible reasons for discrepancies in reported clinical outcomes and EGF receptor expression levels is the paucity of information on alternate forms of the EGF receptor or EGF receptor activation status in ovarian tumor samples. Soluble forms of the EGF receptor (sEGFR) lacking the transmembrane and intracellular domains are detected in ovarian cancer.[5] Although the functions of this form of the EGF receptor are unknown at this time, sEGFR is under investigation as a biomarker for risk assessment, early detection, and/or diagnosis of this disease.[5,47] Another alternate form of the EGF receptor is a constitutively active mutant, EGF receptor variant III (EGFRvIII). EGFRvIII harbors an extracellular domain deletion and is expressed in a number of cancers, most notably glioblastoma.[48–50] Although this specific activating mutation does not appear to be prevalent in ovarian cancer,[51,52] there are numerous EGF receptor mutations identified in human tumors that alter receptor activity[19,20,53,54] but have not been fully explored in ovarian tumors.

There is accumulating evidence that activated (tyrosine phosphorylated) EGF receptor may be a more relevant end point for analysis of EGF receptor functions and prognostics in human tumors. Evidence that EGF receptor phosphorylation status may be an important prognostic indicator is provided in studies in head and neck, lung, and pancreatic cancer.[55–59] In a cohort of patients with locally advanced non–small cell lung cancer, phospho-EGF

receptor (pEGFR) was negatively correlated with overall survival. Patients with high pEGFR levels had median survival of 7.8 months versus 17.7 months for patients with low pEGFR. From this study, it appears that activated pEGFR, but not total EGF receptor, is a better predictor of survival.[55] In stage I non–small cell lung cancer patients, EGF receptor phosphorylation at tyrosine residue 845 proved to be an independent prognostic factor.[56] Analysis of head and neck tumor tissue microarrays found that EGF receptor activation status did not strictly correlate with total EGF receptor levels, and the 10% of patients with high pEGFR had poor outcomes based on disease-free survival.[59] Other studies suggest pEGFR in addition to other phospho-proteins may provide better clinical correlations.[60]

A limited number of studies examine pEGFR in ovarian tumors, and overall, little attention has been given to receptor activation status and disease parameters. In one study, 11.8% of ovarian tumors were positive for pEGFR, but no clinicopathologic parameter or survival differences were noted.[51] In another study, 24 heavily pretreated patients with epithelial ovarian cancer all had detectable EGF receptor and p-EGFR (Y1148), suggesting that EGF receptor activation might be more evident in advanced disease.[61] We conducted a tumor tissue array analysis and found evidence for pEGFR in approximately one third of ovarian tumor samples.[62] EGF receptor activation was statistically positively correlated with matrix metalloproteinase (MMP)-9 expression, a protein associated with tumor invasion and metastasis. In an immunohistochemical analysis of a panel of paired primary tumor and peritoneal metastases obtained from the same patient, approximately one third (35%) of metastases exhibited elevated EGF receptor activation (pEGFR staining) relative to the paired primary tumor, and MMP-9 expression was high in all (100%) pEGFR–positive metastases.[62] Together, these in vivo data indicate that activated EGF receptor is present in ovarian tumor specimens. Because EGF receptor activation stimulates numerous signaling cascades known to drive tumor proliferation and metastasis, further studies to investigate EGF receptor activation and clinical end points are warranted.

Consequences of EGF Receptor Activation in Ovarian Cancer

Cell Growth and Survival

The mitogenic effects of the EGF receptor in different cell types are well documented. EGF receptor activation stimulates numerous signal transduction pathways related to cell growth and survival.[1–4,8–10] EGF increases the growth potential of primary OSE cells in culture[63] and promotes the survival, but not proliferation, of SV40 large T antigen immortalized human OSE cells.[64] Gene expression profiling of normal rat ovarian surface epithelium after EGF treatment demonstrates EGF-dependent activation of genes involved in cell cycle

and proliferation, apoptosis, and protein turnover.[65] In addition, malignant transformation of rat OSE cells results in alteration of downstream effectors of the EGF receptor pathway.[65] Regarding ovarian tumor cells, numerous studies demonstrate that autocrine and paracrine stimulation of the EGF receptor by ligands promote ovarian tumor cell growth.[43,66–73] Furthermore, blockade of EGF receptor signaling or antisense oligonucleotides to EGF receptor ligands inhibits ovarian tumor cell growth and reverses the tumorigenic phenotype.[42–44,74–77] The in vivo relevance is illustrated by the absence of primary ovarian tumor cell xenograft growth in mice depleted of EGF by sialoadenectomy compared with tumor growth in 75% (8 of 12) of xenografts in sialoadenectomized mice supplemented with EGF.[78] Additionally, tumor formation by human ovarian carcinoma cells is enhanced by exogenous expression of pro-HB-EGF and blocked by pro-HB-EGF gene RNA interference or by CRM197, a specific HB-EGF inhibitor.[43] As further evidence that stimulation of the EGF receptor drives ovarian tumor growth, 14 of 19 primary ovarian cancer cell cultures were sensitive to growth inhibition by a 4-anilinoquinazoline inhibitor of EGF receptor activity,[79] and EGF receptor– targeted therapeutics inhibit ovarian xenograft growth in vivo.[72,80]

Increased cell growth and survival upon overexpression or activity of the EGF receptor is associated with resistance to anticancer treatments such as hormone therapy, chemotherapy, and radiotherapy in various tumor types.[81–90] Many experimental studies demonstrate that inhibition of the EGF receptor in cancer cells enhances the effect of conventional chemotherapeutics by increasing apoptosis in vitro or causing arrest of tumor growth in vivo, but the results in clinical trials are mixed.[88–91] The combination of EGF receptor–targeted therapies with conventional radiation or chemotherapeutics has met with best success in head and neck cancer and colorectal tumors, respectively[88–91]. There are few studies in ovarian cancer, but transfection of a dominant negative EGF receptor (lacking the tyrosine kinase domain) into cisplatin-resistant ovarian tumor cells restores sensitivity to cisplatin,[92] and receptor tyrosine kinase inhibitors chemosensitize drug-resistant EGF receptor– expressing ovarian tumor cells.[93,94] In addition, use of an anti-EGF receptor antibody in combination with photodynamic therapy increases survival nearly threefold in a murine ovarian tumor model.[95] Some other studies indicate that EGF enhances ovarian cancer cell sensitivity to chemotherapeutic agents.[96–98] Although these findings are seemingly at odds with the preceding studies, one possible explanation is that EGF causes internalization and degradation of the EGF receptor and therefore a decrease in net EGF receptor activity. A recent study demonstrated that treatment of ovarian cancer cells with EGF and an EGF receptor tyrosine kinase inhibitor (TKI) followed by paclitaxel enhanced cell death.[99] The authors hypothesize that the EGF and TKI combination downregulates the EGF receptor while inhibiting EGF receptor–stimulated signaling pathways, thereby fostering chemosensitization. Other studies suggest that the order of addition for EGF receptor–targeted therapies and conventional chemotherapeutics has an impact on treatment outcomes with synergism evident only when the

anti-EGF receptor agent is administered after the cytotoxic drug.[100–104] The demonstrated success of combining EGF receptor–targeted therapies with conventional chemotherapeutics in certain tumor types[88–91] suggests that further explorations on this strategy may benefit ovarian cancer patients.

Metastasis

In addition to an impact on cell growth, activation of the EGF receptor is associated with stimulation of metastasis-associated cellular responses. Many aspects of tumor metastasis resemble features of epithelial-mesenchymal transition (EMT). EMT transforms relatively immobile epithelial cells to motile cells, and this transformation is accompanied by loss of stable cell:cell contacts mediated by E-cadherin and expression of vimentin intermediate filaments.[105–109] EGF receptor activity is associated with regulation of EMT in normal and tumor tissues, including the ovary.[110–116] In the normal ovary, reversible modulation of ovarian surface epithelium to a fibroblastic form occurs during postovulatory repair of the epithelium.[110] EGF in conjunction with hydrocortisone is an EMT-inducing factor for normal OSE as demonstrated by acquisition of a fibroblast-like morphology, increased cell motility, and production of matrix metalloproteinase (MMP)-2 and -9. These responses are reversed upon EGF withdrawal, resulting in a more epithelial morphology.[110]

The recognition of reversible EMT or phenotypic plasticity in tumor cells is particularly relevant to ovarian cancer and in keeping with known characteristics of the normal tissue described above. Notably, EGF receptor activation is capable of driving EMT-associated events in epithelial ovarian carcinoma cells in culture including migration and invasion,[75,117–124] disruption of E-cadherin–mediated intercellular junctions,[62,119,125,126] and production of matrix-degrading proteinases.[62,117,119,123–131] In contrast with the well-defined events that characterize EMT in development, tumor-associated EMT is currently viewed as a continuum of phenotypic plasticity and gain of mesenchymal characteristics. Tumor phenotype likely reflects the particular complement of EMT regulatory factors expressed in cells or within the tumor microenvironment.[108,109,132] The functional consequences of this phenotypic plasticity are not fully understood but may play a role in modulation of cell survival in suspension (ascites), chemoresistance, and intraperitoneal anchoring of metastatic lesions.[108,111,133]

EGF Receptor in the Ovarian Tumor Microenvironment

The dissemination of ovarian cancer is largely contained within the peritoneal cavity, establishing a unique microenvironmental niche composed of tumor and inflammatory cells and soluble factors including growth factors, bioactive lipids, proteolytic enzymes, extracellular matrix components, and inflammatory

mediators.[134] The primary tumor and metastatic cells maintain direct contact with peritoneal fluid and ascites thereby providing a mechanism for dynamic and reciprocal regulation of the tumor microenvironment. Whereas women with early-stage malignancies (stage I and II) are often free of ascites, the vast majority of women with advanced disease (stage III/IV) produce >500 ml of ascites.[135] Ascites fluid composition is complex with more than 200 different proteins in the soluble fraction and more than 2500 in the combined soluble and cellular fractions detected by proteomics analysis.[136] Three activators of the EGF receptor present in ascites (HB-EGF, endothelin-1, and lysophosphatidic acid) have been studied in some detail and are discussed in the following section. Although these three factors represent only a small subset of the total number of bioactive components in ascites, they illustrate the dynamic interplay between the ovarian tumor microenvironment and the potential for EGF receptor activation.

EGF Receptor Activators in the Ovarian Tumor Microenvironment

There is accumulating evidence that the EGF receptor ligand HB-EGF is particularly important in ovarian cancer biology.[137–139] HB-EGF is elevated in advanced epithelial ovarian cancer tissues[41] and peritoneal fluid[43] when compared with ovarian cyst or normal controls. HB-EGF is present at higher levels than those of other EGF receptor ligands,[41,43] and HB-EGF levels are significantly correlated with clinical outcome.[41] Antibodies against the EGF receptor or HB-EGF suppress the proliferation-stimulating activity in peritoneal fluid from ovarian cancer patients and the growth-promoting activity of HB-EGF in ovarian tumor xenografts.[43] Similarly, tumor formation, ovarian tumor cell growth, and EGF receptor activation are decreased by disruption of HB-EGF through siRNA, inhibitors, or expression of noncleavable forms of HB-EGF.[42,43] These findings illustrate that pathophysiologic levels of an EGF receptor ligand in ovarian peritoneal fluid and ascites regulate EGF receptor activity and suggests that other bioactive components leading to EGF receptor activation may play pivotal roles in responsive tumors.

In addition to direct ligand activation of the EGF receptor, transactivation can occur by stimulation of nonreceptor tyrosine kinases and/or G-protein–coupled receptors (GPCRs).[4,5,139–143] Activation of GPCRs by ligands such as endothelin-1 (ET-1), lysophosphatidic acid (LPA), and others can indirectly activate the EGF receptor through stimulation of the A Disintegrin And Metalloproteinase (ADAM) family of cell surface metalloproteinases, leading to cleavage of membrane-bound EGF family precursors such as HB-EGF.[139–143] An alternate mechanism for EGF receptor transactivation by GPCRs occurs by GPCR-dependent activation of nonreceptor tyrosine kinases such as c-Src.[139–143] ET-1 and LPA are two examples of GPCR ligands that are present in ovarian cancer ascites and contribute to ovarian cancer progression.

ET-1 is receiving attention as a contributor to tumor biology and as a therapeutic target.[144–146] In addition, ET-1 is an important mediator of normal ovarian function[147] and is elevated in ovarian tumors and ascites.[148,149] Treatment of ovarian tumor cells with ET-1 promotes cell proliferation, production of proteolytic enzymes belonging to the MMP and plasminogen activator families, in vitro invasion, and EMT.[150–152] ET-1–stimulated signal transduction is mediated in part by EGF receptor transactivation.[153–155] Dual inhibition of the EGF receptor and endothelin receptor by gefitinib or ZD4054, respectively, provides greater benefit than either agent alone as measured by ovarian tumor xenograft growth.[127] This suggests that targeting both components of a transactivation pathway may improve the therapeutic potential. Similarly, elevated LPA levels are detected in ~90% of ovarian cancer patients, and LPA contributes to aggressive behavior through modulation of proteinase expression and migratory pathways.[156–160] In addition to signaling via Edg/LPA receptors, LPA transactivates the EGF receptor through multiple mechanisms, and, as a consequence, certain LPA-stimulated responses are sensitive to inhibitors of EGF receptor tyrosine kinase activity.[159,161,162] LPA induces ectodomain shedding of HB-EGF leading to enhanced growth of ovarian tumor xenografts, and LPA-induced transactivation of the EGF receptor was abrogated by disruption of HB-EGF activity or expression.[43] These examples of ET-1 and LPA suggest that the EGF receptor is likely to be activated in ovarian cancer, at least in part by receptor transactivation and ligand-dependent mechanisms as a consequence of bioactive compounds in the tumor microenvironment.

EGF Receptor Activation Modifies the Microenvironment

In addition to EGF receptor activation by factors within peritoneal fluid, it is likely that stimulation of the EGF receptor in turn modifies the ovarian tumor microenvironment. Proteinases provide one example as expression and/or activity of numerous proteinases are regulated by activators of the EGF receptor including ET-1 and LPA.[62,110,117,119,151,158,159,163–168] Proteolytic enzymes are implicated in many facets of ovarian cancer pathobiology, and ovarian cancer ascites is rich in proteinases.[163] MMP-2, -9, and -14 are major contributors to pericellular proteolysis in the ovarian carcinoma microenvironment, and there is constitutive MMP-14/MMP-2 activity in primary ovarian carcinoma cells. Although proteinases are commonly expressed by stromal elements, epithelial expression of MMP-9 or MMP-14 correlates with decreased patient survival.[163,164,169–175] Interestingly, MMP-9 is expressed by primary ovarian carcinoma cells derived from the ovary, metastatic implants, and ascites,[174] but MMP-9 expression is rapidly lost with increasing passage in culture.[174] This loss of MMP-9 expression in culture supports the hypothesis that microenvironmental factors including EGF receptor activators contribute to expression of MMP-9 (and potentially other proteinases) in vivo.

Proteinases also contribute to E-cadherin ectodomain shedding,[176] and EGF receptor activation generates an ~80-kDa E-cadherin ectodomain fragment in ovarian tumor cells.[62] EGF-dependent downregulation of E-cadherin is blocked by siRNA specifically directed against MMP-9, and associations between EGF receptor activation, MMP-9 expression, and E-cadherin are evident in human ovarian tumors and paired peritoneal metastases.[62] E-cadherin ectodomain shedding may contribute to ovarian cancer dissemination. The soluble E-cadherin ectodomain itself becomes part of the ovarian tumor microenvironment and has been detected in peripheral blood, ascites, and cystic fluids from ovarian cancer patients, differentiating between benign and malignant tumors.[164,177–179] Furthermore, when this E-cadherin fragment is incubated with ovarian cancer cells at concentrations found in human ovarian cancer ascites, the fragment induces changes characteristic of a phenotypic EMT including altered morphology, disruption of cell-cell adhesion with loss of endogenous junctional E-cadherin staining, and increased cell dispersion.[164] This finding raises the intriguing possibility of a cascade whereby EGF receptor activation leading to elevated MMP expression and E-cadherin ectodomain shedding in the ovarian tumor microenvironment contributes to the EMT that occurs later in epithelial ovarian cancer progression. A greater understanding of the full scope of EGF receptor–mediated changes to the ovarian tumor microenvironment will require further study.

Potential Consequences of Sustained EGF Receptor Activation

The presence of EGF receptor activators in ovarian cancer ascites raises questions about the potential impact of chronic EGF receptor stimulation in ovarian cancer. Typically, experimental studies involve short-term ligand exposures (minutes, hours, or days) and may not fully reflect the outcome after persistent EGF receptor activation as is likely to occur in the ovarian tumor microenvironment. Little is known about the cellular consequences of persistent ligand stimulation of the EGF receptor, but there are some intriguing studies where long-term activation of the EGF receptor led to cancer-relevant responses. In EGF receptor–overexpressing human tumor cells, extended EGF treatment disrupted cell-cell adhesion and caused an EMT due to transcriptional downregulation of caveolin-1 and induction of the transcriptional repressor Snail.[180] Treatment of A431 epidermoid carcinoma cells with EGF for 30 weeks resulted in chemoresistance that was not related to changes in EGF receptor levels or tyrosine phosphorylation but did correspond with a decrease in topoisomerase II expression levels.[181] In another study, extended treatment with TGF-α promoted sequential conversion of mature astrocytes into neural progenitors and stem cells.[182] In each of these examples, the cellular responses observed after persistent EGF receptor activation were distinct from those after transient stimulation.

Some insights into the possible impact and therapeutic implications of sustained EGF receptor activity in ovarian cancer may be gained from models

of mutational EGF receptor activation. A constitutively active, extracellular domain–truncated variant form of the epidermal growth factor receptor (EGFRvIII) provides a model for the consequences of chronic EGF receptor activation. EGFRvIII expression in various cell types confers increased cell survival and resistance to radiation and chemotherapy[183–185] and increased migratory and invasive behavior.[186–189] Introduction of EGFRvIII into an epithelial ovarian cancer cell line (OVCA 433) results in a dissociated, motile phenotype and fibroblastic morphology.[118,126,190] Expression of this mutationally activated EGF receptor leads to a loss of epithelial characteristics including decreased levels of E-cadherin, keratins 7, 8, and 18, and mucins 1 and 4 and gain of the mesenchymal markers N-cadherin and vimentin[126] (Table 10.1). Other consequences include decreased expression of additional adhesion molecules (Table 10.1) and increased trafficking of integrin α2.[118,190] Interestingly, similar changes were detected after extended ligand stimulation of the wild-type EGF receptor (Fig. 10.2). We compared short-term (24–48 hours) with long-term (36 days) EGF exposure of OVCA 433 cells. Distinct differences in

Table 10.1 Consequences of mutationally activated EGF receptor (EGFvlll) expression in ovarian epithelial carcinonma cells

Gene name	Function	Fold change in EGFRvlll-cells	P value	Validated
MMP-7	Protease	↓2	0.017	IF
Maspin	Protease	↓3	0.018	ND
Plakoglobin	Cell-cell contacts	↓3	0.002	IF, IB [126]
E-cadherin	Cell-cell contacts	↓22	0.012	IF, IB RT-PCR[126]
N-cadherin	Cell-cell contacts	↑2.5	<0.001	IB[126]
P-cadherin	Cell-cell contacts	↓3.4	0.009	ND
R-cadherin	Cell-cell contacts	↓1.9	0.015	IF
Integrin α2	Adhesion	↓2.1	0.050	IB, IF[118]
Integrin β4	Adhesion	↓7.8	0.001	IF
Integrin β6	Adhesion	↓2.5	0.014	IF
Integrin β8	Adhesion	↓2.7	0.016	IF
Laminin B1	Adhesion	↓2.4	0.009	ND
Laminin B2	Adhesion	↓1.8	0.032	ND
CD44	Adhesion	↓2.7	0.010	ND
CD24	Adhesion	↓20.2	0.005	ND

IF = immunofluorescence, IB = immunoblot analysis, RT-PCR = real-time PCR, ND = not done. Sample preparation and microarray processing was done according to Affymetrix Expression Analysis Technical Manual (Santa Clara, Ca) using the cancer chip microarray GeneChip Human Cancer G110 Array P/N 900257 (HC-G110). The HC-G110 cancer oligonucleotide expression array contained 1993 oligonucleotides for 1700 genes besides oligonucleotides for control genes (total 2059 oligonucleotides). Analysis of the data was performed using GeneSpring software version 4.2.1 (Silicon Genetices, San Carlos, CA) where an average of the 5 replicates of each cell line was calculated. Down-regulated and up-regulated genes were selected for inclusion in tables if the change was at least 2.0 fold. Statistical comparisons for the expression profiles between cell lines expressing EGFRvIII in comparison to the vector control was done by GeneSpring using a Welch t-test.

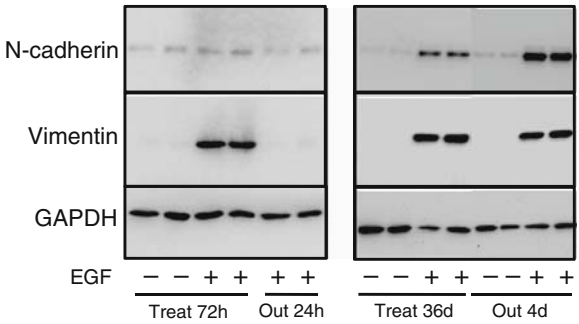

Fig. 10.2 Chronic EGF treatment leads to persistent elevation of mesenchymal markers. OVCA 433 cells were grown as described[117] without (–) or with (+) EGF for 72 hours (*left panel*) or continuously for 36 days (*right panel*). After the indicated exposures, cells were rinsed twice with phosphate-buffered saline and placed in growth medium without EGF for the indicated time points. Protein lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the mesenchymal markers N-cadherin and vimentin were detected by immunoblot analysis. GAPDH was used as a loading control

response were observed for the mesenchymal markers vimentin and N-cadherin. Vimentin expression was elevated within 72 hours of EGF treatment and readily returned to baseline levels after EGF withdrawal. In contrast, no significant increase in N-cadherin expression was detected within this time frame (Fig. 10.2). The migratory and fibroblastic phenotype also reverted to the epithelial morphology within 24 hours of EGF withdrawal after short-term EGF exposure (Fig. 10.3). After 36 days of continuous EGF exposure, both vimentin and N-cadherin were elevated and remained elevated after removal of EGF from the growth medium (Fig. 10.2). These findings suggest that although acute signaling and downstream consequences of EGF receptor activation are largely

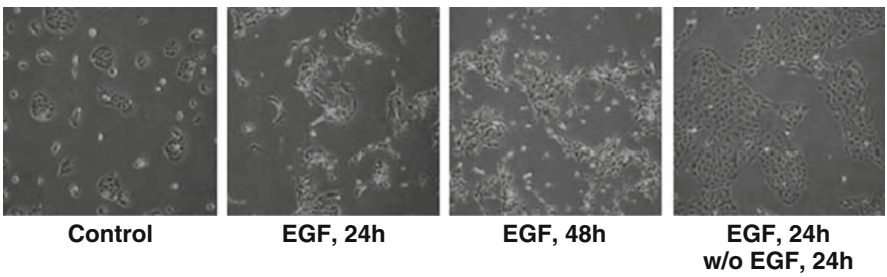

Fig. 10.3 Mesenchymal phenotype is reversible after short-term EGF treatment. OVCA 433 cells were maintained in serum-free medium containing 0.1% bovine serum albumin (w/v) for 24 hours prior to treatment without EGF (control) or with 10 nM EGF for the indicated times. For EGF withdrawal (far *right panel*), cells were treated with EGF for 24 hours, rinsed twice in phosphate-buffered saline, then returned to serum-free medium. Cell phenotype was documented by phase-contrast microscopy and digital imaging

reversible upon ligand withdrawal, chronic EGF receptor signaling may lead to more persistent changes.

There is precedence for conversion from an initially reversible to irreversible phenotype due to an exogenous stimulus. Chronic exposure to either MMP-3 or MMP-9 (but not MMP-2) mediates an EMT and genomic instability in mammary epithelial cells.[191] This MMP-driven EMT is initially reversible but becomes persistent by a mechanism associated with expression of Rac1b, a splice variant of Rac1.[191] This may also occur in vivo as expression of an autoactivating form of MMP-3 leads to the spontaneous development of premalignant and malignant lesions in the mammary glands of transgenic mice.[192] The impact of chronic EGF receptor activation on the development and/or progression of ovarian cancer is unclear at this time, however it is likely that the interplay between bioactive compounds in the ovarian tumor microenvironment and stimulation of EGF receptor signaling pathways is an important aspect of ovarian cancer pathobiology.

Conclusion

There is abundant evidence that EGF receptor activation drives cellular processes linked to ovarian tumor development, tumor cell survival, and metastasis. Because few studies have investigated activated (phosphorylated) EGF receptor in ovarian tumors, we are uncertain about the extent of EGF receptor activation in this disease. Further studies to examine the relationship between pEGFR and patient outcomes is needed to resolve key questions surrounding the clinical impact of EGF receptor expression and activation in ovarian cancer. Recent studies strongly suggest that factors present in ovarian cancer ascites such as HB-EGF and GPCR ligands activate the EGF receptor and may present an environment that fosters persistent receptor stimulation. Greater understanding and identification of ligand and nonligand activators of the EGF receptor in the ovarian tumor microenvironment may offer new therapeutic approaches involving combinatorial therapies to target the EGF receptor and mediators of EGF receptor activation and/or transactivation partners. A number of studies suggest that persistent activation of the EGF receptor contributes to chemoresistance and EMT in human tumor cells, and we find mesenchymal transformation of ovarian tumor cells driven by mutational EGF receptor activation or chronic EGF treatment. The emerging evidence that chronic stimuli such as MMPs or EGF receptor activity can lead to phenotypes that persist after withdrawal of the stimulus may have relevance to explain the disappointing efficacy of EGF receptor–targeted therapeutics observed thus far in ovarian cancer. Overall, current evidence indicates that the EGF receptor and its ligands are important to normal ovarian function and the pathobiology of ovarian cancer. Further studies will be needed to better understand the

dynamic relationship between the ovarian tumor microenvironment, EGF receptor activation, and disease outcome.

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Chapter 11
Ras-Superfamily GTP-ases in Ovarian Cancer

Kwai Wa Cheng, Roshan Agarwal, and Gordon B. Mills

Introduction

Small guanosine triphosphatases (GTPase), together with their associated regulators and effectors, play an important role in signal transduction pathways and as regulators of diverse cellular processes, including differentiation, cell division, cell proliferation, vesicle transport, nuclear assembly, and cytoskeleton formation. The Ras sarcoma (Ras) oncoproteins, including HRas, KRas, and NRas, are the founding members of the Ras-related oncoprotein superfamily. Comparative genomic analyses based on sequence and functional domain homology have revealed that this superfamily has more than 170 members,[1] which can be subdivided into five major branches: the Ras, Rho, Rab, Ran, and Arf subfamilies.[2–6] Variations in structure[7] and posttranslational modifications control specific cellular localization of Ras-superfamily proteins to specific subcellular compartments and recruitment of downstream effectors that allow these small GTPases to function as sophisticated modulators of a remarkably complex and diverse range of cellular processes, including transmembrane signal transduction (Ras), cytoskeletal reorganization (Rho), gene expression (Ras, Rho), intracellular vesicle trafficking (Rab, Arf), and microtubule organization and nucleocytoplasmic transport (Ran). Of the small G-proteins, the Ras subfamily of GTPases is the most studied, in large part because of their critical roles in human oncogenesis.[8] Recently, Rho and Rab GTPases have been implicated in playing a role in cancer and will also be discussed in this review.

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Small GTPase Activation and Posttranslational Modification

Ras-superfamily proteins share a conserved mechanism of operation (Fig. 11.1). The activity of these proteins is determined by the relative amount of GTP-bound (active) versus GDP-bound (inactive) forms, as this induces conformational changes in the switch I and switch II regions that are critical for association with regulatory and effectors proteins, resulting in modulation of binding affinities.[8–12] Although the small GTPases exhibit high-affinity binding for GDP and GTP, the intrinsic GTP hydrolysis and GDP/GTP exchange activities of these proteins is very low in the absence of additional regulatory proteins. In vivo, the GDP/GTP exchange and GTPase activity are regulated by a complex regulatory network consisting of several classes of proteins including guanine nucleotide exchange factors (GEFs),[13] which promote dissociation of bound GDP and formation of the active GTP-bound complex,[14] whereas GTPase-activating proteins (GAP) accelerate the intrinsic GTPase activity to promote formation of the inactive GDP-bound form.[15]

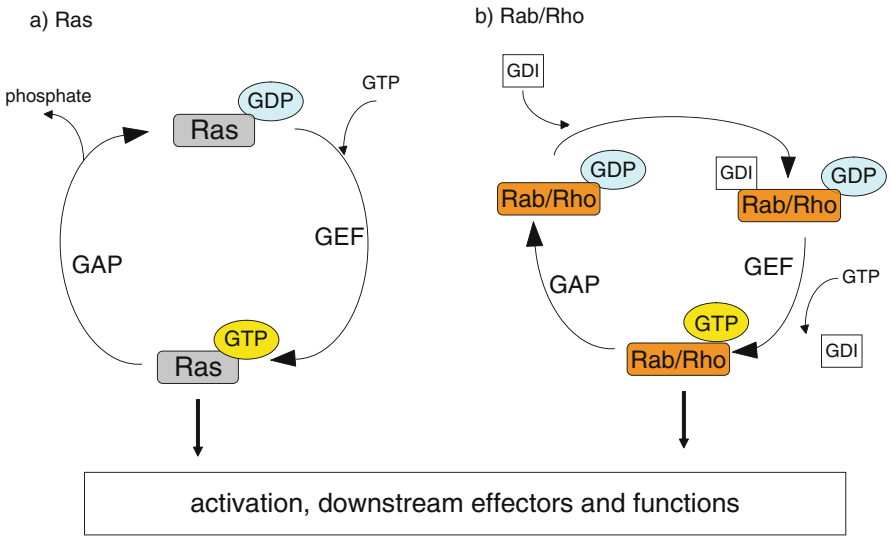

Fig. 11.1 Regulation of Ras-superfamily GTPase activation. (a) Guanine nucleotide exchange factor (GEF) catalyzes exchange of GDP to GTP leading to activation of Ras GTPase, whereas GTPase activating protein (GAP), which stimulates the intrinsic GTPase activity, leads to GTP hydrolysis and inactivation. Cycling between GDP-bound (inactive) and GTP-bound (active) forms is coupled to the transduction of an upstream signal to downstream effectors and functions. (b) Controlling Rab and Rho GTPase activation between GDP- and GTP-bound states is similar to that of Ras by GEF and GAP. However, an addition protein, guanine nucleotide dissociation inhibitor (GDI), binds Rab or Rho GTPases and regulates its activation and membrane-to-cytosol cycling

Posttranslational modifications, such as farnesylation, prenylation, and geranylgeranylation, which are important for protein-protein interactions, protect them from proteolytic degradation and, more importantly, facilitate membrane attachment, direct subcellular localization, and add an additional layer of regulation of function of Ras-superfamily GTPases.[16] The majority of Ras and Rho family proteins have a C-terminal CAAX (C = Cys, A = aliphatic, X = any amino acid) tetrapeptide motif that is the recognition sequence for farnesyltransferase and geranylgeranyltransferase I, which catalyze the covalent addition of a farnesyl or geranylgeranyl isoprenoid, respectively, to the CAAX cysteine residue.[17] In contrast, Rab family proteins terminate in a distinct set of cysteine-containing C-terminal motifs (CC, CXC, CCX, CCXX, or CCXXX) that are similarly modified by geranylgeranyltransferase II. Rho and Rab GTPases are also regulated by a further class of proteins, guanine nucleotide dissociation inhibitors (GDIs), which inhibits GDP dissociation and promotes cytosolic sequestration of these GTPases.[18,19]

Ras in Cancer

The role of the Ras subfamily of GTPases in carcinogenesis is well established. Approximately 20% to 30% of all tumors exhibit mutation of one of the three RAS (KRAS, NRAS, and HRAS) genes, with the relative frequency of involvement estimated to be 85%, 15%, and 1% for KRAS, NRAS, and HRAS, respectively.[20] The selectivity in the relative frequency of mutation of the RAS genes is also paralleled by differences in the specificity of mutations in the three genes in different cancers, as exemplified by KRAS mutations in pancreatic cancer, NRAS in melanoma, and HRAS in bladder cancers, which is thought to reflect their overlapping yet unique tissue-specific expression and function.

Oncogenic RAS mutations in general result in loss of GTPase activity and stabilization of the GTP-bound conformation, preventing timely transition from an active to an inactive state and prolonged stimulation of downstream effectors, with the majority of mutations confined to the GTPases domain and codons 12, 13, and 61.[21] The importance of RAS signaling in cancers is also highlighted by the fact that while a number of tumors do not exhibit RAS gene mutations, nevertheless they do exhibit RAS pathway activation via aberrations of genes involved in regulation of RAS activity such as the GTPase activating proteins GAPs (e.g., NF1) and/or of RAS effector proteins such as the p110alpha subunit of phosphoinositide-3 kinase (PI3K) PI3K (amplified in approximately 30% of ovarian cancers) and v-raf murine sarcoma viral oncogene homolog B1(BRAF) BRAF in melanomas.[22,23] The role of Ras activation in the promotion and maintenance of the malignant phenotype as well as resistance to therapy has been extensively explored in a number of in vitro studies (reviewed in Schubbert et al.[24]). In vivo, in the context of ovarian cancer in particular, mouse models of targeted activation of KRAS have been shown to induce poorly differentiated

invasive ovarian carcinomas albeit in $p53^{-/-}$ transgenic mice with concomitant activation of c-myc and/or akt.[25]

In ovarian cancer, the overall frequency of RAS mutations identified to date at the Sanger center as part of the COSMIC project are 17% for KRAS, 4% for NRAS, and 0% for HRAS, but with significant variation in mutation rates between the various histologic subtypes of ovarian cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/). The majority of mutations of KRAS are observed in serous borderline, mucinous borderline, and invasive mucinous tumors with estimated frequencies of 30%, 50%, and 10%.[24] Occasional mutations of KRAS have also been observed in endometrioid and clear cell carcinomas.[25–27] However, in invasive serous carcinomas, which are responsible for approximately 80% of all ovarian cancers, RAS mutations are rare, being found only infrequently in those uncommon cases with transition of serous borderline tumors to invasive cancers,[25–27] although one study has reported a frequency of 35% in a series of 94 nonmucinous ovarian cancers. Despite this, the current view is that while there is a high preponderance of RAS gene mutations in specific subtypes, the overall prevalence in ovarian cancer is low. Furthermore, the different frequencies of RAS mutations in different ovarian cancer histotypes provides evidence that these tumors arise via distinct pathways and provides a mechanistic basis for differences in the clinical behavior of these tumors.

Although the majority of serous ovarian cancers do not exhibit RAS mutations, frequent activation of the RAS pathway in these tumors has been demonstrated via other mechanisms,[28–31] and occasional RAS gene amplification.[32] Ras protein overexpression independent of gene amplification and/or mutation has been demonstrated in epithelial ovarian cancers relative to normal ovarian surface epithelium and benign tumors.[28–31] While p21 Ras expression in these studies was not associated with tumor stage, grade, or histology, the data with respect to survival is mixed. An association between RAS overexpression and poor prognosis was reported by Scambia et al.[28] in univariate and multivariate analyses, but not in another study by van Dam et al.[33] in a multivariate analysis based on fluorescence-activated cell sorting (FACS) FACS-based quantification of RAS expression, although van Dam et al.[33] observed RAS overexpression in 20% of tumors, with a further increase in expression in residual tumors after chemotherapy or at recurrence, suggesting that RAS expression may be involved in resistance to therapy. In addition, KRAS mutation status was also not associated with survival in a series of 97 invasive nonmucinous ovarian tumors by Cuatrecasas et al.[34] or response to chemotherapy in a series of 74 patients with ovarian cancer.[35]

Rho in Cancer

The Rho subfamily is functionally important primarily in cell migration and invasion. Unlike the Ras subfamily, Rho subfamily gene mutations in tumors are rare, but overexpression is more common.[36] Increased expression of RhoA, RhoC, Cdc42, and Rac1 and Rac2 have been documented in multiple tumors,

including breast, colon, bladder and testicular, melanoma, pancreatic, as well as head and neck cancers.[36–41] In addition, the correlation of increased levels of these GTPases consistently with aggressive histologic features and clinical outcome suggests an important role for these genes in tumorigenesis.[37,39,40] The lack of mutational activation and the frequent increase in of Rho proteins level in cancers suggests that cycling of Rho proteins between active (GTP-bound) and inactive (GDP-bound) conformations may be essential for Rho-mediated biological function in cancer.[42,43]

Among its many functions, RhoA has been shown to play a key role in epithelial cell motility, at least in part through its effector Rho-kinase. Recent studies have indicated a role of Rho and its associated protein in regulating ovarian cancer cell invasion properties.[44,45] The expression levels of RhoA and RhoC were significantly higher in advanced-stage carcinomas when compared with benign and early-stage tumor, respectively,[44] with both RhoA and RhoC mRNA levels higher in metastatic than in primary tumors. In keeping with such a role, in the ovarian SKOV3 cell line model, Rho-specific inhibition has been shown to suppress invasion.[44] Further, p160ROCK, a kinase effector of RhoA-mediator, contributes to RhoA-mediated migration and invasion as demonstrated by the decrease in motility and invasive properties after downregulation of p160ROCK by antisense oligonucleotides and enhancement of cell migration and invasion with transfection of dominant active p160ROCK.[45] Similarly, downregulation of a novel Rho GTPase-activating protein DLC-3 (deleted in liver cancer-3) was has been observed in ovarian as well as in kidney, lung, uterine, and breast cancers[46] further supporting a role for Rho and its regulatory proteins in ovarian cancer.

Rab in Cancer

Rab proteins, first identified as Ras-related genes expressed in rat brain,[47] represent the largest subfamily of the Ras superfamily with more than 70 putative members in human genomes compared with approximately 32 in fruitfly.[1] Studies on Rab GTPases have revealed that Rab proteins are major regulators of intracellular vesicular transport and trafficking of proteins between organelles of the endocytic and secretory pathways.[48] Indeed, the much larger number of Rab proteins in mammals reflects the higher complexity of transport events in higher eukaryotes, as indicated by the fact that several mammalian Rab proteins are expressed only in certain tissues and differentiated cell types, where they participate in specialized transport pathways.[48,49]

Characterization of half of the known Rab GTPases has not only revealed the complexity of membrane trafficking circuits but also showed that Rab GTPases are critical for mediating signaling transduction, control of cell proliferation, and differentiation. Rab proteins are increasingly being found downstream of signaling cascades affecting gene expression and growth control. Rab5, for example, is implicated in epidermal growth factor (EGF) signaling

and thought to sequester APPL1, an adaptor protein involved in chromatin remodeling, apoptosis, and gene expression, on endosomes so it cannot enter the nucleus until activation signals are received.[50,51] APPL1 and 2 are localized to a subpopulation of Rab5-positive endosomes that appear segregated from the well-characterized canonical early endosomes marked by another Rab5 effector EEA1. APPL-harboring endosomes selectively acquire specific endocytic cargo such as the epidermal growth factor receptor (EGFR) but not transferrin receptors, thus raising a possibility that they represent a specialized endosomal compartment devoted to mediating critical signaling events. Further analysis of APPL1 demonstrated that its intracellular distribution is dynamic and changes in response to extracellular stimuli such as EGF or oxidative stress.[51] Therefore, endosomes can be considered as intracellular platforms for active signal propagation, enabling a precise spatial and temporal control of cellular responses.[54] Other Rab subfamily members that signal to the nucleus, such as Rab5, Rab8, and Rab24, might work in concert with the Ran GTPases, which control nucleocytoplasmic shuttling, to bring about rapid responses to signaling that require changes in cell growth or differentiation.[54,55] Rab32, which regulates mitochondrial fission, may participate in adaptation to changing energy requirements during growth.[54,55] In addition, cell proliferation, migration, and differentiation may also be regulated through the coordinated actions of Rab GTPases regulating cell-matrix and cell-cell adhesion and those involved in growth-regulatory signaling and mitosis or apoptosis, such as Rab4a, Rab6a′, Rab8b, Rab11, Rab12, Rab13, Rab21, Rab23, Rab25, and Rab35.[52,58–64] Rab regulatory proteins [guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs)] are also subject to phosphorylation in response to stress and growth factor signaling, adding a further layer of control and complexity in for the regulation of cellular trafficking networks.[50,65]

Given the importance of Rab GTPases in many cellular functions, it is not surprising that altered expression or mutation of Rab proteins and/or their effectors may underlie human diseases. Recent studies have demonstrated multiple links between Rab GTPase dysfunction and associated regulatory proteins in human diseases including neuronal dysfunction (Rab1 and Rab7), retinal degeneration (Rab8), and immune and pigmentation disorders (Rab27 and Rab38).[66–69] For instance, Griscelli syndrome type 2 (GS2), which is caused by mutation of the RAB27a gene,[67] is a rare autosomal recessive disorder that results in pigmentary dilution of the skin and hair, the presence of large clumps of pigment in hair shafts, and an accumulation of melanosomes in melanocytes. Most patients also develop an uncontrolled T-lymphocyte and macrophage activation syndrome, known as hemophagocytic syndrome, leading to death in the absence of bone marrow transplantation. Altered function of multiple RAB regulatory proteins, such as RAB escort protein, RAB geranylgeranyl transferase, and RAB GDP dissociated inhibitor, cause choroideremia (retinal degeneration), Hermansky-Pudlak syndrome (a type of albinism that includes a bleeding tendency and lung disease), and X-linked nonspecific mental retardation, respectively.[63]

Dysregulation of RAB gene expression may also be a generalized component of human tumors. Aberrant expression of the RAB subfamily of GTPases has been documented, with overexpression of RAB5A and RAB7 in thyroid adenomas[68] and RAB1B, RAB4B, RAB10, RAB22A, and RAB24 in hepatocellular carcinomas and cholangiohepatomas.[68,69] RAB25, located on chromosome 1q22, is amplified at the DNA level and overexpressed at the mRNA level in ovarian and breast cancer and associated with a worsened outcome.[70] Indeed, increased RAB25 levels have also been reported in prostate cancer,[71] transitional cell carcinoma of the bladder,[72] invasive breast cancer,[60] liver cancer cells,[69] and Wilms tumor,[73] suggesting a pathologic role of Rab25 proteins in development or progression of tumors of multiple lineages. Further, we and others have demonstrated that the expression of Rab25 increases tumor development, whereas downregulation of Rab25 by RNA interference (RNAi) transfection significantly inhibits ovarian cancer growth in in vitro and in vivo xenograft models.[57,70]

We have recently demonstrated that Rab25 promotes ovarian cancer cell migration within a three-dimensional matrix that is characterized by the extension of long pseudopodia, and the association of the GTPase with alpha5β1 integrins promotes localization of vesicles that deliver integrin to the plasma membrane at pseudopodial tips, as well as the retention of a pool of cycling α5β1 integrin at the cell front,[74] indicating that Rab25 could contribute to tumor progression by directing the localization of integrin recycling vesicles, thereby enhancing the ability of tumor cells to directionally invade the extracellular matrix (ECM) and hence metastasis. Together, these data strongly implicate Rab25 in tumorigenesis and in tumor aggressiveness.

Targeting Ras-Superfamily Protein in Cancer

Several strategies to therapeutically target the Ras superfamily in cancer are being developed (readers are referred to a recent excellent review on this issue by Konstantinopoulos et al.[16]). In brief, the first to enter clinical trials was phosphothioridate-based antisense therapy to HRAS (ISIS2503) and c-RAF1 (ISIS5132). Although they were found to be relatively nontoxic in phase I studies, to date they have not shown any significant efficacy in phase II studies, despite promising activity in preclinicaltudies.[75] Another strategy has been to inhibit Ras-superfamily protein prenylation, which is required for localization of RAS to the plasma membrane and efficient signaling. This can potentially be achieved by inhibition of the mevalonate pathway by statins and bisphosphonates, farnesyltransferase inhibitors (FTIs), and geranylgeranyltransferase inhibitors (GGTIs). FTIs such as SCH115777 despite in vitro ability to revert the transformed phenotype and impressive in vivo activity do not appear to have significant activity in solid tumors in phase III trials.[76,77] This is thought to be due to cross-prenylation of multiple Ras-superfamily

members targeted by geranylgeranyltransferase in the context of FTIs, as well as non-RAS targets of these drugs. More recent work has focused on the identification of inter-facial inhibitors of the interaction of the RAS superfamily with its regulatory proteins such as GAP, GEF, and GDI; development of GTP analogues; and kinase inhibitors of Ras-superfamily effectors as well as upstream receptor tyrosine kinases. However, many of these are still in the early phases of development. Challenges in development of these targeted agents are that (1) Ras-superfamily GTPases and their regulatory proteins within each subfamily are highly homologous; (2) regulatory proteins may target more than one Ras-superfamily member; and (3) each Ras-superfamily GTPase may in turn be regulated by multiple regulatory proteins, making the identification of specific inhibitors difficult. Increased understanding of the molecular mechanism of individual regulatory proteins and GTPase in different tumor types and stage of the disease together with the cellular context is therefore required to enable effective targeting of the Ras superfamily for anticancer therapy.

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Chapter 12
Lipid Generation and Signaling in Ovarian Cancer

Yan Xu, Dongmei Wang, and Zeneng Wang

Introduction

Remaining as one of the most deadly diseases for the past several decades, ovarian cancer will cause an estimated 15,520 deaths in the United States in 2008.[1] From 1991 to 2004, the death rate of ovarian cancer has improved merely 8%.[1] Lack of effective early detection, the highly metastatic nature of the disease, and lack of highly effective therapeutic treatment for the late-stage cancer are the main reasons for the low survival rate of patients with ovarian cancer.[2–4]

The involvement of extracellular lipid signaling molecules, lysophosphatidic acid (LPA) in particular, in ovarian cancer was first shown in 1995.[5,6] Since then, numerous reports have been published demonstrating that LPA regulates almost every aspect of ovarian cancer cell biology, and LPA has been considered as an emerging and important target for ovarian cancer.[7–15] Elevated LPA levels in ascites and blood from patients with ovarian cancer have been reported and supported by recent independent studies.[8,16–18] The functions and signaling pathways of LPA and their receptors in ovarian cancer and reproduction have been rather extensively reviewed in recent years.[7–11,13–15,19–27] LPA production and action in cancer and other cells have also been reviewed in recent years.[7,27–29] The current review will focus on enzymes modulating LPA levels and regulation of these enzymes in ovarian cancer. In particular, the role of phospholipase A_2 (PLA_2) in ovarian cancer has been reviewed in great detail in recent years. In addition, the dual roles of sphingosine-1-phosphate (S1P) in ovarian epithelial cells will be discussed.

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Enzymes Involved in LPA Generation in Ovarian Cancer

The metabolisms of intracellular and extracellular LPA are controlled by many different enzymes. Figure 12.1 summarizes the enzymes involved in the synthesis and degradation of LPA. This review will focus on several enzymes involved in ovarian cancer.

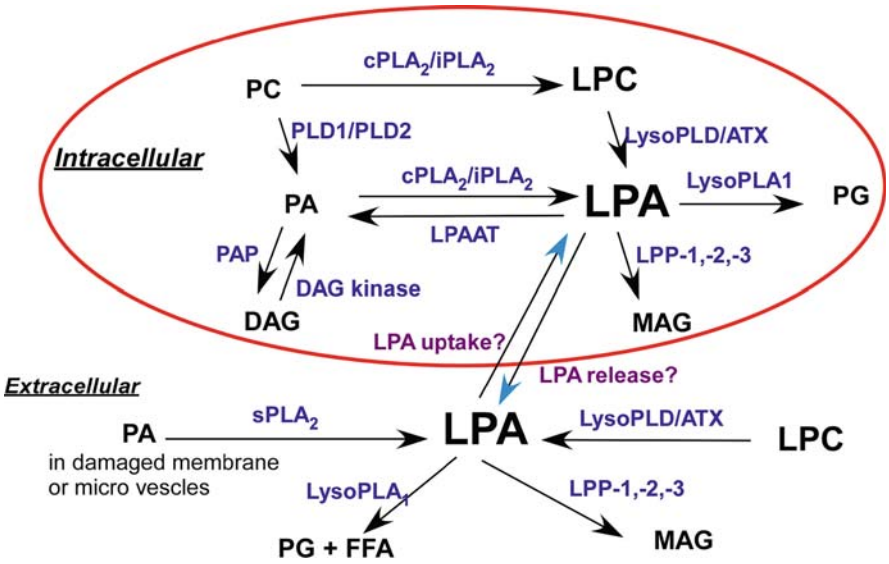

Fig. 12.1 Metabolism of LPA. Intracellular LPA is synthesized by two major pathways: an ATX-mediated conversion of LPC to LPA and/or a $cPLA_2$- and/or an $iPLA_2$-catalyzed production of LPA. $cPLA_2$ and/or an $iPLA_2$ may be involved in both pathways, as the substrate of ATX, LPC, can be produced by a cellular PLA_2 and/or a PLA_1 enzyme. LysoPLD/ATX and LPPs have both intracellular and extracellular activities. The majority of ATX is localized in intracellular organelles, and its extracellular action may be mediated by either the membrane-bound or free form of ATX.[7] LPPs are localized on the plasma, endoplasmic reticulum, and Golgi membranes. On the plasma membrane, the activity sites of these enzymes arefacing extracellular space (ecto-enzymes)[95] and thus are functionally responsible for degradation of LPA and other phospholipids extracellularly. DAG kinase and LPAAT may only function intracellularly. Whether cells can uptake or release LPA and the mechanisms involved are still elusive. $sPLA_2$ and ATX (soluble enzymes) are the two enzymes identified to directly produce extracellular LPA. LPPs, with their ecto-activities, are major enzymes responsible for LPA extracellular degradation. A $lysoPLA_1$ activity is likely to be present in at least some cells, which is responsible for degradation of LPA extracellularly (see text). PC, phosphatidylcholine; PA, phosphatidic acid; DAG, diacylglycerol; MAG, monoacylglycerol; PG, phosphatidylglycerol; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, calcium-independent phospholipase A_2; PLD1/PLD2, phospholipase D1 and phospholipase D2; PAP, phosphatidic acid phosphatase; LPAAT, LPA acyltransferase; lysoPLA/ATX, lysophospholipase D/autotaxin; LPP, lysophospholipid phosphatase; sPLA2, soluble phospholipase A_2

Lysophospholipase D (LysoPLD or Autotaxin)

Autotaxin Activity in Ovarian Cancer

Although lysophospholipase D (lysoPLD) activity, which produces LPA from lysophosphatidylcholine (LPC) and possibly other lysophospholipids, was detected and reported more than two decades ago,[30–32] its molecular cloning and identification were only achieved in 2002.[19,33,34] LysoPLD is identical to autotaxin (ATX), which is a tumor cell motility-stimulating factor, originally isolated from melanoma cell supernatants.[19] Recently, van Meeteren and Moolenaar have extensively reviewed the ATX-LPA axis.[35] Overexpression of ATX and autocrine or paracrine production of LPA by ATX, which contributes to tumor cell motility, survival, angiogenesis, and proliferation, have been shown in multiple cancer types.[34–42] In particular, lysoPLD activity in human peritoneal fluid is significantly elevated under pathologic status in a number of gynecologic cancers, including ovarian cancer, when compared with the serum lysoPLD activity in clinical groups and healthy subjects.[43] ATX also hydrolyzes sphingosylphosphorylcholine (SPC) to produce shingosine-1-phosphate (S1P), another important lysolipid in ovarian cancer.[44] The current review emphasizes several aspects related to ATX in ovarian cancer.

The Cellular Sources of ATX in Ovarian Cancer

The potential sources of ATX activity in the tumor microenvironment include tumor cells and stroma, such as mesothelial cells, as well as inflammatory cells and platelets activated by the proinflammatory tumor environment. Ovarian cancer cell lines, including Dov13, Ovca3, and SKOV3, express ATX.[7] Down-regulation of ATX secretion in SKOV3 cells using antisense morpholino oligomers significantly attenuates cell motility responses to vascular endothelial growth factor (VEGF), ATX, LPA, and LPC.[36] Paradoxically, when an ATX activity was directly measured in several ovarian cancer cells lines, including HEY, SKOV3, and OVCA420 using [^{3}H]LPC as the substrate, we did not observe its conversion to [^{3}H]LPA under different conditions tested (data not shown). Moreover, addition of ovarian cancer cells (such as SKOV3) to cell-free ovarian cancer ascites or leaving the endogenous ovarian tumor cells in human ovarian cancer ascites did not further enhance LPA production when compared with that of cell-free ascites (our unpublished observations). Similarly, we did not detect ATX activity from cultured human peritoneal mesothelial cells in vitro, which are important host cells interacting with ovarian cancer cells in vivo.[45] These observations suggest that other cell types may contribute to the ATX activity observed in human ovarian cancer ascites, which remains to be identified. However, these data do not exclude the possibility that when cancer and/or mesothelial cells are present in the tumor microenvironment in vivo, they may exhibit ATX activity and be responsible for the production of LPA

detected in human ovarian cancer ascites. The cellular source(s) of ascites LPA remain to be further investigated.

ATX Expression in Ovarian Cancer

ATX mRNA levels are increased in a variety of cancers, including ovarian cancer.[7] The www.oncomine.org Web site provides comprehensive data on gene expression in various normal and cancer tissues. Among different cancers, clear cell renal carcinoma had very high levels of ATX (gene ENPP2) expression when compared with many other cancer types, including different subtypes of ovarian cancer ($p = 2.9 \times 10^{-22}$). In ovarian cancer, ovarian adenocarcinomas ($n = 31$) express much higher levels of ATX than normal ovaries ($n = 3$) ($p = 2.44 \times 10^{-13}$). However, decreased ATX levels have been shown in recurrence-positive versus negative ovarian cancer and higher grade/stage versus lower grade/stage of ovarian cancer (www.oncomine.org). These data suggest that (1) ATX and its product, LPA, may be involved in the early stage of tumorigenesis; (2) the activity but not the RNA levels of ATX is more closely related to ovarian cancer; and (3) other enzymes may also be involved in the regulation of production or degradation of LPA in ovarian cancer. The contributions of ATX to ovarian cancer development at different stages need further investigation.

ATX Regulation in Ovarian and Other Cancers

1. *The regulators of expression and/or activity of ATX*
   A link among ATX expression, LPA, and VEGF signaling in ovarian cancer cell lines has been demonstrated.[36] LPA stimulates VEGF secretion from ovarian cancer cells.[46] VEGF-A induces ATX expression and secretion, resulting in increased extracellular LPA production. LPA, via LPA_4, induces VEGF receptor-2 (VEGFR2) expression. Downregulation of ATX secretion in SKOV3 cells using antisense oligomers significantly attenuates cell motility responses to VEGF, ATX, LPA, and LPC, accompanied by decreased LPA_4 and VEGFR2 expression as well as increased release of soluble VEGFR1. This interesting positive feedback loop involving VEGF, ATX, and its product LPA may affect tumor progression in ovarian cancer cells.[36]

   ATX activity can be analyzed using radioactive-labeled substrates (such as [^{3}H] or [^{14}C]-LPC)[47] (Fig. 12.1). Echelon Biosciences Inc. Salt Lake City, UT has developed a fluorogenic lysoPLD substrate (L-2000), which is a doubly labeled analogue wherein the fluorophore is quenched through intramolecular energy transfer. Cleavage of the substrate by lysoPLD produces an increase in fluorescence. ATX uses a single catalytic site for the hydrolysis of both lipid and nonlipid phosphodiesters. Using a sensitive fluorescence resonance energy transfer-based phosphodiesterase sensor assay for ATX, the two ATX products, LPA and sphingosine-1-phosphate, have been shown to be specific and potent inhibitors of ATX in a mixed-type

manner.[48] These results suggest that LPA and S1P negatively regulate their own biosynthesis in the extracellular environment.

ATX activity is inhibited by ethylenediamine tetraacetic acid (EDTA), which can be reversed by Co^{2+} or Zn^{2+}.47 L-Histidine inhibits ATX enzymatic activities in a noncompetitive manner, which can be reversed by 20-fold lower concentrations of zinc salt.[49] In addition, several growth factors/cytokines regulate expression of ATX. In thyroid carcinoma cells, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) stimulate ATX mRNA expression, whereas the cytokines interleukin-4 (IL-4), IL-1β and transforming growth factor β (TGF-β) reduce ATX transcriptional levels.[39] Integrin α6β4 promotes expression of ATX in breast carcinoma cells via transcriptional factor NFAT1, but not NFAT5.[50] Epstein-Barr virus induces autotaxin in Hodgkin lymphoma cells.[51] How ATX expression and/or activity are regulated in ovarian cancer warrants further study.

2. *Hypoxia and ATX activity in ovarian cancer ascites*
Tumor hypoxia is a common feature of solid tumors and is associated with tumor growth, angiogenesis, resistance to apoptosis, and compromise in radiotherapy and chemotherapy, as well as tumor metastasis.[52–54] It becomes a central issue in tumor pathology and cancer treatment. Rapid growth of solid intraperitoneal tumors and large volumes of ascitic fluid characterize ovarian cancer. In particular, large numbers of ovarian tumor cells are present in ascitic fluids. We have found that human ovarian ascites is hypoxic, and hypoxia increases ovarian cancer cellular responses to LPA in cell migration and invasion.[55]

Compared with ascitic fluids from patients with hepatic liver diseases (nonmalignant), ovarian cancer ascitic fluids contain much higher levels of LPA.[56,57] To determine the effect of hypoxia on LPA production, we incubated two pairs of cell-free ascitic fluids (cells were removed by centrifugation and/or filtration through 0.22-μm filters) from patients with ovarian cancer or chronic hepatitis at 37°C under normoxia or hypoxia (1% O_2). At indicated time points, the LPA levels were analyzed using the electrospray ionization mass spectrometry (ESI-MS) method developed previously.[56,57] Ovarian cancer ascites not only contained higher initial levels of LPA but also produced more LPA during incubation when compared with non-malignant ascites (Fig. 12.2A, a–d). Intriguingly, modest but *significantly* higher levels of LPA were produced under the hypoxic condition when ascites was incubated >24 hours in ovarian cancer ascites (Fig. 12.2A, a and b). These results suggest that a soluble LPA-producing enzyme(s) that is sensitive to the hypoxic condition is present in ovarian cancer ascites.

To determine whether a lysoPLD-like activity was present in ovarian cancer ascites, we incubated a radioactive [^{3}H]-labeled LPC with ovarian cancer ascites. We observed an increase in LPA production during incubation of ovarian cancer ascites (Fig. 12.2B, a and b). The kinetics of LPA production detected using either ESI-MS or the radioactive LPC conversion method was

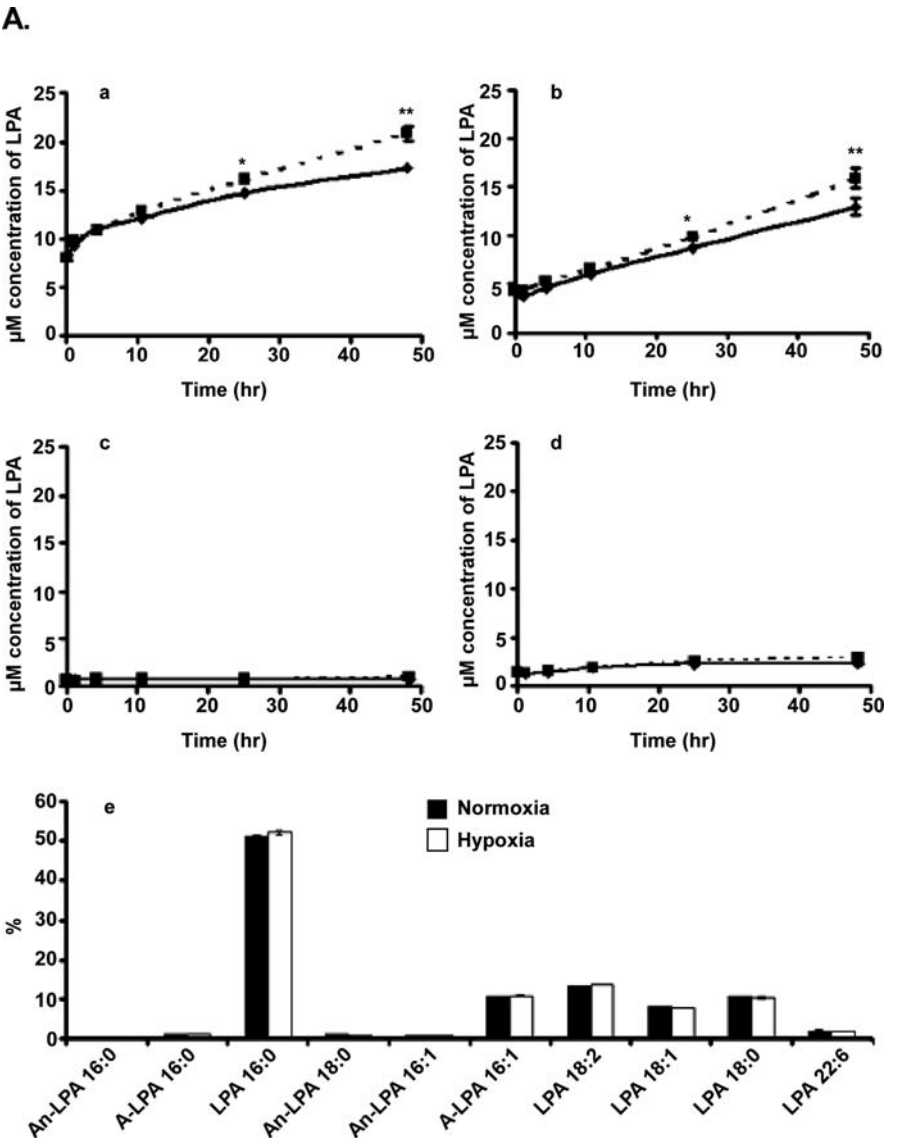

Fig. 12.2 The involvement of lysoPLD-like activity in hypoxia-enhanced production of LPA in ascites. (**A**) Changes in LPA concentrations when ascitic fluids were incubated under normoxic (♦ and solid line) or hypoxic conditions (■ and dashed line, 1% O_2) for the indicated time points. O36 (a) and O37 (b) were ascitic fluids from two patients with ovarian cancer and N30 (c) and N35 (d) were ascitic fluids from two patients with chronic hepatitis. LPA production was analyzed using the ESI-MS method.[56,57] Panel (e) shows the distributions of LPA subspecies in ovarian cancer ascites O36, which were not significantly changed under hypoxic conditions. A-LPA, alkyl-LPA; An-LPA, alkenyl-LPA. Incubation time: 24 hours. (**B**) LysoPLD-like activity in ovarian cancer ascites [(a) O36; (b) O37] during incubation under normoxic and hypoxic conditions. Ascites was labeled with 20 nCi/ml ^{3}H-LPC under normoxic or hypoxic conditions

similar, suggesting that a lysoPLD-like activity may account for the major portion of LPA production activity in ovarian cancer ascites when tested ex vivo. A few more lines of evidence also supported this notion. First, ATP, a known inhibitor for lysoPLD, dose- and time-dependently inhibited LPA production in both normoxic and hypoxic cells (Fig. 12.2B, c and d). Second, the enzymatic activity in ascites was sensitive to both EDTA and Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N′,N′-t etraacetic acid, suggesting that bivalence metal ions are required for the activity. We found that Co^{2+}, an activator of lysoPLD,[34] enhanced the production of LPA (Fig. 12.2B, e). Finally, another soluble enzyme, soluble phospholipase A_2 ($sPLA_2$), has been shown to be able to produce LPA extracellularly.[58] We tested the effect of a specific $sPLA_2$ inhibitor, thioether amide-PC (TA-PC), on LPA production in ascites and found that TA-PC did not have a significant effect on LPA production (Fig. 12.2B, f). Collectively, our data suggest that a lysoPLD or a lysoPLD-like activity is present in ovarian cancer ascites, which is responsible for LPA production ex vivo. These results are consistent with a recent publication showing that lysoPLD activity in human peritoneal fluids is increased when measured by quantifying choline released from exogenous LPC.[43]

Phospholipase A_2

The phospholipase A_2 (PLA_2) family of enzymes catalyzes the hydrolysis of the sn-2 position of phospholipids to generate free fatty acids and lysophospholipids. There are at least 20 different enzymes in this family, which are classified into four groups based on their cellular localization, substrate specificity, and calcium dependence: secreted PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$), the platelet activating factor acetylhydrolase (PAF AHase), and calcium-independent PLA_2 ($iPLA_2$).[59–62]

Fig. 12.2 (continued) for the indicated time points, and results were expressed as percent radioactivity in LPA. The dose-dependent (c) and time-dependent (d) inhibitory effects of ATP on the levels of LPA in ascites (O36) during incubation under normoxia (♦ and solid line) and hypoxia (■ and dashed line) conditions. The dose effect was determined by radiolabeling of ascites (O36) with 20 nCi/ml ^{3}H-LPC under normoxic or hypoxic conditions for 24 hours and results were expressed as percent radioactivity in LPA. The time effect was determined by MS after incubating ascites (O36) in the presence of ATP (10 mM). LPA concentration in O36 incubated in the absence of ATP under normoxia was taken as 100%. (e) Effects of Co^{2+} (1 mM) and metal ion chelating agents EDTA (3 mM) and EGTA (3 mM) on the production of LPA via lysoPLD-like activity in ascites (O37) incubated under normoxic and hypoxic conditions for 24 hours. The radioactive-labeled LPC was used in these studies. (f)Effects of $sPLA_2$ inhibitor TA-PC (10 μM) on the concentrations of LPA in ascites (O36, O37) after incubation under normoxic condition for 24 hours. The concentrations of LPA were determined by ESI-MS and 1-myristoyl-2-hydroxyl-sn-3-phosphate (14:0-LPA) was added as an internal standard.LysoPLD-like activity was expressed as percent radioactivity in LPA after ascites was radiolabeled. Data are presented as mean ± SD for two independent replicates. *$p < 0.05$; **$p < 0.01$ (Student's t-test with two tails)

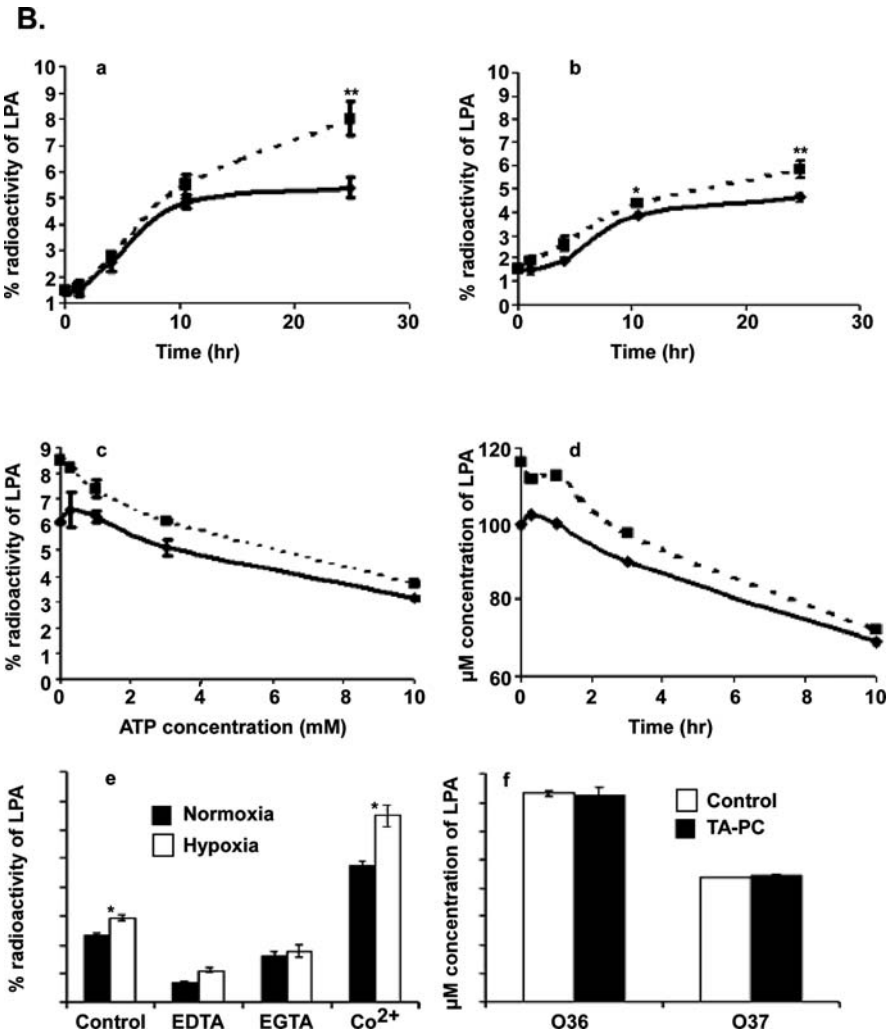

Fig. 12.2(Continued)

Cytosolic PLA_2 ($cPLA_2$)

Group VI PLA_2 ($cPLA_2$; PLA2G6) is believed to play a central role in the release of arachidonic acid (AA) in response to extracellular stimuli.[61] Because all PLA_2 produce both lysophospholipids and fatty acids, $cPLA_2$ also produces cellular lysophospholipids. However, the role of $cPLA_2$ in generating extracellular and signaling lysophospholipids has not been directly demonstrated, although inhibitor studies have suggested that $cPLA_2$ is involved in constitutive LPA synthesis in ovarian cancer cells.[63] The lysophospholipids generated by $cPLA_2$ intracellularly may be quickly metabolized to other lipids, and they may not be released or secreted extracellularly as signaling molecules.

$cPLA_2$ is highly expressed in the ovary versus many other normal tissues. Ovarian clear cell adenocarcinoma expresses higher levels of $cPLA_2$ than do normal ovaries, but ovarian serous adenocarcinoma expresses less $cPLA_2$ than do other ovarian subtypes (www.oncomine.org). Similar to ATX and other PLA_2 (see later), there are increasing lines of evidence to support their roles in cancer,[64,65] which may or may not be correlated with their RNA levels in cancers. It is well known that $cPLA_2$ activity is regulated by phosphorylation and translocation.[61]

$cPLA_2$ is more likely to be involved in mediating LPA signaling than LPA production. We have shown that in HEY ovarian cancer cells, when exogenous LPA was added, it induces cell migration even when $iPLA_2$ activity is blocked, suggesting $iPLA_2$ is involved in LPA production, but not downstream LPA signaling in these cells. In contrast, exogenous LPA cannot reverse the inhibitory effect on $cPLA_2$, suggesting that $cPLA_2$ is involved in LPA signaling leading to cell migration.[66]

Secreted PLA_2 ($sPLA_2$)

$sPLA_2$ may be involved in extracellular LPA production in certain cells, including ovarian cancer cells.[58,61,63] In addition, $sPLA_2$ is involved in cell invasion via regulation of expression and secretion of matrix metalloproteinases (MMPs) in cancer cells.[58] Many different subtypes of $sPLA_2$ have been identified. They belong to I, II, III, IX, X, and XII PLA_2 groups. Group X (PLA2G10) and group IIA $sPLA_2$ are upregulated in colon cancers versus many other cancer types, including different subtypes of ovarian cancer. Group X (PLA2G10) $sPLA_2$ is upregulated in estrogen receptor–positive breast cancer versus estrogen receptor–negative cancer, as well as metastatic prostate cancer versus prostate carcinoma and normal prostate (www.oncomine.org). However, in ovarian cancer, while group X $sPLA_2$ is upregulated in ovarian mucinous adenocarcinoma ($n = 13$) versus normal ovary ($n = 4$) ($p = 7.1 \times 10^{-4}$), it is progressively decreased from stage I to stage IV of ovarian adenocarcinoma ($p = 9.4 \times 10^{-4}$), and lower expression of $sPLA_2$ was detected in the most commonly occurring ovarian cancer type serous adenocarcinoma versus other ovarian subtypes types ($p = 0.001$) (www.oncomine.org). Similarly, lower expression of group IIA $sPLA_2$ was detected in serous ovarian carcinoma versus normal ovaries (www.oncomine.org). These data suggest that (1) group IIA and X $sPLA_2$ may not be involved in ovarian cancer, other than mucinous ovarian adenocarcinoma; (2) other subtypes of $sPLA_2$ may be involved in ovarian cancer; and (3) the activity regulated at the posttranscriptional level(s), but not the RNA levels of $sPLA_2$, may be involved in ovarian cancer. These issues warrant further studies.

Calcium-Independent PLA_2 ($iPLA_2$)

1. *$iPLA_2$ in LPA production in ovarian cancer*
   The mammalian $iPLA_2$ family consists of two members, $iPLA_2\beta$ and $iPLA_2\gamma$, with the former having been more extensively characterized. In

recent years, the involvement of $iPLA_2$ in a broad range of biological processes, including proliferation, apoptosis, inflammation, and atherosclerosis, has been revealed.[59,67,68]

HELSS (the haloenol lactone suicide substrate) is a potent, irreversible, mechanism-based inhibitor of $iPLA_2$[69]. It possesses a 1000-fold selectivity for $iPLA_2$ versus other Ca^{2+}-dependent PLA_2 enzymes.[69] Using this inhibitor, $iPLA_2$ has been suggested to be involved in constitutively active LPA production from ovarian cancer cells.[63] In addition, by using pharmacologic inhibitors, blocking antibodies, and genetic approaches (such as point mutations, dominant negative forms of genes, and siRNAs against specific targets), we show an interesting cascade involving laminin-10/11-β_1-integrin and caspase-3 leading to $iPLA_2$ activation and migration in ovarian cancer cells.[10,15,55,66,70–72] Moreover, we have shown that human peritoneal mesothelial cells (PMCs) constitutively produce LPA, which accounts for a significant portion of the chemotactic activity in the conditioned medium (CM) from PMC to ovarian cancer cells. Production of LPA by PMC and the chemotactic activity in the CM can be blocked by HELSS and $AACOCF_3$ (an inhibitor of both $cPLA_2$ and $iPLA_2$).[45]

2. *The roles of $iPLA_2$ in ovarian cancer*

We have shown that $iPLA_2$ is involved in adhesion, migration, and invasion of ovarian cancer cells.[10,15,45,55,66,71,72] Both sets of products of $iPLA_2$, fatty acids and lysophospholipids (AA and LPA in particular), are likely to be involved in the activities.[66,71] $iPLA_2$ is involved in proliferation of T cells and ovarian cancer cells and is a cell cycle–regulated enzyme in human peripheral blood B or T lymphocytes (PBLs) and ovarian cancer cells.[67,68,73] In PBL and CHO cells, $iPLA_2$ activity is the highest at the G2/M and late S phases and lowest at the G1/S transition, which are inversely correlated with cellular phosphatidylcholine (PC) levels.[67,68] The levels of the full-length 80-kDa $iPLA_2$ protein do not change during the cell cycle, but the expression of an $iPLA_2$ splice variant and the resultant truncated 50-kDa protein is cell cycle dependent and functions as a negative regulator of $iPLA_2$ activity.[67] Interestingly, PBLs mainly express $iPLA_2$ with no or low level expression of $sPLA_2$ and $cPLA_2$.[68] Song et at. have recently shown that inhibition of $iPLA_2$ suppresses proliferation and tumorigenesis of human Ovarian cancer cells, including SKOV3, OVCA3, and Dov13 cell lines.[73] Blocking $iPLA_2$ activity with BEL induces cell cycle arrest in S and G2/M phases independent of the p53 status, with accompanying cyclin B and E increases.[73] Whereas LPA releases S-phase arrest, it cannot release the G2/M phase arrest. Interestingly, LPA partially and significantly reverses BEL-induced inhibition of cell proliferation in two of three OC cell lines. Higher concentrations of BEL (greater than fivefold) are required to inhibit cell proliferation when Fetal bovine serum (containing LPA, LPC, AA, and other potential PLA_2 products) is present, suggesting that $iPLA_2$ products are potentially involved in $iPLA_2$'s cellular functions.[73] In addition, downregulation of $iPLA_2\beta$

expression with a shRNA inhibits cell proliferation and tumorigenesis of SKOV3 cells in nude mice.[73]

3. *The structure and activation of iPLA$_2$*
   The human iPLA$_2$β gene contains 18 exons (17 exons for the coding region) with the full-length cDNA encoding a 806-amino-acid protein with a calculated molecular mass of 88 kDa. The protein contains a lipase motif, GXSXG (at amino acids 511–521), and 8 ankyrin repeats in the N-terminal half of the protein.[59,74,75] In addition, numerous different splice variants of iPLA$_2$ have been reported, and the expression of some of these are cell cycle regulated.[75] Moreover, iPLA$_2$ contains putative caspase-3 cleavage motifs (DVTD^{183}, DLFD^{513}, MVVD^{733}, DCTD^{737} ,and RAVD^{744}). Atsumi et al. first reported that iPLA$_2$ could be cleaved at Asp^{183} in TNF-α/cycloheximide-treated U937 cells, resulting in increased fatty acid release[76] and implying that a caspase-3–mediated activation of iPLA$_2$ is involved. iPLA$_2$ can be cleaved at Asp^{513} and Asp^{733} by caspase-3, resulting in a truncated iPLA$_2$ lacking ankyrin-repeat domains.[71] This truncated iPLA$_2$ has increased activity to produce LPC in UV-induced apoptosis, where LPC serves as a "find me" signal in the phagocytic process for cell clearance.[71] Recent evidence suggests that other proteases may also be involved in the proteolytic activation of iPLA$_2$.[59] In addition, ATP is an activator of iPLA$_2$.[77] A common characteristic of tumor cells is the constant overexpression of glycolytic and glutaminolytic enzymes, accompanying glycolytic ATP production.[59] Moreover, the function of iPLA$_2$ can be regulated by substrate availability, which may be modulated by many factors including oxidative stress.[59] We have shown that in ovarian cancer cells, caspase-3 activates iPLA$_2$ by cleavage at Asp.[71]
4. *Expression of iPLA$_2$ in ovarian cancer*
   iPLA$_2$β has been shown to be upregulated in several other cancers, including melanoma, prostate, and breast cancers when compared with corresponding normal tissue tumorigenesis (www.oncomine.org). There is no data available from this Web site showing differential expression of iPLA$_2$ between OC and normal tissues. However, the importance of the role of iPLA$_2$ in lipid production and ovarian development is implied by published papers as reviewed above.[45,55,63,66,71,72] More importantly, iPLA$_2$ activity can be regulated post-transcriptionally at several different levels as reviewed above, including splicing, protease activation, and regulation by ATP and substrate availability. Together, the data published strongly suggest that increased iPLA$_2$ expression (at either RNA or protein level) in tumor cells/tissues may not be necessary for its role in tumorigenesis. Rather, the activity of iPLA$_2$ in tumor cells and in the microenvironment plays an important role in tumorigenesis.
5. *The intertwining of different PLA$_2$ enzymes*
   Deficiency in neither cPLA$_2$α, iPLA$_2$β, nor sPLA$_2$ in mice is embryonic lethal,[62,78,79] suggesting redundant effects of different PLA$_2$. We have shown that cPLA$_2$ can be activated by iPLA$_2$ via production of LPA in ovarian cancer cells.[66,71,72] In other cancer cells, cPLA$_2$ can be activated by

$sPLA_2$.[58] In addition, $iPLA_2\gamma$ is involved in the inducible expression of group IIA $sPLA_2$ in rat fibroblastic cells. This partial list of interactions suggests that enzymatic activity of any given PLA_2 can be modulated by another PLA_2 enzyme, regardless of whether they belong to the same group or not.

Other LPA-Producing Enzymes

Other enzymes may also be involved in LPA production in ovarian cancer. Phospholipase D (PLD) hydrolyzes phosphatidylcholine into phosphatidic acid (PA), which may in turn be metabolized into LPA via PLA_2 activity. In ovarian cancer cells, PLD activity has been suggested to be involved in LPA production.[63] Promotion of LPA formation by nucleotides is accompanied by stimulation of PLD activity. Nucleotide agonists acting through a P2Y4 purinergic receptor stimulate LPA release from SKOV3 cells via PLD2 and $sPLA_2$ activities.[80] The involvement of phosphatidylserine specific PLA_1 (PS-PLA_1) and lecithin-cholesterol acyltransferase (LCAT) in LPA production from different cell types have been shown,[28,29] but their potential involvement in ovarian cancer is unclear.

The Release/Secretion Issue

LPA exerts many of its cellular effects extracellularly through its plasma receptors LPA_{1-5}. Thus, the "signaling" LPA needs to be either produced extracellularly or secreted from cells. ATX and $sPLA_2$ produce LPA extracellularly and have been considered to be the main enzymes involved in producing signaling LPA. It is well known that fatty acids produced intracellularly can be released/secreted by cells, although the mechanisms of this process are not well understood. Much less is known on whether intracellular LPA can be released from cells and how it is released. Because $cPLA_2$ and $iPLA_2$ are intracellular enzymes, they generate fatty acids and lysophospholipids intracellularly. Therefore, their direct roles in producing extracellular LPA need to be further investigated. Nevertheless, as we have reviewed above, many lines of evidence suggest that $iPLA_2$ or other intracellular enzymes are involved in regulating extracellular LPA levels in ovarian cancer and human ovarian mesothelial cells, where ATX activity was not directly detected.[10,15,45,55,66,71,72] Is it possible that the LPA increase detected extracellularly via modulation of $iPLA_2$ still required a soluble enzyme, such as $sPLA_2$ extracellularly? Regardless whether the role of $iPLA_2$ in regulating extracellular LPA levels is direct or indirect, it is more important that $iPLA_2$ is involved in LPA production in ovarian cancer–related cells. The functional studies of $iPLA_2$ in ovarian cancer further support the notion that it may represent a valid target for ovarian cancer.

LPA can be taken up by cells potentially through a diffusion process.[81] We have shown that intracellular concentrations of LPA are much higher (10- to

30-fold) than those in extracellular spaces in ovarian cancer cells.[10] Is it possible that intracellular LPA is a source of extracellular LPA? We have shown that $iPLA_2$ overexpression in HEY ovarian cancer cells increased LPA but not LPC levels in cell supernatants, but we detect ATX activities in these cells.[71] These results suggest that (1) ATX activity is unlikely to be involved in increased extracellular LPA from these cells tested under the conditions that we have used; (2) $sPLA_2$ activity may be involved and it is regulated by $iPLA_2$ and/or its products; (3) $iPLA_2$ may be able to generate extracellular LPA through an unknown mechanism; or (4) LPA generated by $iPLA_2$ intracellularly is released by the cells. Although LPA release from intact cells has not been clearly demonstrated, which may be related to technical difficulties, two independent release systems for S1P, which is structurally similar to LPA, have been shown to exist in the platelet plasma membrane; an ATP-dependent system stimulated by thrombin and an ATP-independent system stimulated by Ca^{2+}. An ATP binding cassette transporter inhibitor, glyburide, prevents ATP- and thrombin-induced S1P release from platelets.[82] Therefore, it is possible that a diffusion and/or a transporter system are present in cells to release intracellular LPA and/or S1P to extracellular space.

LPA Receptors in Ovarian Cancer

Plasma LPA receptors LPA_{1-3} mediate many effects of extracellular LPA. LPA has been implicated in the migration of different cell types and under different assay conditions. Although LPA_1 plays a very important role in migration of breast and pancreatic cancer cells,[83,84] we and others have shown that LPA_2 and LPA_3 are more important in cell adhesion, migration, and invasion of ovarian cancer cells.[45,55,66,72,85] LPA_2 is also important in mediating the LPA-induced production and secretion of IL-6, IL-8, and GRO-α from ovarian cancer cells.[86,87] These results are consistent with reports showing that LPA_1 is involved in the negative growth regulation in ovarian cancer cells[11] and that LPA_2 and LPA_3, but not LPA_1, expression is upregulated in late-stage ovarian cancer.[12] This latter observation has recently been confirmed by an independent study.[88] They are also consistent with the data in Oncomine, where all four major subtypes of ovarian cancers express significantly lower levels of LPA_1 than do normal ovaries. Ovarian serous, endometrioid, and mucinous adenocarcinomas express significantly higher levels of LPA_2 than do normal ovaries. LPA_3 is overexpressed in serous ovarian cancer versus normal ovaries, and its expression levels are positively correlated with the stages and grade of ovarian cancer (www.oncomine.org). Deregulation of the expression of LPA_2 or LPA_3 is also associated with cancers of the colon, breast, intestine, and glioma.[89]

More LPA actions, including functions of intracellular LPA, beyond the first three LPA plasma receptors (LPA_{1-3}) identified have been detected and investigated. A recent review by Valentine et al. has covered this area rather

extensively.[90] The fourth LPA receptor, GPR23 or LPA_4, is abundantly expressed in normal ovaries but not in ovarian cancer.[91,92] This is consistent with data presented on the Oncomine Web site through independent studies that show LPA_4 is significantly reduced in all major types of ovarian cancer when compared with normal ovaries (www.oncomine.org). GPR92, a G protein-coupled receptor (GPCR) that shares ~35% amino acid identity with LPA_4/GPR23, is a newly identified $G_{12/13}$- and G_q-coupled LPA receptor (LPA_5).[93,94] LPA_5 is highly expressed in the lymphocyte compartment of the gastrointestinal tract,[94] and colon carcinomas express significantly higher levels of GPR92 than many other cancers, including ovarian cancer (www.oncomine.org). The expression of GPR92 in normal ovarian surface epithelium and serous ovarian carcinoma is not statistically different, with endometrioid ovarian carcinomas expressing less GPR92 than do the other three subtypes of ovarian carcinomas (www.oncomine.org). Similar to GPR92, the intracellular nuclear lipid receptor peroxisome proliferator-activated receptor γ (PPARγ) is highly expressed in colon carcinomas when compared with other cancers, including ovarian cancer (www.oncomi ne.org). In addition, ovarian serous and clear cell adenocarcinomas express much less PPARγ than do normal ovaries. A significant negative correlation between PPARγ expression and ovarian cancer stages has been demonstrated (www.oncomine.org). These data suggest that GPR23, GPR92, and PPARγ may not play important promoting roles in the most commonly occurring ovarian cancers. Extracellular instead of intracellular LPA is more likely to be critical for ovarian cancer.

Enzymes Involved in Reducing LPA Levels in Ovarian Cancer

Lipid-Degrading Enzymes Involved in Ovarian Cancer

Lipid Phosphate Phosphatases

The key enzymes responsible for dephosphorylation of LPA, S1P, and phosphatidic acid (PA) are termed lipid phosphate phosphatases (LPPs).[95–98] Dephosphorylation of these lipids either generates lipids without signaling functions or, in some cases, products with different biological activities.They are integral membrane enzymes, and when localized to the plasma membrane, their activity sites are toward the extracellular space, rendering them as the important enzymes regulating the degradation of extracellular levels of signaling LPA, S1P, and PA. LPP activities can be measured using either radiolabeled or fluorescent lipid substrates in intact or broken cell preparations.[99]

Mills' group has demonstrated that the mRNA levels of LPP-1 are decreased in the majority of ovarian cancers and ovarian cancer cell lines when compared with those in normal ovarian epithelium and immortalized ovarian epithelial cells. Ectopically expressed LPP-1 in ovarian cancer cell lines reduces LPA levels, accompanied by reduced cellular effects of LPA.[100] In addition, the

same group has shown that overexpression of LPP-3 decreases the growth, survival, and tumorigenesis of ovarian cancer cells via decreasing LPA.[101] Moreover, this group has reported that a gonadotropin-releasing hormone–responsive phosphatase hydrolyzes LPA within the plasma membrane of ovarian cancer cells. The membrane phosphatase was Mg^{2+} independent and insensitive to inhibition by *N*-ethylmaleimide, characteristics suggestive of phosphatidic acid phosphatase activity.[102] In human ovarian cancer cell line Caov-3, approximately 98% of LPA hydrolysis could be accounted for by the dephosphorylation of LPA to yield monoglyceride, with the deacylation reaction accounting for less than 1% of LPA hydrolysis.[102]

The data from Oncomine show that expression of phosphatidic acid phosphatase type 2A (PPAP2A, LPP-1a) is significantly higher in the ovary than in many other normal tissues, but it is reduced in ovarian serous adenocarcinoma ($n = 41$) versus normal ovaries ($n = 4$) ($p = 1.4 \times 10^{-8}$). In addition, the expression of LPP-1 in ovarian serous adenocarcinoma is significantly lower than that in the other three subtypes of ovarian cancer (clear cell, endometrioid, and mucinous) (www.oncomine.org). Paradoxically, prostate adenocarcinomas, another cancer where LPA has been suggested to play important roles,[103] express much higher levels of LPP1 than do many other types of cancers, including ovarian serous papillary adenocarcinoma ($p = 3.1 \times 10^{-41}$) (www.oncomine.org). In addition, prostate carcinomas express much higher LPP1 than do normal prostates ($p = 1.8 \times 10^{-5}$) (www.oncomine.org), suggesting that the roles of LPPs and consequences of expression of LPPs may be regulated by tissue microenvironments.

Prostatic Acid Phosphatase and Lysophospholipase A_1 Activity

Prostatic acid phosphatase, a nonspecific phosphatase that has been implicated in the progression of prostate cancer, has been shown to inactivate LPA in human seminal plasma, suggesting that this enzyme may be an important regulator of serum LPA concentrations. Its role in ovarian cancer is currently unknown.[104]

When we tested the fate of exogenously added [^{3}H]LPA (labeled in the fatty acid portion) when applied to Swiss 3T3 cells, we have found that approximately 50% of LPA quickly (within 1 hour) converted to fatty acids, suggesting the presence of a lysophospholipase A_1 activity.[10] Whether the LPP and/or lysophospholipase A_1 activities are consistently reduced in ovarian cancer cells when compared with nonmalignant cells remains to be further studied. A lysophospholipase A_1 has been purified from rat brain.[105]

Enzymes Involved in LPA Anabolism

Two cDNAs encoding human LPA acyltransferase (LPAAT), which converts LPA to PA, have been cloned. LPAAT-α is expressed in all tissues with highest

expression in skeletal muscle and LPAAT-β predominately expressed in the heart and liver.[106] Elevated expression of LPAAT-β is associated with reduced survival in ovarian cancer and earlier progression of disease in ovarian and endometrial cancer.[107] Inhibition of LPAAT-β using small interfering RNA (siRNA) or its selective inhibitors induces apoptosis in human ovarian and endometrial cancer cell lines in vitro and enhances the survival of mice bearing ovarian tumor xenografts.[107] LPAAT-β is localized to the endoplasmic reticulum and thus it mainly uses intracellular LPA as its substrate. These data suggest that intracellularly, PA, but maybe not LPA, plays an important role in ovarian cancer, and LPAAT-β may be a potential prognostic and therapeutic target in ovarian and endometrial cancer.

S1P Production and Signaling in Ovarian Cells

S1P in Other Cell Types

The biological effects, signaling, receptors, and metabolism of S1P in cells and animals have been extensively reviewed in recent years.[108–113] S1P affects cell migration through its receptors. To date, five receptors for S1P have been identified, which consist of EDG1 ($S1P_1$), EDG5 ($S1P_2$), EDG3 ($S1P_3$), EDG6 ($S1P_4$), and EDG8 ($S1P_5$).[114–116] Among them, $S1P_1$, $S1P_2$, and $S1P_3$ are widely expressed in various tissues, whereas the expression of $S1P_4$ is mainly confined to lymphoid tissues and platelets and that of $S1P_5$ to the central nervous system. S1P can exert either stimulatory or inhibitory effects on cell migration. In many of the different cell types (mostly nonmalignant cells) studied, $S1P_1$ and/or $S1P_3$ mediate S1P-induced stimulation of cell migration, whereas $S1P_2$ mediates S1P-induced inhibition of cell migration. The migratory response of a particular cell type to S1P has been well correlated with its receptor subtype expression patterns.[117–120] For example, S1P enhances migration of endothelial cells, which predominately express $S1P_1$ and $S1P_3$,[121] whereas it inhibits migration of C2C12 myoblasts[122] and B16 melanoma cells,[120] which have high levels of $S1P_2$. Thus, the expression levels of S1P receptor subtypes appear to be a major factor in determining how cells respond to S1P during migration.

S1P is mainly produced through phosphorylation of sphingosine via sphingosine kinases, SphK1 and SphK2, and extracellular S1P can be dephosphorylated by LPPs. Extensive reviews on these enzymes have been published.[96,115,116,123–129]

S1P in Ovarian Cells

Compared with other cellular/tissue systems, the functions, signaling pathways, and metabolism of S1P in ovarian cancer have only been revealed in

recent years and have not been extensively studied. We have shown that both LPA and S1P stimulate expression and secretion of the proangiogenic factor interleukin-8 (IL-8) in EOC cell lines.[9] Recently, several groups have shown that S1P induces cell migration or invasion in EOC cell lines Dov13 and OVCAR3.[130–132] More importantly, Visentin et al. have shown that an anti-S1P monoclonal antibody (mAb) substantially reduced or eliminated SKOV3 (an EOC cell line) intraperitoneal tumor progression in nude mice.[133] The anti-S1P mAb significantly reduced levels of certain proangiogenic cytokines (IL-6, IL-8, and VEGF) in vivo and in vitro. These data suggest that S1P, similar to LPA, is a potential target of ovarian cancer.[134]

Multifaceted Effects of S1P on Cellular Effects

The roles of sphingolipids, including S1P, are more enigmatic than that of LPA, with the former having both stimulatory and inhibitory roles dependent on the cellular and/or animal systems and the concentrations used.[116,135,136] The latter mainly plays stimulatory roles in most biological activities. The cellular effects of S1P are highly dependent on concentrations of S1P, the type of effect tested, and the cell types used. In ovarian cancer cells cultured in vitro, S1P exhibited a dual effect on growth and/or survival. Low concentrations of S1P ($\leq$1 μM) does not significantly affect ovarian cancer cell proliferation. Higher concentrations of S1P induces cell death when cells are in suspension but stimulates cell growth when cells are attached. The calcium-dependent induction of cell death by S1P is apparently associated with its inhibitory effect on cell attachment and cell adhesion.[137] These concepts may be pathologically relevant, as it is well-known that human ovarian cancer patients produce a large amount of ascitic fluids, which contain many floating tumor cells.

Recently, Simicun et al.[131] have shown that invasion of Dov13 EOC cells is enhanced by low concentrations (0.05–0.5 μM) and inhibited by high concentrations (20 μM) of S1P, and regulation of expression levels of the S1P receptors ($S1P_2$ and $S1P_3$) has been considered to be a factor for the differential effects.[138] In addition, cell-cell adhesion and stress fibers are decreased by LPA and by 0.5 μM S1P but increased by 20 μM S1P in Dov13 EOC cells, which correlated with the stimulatory and inhibitory effect on cell migration.[131] Furthermore, S1P (0.01–1 μM) stimulates chemotactic migration and invasion of OVCAR3 cells in a Pertussis toxin-sensitive manner, which can be completely inhibited by VPC23019 (an $S1P_{1/3}$ antagonist).[132]

Epithelial ovarian cancer cells arise from the epithelial layer covering the surface of ovaries. More recently, we have shown that physiologically relevant concentrations of S1P stimulate migration and invasion of epithelial ovarian cancer cells but inhibit migration of human ovarian surface epithelial (HOSE) cells.[139] In contrast, LPA stimulates migration in both malignant and nonmalignant cells. In addition, S1P inhibits LPA-induced cell migration in HOSE cells but not in epithelial ovarian cancer cells. Lack of expression of "stimulatory" S1P receptors in

HOSE cells is not one of the major factors for the differential effect of S1P, as HOSE cells express functional $S1P_1$. In addition, similar to many other cell types, $S1P_{1/3}$ and $S1P_2$ are involved in the stimulatory and inhibitory effects of S1P on migration, respectively, in both ovarian cancer and HOSE cells.[108,140,141] Interestingly, our data suggest that the expression of these receptor subtypes in cells cannot be the sole factor for determining how these cells respond to S1P. We have found that stress fibers strongly influence the directions of S1P-modulated cell migration. Preexisting stress fibers are likely to play an important role in the differential migratory effects of S1P on ovarian cancer and HOSE cells. All HOSE cells tested displayed strong stress fibers, which were further enhanced by S1P treatment.[139] Cells with strong stress fibers are generally less motile.[142] On the contrary, ovarian cancer cells lost stress fibers and S1P stimulated filopodium-like structures, which have been shown to be related to enhanced cell motility.[143] The functional relationship between the stress fibers and the effects of S1P on cell migration is further supported by restoration of stress fibers in ovarian cancer cells through blocking the mitogen-activated protein kinase/ERK kinase (MEK); mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK); signaling pathway and loss of stress fibers by phorbol 12-myristate 13-acetate (PMA) treatment in HOSE cells, accompanied by corresponding cell migratory behavior changes in response to S1P. Changing of cell morphology (including stress fibers), cell adhesion, and cell motility is a well known and critical event of cellular transformation and tumor metastasis.[144,145] It is also interesting to note that the loss of stress fibers is not a prerequisite for LPA-induced cell migration. Similar to ovarian cancer cells, LPA stimulated cell migration in HOSE cells, but it only weakly reduced stress fibers in these cells.[139] These data are important for further development of novel therapeutic approaches targeting S1P and LPA in ovarian cancer.

S1P Metabolism in Ovarian Cancer

In general, S1P concentrations in animal body fluids and cells are lower than that of LPA.[56,57,113] More recently, using improved extraction and MS detection methods, we have analyzed several pairs of ascites samples from ovarian cancer patients versus ascites samples from patients with benign diseases and showed that both LPA (in a concentration range of 5–40 μM in EOC samples) and S1P (in a concentration range of 60–100 nM in EOC samples) were 5–10 times upregulated in ovarian cancer patients ascites when compared with that in patients with benign liver diseases (Fig. 12.3). In blood samples, S1P concentrations were measured in the 75–1100 nM range.[139] In ovarian cancer ascites, the concentration range of oleoyl-LPA (18:1-LPA) was 1–6 μM.[56,57] To be more pathophysiologically relevant, it is important to conduct in vitro experiments using lipids in these concentration ranges.

Together with other sphingolipid metabolizing enzymes, SphKs regulate the balance of the lipid mediators, ceramide, sphingosine, and S1P.[146] SphK1 and

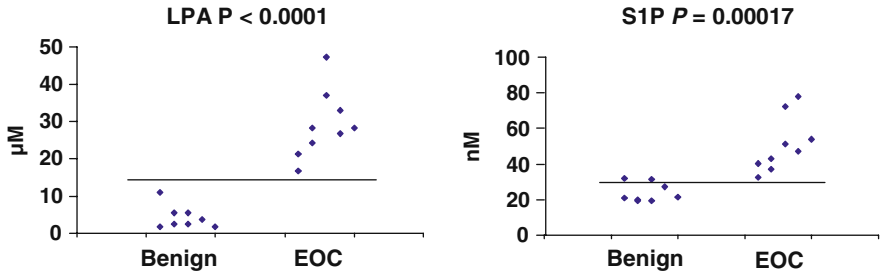

Fig. 12.3 LPA and S1P levels were elevated in EOC ascites compared with that in benign ascites. The lipids were analyzed in eight benign ascites samples from patients with chronic hepatitis and nine ovarian cancer ascites samples. The levels of these lipids were significantly elevated in EOC ascites samples (p values are shown on the illustration)

SphK2 appear to have opposing roles, promoting cell growth and apoptosis, respectively, but they can also substitute for each other, as mice deficient in either SphK1 or SphK2 had no obvious abnormalities, whereas double-knockout animals were embryonic lethal.[146] SphK1 has transformation potential in fibroblasts.[147]

The enzymes involved in S1P metabolism in ovarian cancer have not been directly studied. Ovarian serous adenocarcinoma ($n = 41$), the most common subtype of ovarian cancer, expresses higher levels of SphK1 than do other subtypes of ovarian cancer including clear cell adenocarcinoma ($n = 8$), endometrioid adenocarcinoma ($n = 37$), and mucinous adenocarcinoma ($n = 13$) ($p = 5.8 \times 10^{-4}$) (Hendrix_Ovarian; Cancer Res 2006/02/02; www.oncomi ne.org). SphK1 expression is also positively correlated with the grades and stages of ovarian cancer (Hendrix_Ovarian; Cancer Res 2006/02/02; www.on comine.org). SphK2, the enzyme that may be negatively involved in cell proliferation, is overexpressed in ovarian mucinous adenocarcinoma but downregulated in ovarian endometrioid and serous adenocarcinomas when compared with normal ovaries (Hendrix_Ovarian; Cancer Res 2006/02/02; www.oncomine.org). In addition, a negative correlation of SphK2 expression with ovarian cancer stage has been shown (Hendrix_Ovarian; Cancer Res 2006/02/02; www.oncomine.org). The functional involvement of these SphKs and many other S1P metabolic enzymes in ovarian cancer remain to be investigated.

Future Perspectives

Is LPA and/or S1P production a valid target for ovarian cancer? As it has been reviewed above, multiple synthetic and degradation enzymes of LPA and/or S1P may well be related to a single cancer, such as ovarian cancer. A deregulated balance of these enzymes, but not a single enzyme, may be involved in tumor development. In addition, at least some of these enzymes are likely to be regulated at their activity levels, but not at the RNA or protein levels. To add

more complexity, many of these enzymes are regulated by multiple factors, and they may modulate each other's activity. Moreover, their cellular locations and compartment clearly play a role in modulating their cellular functions. Furthermore, the products of these enzymes may play unexpected roles in cancers. Thus, targeting the production or degradation of LPA and/or S1P can be rather complex and challenging, which has been discussed in a recent review paper.[111] The critical issues related to this include whether we can identify a few enzymes that have an upstream regulatory effect that plays an imperative role in the overall cascade in vivo or a few critical downstream effectors that mediate the most important promoting roles of LPA and/or S1P in ovarian cancer. To this end, whether an enzyme is directly or indirectly involved in LPA or S1P production pertinent to ovarian cancer is less important.

Another important and emerging area of research is that of the effect of the tumor microenvironment on LPA and/or S1P production and function in ovarian and other cancers. If the overall levels of these lipids in the tumor microenvironments are critical, regardless of the source of these lipids, it will be pivotally important to include the contributions of host-tumor interactions to our studies. In addition, because using small molecules to target enzymatic activities is one of the most important approaches for therapeutic development, we need to take host-cell responses and specific/selective targeting into serious consideration. For example, ATX is essential for blood vessel formation during development. ATX knockout mice are embryonic lethal, but none of the LPA receptor mice are embryonic lethal, suggesting that (1) some important biological functions of ATX are unlikely to be mediated by LPA; (2) some effects of LPA may be mediated by receptors other than LPA_{1-3}; (3) some of ATX's effect may be mediated by S1P and its receptors; and (4) inhibiting ATX in general without selective targeting of cancer cells may have detrimental side effects. In contrast, both $cPLA_2$ and $iPLA_2$ knockout mice are viable, although they have reproductive defects.[62,78,148] Moreover, decreased lung tumorigenesis in mice genetically deficient in $cPLA_2$ has been shown, suggesting that $cPLA_2$ from both host and tumor cells may have promoting roles in tumorigenesis.[149] Therefore, targeting $cPLA_2$ using small molecules may be effective. These concepts need to be tested in vivo in preclinical and clinical studies.

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Chapter 13
Lysophosphatidic Acid and Invasion

Fengqiang Wang and David A. Fishman

Introduction

Lysophosphatidic acid (LPA) is a small, bioactive phospholipid produced by activated platelets, mesothelial cells, macrophage, endothelial cells, fibroblasts, adipocytes, and some cancer cells. It is involved in multiple cellular events of almost every mammalian cell type. Upon binding to G-protein–coupled receptors (GPCRs), LPA exerts a myriad of biological effects, including cell proliferation/survival, induction of neurite retraction, inhibition of gap junctional communication, and cell motility. The estimated concentrations of active, albumin-bound LPA in serum are in the range 1–5 μmol/L. Physiologic and pathophysiologic responses to LPA include wound healing, production of angiogenic factors, chemotaxis, neointima formation, tumor cell invasion, metastasis, and cell cycle progression. A large body of evidence suggests that LPA is relevant to the pathogenesis of epithelial ovarian cancer (EOC). LPA is elevated in the blood and ascites of women with ovarian cancer with levels reaching 80 μmol/L. Ovarian cancer cells also produce LPA to maintain an LPA-rich microenvironment.[1] Accumulating evidence suggest that LPA signaling is involved in the initiation, progression, and metastasis of ovarian cancer and imparts a cytoprotective effect to ovarian cancer cells exposed to cisplatin.

Tumor invasion and metastasis constitute a major problem for the treatment of EOC. Approximately 75% of women with ovarian cancer already have widespread peritoneal dissemination and ascites (stage III or IV) at the time of initial diagnosis, with a resulting 5-year survival rate approaching 15% to 30%. Metastatic dissemination is the result of a highly organized process starting from the microscopic initial growth of the originating tumor cells ultimately followed by detachment of select invasive tumor cells from the macroscopic primary tumor mass leading to the

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colonization of other organs. Metastasis is a complex multistage process involving tumor-host interaction, which includes adhesion, proteolysis, and angiogenesis. A metastatic tumor cell must leave the primary tumor and invade host tissue. Unlike other solid tumors, EOC primarily disseminates by direct peritoneal exfoliation rather than the classic hematogenous route where the cells enter the circulation, survive in the circulation, arrest at the distant vascular bed, and extravasate into organ interstitium and parenchyma.[2] LPA induces the production of potent inflammatory cytokines, which further facilitate tumor survival and a more aggressive behavior of tumor cells. Recently, peritoneal mesothelial cells have been reported to constitutively produce LPA, highlighting them as an important source not only of elevated LPA levels found in ovarian cancer ascites but also, more importantly, of the initiation and maintenance of the pathogenic cascade of peritoneal ovarian carcinomatosis through preservation of a reactive microenvironment that is supportive of tumor progression and invasion.[3] Therefore, LPA is not only a potential diagnostic marker but also a likely therapeutic target for the treatment of EOC.

LPA Production Is Elevated in Ovarian Cancer

The original study by Xu and co-workers showed that the LPA levels in the plasma were elevated in 9 (90%) of 10 patients with stage I ovarian cancer and in all patients with stages II, III, and IV ovarian cancer (24 of 24) ($p < 0.001$ compared with controls) or recurrent ovarian cancer (14 of 14) ($p < 0.001$ compared with controls).[4] Although later studies by Baker et al. showed that the levels of LPA in plasma of ovarian cancer patients did not significantly differ from that in control subjects, they did show a significant increase of the LPA levels in malignant effusion samples of ovarian cancer patients compared with that of control subjects.[5] Later studies by Xu et al. further clarified that LPA in plasma samples from preoperative patients with EOC was elevated when compared with that of controls. The mean (SD) values for the combination of 16:0-LPA/20:4-LPA in the plasma samples obtained preoperatively from patients with stages I to IV ovarian cancer were 1.23 (0.52), 0.92 (0.43), 1.23 (0.70), and 0.93 (0.15) μmol/L, respectively, compared with 0.35 (0.17) μmol/L for the controls. The mean (SD) values of total LPA in the plasma samples obtained preoperatively from patients with stage I (7 patients), stage II (3 patients), stage III (31 patients), and stage IV (4 patients) ovarian cancer were 2.57 (0.94), 2.15 (0.71), 2.93 (1.77), and 1.97 (0.27) μmol/L, respectively, compared with 0.90 (0.43) μmol/L for 27 healthy controls ($p < 0.001$), suggesting LPAs as potential biomarkers for ovarian cancer.[6]

LPA Stimulates Ovarian Cancer Invasion In Vitro and In Vivo

LPA Stimulates Ovarian Cancer Invasion and Migration In Vitro

Ovarian cancer (OC) cells are highly migratory and invasive, although their initial dissemination is direct peritoneal exposure rather than lymphatic or

hematogenous. Cellular motility and invasion through the extracellular matrix (ECM) are two of the most critical steps for tumor metastasis, both of which can be studied in vitro. Numerous studies have shown that LPA has the ability to induce migration and invasion as well as the production of neovascularizing factors in vitro as reviewed by Mills and Moolenaar[7] and by Murph et al.[8] Recently, Sengupta et al. reported that LPA induced invasion of OC cells but not borderline tumor cells or normal human ovarian surface epithelial (HOSE) cells. All stage IIIC tumor cells, regardless whether they were derived from the primary or a metastatic site, responded to LPA with an enhanced invasion. Cells from the stage IC tumor had low invasive potential but also responded to LPA. In contrast, the borderline tumor cells and HOSE cells did not invade in the presence or absence of LPA. This is consistent with the nature of these cells, suggesting that LPA-induced cell invasion is malignant cell–specific. Interestingly and surprisingly, these cells responded to LPA in cell migration.[9] There are four most commonly used methods for the evaluation of cell invasion and motility: (1) Matrigel invasion assay (BD Biosciences, San Jose, CA); (2) wound-healing assay or scratch assay; (3) collagen I migration assay; and (4) colloidal gold single-cell migration assay. LPA has demonstrated invasion- and migration-stimulating effects on OC cells in those assays. Recently, Ren and colleagues developed a new invasion-evaluating system. They used an electric cell-substrate impedance sensing (ECIS) system to monitor the invasive process in real-time based on impedance changes due to disruption of the monolayer of cells. Monolayer disruption via the retraction of endothelial cell or peritoneal mesothelial cell junctions induced by tumor cells may be the major or at least part of the causes for the observed electrical resistance changes. The ability of tumor cells to induce these changes reflects their invasive potential. As the metastatic cells invade the endothelial monolayer, they break down the barrier function of the endothelial cell layer, resulting in large drops in impedance. These impedance changes are automatically followed over time and used to quantify the in vitro invasive activities of the cells.[10]

LPA Stimulates OC Invasion and Migration In Vivo

Recent animal studies of potential targeted therapeutics suggest that LPA is critical for the successful completion of the metastatic cascade. Kim et al. provided direct evidence that LPA stimulates OC metastasis in vivo using an orthotopic mouse model. The model that they used clearly distinguishes tumors at the ovary and at the secondary sites, and the tumor spread closely resembles the human disease. They monitored LPA concentrations in mice using mass spectrometry–based methods and found that the LPA concentrations were in the low nanomolar range and induced migration and/or invasion of OC cells in vitro. They also found that LPA at these low concentrations minimally affects primary ovarian tumor growth.[11] Sengupta and co-workers also found that

LPA exerted a significant effect on tumor metastasis using HEY and SKOV3 cell lines in a mouse model. LPA-treated mice had metastases to the mesentery, bowel, liver, body wall, and diaphragm. Phosphate buffer saline (PBS) control mice also had metastases to these organs. However, the size and the number of loci in the LPA group were significantly higher than that of the phosphate buffer saline Phosphate buffer saline (PBS) group. In the SKOV3 cell model, metastases were minimal in the absence of LPA but greatly enhanced in the LPA-treated group.[9]

LPA Is Produced in the OC Microenvironment to Stimulate OC Cell Invasion

OC preferentially metastasizes within the peritoneal cavity, including the surfaces of all abdominal/pelvic organs as well as the peritoneum. Thus, the interactions between OC cells and peritoneal mesothelial cells must play important roles in migration, attachment, and invasion of OC cells to the peritoneal mesothelium as this is a clinical reality. Several adhesion molecules, their receptors, matrix-degrading enzymes, growth factors, and cytokines are involved in interactions between peritoneal mesothelial cells and tumor cells. Ren et al. showed that human peritoneal mesothelial cells constitutively produce LPA via $iPLA_2$ and/or $cPLA_2$ activities. Conditioned medium from peritoneal mesothelial cells stimulates migration, adhesion, and invasion of OC cells.[10] LPA has been shown to be an essential microenvironmental factor in OC. Said and co-workers found that addition of the conditioned media from Meso 301, both heat-sensitive and heat-resistant fraction, to the upper chamber of fibronectin (FN)-coated Transwell inserts (BD Biosciences, Franklin Lakes, NJ, USA) increases the invasion of SKOV3 and OVCAR3 cells relative to medium controls. The biologic activity of the heat-resistant fraction of mesothelial cell conditioned medium is due to the constitutive production of LPA by the action of phospholipases, mainly phospholipase A_2 (PLA_2).[3]

LPA Metabolic Pathways and EOC Invasion

Physiologically significant concentrations of LPA are found in serum, malignant effusions, saliva, follicular fluid, seminal plasma, and in mildly oxidized low-density lipoprotein (LDL). Physiologically, steady-state plasma LPA levels are normally low (less than 100 nM), and its concentration is regulated in the cellular microenvironment by the equilibrium of its production, degradation, and clearance. Emerging evidence indicates that bioactive LPA is generated extracellularly, rather than inside the cell, with subsequent secretion or release. As reviewed by Mills et al. in 2003, a series of secreted and transmembrane ectoenzymes are crucial for the production and metabolism of extracellular LPA. The mechanisms that restrict LPA production in plasma, as well as the triggers

that increase LPA production during pathophysiologic states, remain unclear.[7] In ovarian cancer patients, LPA is present in ascites fluid at between 1 and 80 μM, exceeding levels required to optimally activate LPA receptors. LPA is not produced at significant levels by normal ovarian epithelium, whereas ovarian cancer cells produce increased levels of LPA, suggesting an association between LPA and maligancy. In general, LPA is probably produced locally at the interface between cells and the interstitial fluid and then diffuses to plasma for degradation or clearance. Different processes affect the production and degradation of LPA by platelets and in plasma and thus regulate the invasion-promoting effect of LPA in ovarian cancer.

LPA Production

LPA can be produced by the sequential removal of a fatty acyl chain from phosphatidic acid by phospholipase A1 (PLA1) or phospholipase A2 (PLA2) or by the removal of choline from membrane phosphatidylcholine by ATX (autotaxin)/lysoPLD (lysophospholipase D).

PLA

PLA1 releases fatty acids from the sn-1 position of membrane phospholipids, which, when converted to LPA, produce sn-2 and polyunsaturated LPA isoforms that are selectively active on the LPA3 receptor. Type II secretory phospholipase A2 (sPLA2), which cleaves fatty acyl chains from the sn-2 site, has limited ability to hydrolyze lipids in intact cell membranes, potentially contributing to the low levels of LPA in plasma. sPLA2 selectively hydrolyzes lipids that are present in damaged membranes, membranes of activated cells, or microvesicles such as those released during apoptosis or that produced by cancer cells. Microvesicle numbers are particularly high in malignant fluids such as ascites, potentially contributing to the aberrant production of LPA in cancer patients.[7]

Ren and co-workers demonstrated that human peritoneal mesothelial cells constitutively produce bioactive lipid signaling molecules, such as LPA, via calcium-independent PLA_2 ($iPLA_2$) and/or cytosolic PLA_2 ($cPLA_2$) activities. Conditioned medium from peritoneal mesothelial cells stimulate migration, adhesion, and invasion of OC cells and may play similar roles in vivo. $AACOCF_3$ (an $iPLA_2$ and $cPLA_2$ inhibitor; 25 μmol/L) or HELSS (an $iPLA_2$ inhibitor; 1 μmol/L) blocked peritoneal mesothelial cell LPA production. The pretreatment of the peritoneal mesothelial cell LP9 with HELSS (1 μmol/L, 30 min) and $AACOCF_3$ (100 μmol/L, 30 min) also inhibited invasion of SKOV3 cells into the peritoneal mesothelium, and the inhibitory effect of $AACOAF_3$ was stronger than that of HELSS, suggesting that both enzymes are involved in LPA production. In contrast, $sPLA_2$ and lysophospholipase D do not play a

significant role in the production/secretion of LPA in the conditioned medium of peritoneal mesothelial cells and its migration-promoting effect.[10] Whereas $iPLA_2$ is required for laminin-induced LPA production, $cPLA_2$ is required for cell migration, possibly related to its ability to produce arachidonic acid. They also found that the MEK (mitogen-activated protein kinase/extracellular signal regulated kinase) inhibitor [presumably through inhibiting ERK (extracellular signal regulated kinases)] inhibited cell invasion by both increasing the time required for invasion and reducing the overall extent of invasion compared with control cells. On the other hand, although an Akt inhibitor delayed the invasion process by 5–6 hours, it did not affect the final extent of invasion. To confirm these results, they transiently transfected dominant negative forms of MEK and Akt (also known as protein kinase B) into SKOV3 cells and tested their ability to invade peritoneal mesothelial cells. Results similar to those from the experiments using inhibitors were obtained, supporting a role for MEK-ERK in the invasion process, whereas AKT is involved to a much lesser extent. The work done by Ren et al. provides the first line of evidence that peritoneal mesothelial cells from OC patients possess constitutive PLA_2 activity, which is involved in LPA production.[10]

Sengupta et al. reported that $cPLA_2$ activity is required for LPA-induced OC cell migration. They also observed the effect of cPLA2 in LPA-induced cell invasion.[9,11] Said et al. reported in 2007 that chemotactic and promigratory effects of the heat-resistant fraction of mesothelial cell conditioned medium on ovarian cancer cells were, for the most part, due to PLA_2-induced LPA production/secretion in the conditioned medium.[3]

ATX/lysoPLD

The generation of LPA from lysophospholipids, particularly from lysophosphatidylcholine (LPC), by ATX/lysoPLD ecto-phosphodiesterase represents another regulatory mechanism of LPA production in the extracellular milieu. ATX was originally identified as an "autocrine motility factor" secreted by melanoma cells and was subsequently found to belong to the family of ecto-nucleotide phosphodiesterases (NPPs), which are capable of hydrolyzing phosphodiester and pyrophosphate bonds that are typically found in ATP and ADP. ATX also has lysoPLD activity, and the biological effects of ATX/lysoPLD can be attributed to the production of LPA and, potentially, S1P (sphingosine-1-phosphate). However, ATX seems to be a unique lysoPLD, in that its family members (NPP1 and NPP3) lack a similar phospholipase function. ATX/lysoPLD is widely expressed, with the highest mRNA levels in the brain, ovary, lung, intestine, and kidney, and it is upregulated by certain peptide growth factors. Targeted deletion and transgenic overexpression of ATX/lysoPLD in specific tissues should provide important insights into its pathophysiologic functions. The main substrate for ATX/lysoPLD is LPC. LPC is secreted by the liver and is abundantly present in plasma, where it is predominately bound to albumin and, to a lesser extent, lipoproteins. LPC is also found in the supernatant of cultured cells, presumably as a constituent of microvesicles that have been shed from the plasma membrane. ATX/lysoPLD

converts microvesicle-associated LPC into bioactive LPA, thus providing an explanation for the link between microvesicle shedding and metastasis. ATX/lysoPLD mRNA is upregulated in several human cancers, particularly melanoma, renal-cell carcinoma, and glioma, and studies in nude mice have shown that ATX/lysoPLD enhances tumor aggressiveness. Specifically, *Atx*-transfected, *Ras*-transformed NIH-3T3 cells are more invasive, tumorigenic, and metastatic than *Ras*-transformed control cells. Further, the metastatic capability of breast cancer cells correlates with their ATX/lysoPLD levels. These observations can be explained by its activity on the production of LPA and its effects on protease production, cell motility, chemotaxis, and invasion. By generating LPA (and possibly other bioactive lysophospholipids), ATX/lysoPLD could contribute to tumor progression by providing an invasive and vasculogenic microenvironment for tumor cells.[7] Recently, Ptaszynska et al. reported that exogenous addition of vascular endothelial growth factor (VEGF)-A to cultured cells induces ATX expression and secretion, resulting in increased extracellular LPA production. This elevated LPA, acting through LPA4, modulates VEGF responsiveness by inducing VEGF receptor (VEGFR)-2 expression. Downregulation of ATX secretion in SKOV3 cells using antisense morpholino oligomers significantly attenuates cell motility responses to VEGF, ATX, LPA, and lysophosphatidylcholine, supporting a role of ATX in LPA-induced EOC invasion.[12]

LPA Inactivation

Production of the bioactive LPA by PLA or ATX/lysoPLD is only half of the story: LPA accumulation must be counterbalanced by inactivation mechanisms. Other than the negative regulation on LPA production, an alternative mechanism is provided by inactivation of LPA itself. Recent studies have identified a family of lipid phosphate phosphohydrolases (LPPs) to be responsible for the dephosphorylation of LPA. The LPPs, comprising at least four members, are integral membrane ecto-enzymes, with six putative transmembrane domains. Overexpression of LPP-3 in OC cells decreases colony formation, increases apoptosis, and decreases tumor growth in vitro and in vivo.[13] LPP-1 mRNA is decreased in the majority of OCs. Introduction of LPP-1 into OC cell lines also results in increased LPA hydrolysis associated with a marked inhibition of cell proliferation and colony-forming activity and a marked increase in apoptosis.[14] Interestingly, LPP activity can be increased by gonadotropin-releasing hormone analogues through recruitment of LPPs to the cell membrane. So, LPA signal duration and strength is likely to depend, at least in part, on the expression level of LPPs, which are decreased in OC, and their membrane localization relative to the LPA receptors.[7] Yet, how the degradation of LPA by LPPs will affect LPA-stimulated EOC invasion remains to be tested.

LPA Regulates Growth Factors, Proteases, and Other Proinvasive Biomolecules Within the EOC Microenvironment That Promote Invasion

LPA regulates almost every aspect of ovarian metastasis. In particular, LPA stimulates adhesion, migration, and invasion of OC cells. LPA stimulates secretion of proangiogenic factors, such as VEGF and interleukin (IL)-8. Cell invasion induced by LPA involves protease [such as matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA)] activities.[9] Other biomolecules involved in the OC invasion process are also regulated by LPA, such as COX-2 (cyclooxygenase-2), nuclear factor kappa B (NF-κB), and so on.

Proangiogenic Factors

VEGF

Angiogenesis is essential for tumor growth, and the transition from limited microscopic to rapid macroscopic growth requires neovascularization. The development of new blood vessels acquired from preexisting host venules is dependent upon the production of angiogenic factors released from tumor cells, stromal and immunoregulatory cells within the tumor microenvironment. Among these angiogenic factors, VEGF, also known as vascular permeability factor, binds to vascular endothelial cells and is a potent inducer of angiogenesis. Cancer patients have increased serum VEGF, and elevated VEGF messenger RNA (mRNA) levels occur in the majority of human cancers.[15] In OC, VEGF contributes to the formation of malignant ascites by increasing peritoneal microvessel permeability. In 2001, Hu and colleagues first showed that LPA, in concentrations reported in malignant ascites, stimulated ovarian tumor growth, at least in part, via induction of VEGF expression through transcriptional activation.[16] Using a VEGF neutralizing antibody, we have demonstrated that LPA-induced invasion and migration are VEGF dependent; blocking VEGF activity partially inhibits LPA-induced invasion. The involvement of VEGF in LPA-induced invasion is further supported by evidence showing that exposing OC cells to varying concentrations of SU1498, a specific VEGFR-2 inhibitor, significantly blocked VEGF- and LPA-induced DOV13 cell invasion and migration, suggesting LPA-regulated EOC invasion is at least in part mediated by the binding of VEGF ligand to VEGFR-2. SU1498 also significantly reduced uPA activity and the expression of proMMP-2 as well as the activity of uPA induced by LPA treatment in DOV13 cells, suggesting that the increased secretion and expression of VEGF subsequently induced the MMP and uPA activity to stimulate invasion of EOC cells.[17] Lee et al. investigated the mechanism by which LPA regulates VEGF expression under hypoxic conditions. They reported that LPA induces VEGF via HIF(hypoxia

inducible factor)-1α activation and suggest a critical role of HIF-1α in LPA-induced cancer cell proliferation and angiogenesis.[1] In a later study, they also found that hypoxia enhanced LPA-induced HIF-1α and VEGF expression in human OC cells and resveratrol, a naturally occurring phytoalexin produced by a wide variety of plants in response to stress, injury, and fungal infection, blocked LPA-induced HIF-1α and VEGF expression and cell migration by a wound-healing assay.[2] Malgorzata et al. recently reported that VEGF, via VEGFR-2, stimulates ATX expression in the OC cell lines CaOV3 and SKOV3. An increase in secreted ATX protein increased the conversion of LPC to LPA, which in turn increased expression of VEGF and VEGFR-2. Thus, it forms a positive pathologic feedback loop between ATX, LPA, and VEGF to regulate ovarian carcinoma growth, angiogenesis, and metastatic spread.[12]

IL-8 and IL-6

IL-8 is a 8.4-kDa protein belonging to the CXC subfamily of chemokines, which is characterized by two essential cysteine residues, separated by a third intervening amino acid.[18] There are two major forms of IL-8: the 72-amino-acid monocyte-derived form, predominant in cultures of monocytes and macrophages, and the endothelial form, which has five extra N-terminal amino acids, predominating in cultures of tissue cells such as endothelial cells and fibroblasts. IL-8 is a proinflammatory and proangiogenic factor that has been involved in cancer development. IL-8 enhances both tumor growth and angiogenesis. High expression of IL-8 mRNA is detected in late-stage ovarian carcinomas. Ascites or plasma of patients with OC show significantly higher levels of IL-8 in comparison with those from patients with benign gynecologic disorders.[19] In 2001, Schwartz et al. showed that LPA, S1P, and SPC (sphingosylphosphoryl-choline) regulate the mRNA and protein levels of IL-8 in OC cells.[20] Fang et al. investigated the mechanism by which LPA induced IL-8 production in EOC cells. They demonstrated that the LPA_2 receptor is more effective than the LPA_1 and LPA_3 receptors in the induction of IL-8 by LPA. RNA stability analysis and luciferase assays indicated that LPA induces transcriptional activation of the *IL-8* gene with little contribution from increased stability of RNA, and a synergism between NF-κB and AP-1 (activator protein-1) seems to be critical for LPA-induced transcriptional activation of the *IL-8* gene. They also reported that the p38 MAP kinase and JNK are important intracellular mediators of LPA-induced IL-8 production in ovarian cancer cells.[21] We reported that IL-8 is involved in LPA-induced EOC cell invasion and migration, as blocking IL-8 activity by a neutralizing antibody against it decreased LPA-induced DOV13 invasion. We also found that the regulation of invasion and migration by IL-8 is mediated by the stimulation of MMP-1 and MMP-7.[22] Although LPA also stimulates the production of IL-6, at present no direct evidence exists supporting that IL-6 is involved in LPA-induced EOC invasion.

Proteases

LPA-mediated signal transduction has shown myriad effects, including transient increase in cytosolic-free calcium, phosphorylation of focal adhesion kinase, activation of mitogen-activated protein kinases, and formation of focal adhesions. Among these effects, protease secretion and activation is one of the most important steps that ultimately facilitate EOC cells to invade and migrate through the basement membrane and ECM. Ovarian cancer cells secrete proteolytic enzymes including plasminogen activators and MMPs that degrade extracellular matrix proteins and promote invasion into the proteolytically modified tissue.

Urokinase-Type Plasminogen Activator

Numerous studies indicate the secretion and activation of proteases as one of the most important-steps in cancer cell invasion. Among the large number of proteases involved in cellular invasion, uPA is of particular importance because it converts plasminogen to plasmin and initiates the activation of MMPs. uPA and MMPs confer the ability of cells to degrade the extracellular matrix, thus allowing cells to invade through the physical barrier. Moreover, the interaction of uPA with uPAR (uPA receptor) also promotes cell motility and proliferation, and these processes also impact tumor invasion and metastasis.[23] The association of uPA activity with ovarian tumors is supported by evidence showing that the levels of uPA expression and activity are significantly lower in benign ovarian tumors than in advanced ovarian tumors.[24,25] In addition, high concentrations of uPA in the ascites/plasma correlate with poor prognosis and lack of response to chemotherapy. Experimental models both in vitro and in vivo have demonstrated the effect of uPA on ovarian cancer metastasis. First, it induces OC cell proliferation and migration; second, uPA and uPAR overexpression confer cancer cells with invasive and metastatic potential; and third, the uPA-specific inhibitors significantly decrease OC invasion and metastasis.[26–29] Based on those findings and the role of LPA in OC invasion, one would easily argue whether uPA is regulated by LPA in this process.

In 1999, Pustilnik and co-workers first reported that physiologically relevant concentrations of LPA stimulated uPA secretion in five OC cell lines (OVCAR-3, SKOV-3, OVCA 429, OVCA 432, and OVCA 433), but not from established normal ovarian epithelial (NOE) cells (NOE 033 and NOE 035) or from SV40 large T-antigen–immortalized normal epithelial cell lines (IOSE 29 and IOSE 80). The induction of uPA secretion was only observed using the 18:1 LPA isoform, with 18:0 LPA, 16:0 LPA, or LPC showing no effect, a phenomena possibly due to their difference on LPA receptor stimulation.[30] Five years later (2004), Huang et al. further investigated the effect of LPA receptor overexpression on uPA expression in vivo using transgenic (TG) mice. They developed TG mice that overexpress human LPA_2 in ovarian interstitial tissues. Ovarian cells from TG

mice express greater amounts of uPA and have a lower level of type 2 PA (plasminogen activator) inhibitor than do non-TG mouse ovarian cells, whereas the expressions of the uPA receptor (uPAR) and of tissue-type PA (tPA) show no difference between LPA_2 TG mouse ovaries and non-TG ovaries. In the supernantant of cultured TG ovary cells, secreted uPA activity is about twofold higher than that of non-TG mouse ovarian cells.[31]

The direct evidence showing that uPA expression plays a role in LPA-induced ovarian cancer invasion comes from the Matrigel invasion experiments performed by Li et al. They used small interference RNA (siRNA) against uPA to knock down the expression of uPA in SKOV3 cells and then observed the effect on SKOV3 invasion through the Matrigel. uPA siRNA-3 and siRNA-4 treatment, which greatly reduced uPA protein expression, resulted in approximately a 55% and 62% reduction in LPA-induced invasion in vitro, suggesting that the presence of uPA is essential for LPA-induced in vitro invasion.[23]

So how does LPA induce uPA expression in OC cells? Mechanistic studies by Li et al. reported that G_i-associated signaling is involved in LPA-induced uPA upregulation. In addition, they found that the activities of both Ras and Raf-1, which are downstream effectors of G_i, were required for LPA action. However, the well-established Raf-1 effector MEK1/2 was not significantly involved in LPA-induced uPA expression. Instead, LPA activates NF-κB, and the activity of NF-κB is required for LPA-induced uPA upregulation and uPA promoter activation. Inhibition of Gi, Ras, Raf-1, or nonphosphorylable IκB (inhibitor of NF-κB) by pertussis toxin treatment or infection with Ad (adenovirus) containing dominant-negative H-Ras, Raf-1, or nonphosphorylable IκB in SKOV3 cells followed by Matrigel invasion assay all inhibited LPA-induced cell invasion. These results suggest that the Ras–Raf–NF-κB signaling pathway is essential for LPA-induced in vitro cell invasion.[23] In a recent report, Mahanivong et al. further investigated the signaling components that link Ras to NF-κB for this LPA-induced event. Using specific inhibitors and dominant-negative PKC (protein kinase C) mutants, they showed that PKC-α is necessary for LPA-induced NF-κB activation and uPA upregulation. LPA stimulation led to PKC-α activation, and this activation was abrogated by dominant-negative Ras mutant and Ras inhibitor farnesyltransferase inhibitor (FTI-277). Moreover, Ras and PKC-α interaction induced by LPA was sensitive to the inhibitors of both farnesyltransferase and cPKC (Ca2+dependent PKC). Thus, PKC-α is a signaling component downstream of Ras mediating LPA-induced cellular event. CARMA1, Bcl10, and MALT1 function as part of a signaling complex to bridge PKC to IKKs (IκB kinases), thus facilitating NF-κB activation in lymphocytes. Mahanivong et al. also investigated the role of newly characterized signaling complex CARMA3-Bcl10-MALT1 in LPA-induced NF-κB activation/uPA upregulation. Forced expression of dominant-negative CARMA3 mutant or treatment of cells with siRNAs specifically targeting CARMA3, Bcl10, or MALT1 abrogated these LPA-induced events, implicating the importance of CARMA3-Bcl10-MALT1 in LPA-associated signaling in ovarian cancer cells. In vitro invasion assays using SKOV3 cells

that were infected with Ad containing dominant-negative PKC-α, CARMA3 (CARMA3ΔCARD), or nonphosphorylable IκB (Iκm) upon LPA stimulation showed that dominant-negative PKC-α, CARMA3ΔCARD, and Iκm all inhibited LPA-induced invasion, suggesting that PKC-α–CARMA3 signaling axis plays an essential role in LPA-induced ovarian cancer cell in vitro invasion.[32] Estrella et al. showed that inhibition of p38 mitogen-activated protein kinase (MAPK) signaling by SB202190 completely abrogated LPA-induced uPA secretion, whereas inhibition of the p42/44 MAPK or phosphatidylinositol 3'-kinase (PI3K) pathways with PD98059 or wortmannin and LY294002, respectively, decreased but did not completely block uPA secretion. They also showed that LPA induces uPA secretion from OC cells predominately through the LPA_2 receptor, with LPA_3 contributing to this process. However, whether those inhibitors decrease LPA-induced invasion was not investigated.[33] Other than the pathways mentioned above, we have demonstrated that LPA stimulates uPA secretion through a VEGF–VEGFR-2 mediated signaling pathway, as a VEGFR-2 inhibitor, SU1498, decreases the LPA-induced increase of uPA activity in DOV13 cells and inhibits LPA-induced invasion.[17] Besides the effect on subsequently activated MMPs, uPA is also involved in LPA-induced shedding of an 80-kDa E-cadherin–soluble fragment to decrease cell-cell adhesion and thus promotes in vitro invasion.[34]

Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinase

Matrix metalloproteinases (MMPs), a family of 24 structurally related, zinc-dependent endopeptidases, are capable of directly degrading essentially all components of the ECM and thus are able to expose cryptic sites within the matrix molecules to activate other proteases, growth factors, and cytokines that may facilitate tumor invasion and metastasis.[35] In EOC invasion and metastasis, the roles of several MMP family members have been well documented.[36–38] Gelatinolytic and collagenolytic MMPs, including MMP-2 (gelatinase A), MMP-9 (gelatinase B), and MT1-MMP, were detected in ovarian cancer ascites, cultured cells, and tissues. Furthermore, treatment of animals harboring ovarian cancer xenografts with synthetic MMP inhibitors reduces tumor burden and enhances survival. In addition, the balance between MMP and their tissue inhibitors (tissue inhibitors of metalloproteinase; TIMPs) also delicately regulate MMP activity. Either upregulation of MMP expression or downregulation of TIMPs can lead to aberrant MMP activity.

MMP-2 Secretion and Activation

Numerous studies have shown that MT1-MMP functions in the activation of proMMP-2.[39] Immunohistochemical and in situ hybridization studies have demonstrated that MT1-MMP mRNA and protein are prevalent in ovarian carcinomas, particularly at the invasive front, whereas MMP-2 is produced by both tumor cells and associated normal stromal elements. OC cells with the capacity to bind and activate either endogenous or exogenous MMP-2 have

significantly enhanced invasive activity. Thus, MT1-MMP produced by epithelial ovarian carcinoma cells is suspected to initiate cell surface activation of tumor-derived or stromally derived proMMP-2, thereby facilitating motility, invasion, and metastasis.[40] In addition, clustering of collagen-binding integrins on OC cell membranes induces proMMP-2 activation.[36] We previously demonstrated that LPA at higher concentrations (20–200 μM) stimulates proMMP-2 activation and promotes ovarian tumor cell motility and invasion in an MMP-dependent manner. The increases in pericellular MMP activity induced by LPA correlated with enhanced cellular motility and invasive activity. LPA also upregulates β_1-integrin expression, stimulate integrin phosphorylation and the formation of β_1-integrin–containing focal contacts to promote cellular migration. Our study indicated that LPA-Edg binding and matrix-induced β_1-integrin clustering may function via convergent signal transduction pathways to promote MMP expression and/or processing, with subsequent downstream changes in migratory and invasive behavior.[41] MMP-2 is regulated at multiple levels, as Symowicz et al. reported that inhibition of COX-2 activity decreases proMMP-2 expression and LPA-induced proMMP-2 activation and subsequently inhibits LPA-induced motility and invasive activity.[42] We also showed that LPA-induced MMP-2 secretion and activation is regulated by VEGFR-2 mediated pathway.[17] Do et al. further investigated the mechanism by which LPA stimulates EOC cell migration and MMP-2 activation. We found that cell density affects DOV13 response to LPA stimulation. When cells were cultured at a high density, LPA mediated stress fiber and focal adhesion disassembly and significantly repressed RhoA activity in EOC cells. Inhibition of Rho-kinase/ROCK (Rho-associated coiled-coil protein kinase) enhanced both LPA-stimulated loss of stress fibers and proMMP-2 activation. In contrast, expression of the constitutively active RhoA(G14V) mutant diminished LPA-induced proMMP-2 activation. Interestingly, when cells were cultured at a low density, stress fibers were present after LPA stimulation, and ROCK activity was required for EOC cell migration.[43]

MMP-7

Other than gelatinase and MT1-MMP, we also identified MMP-7 as a critical player in EOC invasion. Distinct from other MMPs, MMP-7 has the minimal domain organization required for secretion and activation and is one of the few MMPs that are overexpressed in ovarian carcinoma rather than normal stromal cells. MMP-7 has broad proteolytic activity against a variety of ECM substrates and is recognized as an important regulator of cell surface proteolysis, binding to cell surface proteins such as E-cadherin, β-integrin, tumor necrosis factor-α, Fas ligand, and heparin sulfate. Overexpression of MMP-7 is seen in many malignancies, including prostate, stomach, colorectal, lung, esophageal, squamous cell carcinomas of the head and neck, and ovary. We show that MMP-7 is overexpressed in EOC and that recombinant MMP-7 promotes EOC invasion in vitro and induces proMMP-2 activation in DOV13 cells. Regulation of

MMP-7 expression in DOV13 cells by MMP-7 sense and antisense gene transfection as well as siRNA show that MMP-7 expression correlates with EOC invasiveness in vitro. Because of the role of MMP-7 in OC invasion and the MMP-dependence of LPA in stimulating OC cell invasion, we speculate that LPA also stimulates the secretion and activation of MMP-7. Indeed, when we stimulated DOV13 cells with LPA (10–80 μM), the total and active forms of MMP-7 are increased in DOV13 cells. Using MMP-7 antisense and siRNA transfection, we show that MMP-7 downregulation significantly decreased LPA-induced DOV13 invasion. However, in addition to the inhibition on MMP-7 expression, MMP-7 antisense transfection seems to have an inhibitory effect on MMP-2 activity. It remains unclear whether this inhibitory effect is nonspecific or is due to the subsequent effect of MMP-7 regulation on MMP-2 activity. The effect of MMP-7 silencing on LPA-induced invasion is also observed in another EOC cell line, R182.[44]

Other MMPs

We have shown that MMP-1 expression is upregulated by LPA stimulation in EOC cells, and MMP-1 secretion is also upregulated by IL-8 stimulation of DOV13 cells, which may also contribute to LPA-induced invasion.[22]

Regulation of TIMPs

At the concentration of 20–200 μM, LPA induces proMMP-2 activation in DOV13 cells. However, in HEY cells at the concentrations that stimulate invasion (50 nM to 30 μM), no significant effect on the expression levels or the proteolytic processing of MMP-2 and MMP-9 was observed. Sengupta et al. analyzed a broader spectrum of MMPs using multiplex ELISA. They showed that LPA at 100 nM, the concentration at which it effectively induces cell invasion, had an inhibitory effect on the secreted levels of MMP-2, and -9 and did not increase the enzymatic activity of MMP-2/MMP-9. Among different MMPs tested (MMP-1, -2, -3, -8, -9, -10 and -13), LPA only increased the expression and enzymatic activity of MMP-3. However, they observed the significant downregulation of TIMP-1 and TIMP-2, two negative regulators of MMP activity. Secreted TIMP-3 levels were also downregulated by LPA as shown by Western blotting. A cDNA array–based study also showed that TIMP-2 was downregulated by LPA at the RNA level to 46%, and TIMP-3 was downregulated by LPA to 64%. TIMP-2 and TIMP-3 significantly reversed the LPA-induced cell invasion, supporting the functional role of these molecules as negative regulators of cell invasion. In addition, TIMP-3 inhibited LPA-induced p38 MAPK phosphorylation, and an upstream activator of p38 MAPK, constitutively active MKK6, transfection reversed the inhibitory effect of TIMP-3 on LPA-induced cell invasion. They also found that VEGF (25 ng/ml) induced approximately ~30% increase in invasion of HEY cells, which could be blocked by TIMP-3, suggesting VEGFR as a

potential target for TIMP-3 in HEY cells. In addition, they showed that TIMP-3 treatment and uPA siRNA had an additive effect and completely blocked LPA-induced cell invasion, suggesting that downregulation of TIMP-3 and upregulation of uPA may be two separate signaling pathways of LPA-induced invasion.[9]

Tumor Necrosis Factor-α Converting Enzyme

Tumor necrosis factor-α converting enzyme (TACE) is also known as a disintegrin and metalloproteinase (ADAM-17), a member of the ADAM family of metalloproteinases. The importance of the role of TACE in the cleavage of these ligands is highlighted by the fact that mice lacking TACE die perinatally and resemble epidermal growth factor receptor (EGFR)-null mice. Knockdown of TACE by TACE siRNA completely prevented chemotactic migration of SCC-9 cells in response to LPA.[45] However, direct evidence of TACE in LPA-stimulated invasion in OC cells has not been shown.

COX-2

COX-2 catalyzes the rate-limiting step in prostaglandin synthesis from arachidonic acid, generating prostaglandin H_2, which is subsequently converted to prostaglandin E_2 and other prostaglandins.[46] Various growth factors, cytokines, and tumor promoters can induce the expression of COX-2 in most cells and tissues. COX-2 inhibits cell apoptosis by changing the levels of proapoptotic and antiapoptotic factors, increases growth factor expression to promote angiogenesis, and enhances cell invasiveness by increasing MMP expression. COX-2 activity is essential for normal ovarian function, and it is thought to be necessary for the rupture of the preovulatory follicle and subsequent release of oocytes during ovulation. It may increase collagenase and proteolytic activity and decrease synthesis of basement membrane components in ovarian granulosa and surface epithelial cells, permitting ovulation.[47,48] A role for COX-2 in ovarian pathobiology is supported by evidence that ascites from ovarian cancer patients contain elevated levels of prostaglandin E_2 compared with that in nonmalignant ascites or ascites from other carcinomas. Symowicz and co-workers evaluated COX-2 immunoreactivity in human ovarian tumors and showed positive COX-2 immunoreactivity in 98% of cases, with 70% displaying moderate to high-level expression, including 50% of borderline ovarian tumors.[42]

The effect of LPA on COX-2 expression was first observed by Ershov et al. in retinal pigment epithelium (RPE) cells.[49] Symowicz et al. showed that treatment of ovarian tumor cells with LPA in vitro induced COX-2 protein expression in a time- and concentration-dependent manner. COX-2 induction was blocked by pertussis toxin, implicating LPA signaling through G_i-protein–coupled receptors in the Edg/LPA receptor family. In addition, they showed that LPA-induced transactivation of the EGFR increased COX-2 expression via the Ras/mitogen-activated protein kinase pathway. Inhibition of COX-2 activity

decreased proMMP-2 expression and LPA-induced proMMP-2 activation and reduced MMP-dependent motility and invasion, suggesting that COX-2 functions as a downstream mediator of LPA to potentiate aggressive cellular behavior in ovarian carcinoma cells. LPA and COX-2 are detectable in OC patients with early-stage disease. The magnitude of LPA-induced COX-2 expression varied among the immortalized borderline and malignant ovarian carcinoma cell lines. Thus, COX-2 inhibitors may be more beneficial in the early stages of cancer or as chemopreventive agents.[42]

Regulation of Adhesion Molecules

It has been suggested that not only do specific MMPs and ADAMs (a disintegrin and metalloproteinase) cleave the extracellular domain of E-cadherin and disrupt intercellular adhesion, but also the 80-kDa soluble extracellular fragment can abrogate cell-cell adhesion and induce invasion into collagen. Plasmin has also been shown to directly cleave E-cadherin and to stimulate cancer progression. Gil et al. explored the role of LPA in the shedding of E-cadherin, the identity of the relevant proteases in shedding, and the potential role that accumulated soluble E-cadherin plays in the microenvironment of ovarian carcinoma cells to promote dissemination. We found that LPA promotes E-cadherin shedding in a uPA-dependent manner and that a soluble recombinant chimera E-cadherin-fc stimulates invasion of OVCA429 cells providing evidence of a novel mechanism by which LPA promotes ovarian metastasis.[34]

LPA and EOC Invasion Under Hypoxic Condition

Tumor hypoxia is associated with tumor growth, angiogenesis, resistance to apoptosis, and compromise in radiotherapy and chemotherapy, as well as tumor metastasis.[50] OC is characterized by rapid growth of solid intraperitoneal tumors and large volumes of ascites (commonly 3–8 L) with millions of ovarian tumor cells. Kim et al. recently found that malignant ovarian ascites is hypoxic, with about a 50% reduction of the O_2 pressure and 87% reduction of oxygen content measured in ascites when compared with normal blood gas values. On the other hand, the CO_2 pressure was increased by only 10%, whereas pH values of malignant ascites fluids were not significantly changed.[11] The rapidly growing and disseminated OC cells within the peritoneal cavity encounter hypoxic conditions under which they acquire the ability to migrate and invade through extracellular matrix proteins. Kim et al. reported that pretreatment of SKOV3 cells under hypoxic conditions enhanced cell migration toward collagen I under normoxic conditions in response to LPA (100 nmol/L to 10 μmol/L). Hypoxia also enhanced SKOV3 and HEY ovarian cancer cell invasion response to LPA. The hypoxia-enhanced effect seems to be LPA specific as the cellular responsiveness to EGF is not increased under hypoxic condition.[11]

HIF-1α is one of the most important factors involved in hypoxia-induced cellular activities. It activates the transcription of genes that are involved in crucial aspects of metastasis, including angiogenesis, cell survival, glucose metabolism, and invasion.[51] Kim et al. found that geldanamycin (10 μmol/L), an effective inhibitor of HIF-1α, did not significantly affect cell migration under normoxic condition, reduced the basal level of cell migration, and completely blocked the enhanced migration and invasion response to LPA in hypoxia-pretreated cells, suggesting the involvement of Hsp90 and HIF-1α in the enhanced cellular responsiveness to LPA in OC cells under hypoxic conditions. This is further supported by evidence showing that siRNA against HIF-1α, but not the control siRNA against GFP (green fluoresent protein), reduced the expression of HIF-1α and LPA-induced cell invasion under hypoxia conditions. The role of HIF-1α in LPA-induced invasion is also supported by the increases of HIF-1α expression by LPA in vivo, which may represent the major mechanism of enhanced LPA effects. Thus, targeting the Hsp90–HIF-1α axis for therapy has been very attractive.[11]

Lee et al. first showed that LPA induced HIF-1α activation in EOC using the cell lines OVCAR-3 and CAOV-3. They found that both the PI3K/Akt/mTOR/p70S6K and p42/p44 MAPK pathways are required for LPA-induced HIF-1α activation in OC.[1] Moreover, they showed that LPA-induced VEGF expression was mediated by HIF-1α.[1] Translation of HIF-1α mRNA seems to be significantly upregulated by LPA and overcomes the rate of normoxic degradation. The direct linkage of LPA to HIF-1α activation and VEGF expression suggested a molecular mechanism for LPA-induced angiogenesis and cell motility under hypoxic conditions.[1] Park et al. also reported that enhanced increases in HIF-1α and VEGF expression by LPA was observed under hypoxic conditions in human OC cells. In addition, this increase in HIF-1α and VEGF expression is efficiently blocked by resveratrol, leading to inhibition of the cell migration of human OC cells. Incubation of OC cells under hypoxic condition increased cell migration. These results imply the significance of LPA-induced cell migration under hypoxic conditions, as direct dissemination from the primary site(s) into the peritoneal cavity is the primary method for OC metastasis.[2]

LPA Receptors

LPA signaling is mediated by at least four G-protein–coupled receptors (GPCRs) referred to as $LPA_{1–4}$. These receptors couple to multiple G-proteins, particularly $G_{12/13}$, G_i, G_q, and possibly G_s, to activate downstream pathways including stimulation of phospholipase C and D, inhibition of adenylyl cyclase, and stimulation of small GTPases, mitogen-activated protein kinases, and phosphoinositide 3-kinase. Receptor-mediated actions of LPA have important influences on cell survival, cytoskeletal remodeling, cell migration, and cell proliferation.[7,52]

Recently, Lee and co-workers identified a new LPA receptor, the orphan GPCR known as GPR92 or LPA_5.[52] The *lpa_1, lpa_2, and lpa_3* genes are structurally related to each other. They are all members of the so-called endothelial differentiation gene (EDG) family of GPCRs and were formerly called EDG2, EDG4, and EDG7, respectively, whereas *lpa_4* (*GPR23*) genes and *lpa_5* (*GPR92*) are phylogenetically far from *lpa_1*, *lpa_2*, and *lpa_3*, and closer to the platelet-activating factor receptor.[53] Five additional members of the EDG-receptor subfamily encode related GPCRs that are specific for the bioactive lysophospholipid sphingosine-1-phosphate (S1P).[7] LPA_1, LPA_2, and LPA_3 have been shown to be expressed in multiple ovarian cancer cell lines, including SKOV-3, and expression of LPA_2 and LPA_3 has been positively correlated with disease progression, whereas overexpression of LPA_1 induces apoptosis in certain OC cell lines. The expression of LPA_4 and LPA_5 has not been reported in specific ovarian cell lines, whereas LPA_4 is highly enriched in ovarian tissue. LPA receptors have been shown in various systems to couple to multiple G-proteins: LPA_1 and LPA_2 to G_q, G_i, and G_{12}; LPA_3 to G_q and G_i; LPA_4 to G_q, G_i, G_{12}, and G_s; and LPA_5 to G_q, G_i, and G_{12}. Thus, LPA signaling is likely mediated by multiple receptors and G-protein subtypes.[54] Another study showed LPA_1 and LPA_3 to be implicated in the LPA-induced cell migration of different types of cancer cells.[55]

One strategy to eradicate the tumor-promoting effect of LPA is to test reagents blocking the receptors for LPA and evaluate the efficacy of blocking each receptor in the treatment of OC both in vitro and in vivo. In vivo experimental models show that blocking LPA-LPA receptor interaction with cyclic LPA inhibits tumor metastasis and development.[13,56] Goetzl et al. investigated the expression and function of different LPA receptor in primary ovarian cultures and normal ovarian epithelium. LPA_2 was predominately expressed in OV202 primary cultures of EOC and in several different established lines of EOC at both mRNA and protein level, whereas it was not detectable in either IOSE 29 or primary cultures of normal. In contrast, IOSE 29 and OSEs (ovarian surface epithelia) had higher levels of LPA_1. EOC, but not IOSEs, responded to LPA, suggesting that LPA_2 transduced effects of LPA on ovarian cells and also mediated the induction of VEGF by EOC and not by OSEs. Recombinant LPA_1 couples principally to G_i, whereas LPA_2 associates with G_q and G_i. This difference in pairing with G-proteins is speculated to explain the different effect they mediated. LPA_1-mediated mobilization of intracellular Ca^{2+} is almost completely inhibited by pertussis (PTX), whereas LPA_2-transduced Ca^{2+} responses are incompletely inhibited. In contrast, rho inactivation by C3 exoenzyme more completely reduces LPA_2-dependent responses than those transduced by LPA_1. A high level of LPA_1 suppresses proliferation and promotes apoptosis.[57] When human LPA_2 is transgenically expressed in mouse ovaries, higher levels of VEGF, isomers of VEGF-A, VEGF receptors 1 and 2, and uPA are produced than that from nontransgenic ovaries, suggesting a potential role of LPA in early development of ovarian cancer.[31]

Ren and colleagues show that LPA in the conditioned medium from human peritoneal mesothelial cells induces migration of OC cells. Using siRNA against different LPA receptors, they show LPA_2 has the strongest effect (~60% inhibition), followed by LPA_3 (30% to 40% inhibition) and LPA_1 (20% to 30% inhibition) in the peritoneal mesothelial cells conditioned medium induced SKOV3 migration. siRNAs against LPA_2 and LPA_3 greatly delayed the invasion process without significantly affecting the extent of invasion. In contrast, siRNA against LPA_1, as well as control siRNAs against GAPDH and GFP, had no significant effect on cell invasion, suggesting that LPA_2 and LPA_3 play important roles in cellular functions of SKOV3 cells.[10]

G_i pathways have been specifically implicated in mediating LPA-stimulated proliferation and migration, as these two LPA effects are blocked by treatment with the G_i selective inhibitor PTX.[54] Targeted deletion of LPA_1 in mice revealed ~50% perinatal lethality. Specific deletion of LPA_2 in mice does not result in blatant phenotypes, but LPA_1 and LPA_2 double knockouts show greater lethality than does the LPA_1 knockout alone. Targeted deletion of LPA_3 and LPA_4 has not yet been reported. Limited information is available regarding the specific functions of each receptor subtype in cancer invasion and metastasis, with LPA_2 and LPA_3 overexpressed in most OC cells compared with normal ovarian epithelial cells.[23] LPA_2 and LPA_3 have been implicated in LPA-induced cell migration for different OC cell lines. LPA_1 is involved in cell migration in other cell types. Malignant transformation resulted in aberrant expression of LPA_2 and LPA_3 in OC tissues, and the ratio of LPA_2/LPA_1 increased markedly during malignant transformation.[10]

Signal Transduction Pathways That Regulate LPA-Stimulated Invasion and Migration

LPA induced cell invasion and motility through LPA receptor–mediated signaling pathways and transcriptional activation of growth factors, cytokines, proteases, and chemokines. However, the exact mechanism by which LPA promotes invasive behavior of OC cells remains to be elucidated. Recent advances have identified multiple pathways that mediate LPA's effect on cell motility and invasion. It has been reported that LPA-induced migration is regulated by several distinct signaling pathways, such as $G\alpha_{12/13}$-mediated activation of RhoA, G_i-mediated activation of phosphoinositide 3-kinase leading to activation of Rac, and G_i-dependent activation of ERK.[58]

Rho and ROCK

Rho and ROCK play essential roles in the migratory process, as evidenced by the inhibition of migration and focal adhesion formation of cancer cells by *Clostridium botulinum* C3 exoenzyme (C3), an inhibitor of Rho, or Y-27632, an

inhibitor of ROCK. LPA also evoked the formation of focal adhesions and tyrosine phosphorylation of focal adhesion kinase and paxillin, all of which were inhibited by C3 or Y-27632, suggesting that LPA induced the migration of OC cells, at least in part, through accelerated formation of focal adhesions mediated by Rho/ROCK-induced actomyosin contractility.[59]

LPA activates $G_{12/13}$, leading to activation of the small GTPase RhoA, which drives cytoskeletal contraction and cell rounding. LPA activates Ras- and Rho-family GTPases to control cell proliferation, migration, and morphogenesis. The Ras- and Rho-GTPases cycle between GDP- and GTP-bound states, with GTP binding being promoted by specific GDP/GTP exchange factors (GEFs); the GTP-bound forms can interact with various downstream effectors to alter cell behavior.

$G_{i/o}$–ERK–Dependent and RhoA-Rho Kinase–Dependent Pathways in Mesenchymal Stem Cells Migration

Rac is required at the front of the cell to regulate cell (re)spreading after initial rounding, lamellipodium formation, and cell migration, and Rho activity in migrating cells is responsible for cell body contraction and rear-end retraction, thereby promoting movement of the cell body and facilitating detachment of the rear end. In addition, G_i-ERK–dependent pathway has shown to be involved in the LPA-stimulated migration of tumor cells. However, the involvement of RhoA and ERK in the LPA-induced migration has not yet been clearly defined. Mesenchymal stem cells (MSCs; also called stromal cells) possess self-renewal capacity, long-term viability, and differentiation potential toward diverse cell types, such as adipogenic, osteogenic, chondrogenic, and myogenic lineages, and they can be isolated from a variety of tissues, such as bone marrow and adipose tissues, suggesting clinical applications of MSCs for regenerative medicine. On the other hand, several recent studies indicate that MSCs could result in an adverse effect that is to favor tumor growth: subcutaneous transplantation of tumor cells together with MSCs exhibited elevated capability of proliferation, rich angiogenesis in tumor tissues, and highly metastatic activity. Furthermore, MSCs have been shown to migrate to various in vivo sites of injury and tumors. Lee and coworkers demonstrated for the first time that LPA is involved in the malignant ascites–induced migration of human MSCs through activation of $G_{i/o}$-ERK–dependent and RhoA-Rho kinase–dependent pathways.[55]

Regulator of G-Protein–Signaling (RGS) Proteins

RGS proteins deactivate heterotrimeric G-proteins by accelerating the rate at which Gα subunits hydrolyze GTP. RGS proteins have been shown to have profound effects on the kinetics and magnitude of multiple in vivo receptor

signaling pathways. Hurst et al. demonstrate that endogenous RGS proteins attenuate signaling of $G\alpha_i$-mediated LPA-stimulated inhibition of adenylyl cyclase and cell migration in ovarian cancer cells.[54] LPA receptors can couple to $G\alpha_{i2}$ in SKOV-3 cells, and endogenous RGS proteins expressed in these cells attenuate LPA signaling through $G\alpha_{i2}$ by accelerating its deactivation. Specifically, mutagenically blocking the association of endogenous RGS proteins with $G\alpha_{i2}$ delayed the deactivation of $G\alpha_{i2}$ activity, resulting in more potent and efficacious LPA effects on cAMP levels. Further, RGS insensitive $G\alpha_{i2}$ mediated more robust cell migration responses to LPA. These findings establish RGS proteins as novel regulators of LPA signaling in ovarian cancer cells.[54]

Signaling Pathways Involved in Migration Regulation

Cell migration is regulated by a combination of different processes: the contraction of actomyosin, the formation of stress fibers, and the turnover of focal adhesions. Contraction of the actomyosin system is important for cell migration, and LPA induces MLC (myosin light chain) phosphorylation through the activation of the small GTP-binding protein Rho, leading to the stimulation of cell contractility and motility. Another fundamental component affecting cell motility is the focal adhesion: cell-ECM adhesions can alter the capacity of the cell to attach and migrate through surrounding tissues. Changes of the expression and activities of the components of focal adhesions could make an important contribution to cancer invasion. At their cytoplasmic face, focal adhesions provide attachment for actin stress fibers. More than just sites of structural linkage between the ECM on the outside and the cytoskeleton on the inside, focal adhesions are regions of signal transduction. Components involved in multiple signal transduction pathways have been identified in focal adhesions, with most attention being directed toward tyrosine phosphorylation at these sites. LPA stimulates cell motility by driving the formation of focal adhesions and elevating tyrosine phosphorylation of focal adhesion proteins such as paxillin, FAK, and Src in fibroblasts and cancer cells.

The Ras proteins, including H-Ras, N-Ras, and K-Ras, are GTP/GDP-binding proteins that play key roles in cellular regulation. Ras can be activated by various extracellular stimuli such as growth factors, cytokines, cellular adhesion signals, and also stress signals, including irradiation and osmotic stress. Ras-involved cellular functions are mediated by Ras downstream effectors such as Raf-1 kinase, Ral-GDS, and PI3K. In addition, MAPK kinase 1 (MEKK1) has been shown to directly interact with GTP-bound Ras, and epidermal growth factor–induced MEKK1 activation requires Ras activity, suggesting that MEKK1 may also act as a Ras downstream effector.

MEKK1 is a serine/threonine kinase that is activated in response to growth factors, cytokines, and chemoattractants. In addition, MEKK1 is also activated in response to changes in cell shape and the microtubule cytoskeleton. MEKK1

is a potent and preferential activator of the c-Jun NH_2-terminal kinase (JNK) group of MAPKs. It also influences the activity of the extracellular signal-regulated kinase (ERK) pathway with little or no effect on the p38 MAPK pathways. Furthermore, MEKK1 has been shown to regulate NF-κB activity by activating IκB kinase-α and -β. A number of studies have demonstrated that MEKK1 plays an important role in cell migration: (a) MEKK1-deficient fibroblasts and embryonic stem cells are defective in cell migration; (b) overexpression of MEKK1 in epithelial cells stimulates lamellipodia formation, a key component of cell migration; (c) MEKK1-JNK signaling cascade is essential in transmission of transforming growth factor β and activin-regulated epithelial cell movement; and (d) MEKK1 interacts with molecules important for cell migration such as Rac/Cdc42, α-actinin, focal adhesion kinase, and p115 Rho GTPase-activating protein. However, it is currently not known how MEKK1 is involved in cell migration. Bian et al. show that LPA stimulates both chemotaxis and chemokinesis of OC cells through a G_i-dependent mechanism. Moreover, they show that the dominant-negative H-Ras mutant (T17N) blocks the ability of LPA to stimulate ovarian cancer cell migration and that the constitutively active H-Ras mutant (G12V) enhances cell migration even without LPA stimulation. However, H-Ras mutants that activate Raf-1 kinase, Ral-GDS, or PI3K were not able to significantly facilitate cell migration, suggesting that a signaling pathway distinct from Raf-1, Ral-GDS, and PI3K is responsible for LPA-stimulated ovarian cancer cell migration. They demonstrate that LPA activates MEKK1 in a Ras-dependent manner and that dominant-negative MEKK1 inhibited LPA-stimulated cell migration in SKOV3 cells. Surprisingly, the well-characterized MEKK1 downstream pathways, namely, MEK1/2-Erk, MKK4/7-JNK, and NF-κB signaling pathways, are not significantly involved in LPA-stimulated cell migration. Instead, G_i–H-Ras–-MEKK1 pathway is involved in LPA-induced FAK (focal adhesion kinase) redistribution to focal contact regions of the plasma membrane. In the course of the migratory response, a FAK-involved dynamics turnover in focal adhesion formation controls the process of cell attachment and detachment, which are required for cell migration. Various research groups have provided evidence that FAK promotes cell migration potentially through the association with other signaling proteins such as Grb7 and SHP-2 or by the increased phosphorylation of p130Cas or paxillin adaptors. In addition, it is a requirement for FAK localization at cellular contact sites (adhesion) to facilitate cell migration.[60] Hall and Nobes also showed that microinjection of Ras-neutralizing mAb blocked cell migration, but addition of MEK1/2 or PI3K inhibitor had only minor effect on cell migration. Recently, the localization of FAK in focal adhesion has also been found to be essential for FAK-mediated adhesion turnover. Bian's work indicates that Ras may regulate cell migration by activating MEKK1 and subsequently facilitating FAK focal contact redistribution.[60]

Small Molecules That Inhibit LPA-Stimulated Invasion

Geranylgeranylacetone (GGA) is an isoprenoid compound that has a similar chemical structure to that of geranylgeranylpyrophosphate, which is in the metabolic pathway of Rho and is essential for geranylgeranylation of Rho. GGA markedly inhibited LPA-induced invasion of human ovarian carcinoma cells by attenuating the activation of Rho. This resulted in changes in cell morphology, loss of stress fiber formation and focal adhesion assembly, and the suppression of phosphorylation of focal adhesion proteins, which are essential processes for cell migration. GGA-induced inhibition of Rho activation and FAK phosphorylation can be restored by geranylgeraniol (GGOH). However, GGOH only partially reduced the GGA-induced inhibition of cancer cell invasion and GGA-induced inhibition of paxillin phosphorylation and stress fiber formation, suggesting other inhibitory mechanism of LPA-induced cancer cell invasion by GGA than only through the inhibition of Rho geranylgeranylation.[61]

Sawada et al. showed that alendronate also markedly inhibited LPA-induced migration of human OC cells by attenuating the activation of Rho. Alendronate inhibits LPA-induced cancer cell invasion by preventing geranylgeranylation of Rho. A significant inhibitory effect of alendronate on LPA-induced Caov-3 cell migration was observed at a concentration of 1 μM, and half-maximal inhibition was estimated to occur at ~3 μM. The addition of GGOH restored the inhibitory effect of alendronate. On the other hand, the addition of farnesol (FOH) did not restore the inhibitory effect of alendronate-induced inhibition of focal adhesion assembly and cell migration despite the partial restoration in Rho activation and MLC (myosin light chain) phosphorylation. This inconsistency suggests two possibilities: (a) FOH might be partially metabolized to geranylgeranylpyrophosphate (GGPP), which activates Rho and MLC phosphorylation to a lesser extent than does GGOH, and lesser phosphorylation of MLC might not be enough to activate focal adhesion assembly and cell motility; and (b) the addition of FOH might have the possibility to induce farnesylation of signaling molecules, which activate MLC phosphorylation but do not promote migration activity, although they could not identify the farnesylated molecule. These results suggest that the formation of focal adhesion assembly, as well as the tyrosine phosphorylation of focal adhesion components, is essential to keep cytoskeletal organization and the resultant onset of migration activity. For the formation of peritoneal dissemination, OC cells need to detach from the primary tumor, attach to the ECM of other tissues, and migrate to form stromal lesions. Once cancer cells migrate into normal stroma, angiogenesis occurs from preexisting venules. The fact that alendronate accumulates in vessels strongly suggests that alendronate has the potential to prevent metastasis of OC cells at concentrations that might be relatively nontoxic to normal cells in comparison with most currently used anticancer agents.[62]

Other Lysolipids Involved in EOC Invasion

S1P

The lysolipid phosphoric acid mediators LPA and S1P are generated enzymatically from membrane lipid precursors in many different normal and malignant cells. Extracellular LPA and S1P both stimulate cellular proliferation and differentiation, enhance cellular survival, and evoke specific cellular functional responses such as migration and adhesion.[57] Smicun et al. show that in OC cells, low-dose S1P stimulated invasion like LPA, whereas high-dose S1P inhibited invasion, which correlated with increased and reduced gelatinase activity in conditioned media. Low and high S1P dose also differently affected the presentation of surface S1P receptors; low S1P dose increased S1P1 and decreased S1P2, whereas high S1P increased S1P3. The dual effect of high and low S1P concentration on invasion was probably caused by the diverse changes to the presentation of surface S1P receptors. The opposite effect of S1P and LPA on expression of each receptor suggests a homeostatic transcriptional mechanism that abrogates the effects of LPA and S1P on EOC cells.[63] They also show cell attachment status affects their response to S1P and LPA. The recovery of membrane N-cadherin and change in cell-cell adhesion and actin stress fibers intensity in response to LPA and S1P inversely correlate with their effects on cellular invasiveness. The decrease of cell-cell adhesion by 0.5 μM S1P and LPA and increase by 20 μM S1P inversely correlate with the effects of S1P and LPA on cell invasion, indicating that changes in cell-cell adhesion induced by S1P and LPA directly impact the invasiveness of these cells, in contrast with the more complex interaction between cell-matrix attachment and invasiveness.[64]

LPE and Other LPA Derivatives

Lysophosphatidyl enthanolamine (LPE) stimulated chemotactic migration and cellular invasion of OC cells. Park et al. suggest the significance of LPE as a bioactive mediator in ovarian cancer. LPE-induced Ca^{2+} response was not desensitized by the treatment of LPA. Furthermore, LPE-induced calcium signaling was not inhibited by two structurally different antagonists for $LPA_{1/3}$ receptors (VPC32183 and Ki16425), though LPA-induced calcium rise was almost completely inhibited by the two antagonists. LPE failed to stimulate NF-κB-driven luciferase activity in HepG2 cells expressing LPA_1, LPA_2, LPA_3, LPA_4, or LPA_5 exogenously. Thus, LPE may bind to its own receptor, which is distinct from LPA receptors, in SKOV3 ovarian cancer cells.[65]

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Chapter 14
Cell Adhesion in Ovarian Cancer

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Introduction

Epithelial ovarian cancer is the most common cause of death among gynecologic malignancies in the Western world.[1] The disease presents late in more than two thirds of patients with spread in the peritoneal cavity on surfaces, intraparenchymal metastases, and in suspension in peritoneal and pleural effusions. Dissemination to and compromise of critical end organs is often the cause of patient morbidity and mortality. Methods of dissemination include the common hematogenous and lymphatic spread, but also shedding into the peritoneal cavity. It is this shedding and subsequent adherence to the serosal and organ surfaces that provides the first step in subsequent carcinomatosis and further metastasis. The advance into the peritoneal cavity, early compared with that seen with other adenocarcinomas of the pelvis and abdomen, is attributed to the ability of ovarian cancer (and fallopian tube cancers) to release tumor cells into the local environment and potential spaces of the peritoneal cavity. Different forms of cell adhesion are necessary to maintain survival signals to the tumor cell and to induce the changes in the local microenvironment needed to sustain and advance tumor development, such as induction or remodeling of local vasculature. As these processes are dissected and more is known, new and exciting advances in therapies targeted to adhesion have emerged.

Cell-Cell Adhesion

Cell-to-cell adhesion is one of several forms of adhesion, all differentially regulated. Ovarian cancer uses multiple forms of adhesion and incorporates these biologic processes into its survival and dissemination activities.

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Heterotypic and homotypic cell-cell adhesion are both involved in ovarian cancer, and both have been modeled in vitro. The most common heterotypic cell-cell interactions in carcinomas are tumor cells binding to endothelial cells. This behavior is necessary for initial entry into the vasculature and subsequent extravasation at secondary sites. Key molecules involved in homotypic and heterotypic cell-cell adhesion have been identified, and in many cases, the molecules or those involved in their downstream signaling pathways have been recognized as potential therapeutic or diagnostic molecular targets (Table 14.1).

Table 14.1 Cell-cell adhesion factors

Cell Adhesion Factors	Cellular Location	Function
E-cadherin	Epithelium	Epithelial cell-cell adhesion
N-cadherin	Neural tissues	Mediates presynaptic to postsynaptic adhesion in CNS synapses
P-cadherin	Peritoneal mesothelium	Cell-cell adhesion
VE-cadherin	Vascular endothelium	Cohesion and organization at intracellular junctions
E-selectin	Endothelium	Recruit leukocytes to site of injury
P-selectin	Platelet	Initial recruitment of leukocytes to site of injury
L-selectin	Lymphocyte	Allows localization of leukocytes

The Cadherins and the Cadherin Pathway

Cadherins are a superfamily of cell surface glycoproteins that participate in cell-cell adhesion and recognition.[2,3] Classic cadherins engage in Ca^{2+}-dependent, homophilic, cell-cell adhesions, are associated with adherent junctions, and are present in almost all solid tissues. They are single-pass transmembrane glycoproteins with unique extracellular domains comprising tandem repeats bearing negatively charged amino acid sequences that bind calcium.[4] Classic cadherins include E (epithelial), N (neural), P (placental), and VE (vascular endothelial) cadherin, named for the tissues in which they were first identified. E-cadherin, important in ovarian cancer, is involved in epithelial cell-cell interactions and is found at the cell membrane in adherens junctions. Cadherin adhesive specificity is mediated by cadherin ectodomains; there is a highly conserved shared cytoplasmic tail that interacts with a range of cytoplasmic proteins linking the cadherin to the cell cytoskeleton and intracellular signaling pathways.[5] In order to put the unusual function of E-cadherin in ovarian cancer into perspective, it is necessary to briefly review its signaling and more common behavior in carcinoma.

Cadherin Signaling

Four proteins comprise the core cadherin-catenin complex. Cadherin partner protein β-catenin binds to the distal end of the cadherin cytoplasmic tail[6] and then to α-catenin.[7] α-Catenin is the direct link to actin filaments.[8–10] p120-Catenin ($p120^{ctn}$) binds to the membrane-proximal region of the cadherin cytoplasmic tail.[11] The role of $p120^{ctn}$, an armadillo protein, in cadherin function is to regulate cadherin surface levels by antagonizing endocytosis and promoting cadherin clustering.[8,12] Plakoglobin, also known as γ-catenin, is another armadillo-repeat protein that binds the cytoplasmic cadherin tail and links the complex to the intermediate filament cytoskeleton.

In addition to mediating outside-in cadherin signaling, β-catenin binding of E-cadherin protects the cytoplasmic domain from rapid degradation,[13] enhancing endoplasmic reticulum to cell surface transport,[14] and recruiting α-catenin. Downregulation of E-cadherin releases β-catenin and $p120^{ctn}$ into the cytoplasm leaving them free for phosphorylation, ubiquitination, and degradation or to regulate transcriptional activity.[15–17] Disruption of adherens junctions releases β-catenin from the cadherin-catenin complex pool, and in the presence of Wnt, stabilized β-catenin mediates target gene expression through participation in a transcription-promoting complex with the DNA-binding factor lymphocyte enhancer factor/T-cell factor (Fig. 14.1).[17,18,20] Target genes include key oncogenes, such as cyclin D1, c-myc, and matrix metalloproteinase 7.[21] Both loss of expression and gain-of-function mutations of β-catenin are found in human cancers, though not commonly ovarian cancer, resulting in loss of cell-cell adhesion and increased gene transcription. Mutations in adenomatous polyposis coli (APC) or axin compromise degradation of β-catenin have been found in colorectal carcinoma and other malignancies.[22] These mutations cause nuclear accumulation of β-catenin leading to aberrant target gene activation. An α-catenin mutation that disrupts binding to β-catenin reducing cadherin/catenin complex linkage to the actin cytoskeleton has been reported in ovarian cancer.[23]

E-cadherin has been shown to interact with receptor tyrosine kinases (RTK) such as epidermal growth factor receptor (EGFR), known to be involved in ovarian cancer.[24] Association with EGFR activation induces tyrosine phosphorylation of β-catenin and plakoglobin and leads to disassembly of the cadherin-catenin complex.[25] Qian et al. described a bidirectional regulation between E-cadherin and EGFR where E-cadherin interacts with EGFR through its extracellular domain and decreases its mobility and ligand-affinity.[26] Cadherin function can be regulated by other cell signals including ubiquitination of the E-cadherin cytoplasmic tail to regulate cadherin endocytosis[27] and signaling by small Rho GTPases that influence cadherin-actin interaction.[28]

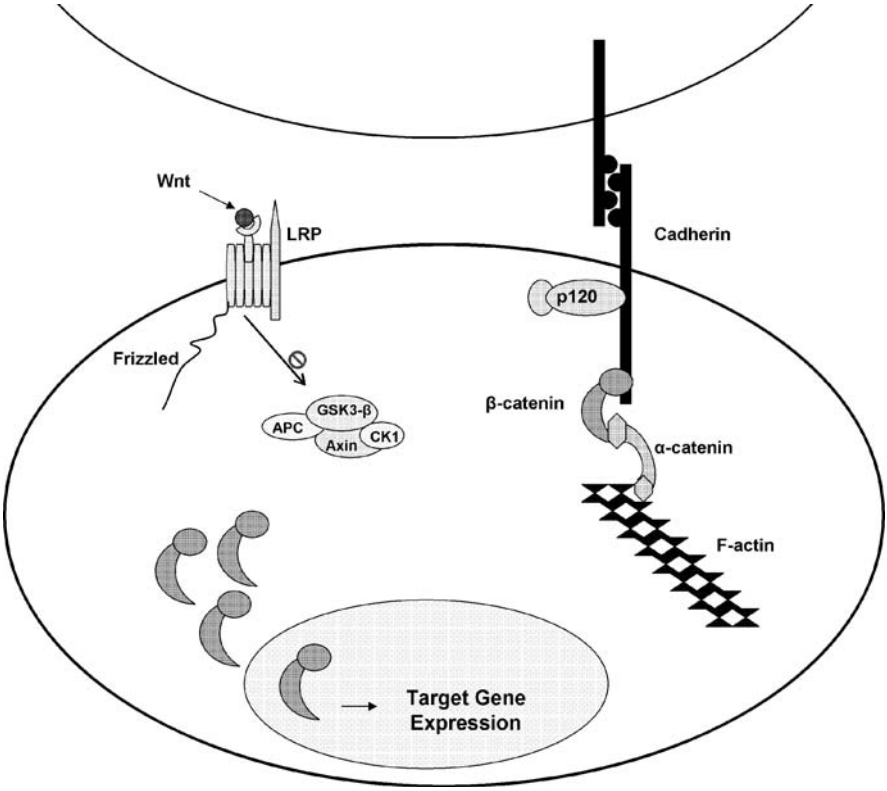

Fig. 14.1 Cadherin-catenin complex. The cadherin-catenin adhesion complex is the major means that cells employ to adhere to one another allowing for different forms of cell-cell interaction including cell-cell cohesion, cell-cell discrimination, and cell-cell locomotion. The complex is a key component of adherent junctions (zonula adherens) and has four components: cadherin, β-catenin, p120-catenin, and α-catenin (which connects the complex to the actin cytoskeleton). In the absence of Wnt signaling, free cytoplasmic β-catenin is targeted for ubiquitination and degradation in the 26S proteosome by paired phosphorylation through the serine/threonine kinases casein kinase I (CKI) and glycogen synthase-3β (GSK-3β) bound to a scaffolding complex of axin and adenomatous polyposis coli (APC) protein.[19,22] Wnt signaling activation inhibits GSK-3β activity and results in accumulation of cytoplasmic β-catenin, which is then free to translocate to the nucleus and induce target gene expression

Cadherins in Cancer

Disruption or loss of the cadherin-catenin complex plays a prominent role in carcinoma and an interesting antithetical role in ovarian cancer. Malignant transformation is often characterized by cytoskeletal remodeling, loss of cell-cell adhesion, and loss of apical/basal polarity. Disruption of normal cell-cell adhesion contributes to enhanced migration, proliferation, and promotion of epithelial-to-mesenchymal transition leading the way to invasion and

metastasis.[29] The majority of cancers of epithelial origin loses E-cadherin–mediated cell-cell adhesion[30] and typically undergoes epithelial-to-mesenchymal transition.[31] E-cadherin was initially identified as a metastasis suppressor.[32] Poorly differentiated, highly metastatic carcinomas commonly have low or undetectable E-cadherin or quantitative or functional loss of any component of the cadherin-catenin complex. This may be due to mutations, promoter hypermethylation, or transcriptional silencing of the E-cadherin gene (CDH1) results in loss of E-cadherin's functions and adherent junctions formation. Mutations can be inherited germ-line mutations (lobular breast cancer and familial gastric cancer[33]) or acquired somatic with loss of heterozygosity and function. Hypermethylation of the CDH1 promoter has been observed in human breast, prostate, and hepatocellular tumors.[34] Aberrant expression of transcription factors that repress CDH1 promoter may result in its transcriptional silencing. These factors include snail, E12/E47, and SIP1, which bind to the CDH1 promoter, inhibit E-cadherin's transcription, and induce an epithelial-to-mesenchymal transition, leading to acquisition of invasiveness (Fig. 14.2).[35,36]

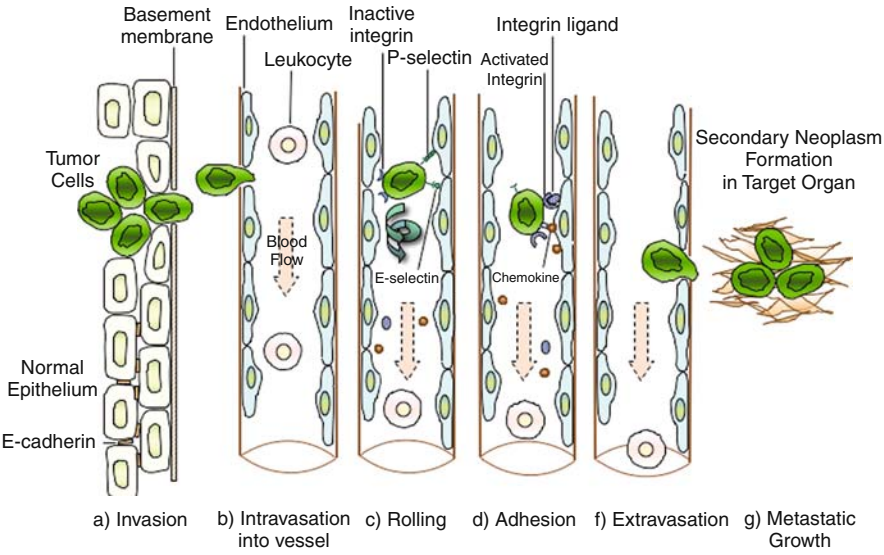

Fig. 14.2 Cancer cell invasion and metastasis. (**a**) Proliferation and local invasion of tumor cells from a primary site through the epithelium, which contrary to its normal physiologic status displays a loss of E-cadherin in cell-cell junctions. (**b**) Tumor cell intravasation from tumor mass and stroma into capillaries. (**c**) The cancer cell survives in the blood circulation, where E-selectin and P-selectin, expressed in activated endothelium, capture it and initiate the process of "rolling." This is similar to leukocyte rolling during inflammation. (**d**) Selectins, integrins, and chemokines coordinate the capture of circulating cancer cells to the vessel wall and promote a firmer adhesion. Heterotypic signaling is activated providing a permissive environment to maintain attachment and promote extravasation. (**e**) The shear flow and the interaction with endothelial chemokines allow transendothelial migration not compromising the integrity of the endothelial barrier. (**f**) Extravasated cancer cells can proliferate in secondary sites

Cadherins in Ovarian Cancer: A Notable Exception

The ovary is surrounded by the capsule, an epithelial cell basement membrane upon which sits a single layer of cuboidal mesothelial-like surface epithelial cells (OSE); a similar single cell layer is found lining the fimbriae of the fallopian tube. This layer generally lacks E-cadherin expression[37,38] and is hypothesized to give rise to most all epithelial ovarian malignancies, especially those of serous and/or papillary histology. Additional epithelium is found lining surface clefts and stromal inclusion cysts, which, in contrast, strongly express E-cadherin, and has a more epithelial-like appearance, and may also be a source of ovarian neoplasms.[39] Ovarian carcinomas and metastatic sites have more epithelial characteristics than do their OSE counterparts and may display a more complex epithelial morphology.[38,40–42] This distinguishes ovarian carcinomas from most other cancers, which in the course of neoplastic progression become less differentiated than the epithelium they originated from.[43–45]

Ovarian epithelial carcinogenesis, a process lacking a clear precursor lesion or well-recognized progression, has been described by some as a process of mesenchymal-to-epithelial transition followed by epithelial-to-mesenchymal reversal. E-cadherin expression is gained during the observed epithelial differentiation of OSE that may herald ovarian carcinogenesis. Mesenchymal-to-epithelial transition was first described in a system where E-cadherin transformed embryonic corneal fibroblasts into stratified epithelium with desmosomes.[46]

The role of cadherins in progression of ovarian cancer to peritoneal dissemination and metastasis remains unclear. One proposed mechanism is switching of the subtype of cadherin expression promoting carcinoma cell detachment and shedding. Peritoneal mesothelium expresses P-cadherin.[47] Advancing ovarian cancer stage has been shown to be associated with a switch from E- to P-cadherin; this would favor homophilic interactions between shed ovarian cancer cells and peritoneal mesothelium at implantation sites. Upregulation of P-cadherin has been described with increasing aggressiveness in breast cancer.[48] These concepts suggest that P-cadherin may be an important potential molecular target to disrupt ovarian cancer dissemination.

Selectins

Heterotypic cell-cell adhesion is important in the interaction of shed and circulating ovarian cancer cells with their microenvironment, promoting success of metastatic foci. Selectins are a family of calcium-dependent type I transmembrane carbohydrate binding proteins.[49] They mediate heterotypic interactions between blood and endothelial cells during leukocyte adhesion and lymphocyte homing[50] and promote early tethering and then rolling of the leukocytes on the endothelium during hydrodynamic vascular shear stress.

Those interactions are characterized by relatively rapid on/off binding kinetics between selectins and their ligands.[51] As with the cadherins, standard nomenclature designates each family member according to the cell type of original identification: E (endothelium; endothelial leukocyte adhesion molecule-1) selectin, P (platelet) selectin, and L (lymphocyte) selectin. Selectins, now shown to mediate endothelial cell interaction with malignant cells, typify heterotypic cell-cell adhesion interactions in cancer.[52–55]

Selectins as Mediators of Heterotypic Cell Adhesion

The heterotypic interaction of endothelial cells with tumor cells and hematopoietic cells has been shown to involve, in part, selectins through their recognition of fucosyl carbohydrate ligands. Structures containing sialyl-Lewis^x (sLe^x) and sialyl-Lewis^a (sLe^a), highly expressed in polymorphonuclear leukocytes and monocytes[56] and less abundantly on natural killer cells,[57] are also expressed on some cancers.[52–55] Selectins have a characteristic extracellular region composed of a domain with structural homology to calcium-dependent lectins, an EGF-like domain, and two to nine short consensus repeats homologous to complement regulatory proteins, a membrane spanning region, and a cytoplasmic tail.[49] Cell binding is mediated through the selectin lectin domains and cell surface carbohydrate ligands.[58]

Selectin-carbohydrate adhesion is an early event in leukocyte adhesion responses that promotes the attachment of nonactivated leukocytes to activated endothelium.[59] Selectins are responsible for the leukocyte rolling, a transient stage of adhesion that is a prelude to firm attachment to the vascular endothelium collaboratively with other cell adhesion molecules and leukocyte integrins.[60,61] Synthesis of E-selectin is induced rapidly after endothelial cell stimulation by proinflammatory cytokines and translocates to the endothelial cell luminal surface.[62]

Selectins and Their Ligands in Cancer

The functional importance of selectins in cancer revolves around cancer cell adhesion to both endothelium and platelets. Dysregulated glycosylation is a known feature of malignancies, and sialylated fucosylated selectin ligands have been shown to be expressed and/or secreted in cancer.[63,64,52,53,55] The sialylated Lewis^a antigen is a well-characterized selectin ligand approved as a tumor marker (recognized by CA-19-9 antibody) for colon and pancreatic cancers. Soluble E-selectins have been shown to be significantly higher when compared with controls in ovarian, breast, and gastrointestinal cancers. High levels of circulating E-selectin are observed in ovarian cancer patients[65]; however, no differential in blood E-selectin concentration was observed between benign and malignant ovarian masses suggesting limited clinical value for serum E-selectin as a diagnostic marker in epithelial ovarian cancer.[66]

N-acetylglucosamine 6-*O*-sulfotransferase 2 (GlcNAc6ST-2), a member of the carbohydrate 6-*O*-sulfotransferase family, is ectopically expressed in malignant ovarian mucinous adenocarcinoma tissues but not in adenomas.[67] GlcNAc6ST-2 participates in the biosynthesis of a L-selectin carbohydrate ligand (GlcNAc-6-*O*-sulfated sialyl-Lewis^x)[68] and is highly expressed in ovarian papillary serous and clear cell adenocarcinomas, whereas the solid serous and endometrioid subtypes have a lower expression.[67] Mucinous, clear cell, and papillary serous subtypes represent more than 80% of the ovarian epithelial adenocarcinomas, and drug resistance occurs rapidly in patients with advanced disease. For this reason, GlcNAc6ST-2 has been proposed as a novel ovarian cancer tumor antigen. Ovarian mucinous adenocarcinomas express sulfated glycans, including L-selectin ligands, sialyl-Lewis^x,[69] sialyl-Tn (sTn), Le^a, and sialyl-Lewis^a antigens more frequently than do serous and endometrial adenocarcinomas.[70] Lewis^x-related structures have been associated with metastasis, making the role of selectins in ovarian cancer metastasis and dissemination intriguing.

CA-125 is the only approved serum biomarker for ovarian cancer, but it lacks the sensitivity and specificity to act alone in a screening test, especially for detection of early-stage disease,[71] and it has not been validated as a prognostic marker. CD24, a ligand for P-selectin, has been proposed as an independent molecular marker of patient survival.[63] Its intracytoplasmic expression can be used to differentiate benign and malignant tumors[63,72]; however, further testing is needed to assess its clinical value.

Hematogenous metastasis, common in solid tumors and sarcomas, occurs albeit late in ovarian cancer; it is a complex and dynamic process. Both detachment from neighboring tumor and endothelial cells and stromal compartments followed by migration are required for initiation, and similar events are required for extravasation at the secondary site. Selectins are involved in adhesion of tumor cells to endothelium similar to their function in inflammation.[73] In this adhesion, selectin ligands of the invading tumor cells interact with their endothelial selectin receptors.[64,74] E-selectin has been shown to be critical in mediating cell tethering and rolling of adenocarcinoma cells,[75] and silencing of the tumor-induced activation of host E-selectin through antisense nucleotides was shown to result in a decreased number of metastases.[76]

P-selectin is involved in tumor cell–platelet interaction facilitating metastasis.[77–79] Both in vitro and in vivo studies have provided evidence that platelets promote metastasis. CT-26 colon adenocarcinoma cell line–induced pulmonary metastasis in C57Bl/6 mice was inhibited by induction of thrombocytopenia, and this inhibition was reversed by platelet infusion.[79] P-selectin knockout mice have elegantly demonstrated that the lodging of metastatic cells was dramatically attenuated compared with wild-type control mice.[80] Several hypotheses to address this mechanism have been generated including release of stimulatory factors from activated platelets, platelet protection of cancer cells from NK cell lysis, and enhancement of tumor cell extravasation by platelets.[81,82] Increased levels of soluble P-selectin indicate platelet activation, and they occur typically in hematologic malignancies.[83]

Selectins and Angiogenesis

Angiogenesis is a complex multistep process of blood vessel proliferation involving proteases, angiogenic growth factors, and endothelial adhesion molecules.[84] Selectin-endothelial interactions have been shown to be important not only in metastasis but also in angiogenesis.[73] Circulating E-selectin is considered evidence of endothelial activation, and endothelial cell expression increases with capillary tube formation. It is not clear if soluble E-selectin may be used to follow effect on disease of therapeutic modulation of angiogenesis. Furthermore, in the proximity of tumor microenvironment, proinflammatory cytokines released by immune cells augment selectin expression, suggesting inflammation as a cancer-promoting process.[51,85] Interference with binding of E- and P-selectin to their ligands caused by synthetic analogues of sialyl-Le^x or by anti-Le^x was shown to inhibit angiogenesis.[97] Co-transplantation of F-2 endothelial cells with human cancer cells in nude rats formed extremely vascular tumors. The vascularization and the size of tumors were decreased with administration of anti-$Le^{a/x}$ antibodies, further supporting the hypothesis that selectins play an important role in the angiogenesis.[87]

Selectins as Therapeutic Targets

The importance of selectins as pharmacologic targets stems from their significance as early mediators of tumor progression, metastasis, and inflammation. Three main categories of intervention have been proposed: anti-selectin antibodies, antibodies against selectin ligands, and low-molecular-weight antagonists.[88] Humanized anti-selectin antibodies have been developed and clinically tested although not with success.[89,90] Antibodies against selectin ligands have been developed to inactivate P-selectin glycoprotein ligand-1 (PSGL-1) expressed in leukocytes. A beneficial effect of anti-PSGL-1 was demonstrated in an ex vivo rat model of hepatic cold ischemia and reperfusion. Increased bile production, reduced hepatocellular damage, and enhanced portal venous flow resulted.[91]

Glycoproteins are the principal selectin ligands leading to the design and testing of glycomimetics antagonists to E- and P-selectins.[88] Those glycomimetics include a pentasaccharide with a sialyl-Le^x substructure (CY1503), a modified trisaccharide sialyl-Le^x, and a conjugated monosaccharide dimer bimosiamose (TBC1269).[92,88,93] PSI-697, a quinoline salicylic acid class of compounds, is an orally available small molecule developed as a P-selectin antagonist, blocking PSGL-1 interactions.[94,95]

Cell-Matrix Interactions

Heterotypic cell-matrix interactions are the second major adhesion event in ovarian and other solid tumor biology. The extracellular matrix (ECM) is an important source of positive and inhibitory regulation for the cancer

microenvironment. Several families of interaction molecules and ligands have been described. These have key roles in malignant dissemination and the vascular response to the cancer, and many have been the target of pharmacologic intervention. The combination of cell-cell adhesion and cell-substratum interactions are those that provide the trophic and stabilization signals to normal and normally remodeling tissues. Alterations in one or both are the hallmark of the flexibility of malignant cells.

Matrix Scaffolding

Malignant cells and activated stromal cells have the capacity to alter the type and amount of matrix molecules they produce, secrete, and modify in their local microenvironment. Further, they have varied receptors capable of responding to matrix and matrix fragments and the other molecules within their noncellular microenvironment. These events result in both stimulatory and inhibitory signals. This also occurs in ovarian cancer whether in solid phase or in effusions.[96–98] Outside-in signaling is propagated by cell-substratum interactions as with cell-cell adhesion. Cell-matrix adhesion can occur with or without ligand binding. Actin filaments in lamellipodia and filopodia can drive β1-integrin membrane clustering and adhesion seeking independently of ligands.[99] Shear stress and force on the cells in an adherent setting also may enhance or mimic tumor-matrix binding signals. Shear force was shown to cause colon cancer cells to adhere via a β1-integrin–mediated mechanism, with resultant activation of downstream survival and adhesion signaling.[100–102]

Integrins

Integrins are fundamental to cellular growth and survival, migration, differentiation, and signal transduction. They are a family of membrane-spanning molecules composed of noncovalently linked α and β heterodimers.[103,104] Integrin structure consists of three primary components: an extracellular receptor, a transmembrane domain, and a cytoplasmic domain. Integrins are mediators of cross-talk between the ECM and cytoplasm via signal propagation.[105] Integrin activation is vital to normal cell activity and also promotes aberrant growth and survival, migration, invasion, and adhesion in cancer. Integrin engagement is associated with generation of a focal contact that becomes a signalosome to traffic information from the ECM to the cell. Integrin heterodimers interact with a variety of matrix and other scaffolding molecules to regulate the cell interaction with its microenvironment.

Integrin Signaling

Upon stimulation either by extracellular matrix or other ligands, or indirectly, integrins aggregate and trigger downstream signaling. This occurs through

formation of a signalosome, a submembrane "organelle" consisting of the integrin cytoplasmic domain and its recruited signaling proteins, such as integrin-linked kinase, focal adhesion kinase (FAK), and phospholipase C.[106,107] The signalosome processes information driving changes in cell function. This is sent to molecules such as RhoA, ras, the MEK/ERK mitogen activating kinase pathway, and the Jun kinase activating kinase pathway.[108,109] FAK is a nonreceptor kinase involved in signaling between ECM and the cell and now an important new molecular target.[110] FAK responds to growth factors and ECM ligands by autophosphorylation and downstream signaling to promote cell adhesion and spreading, migration, and invasion. It has been shown to be important in ovarian cancer. Judson et al. found there was a fourfold increase in FAK expression by immunohistochemistry and Western blot in ovarian carcinoma cases compared with that in normal ovarian epithelium.[111] Further, a marked reduction in migration and invasion was observed in ovarian cancer cell lines transfected with a kinase-deficient FAK.[112] New FAK inhibitors have been shown to reduce activation of FAK, ovarian cancer cell growth, and also tumor burden in xenografts. It has been shown to combine safely and effectively in preclinical studies with taxanes.[110]

FAK is recruited early in outside-in signaling, proximate to the cell membrane. Its autophosphorylation results in sites for partner protein binding for src homology 2 (SH2) domain-containing proteins and src kinases, which are critical in signaling pathways promoting survival, proliferation, and metastasis.[113,114] Src is also a nonreceptor tyrosine kinase critically involved in ovarian cancer tumor progression. It is both upstream and downstream of FAK. Src may be upregulated in late-stage ovarian cancers.[115] A mouse model of spontaneously transformed ovarian epithelium demonstrated increased src expression with increased Phosphoinositide 3-kinase (PI3K) and ras activation, which translated to resistance to paclitaxel. A new small molecule that inhibits src and abl kinases, dasatinib, has been approved for use in imatinib-resistant chronic myelogenous leukemia and select acute lymphoblastic leukemia.[116] Dasatinib is a potent inhibitor of src kinase activity and is therefore a potential therapeutic for cancers such as ovarian cancer where src activity is upregulated. The intramural National Cancer Institute will be running a phase I clinical trial of dasatinib and bevacizumab for patients with solid tumors.

FAK autophosphorylation yielding an SH2 domain ligand causes activation of PI3K.[117] *PI3KCA*, encoding for the catalytic subunit of PI3K, is genomically upregulated, overexpressed, and results in increased protein expression and activation ovarian carcinomas.[118,119] Activation of PI3K induces cell-cell and cell-substratum contact in vitro and as shown by enhanced metastatic potential and resistance to apoptosis.[120,121]

Integrin Activation of the Invasive Phenotype

Adhesion molecules such as integrins communicate with the ECM and the cytoplasmic signaling network to produce and/or activate proteases and other

molecules involved in the invasive and metastatic phenotype. Ovarian cancer cells floating in ascitic fluid have survival and invasive phenotypes, activated due to the loss of their substratum interactions. Cells found in ascites and pleural effusions have a different phenotype compared with solid primary and metastatic tumors.

Davidson et al. have examined differences between effusions and primary solid tumor and metastases. They have demonstrated an increased expression of αv- and β1-integrin subunits in carcinoma cells bathed in ascitic fluid compared with solid tumors. The differential integrin subunits contained by these cells bind components of mesothelium, likely aiding in their invasive and metastatic potential.[122] Furthermore, in vitro studies of ovarian cancer cell aggregates, or spheroids, confirm the distinct phenotype of cells immersed in ascitic fluid compared with solid primary or metastatic lesions. Similar to the effusions, the spheroid phenotype is mediated by $\alpha_5ß_1$-integrin.[123,124]

Integrin ECM engagement upregulates invasion signaling pathways resulting in increased secretion of proinvasive proteins. Ligation of integrin receptor α5β3 to vitronectin causes activation of the urokinase-type Plasminogen Activator uPA-uPAR Urokinase receptor pathway resulting in increasing plasmin activation and invasion of malignant melanoma cells.[125] Soluble ECM proteins and fragments, such as fibronectin, can activate integrins, which results in increased MMP-9 secretion.[126] Treatment of ovarian carcinoma cells with a blocking antibody for α_5-integrin caused decreased MMP-9 secretion and invasion.[126] Additionally, proteases such as MMP-2 can bind integrin α5β3 directly supporting the concept of cell-mediated proteolysis.[127] Secreted protein acidic and rich in cysteine (SPARC), interacting with the ECM, reduced peritoneal dissemination through negative regulation of VEGF and subsequent decrease in MMP-2 and MMP-9 production.[128]

A component of the integrin role is connection to the actin cytoskeleton to facilitate migration and invasion. Upon adhesion and migration stimulation and signaling, actin is rearranged into actin filaments and drives the cell for adhesion and spreading and/or lamellipodial protrusion for migration. One downstream protein involved in actin rearrangement and now shown to have importance in ovarian cancer is actinin-4, an isoform of α-actinin. Histologic staining of actinin-4 in ovarian carcinoma is associated with late stage, increased tumor residual, and decreased 5-year survival rate.[129]

Angiogenesis: An Application of Cell-Cell and Cell-Substratum Adhesion

Angiogenesis is a form of physiologic invasion, involved in normal growth, development, and wound healing.[130] The branching and formation of new blood vessels occurs from existing vasculature in response to stimulation that may include circulating factors, matrix molecules and fragments, and cells, such as tumor cells. Tumor progression is dependent upon nutrient supply and waste

removal[131] and access to the endothelial and lympha-endothelial vasculature as a conduit.[132] Ovarian cancer is unique in that its initial dissemination occurs via shedding, then later by hematogenous and lymphatic spread.[133] Vessel formation requires an invasive-type behavior where there is a sprout that forms, extends, canalizes, then has flow. Normal and tumor angiogenesis follow the same multistep process characterized by the invasive characteristics of metastatic cancers.[134–137]

The importance of angiogenesis in ovarian cancer has been demonstrated at multiple levels including clinical correlates,[138] effective clinical investigation,[139–142] laboratory studies demonstrating the activation of vasculature upon tumor cell adhesion to the endothelial cells lining the vessel,[141] and, finally, recognition that understanding this heterotypic cell adhesion may uncover new therapeutic and imaging targets.

Microenvironment Cross-talk

Ovarian cancer interacts with its local microenvironment physically thorough heterotypic and homotypic cell-cell binding and cell-substratum interaction and also by production and secretion of factors to activate the local microenvironment and to subvert its function to respond to the tumor. A critical and potent pro-angiogenic factor, vascular endothelial growth factor (VEGF), was first identified from ovarian cancer ascites for its role in promoting vascular leakage.[134,142] It is a mitogen and motogen for vascular development and neoangiogenesis and functions to promote survival of neovessels during their formation.[134,143]

The role of VEGF in ovarian cancer has been demonstrated in multiple ways. Expression of VEGF and its receptors has been shown to correlate with poor prognosis in ovarian cancer.[144] Ovarian tumor cells express VEGF, and some have been shown to express VEGF receptors 1 and 2.[145] The relationship between the ovarian cancer and the vasculature is interactive paracrine. Preclinical data show that ligand binding and/or inhibition of Vascular endothelial growth factor receptor 2 (VEGFR2) have significantly reduced ovarian cancer mass and ascites formation in mice.[146,147]

The preclinical data translated successfully to intervention using bevacizumab, the monoclonal neutralizing VEGF antibody, and more recently VEGFR inhibitors. Bevacizumab is a humanized monoclonal antibody against VEGF. It has been the first drug to show significant activity against solid tumors including epithelial ovarian cancer. Bevacizumab has been reported to bind to all isoforms of VEGF, effectively preventing the ligand from binding to VEGFR.[148] In addition to monoclonal antibodies, small-molecule inhibitors of VEGFR receptor tyrosine kinase represented another approach to blocking VEGF-mediated angiogenesis. These inhibitors are ATP mimics, blocking the kinase activity.

Sorafenib is a small-molecule tyrosine kinase inhibitor that inhibits raf kinase, VEGFR2, and platelet-derived growth factor (PDGF) receptor (PDGFR)-β. Sorafenib has shown minimal single-agent results. The National Cancer Institute (NCI) phase I trial of bevacizumab in conjunction with sorafenib has yielded partial responses, and efficacy is now being tested in phase II. Translational results show inhibition of angiogenesis by imaging.[149] A interactive increase in hand-foot syndrome and hypertension was observed and no consistent validity of CA-125 as a measure of disease response.[150]

Angiogenesis Mitogens and Motogens

Many pro- and anti-angiogenic factors exert their effects through modulation of both the mitogenesis and migration of endothelial cells. Both processes require activation of many or all of the adhesion events defined above. Expression of these endothelial cell mitogens and motogens frequently occurs in parallel and is generally paracrine, a result of the complex activation of the local microenvironment. As such, regulation of mitogenesis of the ovarian tumor, such as with paclitaxel, will dysregulate mitogenesis, cell-cell and/or cell-substratum adhesion, and migration of both tumor cells and endothelium.[151,152]

The angiopoietin (Ang) family of growth factors contains four members that associate with the Tie-2 endothelial cell receptor tyrosine kinase. Ang-1 and Ang-4 activate the Tie-2 receptor, and Ang-2 and Ang-3 inhibit Ang-1–induced Tie-2 phosphorylation. Similar to VEGF, Ang-1 is essential for vascular development in the mouse embryo. The absence of Ang-1 and Tie-2 receptors results in severe vascular abnormality in mouse embryos.[153,154] Ang-2, in the presence of VEGF, causes loosening of cell-cell and cell-matrix interactions, which allows for endothelial cell migration and subsequent angiogenesis. An interaction between the angiopoietins and VEGF in regulation of ovarian cancer has been reported.[155] Hata et al. have determined that Ang-1 gene expression and Ang-1/Ang-2 gene expression ratio in normal ovary with corpus luteum (CL) had significantly higher levels compared with that in ovarian cancer.[156]

The fibroblast growth factor (FGF) family consists of 22 members similar to one another in primary sequence and heparin binding.[157] FGF-2, also known as basic FGF (bFGF), is a heparin-binding protein that has been long known to have angiogenic activity.[158] It has been identified to induce ovarian cancer cell migration, proliferation, and vascularization. Serum and ascites samples from patients with advanced epithelial ovarian cancer have been shown to contain elevated levels of FGF-2.[135] Furthermore, several ovarian cancer cell lines have shown increased FGF-2 transcripts,[159] and patients with ovarian cancer have high FGF-2 amounts in urine samples in comparison with other tumors.[160] These findings have been reiterated recently confirming that there is differential FGF expression in ovarian cancer and confirming that FGF-1 (acidic FGF) is

involved in endothelial migration in modified Boyden chambers, a process that requires cell-substratum adhesion.[161] The FGF receptor(s) has been shown to be inhibited by some of the promiscuous kinase inhibitors such as NP603 and PD 161570 (a FGF receptor 1 inhibitor),[162,163] as FGF receptors such as FGF-1 receptor have been shown to mediate chemotaxis,[164] thus making FGF receptor a potential therapeutic target in cancer.

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Chapter 15
Microenvironmental Regulation of Ovarian Cancer Metastasis

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Introduction: Ovarian Carcinoma Metastasis

Tumors arising from the ovarian surface epithelium (OSE) account for the vast majority of ovarian malignancies; however, the etiology of epithelial ovarian cancer (EOC) remains poorly understood,[1] and the analysis of early events in ovarian carcinogenesis is limited by the relative lack of early-stage tumors for study. The normal OSE is a single layer of mesodermally derived cells that exhibit the remarkable ability to transition between epithelial and fibroblastic phenotypes in response to microenvironmental cues.[2–4] Such phenotypic plasticity is usually limited to immature, regenerating, or neoplastic epithelium. Unlike most carcinomas that initially de-differentiate during neoplastic progression, ovarian carcinomas undergo a mesenchymal-epithelial transition and acquire a more differentiated epithelial phenotype resulting in significant morphologic heterogeneity as tumors acquire increasingly complex differentiation reminiscent of the highly specialized epithelia of Müllerian duct origin.[5,6] Differentiated primary ovarian tumors acquire morphologic characteristics of the fallopian tube (serous carcinoma), endometrium (endometrioid carcinoma), endocervix (mucinous carcinoma), and vagina (clear cell carcinoma).[5,6] More recently, classification of ovarian tumors into low-grade (type I) versus high-grade (type II) malignancies has been proposed based on presumed pathways leading to tumorigenesis, rather than histopathologic characteristics.[7] Low-grade carcinomas are more indolent, develop from a recognized precursor lesion, and are often confined to the ovary at diagnosis. In contrast, high-grade tumors are clinically aggressive at initial presentation, are not associated

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with a morphologically recognizable precursor, metastasize early, and are associated with poor clinical outcome. Molecular analyses have established distinct molecular changes that distinguish type I and II tumors, supporting the concept of two pathways of ovarian carcinogenesis.[1]

It is predicted that more than 15,500 women will die this year from complications related to epithelial ovarian cancer metastasis.[8] As 75% of women with EOC are initially diagnosed with previously disseminated intra-abdominal disease, a more detailed understanding of the cellular and biological and biophysical factors that promote successful metastatic dissemination clearly can ultimately improve patient survival. In addition to genetic alterations that promote tumorigenesis, the contribution of the unique ovarian carcinoma microenvironment to the development of metastatic disease is gaining increasing recognition. Unlike other solid tumors, hematogenous dissemination of ovarian cancer cells is uncommon. Instead, an early event in EOC metastatic dissemination is the exfoliation of cells from the primary ovarian tumor into the peritoneal cavity (Fig. 15.1A, B). Shed tumor cells are believed to block peritoneal lymphatics[9] and, together with expression of vascular endothelial growth factor (VEGF), contribute to the build-up of peritoneal ascites.[6,10] Individual tumor cells as well as multicellular aggregates (MCAs) or spheroids have been detected in ovarian cancer ascites.[11–13] Shed tumor cells interact with mesothelial cells lining the inner surface of the peritoneal cavity (Fig. 15.1C), whereupon cell-cell and cell-matrix adhesion molecules contribute to anchoring of tumor cells to establish secondary lesions.[14,15] The dissemination of ovarian cancer is largely contained within the peritoneal cavity, establishing an unique microenvironmental niche composed of tumor cells, inflammatory components, and a host of soluble factors secreted by—or in response to—tumor cells. These include growth factors, bioactive lipids, proteolytic enzymes, extracellular matrix components, and inflammatory mediators.[16–18] The primary tumor as well as both suspended and anchored metastatic cells maintain direct contact with ascites, providing a mechanism for dynamic and reciprocal regulation of the tumor microenvironment.

Pericellular Adhesive Microenvironment

Ovarian tumor progression is accompanied by changes in the pericellular adhesive microenvironment that may reflect, in part, the unique phenotypic plasticity of the normal OSE. The OSE is composed of a single layer of simple squamous, cuboidal, or columnar epithelium separated by a basement membrane from the underlying connective tissue layer, the tunica albuginea.[19] OSE displays both epithelial and mesenchymal characteristics in situ, expressing epithelial markers such as keratin, desmosomes, and basement membrane as well as mesenchymal markers including vimentin and interstitial collagens.[2,20] Reversible modulation between epithelial and fibroblastic phenotypes occurs

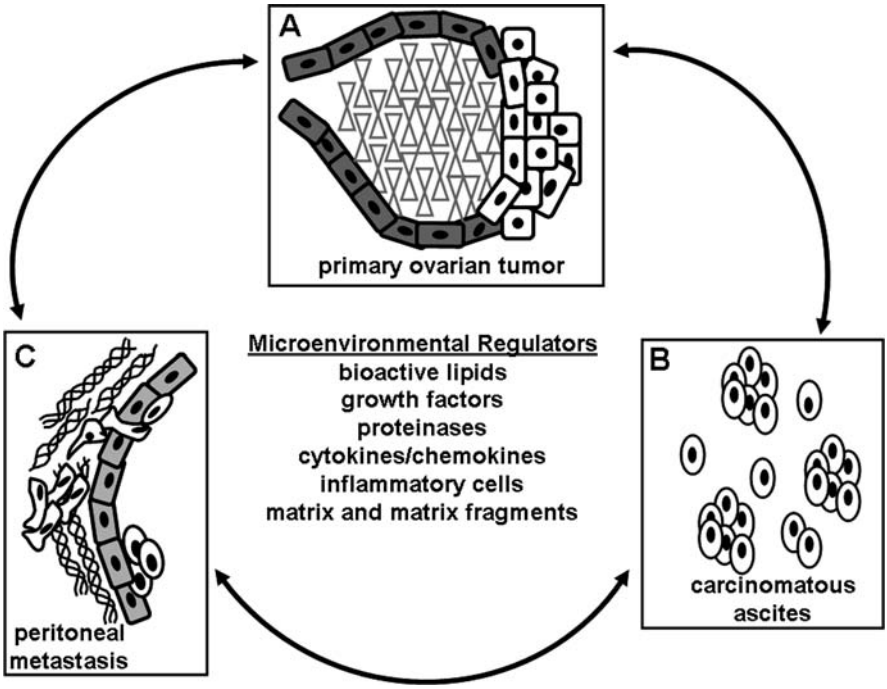

Fig. 15.1 Schematic model of distinct adhesive microenvironments in epithelial ovarian cancer (EOC) metastasis. (**A**) The primary tumor likely forms from malignant transformation of ovarian surface epithelial (OSE) cells. While the OSE expresses primarily N-cadherin, primary ovarian tumors undergo a mesenchymal-epithelial transition and acquire expression of the epithelial cell-cell adhesion molecule E-cadherin. (**B**) Metastatic cells are shed from the primary tumor as single cells and multicellular aggregates (MCAs). Formation of malignant ascites is common, particularly with advanced-stage tumors. Tumor cells in ascites lack integrin-matrix contacts, whereas cadherin-based adhesion is prevalent. (**C**) Peritoneal metastasis involves reestablishment of integrin-matrix contacts as single cells or adherent MCAs interact with submesothelial types I and III collagens. Tumor cells proliferate to established anchored secondary lesions containing cell-matrix binding integrins such as $\alpha 2\beta 1$ and $\alpha 3\beta 1$ as well as cell-cell binding cadherins, predominately N-cadherin. The role of this distinct adhesive milieu on cellular response to microenvironmental regulators including growth factors, cytokines, and bioactive lipids is under investigation

during postovulatory repair and includes alterations in cell shape, cell-cell and cell-matrix contacts, and altered expression of differentiation markers.[3,4] Similarly, reversible modulation of cellular adhesive events likely plays a critical role in remodeling of the OSE during tumor progression.

Cell-matrix and cell-cell adhesion, mediated by integrins and cadherins, respectively, are required for the maintenance of tissue integrity. Moreover, modulation of the expression and/or function of adhesive proteins may contribute to metastatic dissemination. The expression of integrins, heterodimeric transmembrane receptors for extracellular matrix components, has not been

extensively evaluated in OSE; however several integrins including α2, α3, α5, αv, β1, and β3 have been detected.[21–23] Localized expression of α6β4-integrin on the basal epithelial surface of OSE, which is lost in progression to EOC, has also been reported.[23,24] Adhesion mediated by β1-integrins may represent a potential therapeutic target, as this integrin subunit has been implicated in binding of individual tumor cells and MCAs to peritoneal mesothelium or submesothelial matrix and promoting MCA disaggregation[11,12,25–31]

In marked contrast with other epithelia that express E-cadherin, normal OSE epithelial cell-cell integrity is maintained by N-cadherin, and moderate N-cadherin staining of some EOC tumors has been reported (reviewed in Hudson et al.[14]). The epithelial cell-cell adhesion molecule E-cadherin is largely absent in OSE yet becomes more abundant in primary differentiated EOC, suggesting a role in early events leading to cellular transformation.[14] Reduced E-cadherin staining is found in late-stage carcinomas and ascites-derived tumor cells,[32–34] whereas N-cadherin immunoreactivity is elevated in advanced tumors and peritoneal metastases.[14] This reacquisition of mesenchymal-type adhesive features in advanced tumors may change the morphology of MCAs by redistribution (sorting) of E- and N-cadherin presenting cells, thereby modifying the biomechanical properties of the aggregate (such as surface tension)[35–37] and contributing to intraperitoneal anchoring of metastatic lesions.

Reversible changes in the expression and/or function of cellular adhesive proteins occur during ovarian tumor progression (Fig. 15.1). Initial dissemination of cells from the primary tumor on the ovarian surface requires disruption of both cadherin-mediated cell-cell contacts and integrin-matrix interactions. Tumor cells in ascites lack integrin-matrix contacts, yet cadherin-based adhesion is prevalent in MCAs. Integrin-matrix adhesion is reestablished during peritoneal anchoring, and subsequent tumor cell proliferation generates cadherin-expressing metastatic colonies. The influence of this reversible modulation in the pericellular adhesive milieu on tumor progression and metastatic competence is largely unexplored. Furthermore, the adhesive milieu may also alter the response of tumor cells to soluble and/or matrix-associated microenvironmental regulators such as growth factors, bioactive lipids, and ECM proteins.

Microenvironment of Ascitic Tumor Cells

The peritoneal cavity of normal women contains 5–20 ml of serous exudate including many plasma proteins and free-floating cells such as macrophages, desquamated mesothelial cells, lymphocytes, and mast cells.[38] In women with ovarian cancer, obstruction of peritoneal lymphatics together with enhanced vascular permeability often results in abdominal distension caused by accumulated malignant ascites fluid in the peritoneal cavity ranging from $<$500 ml to $>$2 L. Whereas women with early-stage malignancies (stage I and II) are often

free of ascites, the vast majority of women with advanced disease (stage III/IV) produced >500 ml of ascites.[39] In women with advanced ovarian cancer, the presence of ascites is an independent adverse prognostic factor[40] and is correlated with both intraperitoneal and retroperitoneal tumor spread.[41] Together, these data indicate that presence of ascites is highly predictive of ovarian malignancy in women with a pelvic mass.[39]

The complexity of ascites fluid is highlighted by a recent proteomic analysis that identified more than 200 proteins in the soluble fraction of ascites and more than 2500 in the combined soluble and cellular fractions.[42] Soluble components include the bioactive lipid lysophosphatidic acid (LPA), synthesized by both platelets and activated mesothelial cells,[43,44] cytokines including interleukins[17,45] and macrophage migration inhibitory factor,[46] growth factors such as EGF, VEGF, and HB-EGF,[47–50] and newly synthesized as well as degraded ECM proteins such as collagens types I and III.[51–53] These soluble factors can have dramatic consequences on tumor cell behavior and metastatic progression. For example, LPA can directly interact with tumor cell LPA receptors[54] to activate cellular motility pathways (Fig. 15.2).[55,56] Tumor cell dissemination

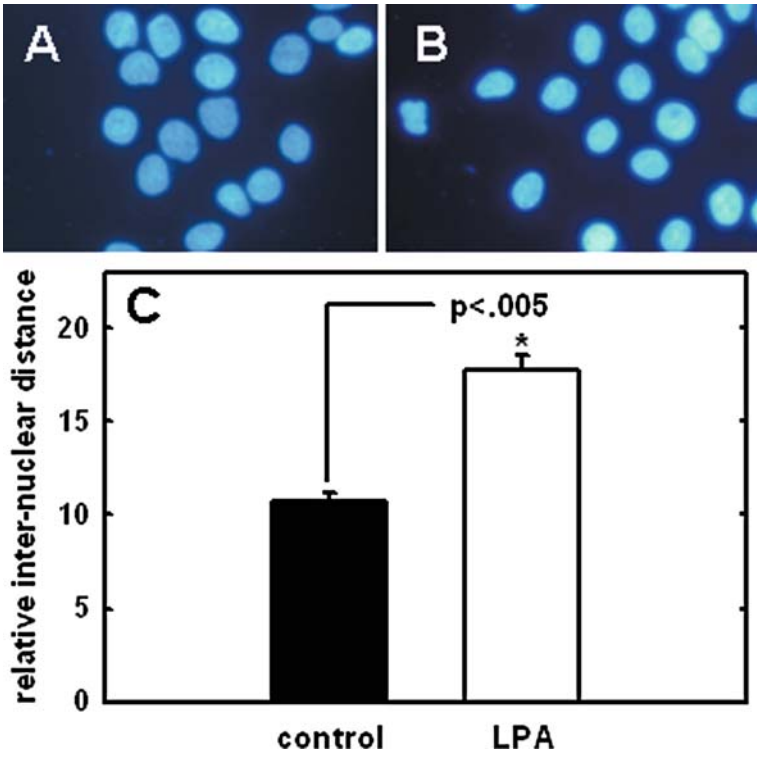

Fig. 15.2 LPA promotes cell migration. OvCa429 cells were treated with vehicle or LPA (30 μM) for 18 hours, and cell motility was quantified by measuring internuclear distance. (**A**) Vehicle control, (**B**) LPA (30 μm), (**C**) quantitation of internuclear distance

may be promoted through other mechanisms such as regulating the expression and/or activation of metastasis-associated proteinases in the matrix metalloproteinase (MMP) and plasminogen activator families.[54,55,57] Alternatively, LPA may also influence tumor progression through altered expression of secondary regulators of tumor cell behavior including interleukins and chemokines.[45,58–60]

Cellular components including activated mesothelial cells and inflammatory cells are also prevalent in ascites. Stromal cells in the tumor microenvironment may contribute to regulation of EOC metastasis, and recent studies support a role for inflammatory cells in promotion of intraperitoneal disease. Tumor cell dissemination from the ovaries was found to correlate temporally with enhanced peritoneal inflammation[61] and direct intraperitoneal injection of tumor cells also showed a prometastatic effect on inflammation. Depletion of peritoneal macrophages reduced metastasis, suggesting that inflammation facilitates metastasis via a macrophage-mediated mechanism.[61] This is supported by data demonstrating that co-culture of ovarian cancer cells with macrophages led to dynamic changes in macrophage expression of IL-10, -12, and -6, TNF-α, and CSF-1.[47,62] Macrophage-secreted TNF-α increased tumor cell invasiveness,[47] providing an example of stromal-epithelial cross-talk in the control of EOC metastasis via dynamic regulation of the cytokine network.

Does Cadherin Expression Contribute to Intraperitoneal Survival?

A unique feature of tumor cells in ascites is the ability to survive the lack of cell-matrix contact and proliferate as a floating tumor population (Fig. 15.1B). It is interesting to speculate that these conditions favor the survival of a subpopulation composed of highly neoplastic cells.[63] In support of this hypothesis, recent studies have demonstrated that cells isolated from murine ascites proliferate more readily and are more aggressive relative to the parental cells when re-injected in vivo.[64] In gene expression profiling studies comparing cells cultured as monolayers, MCAs, or tumor xenografts, MCA expression profiles were found to cluster with tumor xenografts rather than with monolayer cells.[65] These data suggest that MCAs represent a more advanced stage of malignancy relative to the parental primary tumor cells. A self-renewing population of ovarian cancer initiating cells that form self-renewing spheroids was recently isolated from solid primary ovarian tumors.[66] When cultured under stem cell selective conditions, these MCAs displayed a 100,000-fold increase in tumorigenicity, resulting in tumors that metastasized to the omentum and colon. These data support the hypothesis that the nonadherent MCA population found in human ovarian ascites is the primary source of intraperitoneal metastatic lesions.

Although survival of single cells and MCAs in ascites is likely influenced by ready access to soluble growth factors,[48,49] recent data support a role for

cadherin expression in MCA survival. MCAs in human ovarian carcinomatous ascites are relatively heterogeneous, ranging in size from 30 to 200 μm.[11,12] As summarized above, primary ovarian carcinomas gain expression of the cell-cell adhesion molecule E-cadherin relative to unaffected ovarian surface epithelium, as tumor cells undergo an initial mesenchymal-epithelial transition in early carcinogenesis.[14] It has been suggested that acquisition of E-cadherin–mediated adhesion early in tumor progression functions to suppress anoikis, a type of programmed cell death resulting from loss of integrin-based cell-matrix contacts as cells are shed from the primary tumor into the peritoneal cavity.[14] This is supported by data showing that E-cadherin engagement activates Akt via PI3-kinase signaling,[66] and downregulation of E-cadherin leads to loss of viability.[66] Thus, proliferative signals downstream of E-cadherin activation may contribute to ovarian cancer cell survival. Whereas reduced E-cadherin staining is often found in late-stage carcinomas and ascites-derived tumor cells, N-cadherin staining persists in late-stage tumors and paired peritoneal metastases[14]; however, the role of N-cadherin in MCA survival has not been explored. Interestingly, N-cadherin expression levels of glioblastoma cells cultured as MCAs correlate with surface tension of the aggregate, indicative of stronger aggregate cohesion.[67] Furthermore, the effect of microenvironmental regulators such as bioactive lipids, cytokines, growth factors, and matrix fragments on MCA survival and dissemination has not been extensively investigated. In this context, it is interesting to note that treatment of ovarian cancer MCA cultures with LPA induces expression of the mesenchymal marker vimentin (Fig. 15.3), suggesting that LPA may be a modulator of the late epithelial-mesenchymal transition (EMT) observed in progression to metastasis.[14]

Microenvironment of Peritoneal Metastatic Lesions

A major structural component of the ovarian cancer microenvironment is the peritoneum, a large membranous sheet that covers the abdominal organs and viscera. Involvement of the peritoneum or serosal surfaces is observed in at least 80% of women with EOC[18] and is a significant predictor of poor prognosis. Whereas women whose tumors lack peritoneal involvement have a 5-year survival of 90%, survival is decreased to 20% over 5 years in women with microscopic or visible metastatic foci.[18]

The peritoneum is composed of a single layer of mesothelial cells with abundant long microvilli that form an extensive serous membrane with approximately the same surface area as the skin.[38] An associated basement membrane overlays a stromal compartment composed predominately of an interstitial (types I, III) collagen-based matrix. Elastic fibers, fibronectin, laminin, glycosaminoglycans, fibroblast-like cells, lymphatics, nerves, and capillaries are also found here.[68] Tight junctions and desmosomes connect peritoneal mesothelial cells; however, intercellular spaces are also present.[18,68] Direct

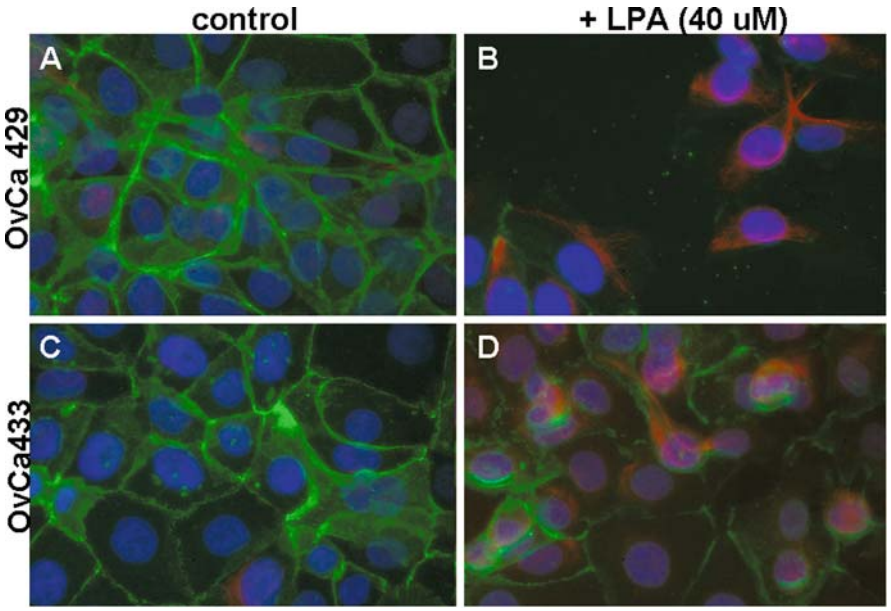

Fig. 15.3 LPA promotes epithelial-mesenchymal transition in ovarian cancer cell MCAs. MCAs formed from (**A**, **B**) OvCa 429 or (**C**, **D**) OvCa 433 cells were treated with LPA (40 μm) for 72 hours prior to processing for immunofluorescence microscopy. *Green*, E-cadherin; *red*, vimentin; *blue*, DAPI (nuclei). Note that LPA treatment induces loss of epithelial characteristics (E-cadherin) and a gain of mesenchymal phenotype (vimentin)

communication between the mesothelium and lymphatic endothelium has been observed through stomata, represented by gaps in the basal lamina of both the mesothelium and lymphatic endothelium at points of contact between mesothelial cells and terminal lymphatics.[38,68] It has been proposed that these stomata may permit transfer of molecules or cells between the stroma and peritoneal cavity.[18]

There is increasing evidence that soluble products derived from the primary tumor may induce critical alterations in the peritoneal surface that prime mesothelial tissues for tumor spread. Phenotypically, mesothelial cells from advanced EOC patients display as a discontinuous layer of rounded cells relative to the continuous flat mesothelial layer observed in non-EOC patients,[69] suggesting that soluble factors in ascitic fluid modulate mesothelial cell function. This is supported by transcriptional profiling studies of peritoneal biopsies from women with EOC versus benign disease. Enhanced expression of genes associated with inflammation, catalytic activity, cell adhesion, and extracellular matrix was observed in samples obtained from EOC patients.[18,70] These data support the concept that soluble factors released from malignant tissues induce a mesothelial response that favors peritoneal implantation.

Metastatic ovarian tumors arise as a consequence of CD44- and integrin-mediated intraperitoneal adhesion and localized invasion into the interstitial collagen-rich submesothelial matrix to anchor secondary lesions (Fig. 15.1).[11,12,15,27,28,71,72] After attachment of disseminated ovarian tumor cells to the peritoneal mesothelium, cells extend cytoplasmic processes through the junctional margins of neighboring mesothelial cells, inducing cellular retraction and exposure of the underlying submesothelial ECM.[73,74] Metastasizing ovarian cancer cells encounter an interstitial collagen-rich environment, as the submesothelial matrix is composed primarily of types I and III collagen.[51,68,73,74] Ovarian cancer cells adhere preferentially to interstitial collages using α2β1- and α3β1-integrins.[27–29] Such affinity for interstitial collagens likely reflects the phenotypic plasticity and mesenchymal origin of the ovarian surface epithelium.[53,75,76] Matrix binding induces MCA dissociation, as multivalent cell-matrix contacts replace cell-cell adhesive interactions.[11–13,77] β1-integrin–mediated submesothelial adhesion represents an important early event unique to ovarian cancer metastatic dissemination,[12,13,27–29,30,77] and the resulting alterations in integrin signaling may contribute to metastatic success.

Integrin-Matrix Interaction in Peritoneal Metastasis

There is increasing evidence that the peritoneal microenvironment actively promotes progression of metastatic ovarian carcinoma.[15] Ovarian cancer cells that encounter this matrix barrier respond to de novo integrin signaling, resulting in changes in gene expression that may ultimately potentiate metastasis (Fig. 15.4B). Using three-dimensional cell culture systems to model adhesive events in intraperitoneal metastasis, our laboratory has shown that aggregation of collagen-binding integrins activates integrin-mediated signaling via Src kinases to induce expression of the early growth response gene Egr-1.[30] The resulting Egr-1–mediated transcriptional activation of the membrane-type 1 matrix metalloproteinase (MT1-MMP) promoter stimulates expression of MT1-MMP, leading to enhanced collagen invasion.[29,77] Acquisition of MT1-MMP collagenolytic activity may be key to metastatic success, as it was recently reported that MT1-MMP–driven collagenolysis is necessary to remove matrix barriers to allow for the cytoskeletal reorganization necessary to drive a proliferative response.[78] Indeed, the ability of ovarian cancer cells to survive long-term and proliferate in three-dimensional collagen gels is substantially enhanced by MT1-MMP expression (Fig. 15.5A–C), and MT1-MMP expression is prevalent in peritoneal metastases from ovarian cancer patients (Fig. 15.5D, E). Additional metastasis-associated proteinases such as MMP-9 are also induced by integrin-collagen contact.[79] MMP-9 participates in E-cadherin ectodomain shedding, a process that likely contributes to disaggregation of cells from the primary tumor or ascitic MCAs to facilitate subsequent intraperitoneal anchoring and metastatic dissemination.[79]

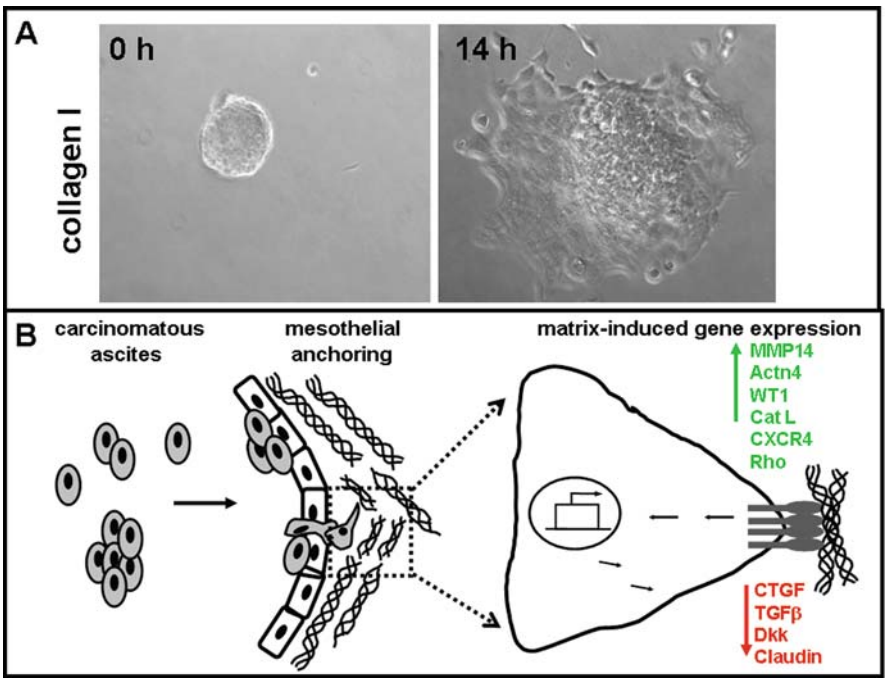

Fig. 15.4 Interaction of ovarian cancer cells with three-dimensional collagen gels. (**A**) Multicellular aggregates from Dov13 cells were plated on three-dimensional gels composed of interstitial type I collagen. Note collagen-induced MCA disaggregation and cell spreading. (**B**) In an in vitro model of intraperitoneal metastasis, cells are plated on three-dimensional collagen gels prior to analysis of collagen-induced changes in gene expression using cDNA microarray. Three-dimensional collagen culture results in upregulation and downregulation of genes involved in invasion, motility, proliferation, and survival

In addition to proteinases, other genes involved in transcriptional control, proliferation, and motility are also altered by engagement of collagen-binding integrins. For example, the motility regulatory protein actinin alpha-4 (ACTN4) is upregulated by three-dimensional collagen culture conditions (Fig. 15.4),[80] and enhanced expression of ACTN4 is found in peritoneal metastases relative to paired primary tumors from the same patient.[80] ACTN4 is enriched in the cytoplasm of cells on the migratory front of cell clusters. Moreover, cells in which ACTN4 is silenced with specific siRNAs display significantly reduced migration and collagen invasion, confirming a role for ACTN4 expression in ovarian cancer motility and invasion. A robust upregulation of Wilms tumor gene product 1 (WT1) is also observed in cells cultured on three-dimensional collagen gels, and expression of WT1 is associated with advanced and metastatic human ovarian carcinoma.[81] In contrast, expression of connective tissue growth factor (CTGF) is downregulated by three-dimensional collagen culture. EOC cells adhere avidly to CTGF/collagen I surfaces, such that

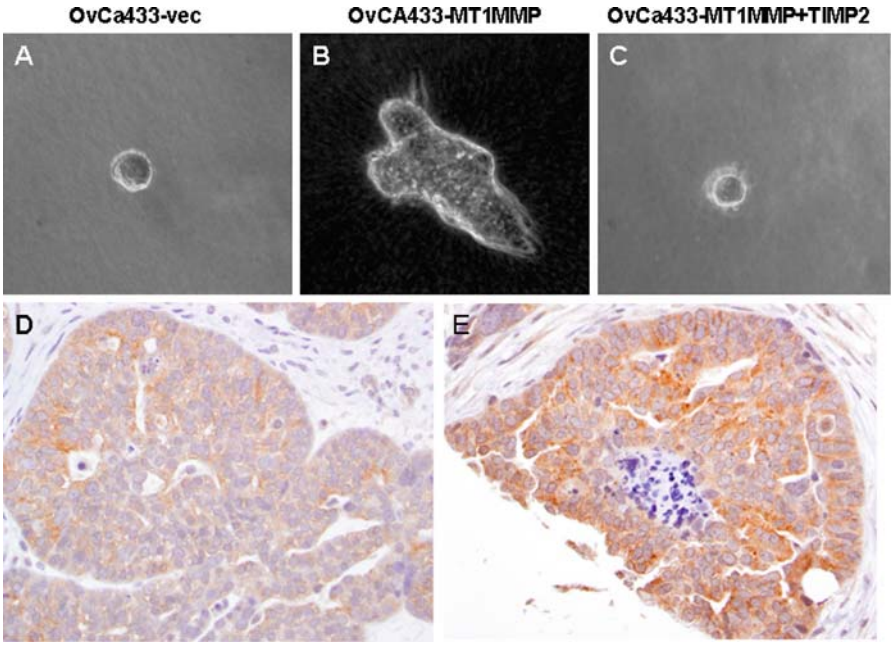

Fig. 15.5 Acquisition of MT1-MMP expression promotes proliferation in a three-dimensional collagen microenvironment. OvCa 433 cells were transfected with (**A**) vector control or (**B**, **C**) vector expressing MT1-MMP prior to plating as single cells encased in a three-dimensional collagen gel for 10 days. The gel in (**C**) also contained the MMP inhibitor TIMP-2. Note the lack of proliferation in the three-dimensional collagen microenvironment in the absence of MT1-MMP expression (compare **A** and **B**) or in the presence of an MT1-MMP inhibitor (compare **B** and **C**). (**D**, **E**) Immunohistochemical analysis of MT1-MMP expression in primary ovarian tumors and paired peritoneal metastases from the same patient. Analysis was done on tumor tissue microarrays prepared with Institutional Review Board approval by the Pathology Core Facility, Northwestern University. The microarray tissue specimens included 16 paired primary and metastatic ovarian cancer tissues obtained during the same surgical procedure. Staining was done according to standard procedures, and stained tissues were scored on a 0–3 scale. Expression of MT1-MMP at the metastatic site was greater than or equal to that of the primary tumor in 81% of cases. Examples shown were scored as (**D**) 1+ and (**E**) 2+

loss of CTGF expression promotes a proinvasive phenotype.[82] Additional genes differentially regulated by collagen engagement of EOC integrins have not been reported in association with EOC but are known to have functional significance in other tumor models. Genes downregulated by collagen contact (Fig. 15.4B) include the tight junction protein claudin-1, loss of which is correlated with high tumor grade in recurrent breast and prostatic adenocarcinoma.[80,83,84] Downregulation of TGF-β2 has been reported for metastatic oral squamous cell carcinoma.[80,85] These data suggest the potential utility of three-dimensional and organotypic models of extraovarian metastasis for the identification and verification of potential novel targets for therapeutic intervention.

Conclusion

An early event in ovarian cancer metastasis is shedding of cells from the primary tumor into the peritoneal cavity as anchorage-independent MCAs that survive in suspension (ascites) and are exposed to specific microenvironmental cues that promote metastatic implantation into the submesothelial matrix to anchor secondary metastatic lesions throughout the peritoneal cavity. This is distinct from most other solid tumors that metastasize hematogenously and presents a distinct set of therapeutic challenges. In most carcinomas, metastasis is associated with a dysregulated adhesion phenotype, typically characterized by a loss of epithelial (E)-cadherin. In contrast, EOC is unusual because early-stage disease reflects an initial *gain* of epithelial characteristics (mesenchymal-epithelial transition, or MET), including E-cadherin expression. It is becoming apparent that MET and reestablishment of epithelial characteristics is important to later stages of metastasis; however, the MET of ovarian cancer occurs early in disease progression, suggesting that this event confers a competitive advantage. Most cells that lose their contacts to the extracellular matrix undergo apoptosis (anoikis) and die, however EOC cells survive in suspension (ascites). It is generally assumed that the cell-cell contacts (cadherins) that hold MCAs together also help to ensure tumor cell survival. The role of additional soluble, cellular, and matrix factors in the ovarian tumor microenvironment in promoting MCA survival, reversion to a mesenchymal phenotype (EMT), and ultimate metastatic success is currently under active investigation. A molecular-level understanding of how tumor cells metastasize is necessary for the development of novel therapies to inhibit intraperitoneal spread and thereby improve the long-term survival of thousands of women with EOC. Development of models that accurately reflect the metastatic competence of EOC presents a remarkable scientific challenge, because the EOC metastatic microenvironment involves a novel shedding of MCAs into a cavity as anchorage-independent, chemotherapy-resistant spheroids. In this unique niche, it is currently unknown what regulates the transition from free-floating MCA to anchored metastatic lesion. Understanding this transition will enable novel means of targeting intraperitoneal therapies to appropriate multicellular populations.

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Abbreviations EGF - epidermal growth factor; HB-EGF - heparin binding EGF; CSF-1 - colony stimulating factor-1; DAPI - 4′ 6-diamidino-2-phenylindole; Akt - protein kinase B; PI3-kinase - phosphoinositide-3-kinase; VEGF –vascular endothelial growth factor; MCA- multi-cellular aggregate; EOC- epithelial ovarian cancer; OSE- ovarian surface epithelium; LPA- lysophosphatidic acid; TNF- tumor necrosis factor; MMP- matrix metalloproteinase

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Chapter 16
Organotypic Models of Metastasis: A Three-dimensional Culture Mimicking the Human Peritoneum and Omentum for the Study of the Early Steps of Ovarian Cancer Metastasis

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Introduction

Because most ovarian cancer (OvCa) patients present at a late stage, when metastasis has already occurred, the study of early events in peritoneal dissemination is difficult. One problem has been the lack of adequate model systems for the study of ovarian tumor transformation and metastasis.[1,2] Current models in use include co-cultures, whole tissue cultures, and immunocompromised and genetic mouse models. All of these have unique advantages; however, none of them replicates the human in vivo situation. The development and use of a three-dimensional (3D) organotypic model of OvCa has the potential to bridge the gap between the current models of OvCa and the human disease.

Ovarian Cancer Metastasis

Most patients with OvCa present with advanced disease metastatic to the peritoneum. Despite aggressive surgery and chemotherapy, patients with intra-abdominal, widely disseminated OvCa rarely achieve long-term cures.[3] The key to improved treatment of OvCa is a better understanding of the molecular mechanisms governing peritoneal dissemination.

OvCa metastasis is predominately confined to the abdominal cavity and, unlike breast, colon, or lung cancer, rarely metastasizes hematogenously. Once an ovarian epithelial cell undergoes neoplastic transformation, it can freely

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disseminate throughout the peritoneal cavity carried by the flow of peritoneal fluid, which is resorbed by the mesothelial cells of the omentum and peritoneum. Most published studies have focused on metastasis of OvCa cells to the abdominal peritoneum, under the assumption that similar mechanisms are involved in metastasis to the omentum. Given the differences in the structure of omentum and peritoneum, and that the omentum *per se* is the target of metastasis in 80% of advanced-stage cases, a model system focusing on the omentum is vital but is currently understudied.[4] Because most OvCa patients present at a late stage, when metastasis has already occurred, the study of early events in peritoneal dissemination is difficult. The potential of a tumor cell to metastasize depends on many factors, including interactions with the microenvironment at the metastatic site, which can promote cell adhesion, growth, survival, angiogenesis, and invasion.[5] Adequate model systems, therefore, are vital to address the study of early regulatory steps in metastasis.[1,2] Traditional models that have been widely used include whole tissue cultures, co-culture, and immunocompromised and genetic mouse models, all of which have their unique advantages and disadvantages. Although no model has been able to comprehensively recapitulate the tumor microenvironment to study early steps in metastasis, our newly described 3D organotypic model of OvCa[6] has the potential to bridge the gap between the existing models of OvCa and the nascent human environment.

Characteristics of the Ovarian Cancer Microenvironment in the Omentum

The omentum is a visceral fold of the peritoneum that is divided into two regions: an adipose-rich and a membranous/translucent region. The adipose-rich region, also known as the greater omentum, makes up most of the human omentum and is a fatty apron-like structure that is positioned on top of the abdominal organs like a blanket.[7–9] Its clinical function is to provide protection from blunt trauma, to promote healing, to wall off intra-abdominal infection, store lipids, and act as a regulator for fluid exchange.[12] The normal human omentum and peritoneum are covered by a confluent layer of mesothelial cells (see Fig. 16.1). This surface mesothelium, which is the first contact site for OvCa cells, forms a low-friction, nonadhesive surface and selective barrier, which is involved in fluid metabolism between the abdominal cavity, the interstitial space, and the vasculature.[10] Tight junction proteins (zonula occludens protein 1, occludin, and claudin-1) and the adherens junction protein β-catenin hold the continuous single layer of mesothelial cells together.[11] A number of adhesion molecules play an integral role in normal mesothelial cell physiology. The integrins, for instance, play a important role in mesothelial cell-cell and cell-ECM adhesions.[12] Adhesion of OvCa cells to mesothelial cells is also mediated by different proteases, notably matrix metalloproteinase (MMP)-2, and

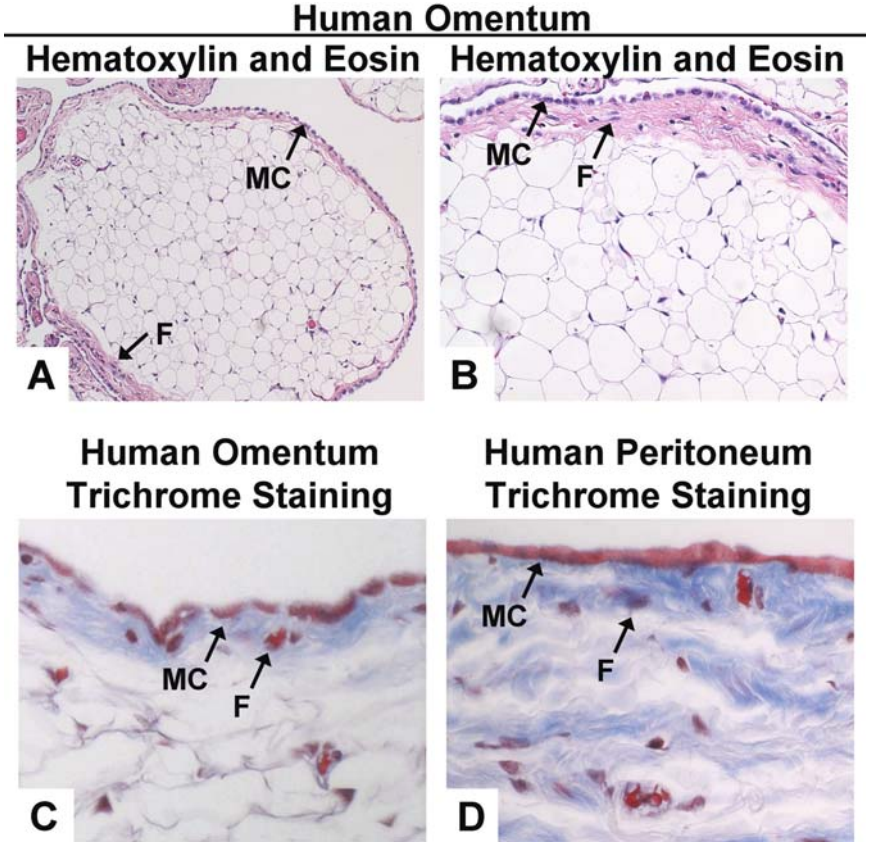

Fig. 16.1 Histology of human omentum and peritoneum. Hematoxylin and eosin staining of human omentum at (**A**) 100× magnification and (**B**) 200× magnification. Trichrome staining for the detection of collagen in human (**C**) omentum and (**D**) peritoneum. Collagen fibers are stained in *blue*, and cells are stained in *red*. MC, mesothelial cells; F, fibroblasts

CD44.[6,13–16] In addition, mesothelial cells secrete fibronectin, which plays a role in the adhesion and invasion of OvCa cells.[6,16–18] Fibroblasts, which are embedded in the layer of extracellular matrix (ECM) underlying the mesothelial cells (Fig. 16.1), constitute another major cell type that interacts with OvCa during adhesion and invasion to the peritoneal cavity. Many reports confirmed the hypothesis that stromal fibroblasts play an important role in cancer cell progression and metastasis (reviewed in Refs. [19] and [20]).

The mesothelial cell layer is attached to a basement membrane (BM) predominately composed of collagen I and IV, fibronectin, vitronectin, and laminin.[18,21,22] OvCa cells have a predisposition to adhere to collagen,[23–25] an interaction that can be blocked with an α2β1-integrin antibody.[25] The laboratory of Dr. Sharon Stack has, as early as 1994, shown that collagen I is important in OvCa progression and invasion.[23,26–32] In the omentum and

peritoneum, a thick network of collagen fibers are entwined between fibroblasts just below the mesothelium as shown in paraffin-embedded human tissue stained with trichrome to detect collagen (Fig. 16.1C, D).

Models to Study Ovarian Cancer Biology

Most models used to study OvCa utilize human OvCa cell lines established from ascites or pleural effusions that grow on plastic as a flat monolayer in two dimensions. The advantages of using cell lines are that they are readily renewable sources, amenable to genetic manipulation, and relatively inexpensive. The small number of OvCa cell lines available have allowed for extensive characterization by many laboratories. Indeed, studies using OvCa cell lines have made invaluable contributions to our understanding of ovarian tumor biology, especially of the signaling pathways involved in this disease.[33,34] However, two-dimensional (2D) models have clear limitations. Since they lack the microenvironmental context needed to study the earliest stages of metastasis as they occur in vivo.[35,36] Ovarian tumors are not composed exclusively of malignant cells but of a mixture of fibroblasts, mesothelial cells, and inflammatory cells. Therefore, 2D cultures on plastic do not mimic the complexities of the "tumor microenvironment" and the cross-talk that may take place between and among components of the microenvironment. Hence, the results obtained using them may not be as relevant to the clinical situation as they could be. Incremental improvements have been made to OvCa 2D cultures by co-culturing the cancer cells on one ECM protein or with one stromal cell type. However, these stromal cells are often from a site other than the peritoneum/omentum (e.g., foreskin fibroblasts) or from a different species altogether (e.g., rat) raising the question of tissue or host specificity.[37–39]

Xenograft mouse models are the second most widely used research models for the study of OvCa and were first described by Rygaard in 1969. They are powerful tools for investigating late-stage OvCa metastasis and for testing new therapies.[40] However, they have several deficiencies that make extrapolation to human tumor biology problematic. Human cancer cells are injected into a young, immunocompromised mouse host that lacks factors, such as IL-8, that are present in humans. Moreover, the mouse omentum is anatomically adjacent to the pancreas and has a different histologic appearance than that of human omentum.[7] These differences may be just one reason why only 27% of chemotherapeutic agents showing efficiency in a xenograft model ultimately have clinical activity in phase II clinical trials.[41]

Given the deficiencies of 2D cell cultures and xenograft mouse models, we and others hypothesized that establishment of a 3D organotypic model of OvCa metastasis might provide a better method for the study of the interaction of cancer cells with their omental microenvironment. The advantages of this model are that it contains all the key regulatory components of a particular

metastatic site (e.g., omentum), it is reconstituted from primary human cells, and it can be easily manipulated and evaluated.

Current In Vitro Models of Ovarian Cancer

A number of co-cultures have been established (Fig. 16.2) using OvCa cell lines and primary cells, which were added to a matrix as either single cells or as tissue-like aggregates (spheroids). Three-dimensional matrices have been generated from purified molecules such as collagen type I and from native extracellular matrices.[42] In 1987, Niedbala et al. were the first to use primary human OvCa cells to investigate degradation of ECM from bovine corneal endothelial cells

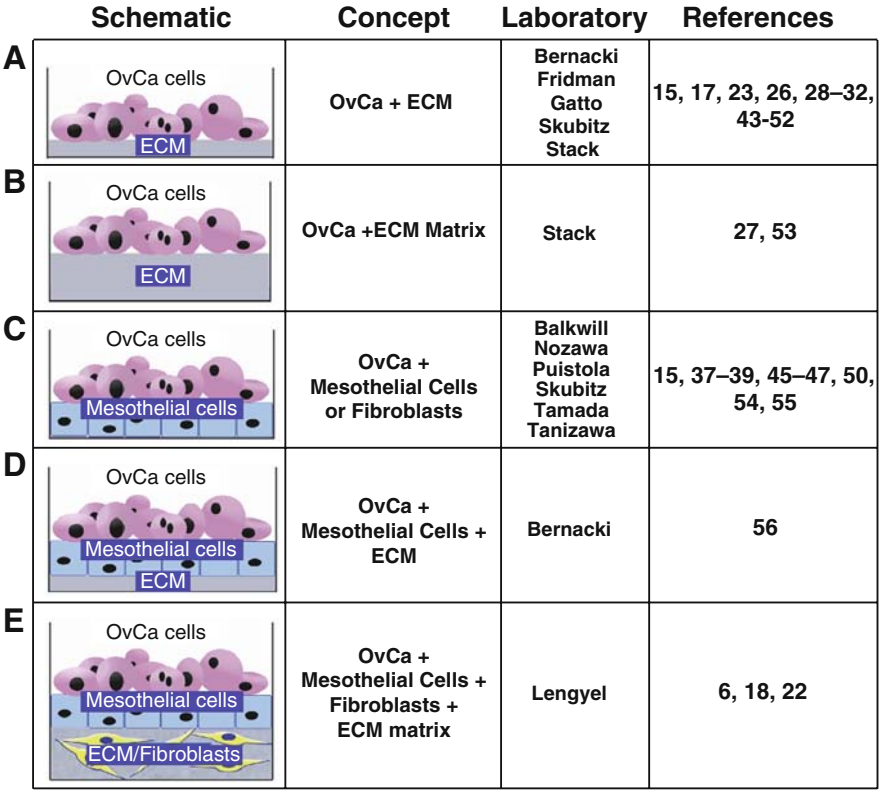

Fig. 16.2 Concepts of various 3D models to study ovarian cancer cell adhesion/invasion. (**A**) OvCa cells cultured on a thin layer of ECM. (**B**) OvCa cells cultured on a thick layer of collagen. (**C**) OvCa cells cultured on a single layer of mesothelial cells or fibroblasts. (**D**) OvCa cells cultured on a single layer of mesothelial cells plated on ECM. (**E**) OvCa cells cultured on a 3D organotypic model of human omentum. The 3D model is composed of fibroblasts embedded in a thick ECM layer and covered by primary human mesothelial cells

(Fig. 16.2A).[43] Kanemoto et al. investigated the adhesion, invasion, and colony formation of OVCAR-3 cells with a number of ECMs and reported that a Arginine-Glycine-Aspartate (RGD) peptide inhibited adhesion to fibronectin and colony formation.[44] Rieppi et al. used conditioned media from mesothelial cells and found that it stimulated OvCa invasion through fibronectin.[17]

The laboratories of Dr. Sharon Stack[23,26–32] and Dr. Amy Skubitz[15,45–52] have made very significant contributions to our understanding of integrin function in the interaction of OvCa cells with the ECM. Casey et al. blocked adhesion to fibronectin and spheroid formation in an OvCa cell line with a β_1-integrin blocking antibody.[51] Burleson et al. quantified OvCa cell spheroid (ascites-derived or cell line–derived) adhesion to and desegregation on a variety of ECMs.[45–47] They found that ascites-derived OvCa spheroids adhere to fibronectin and collagen type I, with reduced adhesion to collagen type IV and laminin, which can be partially inhibited by treatment with a β_1-integrin antibody.[46] Dr. Barbolina from the Stack laboratory cultured OvCa cell lines in collagen (Fig. 16.2B)[53] and found that OvCa cell lines grown in a 3D collagen microenvironment showed increased expression of the transcription factor early growth response protein and subsequently membrane type 1 matrix metalloproteinase expression when compared with cells grown on a thin layer of collagen (2D).[27]

Several studies have explored OvCa cell adhesion and invasion through mesothelial cell monolayers (Fig. 16.2C).[15,54,55] Lessan et al. discovered that CD44 and β_1-integrin mediate OvCa cell adhesion to an immortalized peritoneal mesothelial cell line (LP9).[15] Suzuki et al. found that treatment with an immunoglobin M that recognized a glycoprotein on the cell surface inhibited OvCa cell adhesion to mesothelial cells.[54] Kishikawa et al. investigated colony formation and invasion of six OvCa cell lines that interacted with primary omental mesothelial cells.[55] They classified the OvCa cells into two groups, adhesive-type and invasive-type, based on their interaction with mesothelial cells. Interestingly, blocking antibodies to α_2- and β_1-integrins only inhibited invasion of the invasive-type cancer cells through the mesothelial monolayer. Casey et al. employed an in vitro assay to measure OvCa cell line invasion through permeabilized mesothelial cell line monolayers.[50] Using a dye-based assay, they found an inhibitory effect of a β_1-integrin antibody, hyaluronan, and GM6001, a MMP inhibitor, on OvCa cell invasion through mesothelial cells.[50]

In the latest studies, Burleson and colleagues investigated OvCa cell spheroids (ascites-derived or cell line–derived) dissemination on and invasion into normal human mesothelial cell monolayers.[45–47] They found that ascites-derived spheroids adhere to live, but not fixed, human mesothelial cell monolayers, and this adhesion is partially inhibited by a β_1-integrin antibody.[46]

These previously described studies investigated the interaction of OvCa cells either with an ECM or with mesothelial cells alone, with the exception of a very elegant study published in 1985. Niedbala et al. investigated the interactions of human OvCa tumor cells with human mesothelial cells grown on ECM (Fig. 16.2D).[56] Primary human mesothelial cells were cultured on top of bovine

corneal endothelial cell–derived ECM. Then, radiolabeled ascites-derived primary human OvCa cells were added and attachment measured.[56] Results of this study indicated that OvCa cells exhibit a more rapid and firmer attachment to ECM than to mesothelial cells or to plastic alone.[56]

Three-dimensional Models of Carcinogenesis

The rationale for growing cells in three dimensions is that such a culture provides an environment that more closely mimics the human omentum, possibly making the results more relevant to understanding the biology of OvCa in humans. Epithelial cells grown in three dimensions exhibit a number of features comparable with their in vivo counterparts. When primary human cells at low passage number are used to assemble the 3D culture, it is likely to be the closest approximation to the human in vivo situation currently known.

Cultures of cancer cells grown in 3D extracellular matrices began with the work of Bissell, Petersen, and colleagues.[57–64] Culturing immortalized breast cells in three dimensions leads to lobule formation very similar to the normal breast lobules seen in a human breast.[64] One of the most important early findings of these studies was that epithelial cells cultured within a thick ECM undergo glandular differentiation, whereas cells cultured on a thin ECM on tissue culture plastic do not.[65] The study of breast tumor cells in three dimensions also allowed investigators to unravel the importance of cell-adhesion proteins in modulating the tumor phenotype. For example $\alpha_2\beta_1$-integrin is constitutively expressed in normal ductal mammary cells but lost once they transform into a malignant breast cancer.[66] Reexpression of $\alpha_2\beta_1$-integrin restores the ability of cancer cells to differentiate into glands, reversing the malignant phenotype.

A comparable anatomic situation to that of OvCa is skin cancer. Melanomas grow and invade on a surface, the skin, just as OvCa grows and invades the surfaces lining the peritoneal cavity. A human skin reconstruct model was pioneered by the laboratory of Dr. Meenhard Herlyn combining collagen type I, fibroblasts, and keratinocytes with cancer cells to simulate human melanoma.[67–69] Haass et al. manipulated the multiple layers of the 3D skin model to identify key cell-cell adhesion and cell communication molecules involved in the development of melanoma and found that E-cadherin, N-cadherin, and integrins are involved in melanoma growth and invasion.[69]

Designing the 3D Organotypic Model of Ovarian Cancer

Because the peritoneum and omentum are the major sites of OvCa metastasis, we sought to develop a 3D organotypic model to mimic the key components of the peritoneal/omental surface microenvironment (Fig. 16.2E).[6] Our first step was to review and characterize the histology of the superficial layer of human

peritoneum/omentum. The surface of the omentum is covered by a layer of mesothelial cells on top of a matrix containing collagen fibers and primary fibroblasts (Fig. 16.1C). To mimic the human omentum as closely as possible in three dimensions, we extracted primary human fibroblasts and mesothelial cells from nondiseased human omentum removed at surgery from the same patient. The 3D model was assembled by embedding early-passage primary human fibroblasts in ECM (collagen type I) (Fig. 16.3A), which was covered by a layer of early-passage primary human mesothelial cells in a 1:8 ratio (Fig. 16.3B). The 1:8 ratio of fibroblasts to mesothelial cells was extrapolated by studying normal human omentum. Collagen I was used as the matrix because it is the major structural ECM protein present in the omentum (Fig. 16.1C).[23,26–31]

In order to understand early OvCa metastasis, we cultivated OvCa cells with the 3D organotypic model of the peritoneal microenvironment (Figs. 16.2E and 16.3C) and optimized the model to investigate OvCa cell adhesion and invasion. To investigate adhesion, a thin layer of collagen I (0.5 mg/0.3 cm^2) was used, while a thicker layer of collagen (15 mg/0.3 cm^2) was applied to the 3D organotypic model to investigate invasion. After addition of OvCa cells, the histologic appearance of the 3D organotypic model mimicked microscopic metastases to the omentum from patients with OvCa (Fig. 16.4).[6] Two different OvCa cell lines (HeyA8, SKOV3.ip1) and primary human OvCa cells were added to the 3D organotypic model to evaluate the contribution of various cell types and ECMs to OvCa cell adhesion and invasion. Direct contact of OvCa cells with mesothelial cells inhibited adhesion and invasion when compared with OvCa cell interactions with fibroblasts and ECMs (Fig. 16.5).[6] The principal inhibitory effect of mesothelial cells on OvCa cell adhesion and invasion is mediated by direct cancer cell–mesothelial cell contact because pretreatment of cancer cells with mesothelial cell conditioned media only minimally inhibited adhesion and invasion.[6] This finding suggests that the mesothelial cell layer in the peritoneum and omentum serves as a protective layer blocking initial OvCa metastasis.

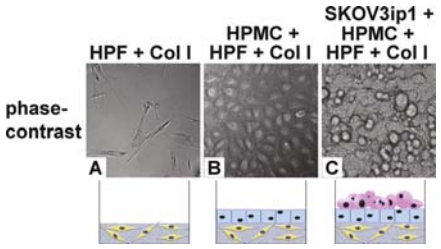

Fig. 16.3 Construction of a 3D organotypic model of ovarian cancer. Phase-contrast pictures of (**A**) fibroblasts in a collagen gel, (**B**) a confluent layer of mesothelial cells overlaying fibroblasts in a collagen gel, and (**C**) SKOV3ip1 OvCa cells adhering to the 3D model. Col I, collagen I; HPMC, human primary mesothelial cells (*blue*); HPF, human primary fibroblasts (*yellow*), SKOV3ip1 OvCa cells (*pink*)

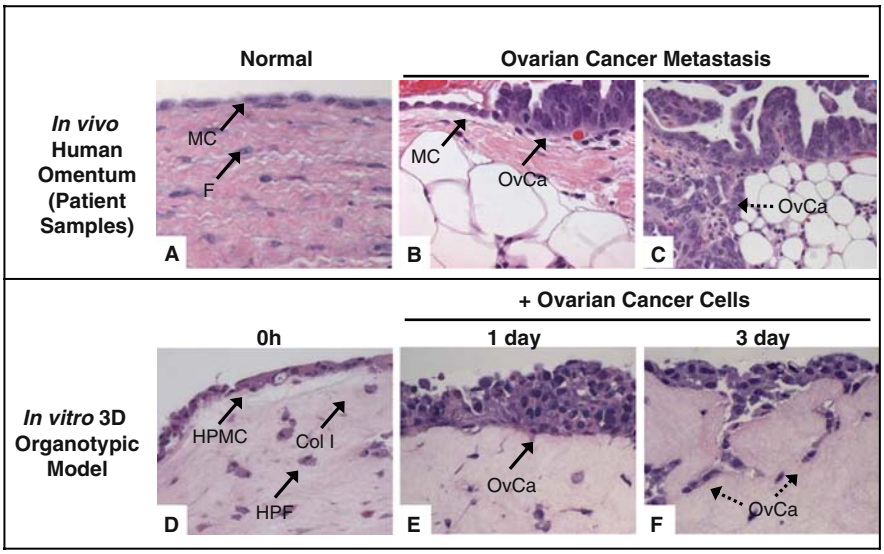

Fig. 16.4 A 3D organotypic model of ovarian cancer metastasis. Hematoxylin and eosin staining of (**A**) the normal human omental surface and (**B**, **C**) early OvCa cell metastasis to the omentum. First, OvCa cells attach and proliferate on the surface of the omentum (**B** and **C**, *closed arrows*). Second, OvCa cells invade the basal membrane and invade the omental tissue (**C**, *dashed arrow*). Hematoxylin and eosin staining of (**D**) the 3D organotypic model without cancer cells, (**E**) with SKOV3ip1 OvCa cells for 24 hours and (**F**) 120 hours. First, OvCa cells attach and proliferate on the surface of the 3D organotypic model (**E**, *closed arrow*). Second, OvCa cells invade the basal membrane of the 3D organotypic model (**F**, *dashed arrow*). MC, mesothelial cells; F, fibroblasts; Col I, collagen I; HPMC, human primary mesothelial cells; HPF, human primary fibroblasts

Stromal fibroblasts play an important role in cancer cell progression.[19,20] They undergo a transformation from quiescent cells to proliferating and excessively matrix-producing cells, a process known as "fibroblast activation," which results in cancer-associated fibroblasts. To determine the role of omental fibroblasts in omental metastasis, we studied the adhesion and invasion of cancer cells in both the presence and absence of primary human omental fibroblasts. In contrast with our findings with mesothelial cells, OvCa cells are significantly more adhesive and invasive when cultured on fibroblasts (Fig. 16.5). Moreover, fibroblast-conditioned media increased OvCa cell adhesion and invasion.[6] Therefore, we saw an increase in OvCa cell adhesive and invasive activity that involved both soluble mediators and direct interaction(s) between tumor cells and fibroblasts.

Our findings from testing the 3D organotypic model of OvCa metastasis imply that the stromal cells are more important than the ECM in determining OvCa cell attachment and invasion. However, we cannot exclude the possibility that the effect of stromal cells is, at least in part, mediated by other ECM proteins that are laid down by the mesothelial cells or the fibroblasts during the time the 3D organotypic model is assembled. Mesothelial cells secrete

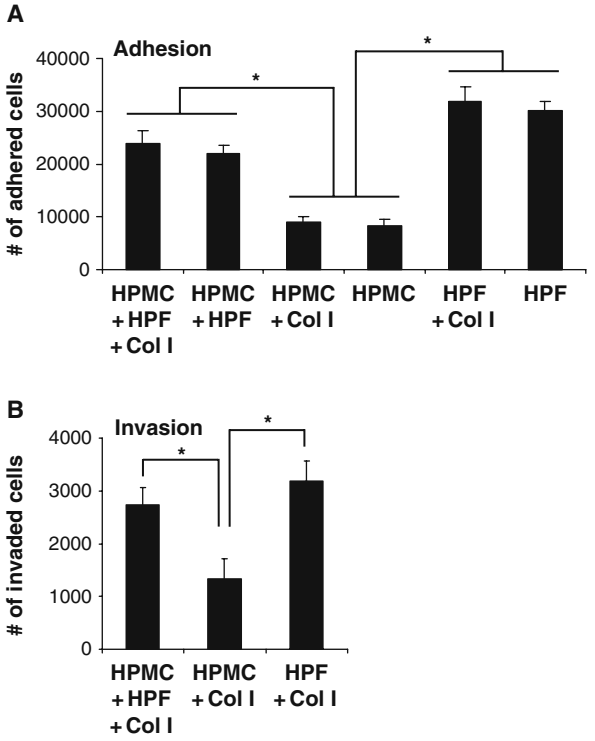

Fig. 16.5 Mesothelial cells inhibit whereas fibroblasts induce ovarian cancer cell adhesion and invasion. (**A**) Adhesion assays were performed with fluorescently labeled HeyA8 cells for 4 hours to primary human fibroblasts, mesothelial cells, or a combination of both with or without collagen I. (**B**) Invasion assays were performed with fluorescently labeled HeyA8 cells for 24 hours to primary human fibroblasts on collagen I, mesothelial cells on collagen I, or the 3D organotypic model. Asterisk (*) denotes a significant change ($p < 0.01$) in number of bound or invaded cells between conditions. Each bar represents the average of three wells and standard deviation. Col I, collagen I; HPMC, human primary mesothelial cells; HPF, human primary fibroblasts

fibronectin and collagen, which are also part of the omental basement membrane underlying the mesothelial cells.[25] In the 3D organotypic model, stimulation of invasion by collagen I can be abrogated by mesothelial cells or further induced by fibroblasts, suggesting that, at least in the early phases of omental metastases, stromal cells modulate the proinvasive signals of the ECM.

A third of OvCa patients present with ascites[70] containing OvCa cells as single cells, aggregates, or as spheroids.[71] Ascites fluid can either stimulate or inhibit OvCa cell invasion, proliferation, spheroid formation, and gene expression.[71] Similarly, we confirmed that ascites fluid either increased or decreased OvCa cell adhesion to the 3D organotypic model depending on individual cases (Fig. 16.6A). However, OvCa cells were significantly more invasive through the 3D organotypic model when stimulated with ascitic fluid (Fig. 16.6).

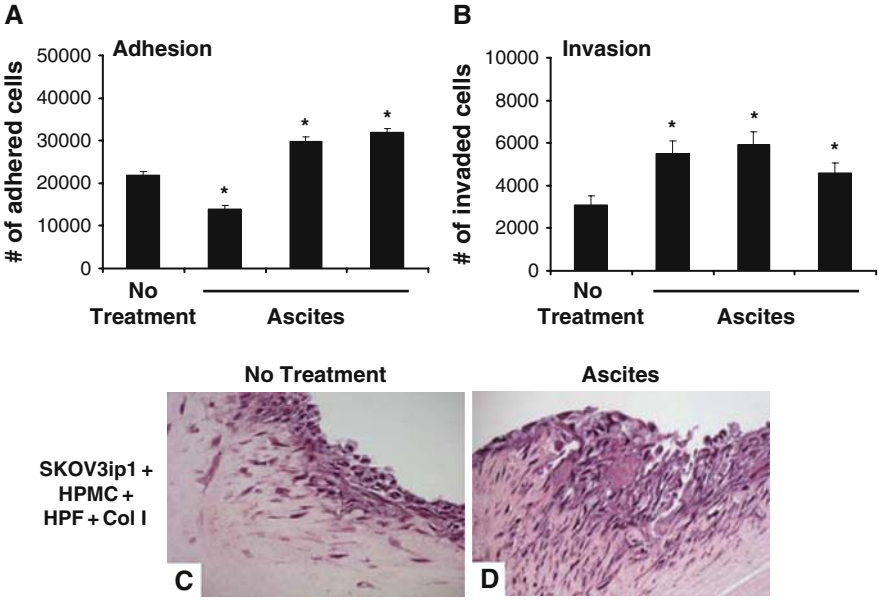

Fig. 16.6 The effect of ascites on ovarian cancer cell adhesion and invasion. Treatment of SKOV3ip1 OvCa cells with ascites during (**A**) adhesion to and (**B**) invasion through the 3D organotypic model. Three independent patient ascites samples were tested. Asterisk (*) denotes a significant change ($p < 0.01$) in number of bound or invaded cells between treatments. Each bar represents the average of three wells and standard deviation. Hematoxylin and eosin staining of the 3D organotypic model with SKOV3ip1 OvCa cells for 48 hours in (**C**) absence or (**D**) presence of ascites. Col I, collagen I; HPMC, human primary mesothelial cells; HPF, human primary fibroblasts

In our next study, we sought to determine if the 3D organotypic model could be used to unravel novel molecular mechanisms involved in metastasis of ovarian cancer to the omentum and peritoneum. The 3D culture was assembled with fibroblasts, mesothelial cells, and ECM (Fig. 16.4) and fluorescently labeled OvCa cells added and allowed to interact. The co-culture was harvested and the labeled OvCa cells sorted by fluorescence-activated cell sorting (FACS), allowing us to study the molecular changes in OvCa cells occurring after adhesion to the 3D culture. We found that after adhesion, MMP-2 is transcriptionally upregulated in OvCa. Cancer cell–derived MMP-2 then cleaved the ECM proteins, fibronectin and vitronectin, allowing for even stronger adhesion of OvCa cells to the ECM fragments through $\alpha\beta_1$- and $\alpha_v\beta_3$-integrins.[22]

In another project, our laboratory used the 3D culture to determine which integrin is important for the initial step of OvCa metastasis. OvCa cells were treated with antibodies against the most common integrins, and adhesion was measured. We reported that α_5-integrin, but not $\alpha_v\beta_3$-integrin, plays an important role in the initial adhesion of OvCa cells to the 3D culture.[18] These findings

were confirmed in vivo when inhibition with the α_5-integrin antibody blocked adhesion to mouse peritoneum and omentum and decreased tumor growth and metastasis in a xenograft model of OvCa.[18]

We believe that the organotypic omental or peritoneal 3D culture presents a number of advantages. The 3D culture can be assembled to be histologically very similar to the superficial layers of human omentum (Fig. 16.4) or peritoneum. It can be assembled with primary human omental mesothelial cells and primary human omental fibroblasts, approximating the human tissue as close as possible. Though we have not yet done so, it is certainly feasible to extract mesothelial cells, fibroblasts, and OvCa cells from the same patient and study patient-specific metastatic biology. After extracting primary cells from many patients, we found the results from various preparations of primary cells to be very reproducible (e.g., inhibition of OvCa cell adhesion and invasion by mesothelial cells). This model can also be used as a predictive preclinical model that allows for drug testing and might allow one to predict if an ovarian cancer cell will respond to therapy. The fact that inhibition of adhesion with an MMP inhibitor[22] or an α_5-integrin antibody[18] could be confirmed in other models shows that drug sensitivity patterns can be recapitulated in the 3D organotypic model. From our perspective, the chief strength of the 3D culture may be that labeled OvCa cells can be added to the culture and that after interaction, cancer cells can be isolated and used to study signaling, cell cycle progression, protease expression, or other early regulatory pathways.[6]

Clearly, there are limitations to the omental/peritoneal 3D culture described herein. While it attempts to mimic the omental surface, it is only an approximation of the in vivo omentum. The 3D model lacks the host immune cells, vasculature, and other ECM proteins present in vivo. The culture is viable only for 1 week and thus provides reproducible conditions only for a short period of time (Fig. 16.4). Given the intrinsic complexity of the invasion process, this limits study of invasion to its earliest steps, such as the breakdown of the basement membrane or the early interaction with stromal cells. Also, only a limited number of investigators have access and approval to use human peritoneum or omental tissue for the isolation of primary cells, which from our perspective is necessary for a close approximation to the in vivo situation. Still, the model can be assembled by isolating mesothelial cells and fibroblasts from mouse or rat omentum/peritoneum, and/or by using immortalized mesothelial (e.g., LP-9)[15] or fibroblast[39] cell lines. Alternatively, a full organ culture could be used to study ovarian cancer metastasis, as described by Hotary et al.[72] For this set-up, full rat peritoneum is stripped of overlying mesothelial cells, and the basement membrane is cultured in a Transwell dish (Corning, Inc.).

Future Perspectives

Because of its modular concept, the 3D organotypic model of OvCa metastasis allows for the manipulation of individual culture conditions. The contribution of endothelial cells, inflammatory cells, and ECMs present in human peritoneum

and/or omentum could be analyzed by including them into the culture. These cells have been shown in other cancers to contribute to the metastatic process.[73] Another feature of the 3D model is that it could allow investigators to study OvCa spheroid adhesion and invasion, which appears to differ from the adhesion and invasion of single cells. Lu et al. found that A2780 cells grown in spheroids were more resistant to an anticancer drug associated with the overexpression of antiapoptosis protein bcl-2 and the downregulation of caspase-3 activity.[74] Gene expression analysis of three OvCa models from OvCa tumors and ascites showed diverse expression profiles between cells grown as spheroids, xenografts, or monolayer cultures.[75]

Recently, Kurman et al. have proposed to subclassify OvCa into two major subtypes after correlation of molecular genetic studies with clinical and histopathologic findings.[76] Type I cancers are slow-growing tumors that are often confined to the ovary and harbor mutations in KRAS, BRAF, PTEN, and beta-catenin. Type II cancers are highly aggressive tumors that are highly metastatic and often have p53 mutations and a high level of genetic instability. The 3D organotypic model could allow investigators to study type I and type II OvCa adhesion, invasion, proliferation and the molecular mechanisms involved in these pathways. Lastly, the 3D organotypic model may enable us to discover unknown mechanisms of ovarian tumor metastasis to mesothelial cell–covered surfaces. It is hoped that by identifying key molecules involved in the attachment, growth, or invasion of OvCa cells, researchers will discover new OvCa-specific targets and, ultimately, develop new site specific therapies.

Conclusion

The spread of cancer cells within the abdominal cavity leads to innumerable tumor nodules that can cause a significant tumor burden, bowel obstruction, and will ultimately lead to death. The omentum and peritoneum, which covers the entire abdominal cavity, provides a rich medium for the attachment, spread, growth, and invasion of OvCa cells. To make further progress in the treatment of OvCa, a better understanding of OvCa metastasis is absolutely essential. The 3D organotypic model of OvCa metastasis provides a physiologically relevant approximation to the human omentum and peritoneum allowing researchers to investigate various aspects of tumor biology regulated by the peritoneal microenvironment. We are hopeful that with all the models currently available, we will advance our understanding of OvCa dissemination and find new therapies that regulate metastasis, thereby prolonging the survival of OvCa patients.

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Chapter 17
Animal Models of Ovarian Cancer

Denise C. Connolly

Introduction

As contributors have discussed in previous chapters, our understanding of epithelial ovarian cancer (EOC) biology and clinical management of ovarian cancer patients has improved significantly over the years. Yet in spite of all of our advances, the overall improvements in patient outcomes have been incremental. The majority of patients are still diagnosed at advanced stage when the probability of disease recurrence and complications that ultimately result in death are quite high. The typical late-stage diagnosis of EOC is associated with a complex array of genetic and epigenetic alterations present in tumors. As a result, it has been difficult to determine the sequence of events that are involved in tumor initiation, progression, and maintenance. In addition, there are several morphologic variants of EOC including multiple histologic subtypes (e.g., serous, endometrioid, mucinous, clear cell, mixed Müllerian tumors) as well as tumors of low malignant potential. Most researchers in the field believe that models in which each stage of EOC development and progression including initiation, progression, or clonal expansion and invasion and metastasis are represented are critical to our better understanding of EOC. This belief is reflected in the enormous effort directed toward the identification, development, and analysis of animal models of EOC. As in humans, spontaneous or induced ovarian cancers can arise from the epithelium, stroma, or germ cells of the ovary. For the purposes of this chapter, the discussion is largely limited to animal models of EOC, including animal models of spontaneous EOC, human tumor xenografts, chemically induced EOC, spontaneous and genetically induced transformation of the ovarian surface epithelium (OSE), and genetically engineered mouse (GEM) models. We conclude with a discussion of methods and technologies for small-animal imaging for improved analysis of animal models of EOC.

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Spontaneous Models

Aging Hen

Chickens are among the few animals that develop spontaneous ovarian tumors with relatively high incidence (reviewed in Refs. 1–3). Early studies of ovarian cancer development in hens were complicated by several issues including the lack of distinction between tumors of the oviduct and those of the ovary as well as inherent differences in ovarian histology and physiology between birds and mammals.[2] In a comprehensive analysis of 466 White Leghorn hens, Fredrickson showed that 149 of 466 (32%) hens developed age-associated ovarian tumors and that the majority of these cases, 112 of 149, or 75%, were ovarian adenocarcinomas.[2] Thus, the overall incidence of ovarian adenocarcinomas in hens was ~24% (112 of 466 hens in the study).[2] Ovarian adenocarcinomas in hens originate as tumor nodules within the ovary stroma or on the surface of follicles and ultimately replace the normal ovary.[2] Tumor cells are shed into the peritoneal cavity, implant and grow on serosal surfaces of the oviduct, mesentery, intestines, and pancreas, and cause the production of ascites.[2] Like human EOC, ovarian adenocarcinomas in chickens rarely metastasize via hematogenous routes.[2] Although no associations with ovulation or hormones were found in this analysis,[2] a subsequent study[4] of two independent strains of White Leghorn hens showed increased levels of plasma estradiol were associated with the strain showing increased incidence of spontaneous ovarian carcinoma. In a study to assess the chemoprotective effect of medroxyprogesterone acetate against the development of reproductive tract carcinomas, Barnes et al.[5] showed a 15% risk reduction in treated animals compared with controls. These results suggested the potential utility of this model in chemoprevention studies. Although ovarian adenocarcinomas in hens share common features and expression of molecular markers[6–8] with human EOC, limitations to the common use of aging hens as a model system include intrinsic differences in reproductive tract anatomy and physiology as well as the need for very large cohorts of animals (e.g., hundreds of animals) to obtain sufficient statistical power.

Non-human Primates

Non-human primates do not develop spontaneous EOC with significant incidence; however, they do bear close similarity to humans in anatomy, physiology, and genetics, as well as the structure and hormonal regulation of the reproductive tract. For these reasons, non-human primates have been employed as models for chemoprevention and therapeutics studies. Oral contraceptive pills (OCPs) have been shown to confer a protective benefit against the development of ovarian cancer.[9] Using cynomolgus macaques as a model,

Rodriguez and colleagues investigated the mechanisms by which oral contraceptives confer protection and showed that progestin induces apoptosis in the ovarian epithelium, perhaps mediated by differential regulation of TGF-β isoforms.[10,11] Similarly, Brewer and colleagues used rhesus macaques to study the chemoprotective effects of a combination of 4-(*N*-hydroxyphenyl) retinamide (4-HPR) and OCPs by fluorescence spectroscopy and showed increased levels of the coenzymes NAD and FAD suggesting increased redox potential and less hypoxia in the ovary.[12–14] The effects of high-dose estrogen and selective estrogen receptor modulators (SERMs) on rhesus macaque ovarian surface epithelium proliferation have been studied in vitro and shown to induce cell cycle arrest via induction of Rb, p53, and p21.[15–17] In addition to studies related to chemoprevention, the cynomolgus macaque is also a useful model to test the toxicologic properties of novel therapeutics such as monoclonal antibodies and toxin-conjugated monoclonal antibodies.[18,19]

Xenograft Models

The availability of immunodeficient strains of mice such as athymic nude mice lacking T lymphocytes[20] and severe combined immunodeficient mice (SCID), which lack functional T and B lymphocytes,[21] afforded researchers the opportunity to grow cancer cells established from human tumors in vivo. The significance of xenograft models of human ovarian carcinoma is underscored by the voluminous literature in the field (a PubMed search using the terms "xenograft" and "ovarian carcinoma" conducted while writing this chapter returned 1023 publications). Clearly, it would be impossible to discuss this vast body of research within a single chapter, but highlighted below are selected historical examples of the development and use of xenograft models to study human EOC. These models have relied on the use of subcutaneous (s.c.) and intraperitoneal (i.p.) as well as orthotopically implanted xenografts of human ovarian carcinoma cell lines.

Subcutaneous

Initial methods for xenografting human ovarian cancer cells relied on s.c. implantation of tumor cells in nude mice.[22–25] Although this was a technical advance, tumor formation in subcutaneously injected mice rarely resulted in the peritoneal tumor metastasis and ascites production that is commonly observed in clinical EOC. In addition, early studies suggested that i.p. or orthotopic implantation of ovarian carcinoma cells provided a more relevant tumor microenvironment, as tumor "take" was significantly less efficient in subcutaneously injected mice.[26] However, subcutaneous tumor formation is advantageous in that tumors can be readily measured with calipers to quantify tumor volume, and for this reason this method has been extensively used for the evaluation of therapeutic efficacy of novel drugs and combination therapies.

Intraperitoneal

An early model of a transplantable murine teratoma in which i.p. injection resulted in peritoneal dissemination and the production of ascites was used to show the potential advantage of i.p. administration versus intravenous (i.v.) administration of chemotherapy.[27–29] Significantly, these studies became the basis for early clinical trials of i.p. therapy in ovarian cancer patients,[30] a topic that has garnered renewed interest in recent years. The pros and cons of i.p. chemotherapy for EOC were recently reviewed in detail (in this volume and Refs. 31, 32). Limitations regarding the clinical relevance of the murine teratoma model to human EOC were immediately recognized, and subsequent studies used cell lines derived from the tumors and/or ascites isolated from patients with EOC.[23,24,33–35] In 1984, Hamilton and colleagues described methods for the generation of a mouse model of EOC in which mice developed malignant ascites and intra-abdominal carcinomatosis.[36] In this study, a highly malignant subpopulation of the tumorigenic, drug-resistant NIH:OVCAR-3 cell line[24] was isolated by serial in vivo and in vitro selection of cells grown as s.c. tumors in nude mice and as colonies in agarose, respectively.[36] This malignant subpopulation of NIH:OVCAR-3 cells was injected intraperitoneally into athymic nude mice resulting in the production of malignant peritoneal ascites and carcinomatosis.[36] Tumor nodules were adherent to the peritoneal wall, mesentery, and diaphragm, ranging in size from 0.1 to 2 cm. When visualized under light microscopy, tumor cells appeared to grow as solid sheets with areas of glandular and papillary structures.[36] Because NIH:OVCAR-3 cells were isolated from a patient with drug-refractory disease, this model has subsequently been used to investigate alternative therapeutic agents and/or drugs that sensitize chemoresistant cells to cytotoxic agents.[37] This model is still widely used in ovarian cancer research today and is the paradigm for studies involving growth of a wide variety of human ovarian carcinoma cell lines as xenografts in immunocompromised mice (examples including Refs. 38–40).

Orthotopic

To more accurately model EOC growth initiating in the ovary and circumvent the need for in vivo selection of tumorigenic cell lines, methods were developed for orthotopic (i.e., grafting a tissue in a natural position) implantation of human ovarian tumor specimens adjacent to the ovaries of mice. Orthotopic implantation of ovarian tissue in rodents is facilitated by the presence of an accessory anatomic structure, the ovarian bursa, that is absent in humans. The bursa is a thin, fluid-filled membrane or sac that surrounds the ovary and provides a convenient compartment for the deposition of tissue, cells in suspension, or infectious viral particles. Fu and Hoffman first described orthotopic implantation of human ovarian tumor tissue in 1993.[41] These investigators used

two approaches: (1) direct transplantation of minced tumor tissue into the intrabursal space followed by closure of the bursa with a suture and (2) an "onplantation" method involving direct attachment of human tumor tissue to mouse ovary with sutures, leaving the bursa open.[41] Using these methods, tumor tissue could be grown orthotopically in nude mice with preservation of tumor histology and the capacity for metastatic peritoneal growth in the recipient mice.[41] A subsequent study[26] from the same laboratory showed that orthotopic implantation of tumor tissue derived from human RMG-1 cells grown as a s.c. xenograft resulted in tumor formation in 100% of recipient mice. The tumor take rate was higher in the orthotopic setting, suggesting a more favorable microenvironment for tumor growth. Tumors grew locally at the site of implantation at early stages (6 weeks after injection) but exhibited increased metastasis and invasion of the contralateral ovary, retroperitoneum, mesentery, peritoneum, and abdominal organs at later stages (12 weeks after injection). We and others have since shown that suspended human ovarian cells can be implanted orthotopically by direct injection within the ovarian bursa via the infundibulum.[42,43]

With regard to the accurate recapitulation of tumor growth, progression, and metastasis in xenograft models of human cancer, the importance of the site of implantation of human tumor cells has been demonstrated.[44] For example, many metastatic human tumor cell lines, including ovarian, do not exhibit metastatic spread that is characteristic in patients when injected subcutaneously, intravenously, or at other sites.[44] Most studies support the idea that i.p. or orthotopic implantation of human ovarian carcinoma cells results in tumor formation that more accurately reflects the clinical course of EOC. However, estimating the volume and extent of tumor burden in mice with widespread peritoneal disease resulting from i.p. or orthotopically implanted tumor cells is technically challenging. For this reason, many preclinical therapeutic studies have relied on s.c. xenograft models. Difficulties related to quantitation of disseminated tumor can be circumvented by the use of secreted surrogate tumor biomarkers[42] and/or the use of fluorescent or bioluminescent reporters in conjunction with optical imaging (described in greater detail in the "In Vivo Imaging" section at the end of this chapter).

Xenograft models in athymic nude or SCID mice are imperfect to the extent that interactions between the murine host tumor microenvironment (including the stroma, vasculature, and extracellular matrix) with human tumor cells cannot be assumed to be the same as they are in situ in patients. It is well recognized that host immune factors are an integral component of tumor development and progression, but by definition these interactions are incomplete in immunocompromised hosts. Moreover, though xenograft models of human cancer have been used extensively to evaluate the potential efficacy therapeutic strategies in preclinical studies, they have been shown to have limited predictive value.[45,46] For these reasons, significant efforts have been directed toward the development of spontaneous and genetically induced animal models of human EOC.

Chemically Induced Models

In order to study ovarian tumorigenesis in situ, rodent models of chemical carcinogen–induced or hormone-induced EOC have been developed. A commonly used strategy involved surgical implantation of sutures impregnated with carcinogens such as 7,12-dimethyl benz[*a*]anthracine (DMBA) or *N*-methyl-*N*-nitrosourea (MNU) within the ovarian cortex of rat.[47–49] This strategy resulted in the frequent development of ovarian tumors including adenomas, adenocarcinomas, squamous carcinomas, and sarcomas.[47,49] The adenocarcinomas arising in DMBA-treated rats were in some cases similar to serous and endometrioid carcinomas and therefore to some extent reflect the Müllerian histology observed in human EOC.[47] A subtle variation on this strategy involving implantation of sutures with significantly reduced doses of DMBA resulted in the induction of preneoplastic epithelial lesions with progressive histology including epithelial cell preneoplasia, serous low-malignant-potential tumors, and invasive carcinomas.[50] Molecular analyses of preneoplastic lesions and ovarian tumors showed a high frequency of *Ki-Ras* [4 of 12 (30%) benign lesions and 1 of 4 (25%) invasive lesions] and *p53* [4 of 12 (30%) benign lesions and 4 of 4 (100%) invasive lesions] mutations.[50] Underscoring the importance of p53 mutations in ovarian tumor development in DMBA-induced models, a recent study demonstrated that mice harboring a single mutant allele of *p53* ($p53^{Ala135Val/WT}$) were more susceptible to DMBA-induced ovarian tumor formation.[51] Mice carrying the $p53^{Ala135Val/WT}$ germ-line mutation developed tumors with shorter latency and increased frequency compared with $p53^{WT/WT}$ controls.[51] Tumor histology in DMBA-treated mice included adenocarcinomas (23%) and/or sarcomas.[51] Studies to evaluate the role of hormonal factors in ovarian tumor induction in guinea pigs demonstrated an increase in benign and occasionally malignant ovarian neoplasms in animals treated with testosterone, estrogen, or estrogen and diethylstilbestrol.[52,53] Although chemically or hormonally induced models have the advantage of spontaneous disease induction that should represent all stages of EOC development, such strategies infrequently result in disease induction in all treated animals. Moreover, the histopathologic subtype of ovarian tumors is not completely predictable, and the individual molecular alterations underlying disease initiation and progression and the sequence in which they occur remain largely unknown.

Models of Ex Vivo Transformation of the Ovarian Surface Epithelium

In 1971, Fathalla[54] proposed the "incessant ovulation" hypothesis as a model of EOC etiology, based on the observations that (1) nulliparity increases and multiparity decreases risk of EOC in humans, and (2) incessantly ovulated

hens have a high frequency of spontaneous ovarian carcinomatosis. This model postulates that the lack of pregnancy-induced rest periods contribute to EOC because repeated cycles of follicle rupture during ovulation cause local inflammation and release of reactive oxidants at the ovarian surface. Inflammation and the induction of DNA synthesis and cell division to repair the postovulatory wound can thus promote acquisition of critical mutations, genomic instability, and ultimately local expansion of transformed and/or tumorigenic populations of epithelial cells (reviewed in Ref. 55). Notably, studies in mice suggest that an increased incidence of morphologic changes in OSE, including pseudostratification, invaginations, and inclusion cysts, is related to the total lifetime ovulation.[56,57] Because the majority of EOCs are thought to arise from this single layer of epithelial cells that cover the ovary,[58,59] significant effort was directed at the isolation, in vitro propagation, and characterization of these cells. The isolation and in vitro cultivation of ovarian germinal epithelium was reported as early as 1940.[60] Subsequently in 1980 and 1981, Hamilton et al.[61] and Adams and Auersperg[62] described detailed methods for isolation and in vitro culture of pure populations of primary OSE cells from rats. These methods have been adapted for isolation of primary cultures of OSE cells from human,[63–65] rabbit,[66,67] mouse,[68,69] and, more recently, chickens.[4] With the ability to isolate the putative precursor cells of EOC, numerous studies followed with the goal of inducing both in vitro and in vivo properties transformation and tumorigenicity by the introduction of oncogenic viruses, by repeated passaging in culture, and by the induction of specific genetic alterations.

Immortalization and Transformation by Oncogenic Viruses

Rat

Adams and Auersperg[62] showed that primary cultures of rat OSE (ROSE) cells could be transformed by infection with the Kirsten murine sarcoma virus (Ki-MSV). Retroviral infection of ROSE cells with Ki-MSV resulted in enhanced proliferation and focus formation in vitro and tumor formation when transduced cells were implanted subcutaneously or intraperitoneally in irradiated syngeneic rats.[62] Transfection of early-passage primary ROSE cells with the simian virus 40 early region including the T antigen (SV40 TAg) genes resulted in immortalization and a slight enhancement of the capacity of these cells to form colonies in soft agar.[70] Expression of SV40 TAg in spontaneously immortalized ROSE cells (ROSE 199)[71] resulted in a pronounced enhancement of cloning efficiency in soft agar.[70] In both primary ROSE and ROSE 199 cells, expression of SV40 TAg resulted in malignant transformation as evidenced by the capacity to form tumors in subcutaneously injected athymic mice.[70]

Mouse

Kido and Shibuya[68] described the establishment of an immortal transformed mouse ovarian surface epithelial (MOSE) cell line by expression of the SV40 TAg in cultured MOSE cells isolated from C3H/He mice. These cells were tumorigenic when injected into immunocompromised recipient mice.[68] Independent experiments in our own laboratory confirm that transfection of the SV40 TAg into primary cultures of MOSE cells isolated from C57Bl/6 mice results in immortalization and malignant transformation of these cells (D.C. Connolly and T.C. Hamilton, unpublished observations).

Human

Rodent OSE cells are susceptible to transformation by expression of viral oncogenes, perhaps due in part to the constitutive expression of telomerase.[72] Human OSE (HOSE) cells are much more resistant to malignant transformation by oncogenic viruses. Introduction of SV40 TAg[73] or human papilloma virus 16 (HPV 16) E6 and E7 genes[74] resulted in a somewhat prolonged life span of HOSE cells in culture with most cells eventually undergoing crisis. After continued culture of SV40 TAg or HPV16 E6/E7 transfected cells, populations of spontaneously immortalized cells occasionally emerged,[74,75] with some cell lines exhibiting a slight tendency to form colonies in soft agar.[75] At early passage, SV40 TAg or HPV16 E6/E7 immortalized HOSE cells did not result in tumor formation in immunocompromised mice.[74,75] However, after continuous growth in culture, late passage (>70 passages) HPV16 E6/E7 cells ultimately acquired the capacity to form colonies in soft agar, and occasionally cells isolated from these colonies were able to form tumors in SCID mice.[76] These results suggest that additional spontaneous genetic alterations are required to induce malignant transformation of HOSE cells. This idea is supported by experimental studies described in detail below.

Spontaneous Immortalization and Transformation

Rat

In order to recapitulate the repeated cell division and wound repair associated with incessant ovulation in vitro, Godwin et al.[77] developed a model for spontaneous transformation of OSE cells. In this model system, parallel cultures of primary ROSE cells were subjected to repeated subculture, where after 20–30 passages, a subset of the cultures (~30%) lost contact-mediated growth inhibition and exhibited features of spontaneous transformation including substrate-independent growth, cytogenetic abnormalities, and tumor formation

in immunodeficient animals.[77,78] In some cases, well to moderately differentiated adenocarcinomas were identified, while the majority of transformed ROSE cell lines resulted in the development of poorly differentiated adenocarcinomas.[78] The degree of cytogenetic abnormality in the transformed cell lines closely paralleled the degree of differentiation observed in tumors; that is, late-passage cell lines gave rise to the most poorly differentiated tumors in athymic nude mice, and the cell lines derived from these tumors exhibited the most complex karyotypes.[78] Subsequent genetic and gene expression analyses of these transformed rat OSE and tumor-derived cell lines have identified specific molecular alterations thought to contribute to the transformation of OSE.[79–82]

Whereas the ability to grow human or rodent cell lines as xenografts and/or allografts in immunocompromised rodents was a significant advance in the development of animal models of EOC, these models were clearly limited in their potential to study host immune contributions to tumor development and progression as well as the efficacy of novel strategies for immunotherapy. In addition, xenograft models may differ substantially in interactions between tumor cells and host microenvironment (including stromal contributions) based on inherent differences between species. The ability to cultivate and transform rodent primary OSE cells by extended passaging in culture (spontaneous transformation) or by direct genetic manipulation provided a means to circumvent some of these limitations by the establishment of syngeneic in vivo tumor models.

A syngeneic model of EOC was described in 1996 by Rose and colleagues.[83] As noted above, injection of athymic nude mice with Fisher 344 rat–derived, spontaneously transformed OSE cell lines frequently gave rise to ovarian adenocarcinomas in the immunocompromised recipients, and rat ovarian carcinoma cell lines were established from these tumors.[78] To develop a syngeneic model of EOC, one such cell line (designated NuTu 19) was allografted into naïve Fisher 344 rats by i.p. injection of cells at a range of concentrations.[83] Peritoneal implantation of NuTu 19 cells resulted in widespread disease with tumor nodules adherent to serosal surfaces and abdominal organs and the formation of malignant ascites.[83] Tumor formation and overall survival of Fisher 344 rats was dependent on the total number of NuTu 19 cells injected with as few as 10^4 cells resulting in 70% tumor take rates and 10^5 cells resulting in 100% tumor take.[83]

Mouse

Roby and colleagues[69] used a similar strategy to develop a syngeneic mouse model of EOC. These investigators isolated mouse ovarian surface epithelial (MOSE) cells, subjected them to repeated passage in culture, and compared tumorigenicity between early-passage (<20) and late-passage (>20) cells.[69] Tumorigenicity of the cells was initially evaluated in athymic mice and demonstrated that only late-passage MOSE cells resulted in tumor formation in subcutaneously or intraperitoneally injected mice. Tumorigenicity of the

late-passage MOSE cells was subsequently confirmed in syngeneic C57Bl/6 mice. Notably, mice in which tumor cells were injected subcutaneously had a much longer latency than did those that were injected intraperitoneally (4 months in subcutaneously injected vs. 33 days in intraperitoneally injected), providing further evidence that the pseudo-orthotopic i.p. injections provide a more favorable environment for growth of the tumor cells. Tumor formation in intraperitoneally injected mice was widespread, affecting omentum, bowel, diaphragm, peritoneal wall, and the surface of kidney, pancreas, stomach, and spleen and was accompanied by the formation of malignant ascites.[69] Clonal cell lines were established from the spontaneously transformed late-passage MOSE cells[69] and have been used in a number of subsequent studies.[84–91] Using the same experimental strategy, researchers from an independent laboratory showed that neoplastic progression of spontaneously transformed MOSE cells was characterized by remodeling of the actin cytoskeleton and focal adhesions and decreased expression of E-cadherin and connexin-43.[92]

The clonal cell lines isolated by Roby et al.[69] (e.g., ID8 cells) have been commonly used to study ovarian tumor biology. In subsequent studies, ID8 cells were shown to express constitutively activated Src.[87] Inhibition of Src in ID8 cells by pharmacologic agents or direct RNAi resulted in diminished phosphorylation (e.g., activation) of Akt, focal adhesion kinase (Fak), and Foxo1.[87] Interestingly, pharmacologic inhibition of Src also resulted in sensitization of ID8 cells to both paclitaxel and cisplatin via activation of caspase-3.[84,87] This cell line has also been modified by retroviral transduction to stably overexpress the murine *Vegf 164* isoform.[90] Expression of this secreted isoform of *Vegf* potentiated the malignant phenotype resulting in decreased tumor latency, markedly enhanced (12.9-fold greater) ascites production, 2.6-fold greater serum VEGF levels, increased tumor microvessel density, and decreased tumor cell apoptosis.[90]

Genetically Induced Transformation

The identification of individual genetic alterations thought to contribute to EOC coupled with improvements in the technologies for in vitro manipulation of gene expression (i.e., gene delivery strategies) made it possible to directly evaluate the consequences of specific genetic alterations on EOC development. Below are several examples of manipulation of genes implicated in EOC in otherwise normal or non-transformed OSE isolated from rabbit, rat, human, or mouse ovaries.

Rabbit

An early example involved transformation of primary cultured rabbit OSE cells by ectopic expression of a growth factor receptor. Previous reports demonstrating high levels of expression of insulin-like growth factor-1 (IGF-1) and

insulin-like growth factor-1 receptor (IGF-1R) in human ovarian carcinoma cell lines suggested that this signaling axis contributes to EOC development.[93,94] In a direct test of this hypothesis, Coppola et al.[95] showed that constitutive expression of a cDNA construct encoding the IGF-1R in primary cultures of rabbit OSE resulted in anchorage-independent growth in soft agar and tumor formation in nude mice. This study established a direct a role for the IGF-1/IGF-1R pathway in EOC and is further supported by the analysis of IGFs and IGF-1R expression in human primary tumor specimens.[96,97]

Rat

Transfection of a spontaneously immortalized ROSE cells, with mutant *c-H-Ras* resulted in malignant transformation and sarcoma formation in subcutaneously injected athymic mice and immunocompetent rats.[70] Davies et al.[98] also using ROSE 199 cells, showed that retroviral transduction with activated ErbB2/Neu resulted in enhanced migration, loss of contact inhibition, and in vivo tumor formation in syngeneic rats injected intraperitoneally or within the ovarian cortex. In a study designed to test whether acquisition of an angiogenic phenotype affects cell growth and tumorigenic capacity, ROSE 199 cells were transfected with a secreted form of the vascular endothelial growth factor (VEGF 165).[99] Overexpression of VEGF 165 in ROSE 199 cells had very little effect on proliferation, focus formation, or soft colony formation in vitro, but profound effects were observed in vivo.[99] Athymic nude mice injected subcutaneously with VEGF165 expressing ROSE 199 cells developed tumors with high frequency (17 of 20 mice), and when these cells were injected intraperitoneally, 100% (20 of 20 mice) developed malignant ascites and peritoneal tumors.[99] Pharmacologic inhibition of VEGFR receptor (VEGFR) signaling with SU5416 or inducible expression of endostatin in these cells was sufficient to reverse the angiogenic phenotype and tumorigenicity in vivo.[99]

Human

As noted above, expression of the SV40 TAg in primary human OSE cells results in a prolonged life span in culture, and after continuous culture some cells become immortalized, but not transformed.[73] Expression of human telomerase reverse transcriptase (hTERT) in conjunction with expression of SV40 TAg[100,101] or RNAi-mediated knockdown of p53 [102] is sufficient to result in immortalization of human OSE cells. The additional expression of oncogenes implicated in EOC can mediate complete transformation of immortalized human OSE cells. For example, expression of E-cadherin in SV40 TAg immortalized cells resulted in induction of characteristics indicative of mesenchymal to

epithelial transition, markers of metaplastic and neoplastic transformation, and anchorage-independent growth of cells in culture as well as tumor formation in vivo.[103,104] Similarly, introduction of oncogenic mutant alleles of either human *HRAS* (*HRAS*^{V12}) or *KRAS* (*KRAS*^{V12}) into SV40 and hTERT immortalized human OSE cells resulted in tumor formation in vivo after s.c. or i.p. injection.[101] The oncogenic potential of *HRAS*^{V12} was apparently greater as tumors arose with higher frequently in mice injected with cells expressing *HRAS*^{V12} than *KRAS* ^{V12}.[101] Tumors expressing *HRAS*^{V12} were classified as undifferentiated adenocarcinomas with focal papillary growth.[101] Although *KRAS* mutations are often associated with borderline and invasive mucinous tumors and *KRAS* pathway activation (e.g., via *KRAS* or *BRAF* mutations) is associated with borderline serous tumors,[105–111] *KRAS*^{V12} tumors arising in this model were poorly differentiated with components of carcinoma and sarcoma, similar to mixed Müllerian tumors of the ovary.[101] Gene expression profiling of parental *SV40 TAg* and *hTERT* immortalized OSE cells and their transformed derivatives expressing *HRAS*^{V12} and *KRAS*^{V12} were shown to express several markers of human EOC, including CA-125, mesothelin, and several cytokines including interleukins (IL)-1B, IL-6, and IL-8.[101]

Mouse

Using a retroviral transduction strategy, Orsulic and colleagues[112,113] developed a mouse model of ovarian carcinoma with defined genetic lesions. By using transgenic mouse strains engineered to ectopically express the avian retroviral receptor TVA,[114] somatic cells isolated from the transgenic mice can be infected with recombinant avian proviral vectors.[115,116] The use of replication competent, avian leukemia virus-derived LTR, with splice acceptor (RCAS) retroviral vectors[116] allows for stable integration and expression of the oncogene introduced into the mouse genome. This retroviral delivery system allows for flexible delivery of single and/or multiple oncogenes and has been used to model a variety of cancers in mice, including brain, lung, hepatic, pancreatic, and mammary cancers.[117–123]

At the time Orsulic and colleagues initiated efforts to develop a mouse model of EOC, there were no specific promoters available to generate transgenic mice expressing the TVA in the ovarian epithelium; therefore, these investigators isolated somatic ovarian cells from transgenic mice expressing the TVA receptor under the transcriptional control of the β-actin[115] or keratin 5 (K5)[113] gene promoters and subsequently infected them with recombinant oncogenic proviral constructs ex vivo. Infection of ovarian explants with recombinant retroviral constructs expressing human *c-MYC*, mutant murine *K-Ras*(*K-Ras*^{G12D}), or myristoylated murine *Akt1* singly or in any combination of two or all three oncogenes was insufficient to produce tumors in subcutaneously injected recipient mice. However, when β-actin-TVA or K5-TVA mice were crossed into a *p53*$^{-/-}$ background, infection of β-actin-TVA;*p53*$^{-/-}$ or K5-TVA;*p53*$^{-/-}$ ovarian cells with retroviruses containing any

combination of two or three oncogenes (*c-MYC*, *K-Ras*^{G12D} and/or *Akt1*) resulted in rapid tumor formation (e.g., by 8 weeks) after s.c. injection.[113] Analysis of s.c. tumors arising from β-actin-TVA;*p53*$^{-/-}$;RCAS-*c-MYC* and RCAS-*K-Ras*^{G12D} or K5-TVA;*p53*$^{-/-}$;RCAS-*c-MYC* and RCAS-*Akt* ovarian cells showed that the tumor cells were epithelial, as evidenced by expression of cytokeratin 8, and expressed the TVA receptor.[113] Notably, the stromal component of these s.c. tumors did not express the TVA receptor, indicating that the stroma was host derived. These results suggest that regardless of the gene promoter driving the TVA receptor transgene, the epithelial component of the ovarian explants is more susceptible to transformation by the combined loss of p53 and expression of two additional oncogenes than the stromal component of the explant. To study the capacity of tumor formation in a more relevant microenvironment, these cells were subsequently implanted orthotopically by replacement of the normal ovary with infected ovarian cells within the ovarian bursa of recipient mice. Tumors developed rapidly at the site of the orthotopic injection and resulted in peritoneal spread with implants on or within the contralateral ovary, intestines, liver, pancreas, kidney, mesothelium, omentum, and diaphragm. Histopathologic evaluation of these tumors identified them as poorly differentiated carcinomas with regions of papillary structures similar to human papillary serous carcinoma (Fig. 17.1A, B and Ref. 113).

Subsequent studies using this model to test the mechanisms of tumor sensitivity to pathway and molecular targeted therapy showed that the mammalian target of rapamycin (mTOR) inhibitor rapamycin was effective in inhibiting cells and tumors dependent on the Akt pathway for survival and proliferation (i.e., *p53*$^{-/-}$;RCAS-*myr-Akt1 and c-MYC* or RCAS-*K-Ras*^{G12D}) but cells not dependent on the Akt pathway (i.e., *p53*$^{-/-}$;RCAS-*c-MYC* and RCAS-*K-Ras*^{G12D}) were resistant to inhibition by rapamycin.[124] Introduction of RCAS-*myr-Akt1* into *p53*$^{-/-}$;RCAS-*c-MYC*; RCAS-*K-Ras*^{G12D} ovarian cells and subsequent treatment with rapamycin did not inhibit in vitro proliferation of cells or in vivo tumor formation but did inhibit ascites formation in mice.[124] Conversely, introduction of an additional

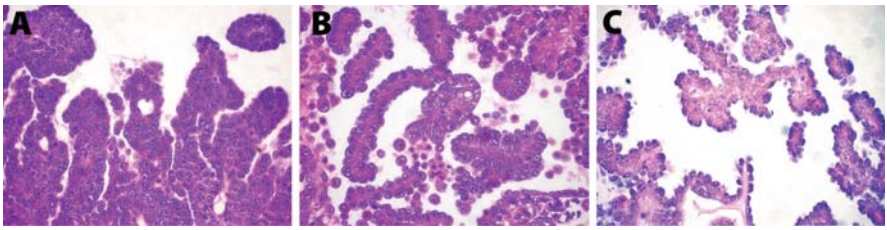

Fig. 17.1 Hematoxylin and eosin (H&E)-stained sections of tumors arising in mice injected intraperitoneally with ovarian explants derived from p53$^{-/-}$ mice and transduced with oncogenic retroviruses. (**A**) Tumor from K5-TVA;*p53*$^{-/-}$;*C-Myc;K-Ras*^{G12D} ovarian cells, (**B**) tumor from K5-TVA;*p53*$^{-/-}$;*C-Myc;myr-Akt1* ovarian cells, and (**C**) tumor from K5-TVA;*p53*$^{LoxP/LoxP}$;*Brca1*$^{LoxP/LoxP}$;*C-Myc* ovarian cells. Each tumor exhibits differentiation similar to serous adenocarcinomas in women. The images are magnified 400×. (Images provided courtesy of Dr. Sandra Orsulic, Cedars-Sinai Medical Center, Los Angeles, CA.)

oncogene (e.g., *K-Ras*^{G12D} or *Her-2*) to *p53* $^{-/-}$;RCAS-*myr-Akt1;*RCAS-*c-MYC* cells resulted in an increase in resistance to rapamycin in vitro and in vivo.[124] Although MAPK/ERK Kinase (MEK) is thought to be a key mediator of Ras activation and transformation, treatment of *p53* $^{-/-}$;RCAS-*myr-Akt1;*RCAS-*c-MYC*;RCAS-*K-Ras*^{G12D} cells with the MEK inhibitor PD98059 did not inhibit in vitro proliferation.[124] However, simultaneous inhibition of both mTOR and MEK by combination treatment with rapamycin and PD98059 resulted in diminished proliferative capacity of these cells.[124] Taken together, these results elegantly demonstrated that molecular and pathway targeted inhibitors may be effective in a subset of tumors with activation of the relevant pathway, but tumors with alteration of multiple proliferative and/or survival pathways may require combination treatments to achieve therapeutic efficacy.

Using a similar experimental strategy to develop a model of inherited EOC, expression of human c-MYC in conjunction with conditional loss of both *p53* and *Brca1* resulted in transformation of murine OSE in vitro and in vivo.[125] Transgenic K5-TVA mice were crossed with mice harboring Lox P flanked (floxed), conditionally expressed alleles of *Brca1* (*Brca1* $^{LoxP/LoxP}$ mice),[126] and *p53*(*p53*$^{LoxP/LoxP}$ mice)[127] to generate *K5-TVA;Brca1*$^{LoxP/LoxP}$ and *K5-TVA;p53*$^{LoxP/LoxP}$ mice, respectively.[125] These mice were subsequently crossed to generate triple transgenic *K5-TVA;Brca1*$^{LoxP/LoxP}$*;p53*$^{LoxP/LoxP}$ mice.[125] Ovarian explants were subsequently infected with recombinant RCAS retroviruses, including RCAS Cre-recombinase (to mediate excision of floxed sequences) and RCAS-*c-MYC*, RCAS-*Her-2*, RCAS-*K-Ras*^{G12D} and/or RCAS-*Akt1*. Expression of *c-MYC*, but not *Her-2*, *K-Ras*^{G12D}, or *Akt1*, resulted in transformation of *Brca1* and *p53* deficient ovarian cells. However, expression of *c-MYC* in ovarian cells deficient for either *Brca1* or *p53* alone was insufficient for transformation. Mice injected intraperitoneally with *K5-TVA;Brca1*$^{LoxP/LoxP}$*;p53*$^{LoxP/LoxP}$;RCAS-*c-MYC* cells developed tumors with implants on the peritoneum, intestines, pancreas, and diaphragm and the production of hemorrhagic ascites.[125] Tumors arising in intraperitoneally injected mice (Fig. 17.1C) were epithelial in origin (confirmed by expression of cytokeratin 8) and characterized by papillary structures characteristic of human serous papillary tumors arising in BRCA1 deficient patients.[125] Also consistent with *BRCA1*-associated human EOC, tumors arising in this experimental model exhibit enhanced sensitivity to cisplatin.[125,128]

Results from these studies illustrate that ex vivo retroviral transduction of ovarian explants is a flexible strategy that can be employed to generate in vivo models to test the specific role of oncogenes and tumor genes in EOC etiology and to test the efficacy of molecular and pathway targeted therapeutic agents.

Genetically Engineered Mouse Models of EOC

Methods for manipulation of the mouse germ line have been available for decades. The first successful attempts to make transgenic mice, in which cloned DNA sequences were introduced in the mouse germ line, were described in the

early 1980s (Refs. 129–132 and reviewed in Ref. 133). Soon after the technology was developed, transgenic mouse models with hereditary cancer were described.[133] The 1990s ushered in the technology to generate targeted germline deletion or knock-out of genes in mice,[134] a critically important strategy for manipulation of the expression of tumor suppressor genes in vivo (Refs. 135–137 and reviewed in Ref. 138). Yet nearly 20 years after the development of the first genetically engineered mouse (GEM) models of tumor-prone mice, there were no models of human EOC. The reasons for this include a lack of understanding of the epithelial precursor of EOC and of the genetic alterations involved in initiation, progression, and maintenance of ovarian cancer. Over the past several years, significant inroads have been made toward the development of GEM models of EOC as described below.

Transgenic Models

The greatest obstacle to the development of transgenic models of EOC was the lack of a suitable gene promoter to target transgene expression in a tissue-specific and -restricted manner to the ovarian epithelium. Several attempts to develop transgenic models were made using gene promoters such as the mouse metallothionein-1 promoter, the bovine glycoprotein hormone alpha subunit, murine inhibin-α, Müllerian inhibiting substance (MIS)/anti-Müllerian hormone (AMH), and an ovary-specific promoter-1 derived from endogenous retroviral sequences present in rat.[139–145] Although these efforts successfully resulted in the production of mice that developed ovarian tumors, the tumors were sex-cord stromal in origin rather than epithelial.[139–144]

In early 2001, our laboratory began to evaluate candidate gene promoters for the development of a transgenic model of EOC. Among the candidates, we focused on proteins involved in female reproductive tract development. During embryogenesis, secondary sex determination is dependent on the presence or absence of Müllerian inhibitory substance (MIS) and its cognate receptors, MIS type I and type II receptors (reviewed in Refs. 146, 147). In the male animal, MIS is secreted from Sertoli cells of the developing testes and stimulates the regression of the Müllerian duct. In the absence of MIS in the developing female embryo, the Müllerian duct persists and differentiates into the secondary structures of the female reproductive tract including fallopian tube, uterus, and upper vagina.[146] Although MIS signaling is not directly involved in the development of the female reproductive tract, functional receptors are present in adult animals. Based on the presumption that OSE cells arise from the same coelomic epithelial cells that give rise to the Müllerian duct (reviewed in Ref. 148), we hypothesized that the *MISRII* would also be expressed in the ovarian epithelium. Further evidence to support this hypothesis existed from a previous study[149] showing that the *MISRII* gene is expressed in established ovarian cancer cell lines and cell lines derived from the ascites of patients with ovarian carcinoma.

After confirming that the *MISRII*, gene was expressed in normal mouse OSE, we made transgenic mice by expressing the early region of the SV40 TAg gene under transcriptional control of the *MISRII* gene promoter.[150] Female Tg*MISIIR-TAg* transgenic mice develop bilateral ovarian cancer, malignant ascites, and disease dissemination that is typically restricted to the peritoneum.[150] Histopathologic analysis of these tumors demonstrated that they were epithelial in origin and resembled human serous EOC. Whereas 50% of female transgenic founder mice exhibited the ovarian cancer phenotype, none of those mice were fertile.

Subsequent to our initial report,[150] we isolated a stable transgenic line of mice from a male transgenic founder.[151] Female offspring from this stable transgenic line (Tg*MISIIR-TAg-DR26*) develop bilateral ovarian carcinomas resembling human serous EOC (Fig. 17.2) with 100% penetrance and have an average life span of 5–6 months. To date, this is the first transgenic mouse model that develops spontaneous ovarian cancer with pathologic features of serous EOC. Like human EOC, mice with significant tumor burden exhibit few or no symptoms of illness. In addition, tumor cells share many molecular features with human tumors including expression of cytokeratins, MUC16 (the murine equivalent of CA125), vascular endothelial growth factor (VEGF), COX-1, and activated AKT, STAT3, and Src (Refs. 150–156 and unpublished results). We have established a large panel of murine ovarian carcinoma (MOVCAR) cell lines from malignant ascites and primary tumors of Tg*MISIIR-TAg* mice.[150,151] These cell lines are tumorigenic in SCID and syngeneic recipient mice and are useful for in vitro and in vivo tumor biology and preclinical therapeutics studies.[152–157] We have confirmed the potential utility of this model for preclinical therapeutic studies by testing the efficacy of a standard combination therapy consisting of cisplatin and paclitaxel[151] and showed that tumors regress in response to drug treatment and that drug-treated mice live significantly longer. As tumors arising in the Tg*MISIIR-TAg* mice frequently express activated Akt, we also tested the therapeutic efficacy of molecular targeted inhibition of the Akt pathway using the mTOR inhibitor everolimus (RAD001).[153]

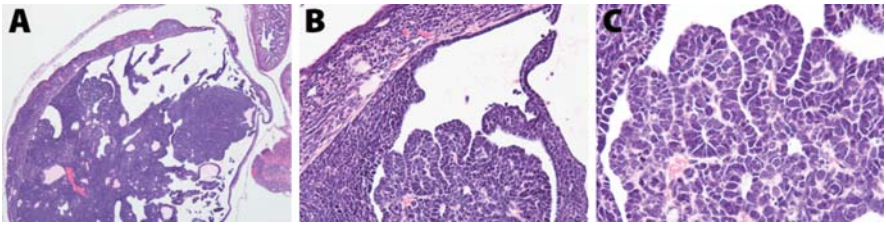

Fig. 17.2 Hematoxylin and eosin (H&E)-stained sections of a spontaneous ovarian tumor arising in a Tg*MISIIR-TAg* transgenic mouse. The invasive tumor is apparently contained in a cystic structure within the ovary and exhibits morphologic features similar to serous cystadenocarcinomas. (**A**) Low-power image showing the tumor within the cortex of the ovary. The arrow points to a normal follicle. The same tumor is magnified at (**B**) 200× and (**C**) 400×

Treatment with RAD001 significantly delayed tumor development and ascites production in vivo in Tg*MISIIR-TAg-DR26* transgenic mice.[153] These effects are likely mediated at least in part by diminished expression of VEGF and matrix metalloproteinase-2 (MMP-2) and decreased invasiveness of tumor cells.[153]

Although there is no known association of SV40 TAg with human EOC, many transgenic tumor models were developed by expression of the large and small TAg genes, which result in functional inactivation of Rb and p53 as well as PP2A.[158–164] The continued utility of these models in studying cancer is underscored by seminal contributions to an understanding of the "angiogenic switch"[165–169] and tumor progression and invasion.[170] Importantly, a recent study[171] identified an integrated gene expression signature from three distinct TAg mouse models (i.e., mammary, prostate, and lung cancer models) that is comparable with a signature associated with the aggressive biological behavior and prognosis for several human epithelial tumors, including breast cancers. Results from this study show that tumors arising in TAg-based mouse models share common features of gene expression with human cancer and are relevant preclinical models.[171]

Germ-Line Deletions

Germ-line deletion of the follicle-stimulating hormone receptor (FSHR) has been reported to result in ovarian tumor formation. One report of follitropin receptor knockout (FORKO) mice described the development of ovarian sex-cord stromal tumors with Sertoli-Leydig cell differentiation after 12–15 months.[172] Subsequently, these investigators showed that tumors arising in these mice commonly contain sex-cord stromal differentiation as well as an epithelial component resembling serous papillary adenoma of the ovary.[173] Changes in the ovarian epithelium appeared to occur early as increased levels of cytokeratin expression in the OSE were observed as early as postnatal day 2, and cytokeratin-positive cells were detected within the ovarian cortex by 24 days.[173] FORKO mice do not ovulate due to the absence of the FSHR; therefore, the development of serous adenomas in these mice suggests that tumor development is not related to ovulation in this model. Other investigators have generated both follicle-stimulating hormone receptor knockout (FSHRKO) and follicle-stimulating hormone-β subunit knockout (FSHβKO) mice that develop age-related ovarian hypertrophy, epithelial inclusion cysts, tubular structures, and ovarian pathology similar to serous cystadenocarcinomas.[174]

Conditional Genetic Models

Germ-line loss of tumor suppressor genes can in some instances result in embryonic lethality; therefore, methods for conditional expression of tumor suppressor genes have been used to facilitate the development of GEM models

of human cancers. One common method is the use of Cre-recombinase–mediated excision of LoxP flanked (floxed) sequences contained within endogenous tumor suppressor alleles in mice. This approach has been successfully used to develop GEM models of a number of human malignancies including mammary gland, brain, lung, and pancreas.[126,127,175–178] Tissue-specific delivery of Cre-recombinase can be accomplished by crossing mice harboring conditional floxed alleles of the gene of interest with transgenic mice expressing Cre-recombinase in a tissue-restricted fashion. An alternative method involves the localized administration of recombinant adenovirus expressing Cre-recombinase (Ad-Cre). The latter method is particularly useful in modeling cancers for which there are few or no promoters available to generate transgenic mice that express Cre-recombinase in the tissue of interest or for anatomic sites that are amenable to viral delivery. There are currently no available transgenic strains of mice that express Cre-recombinase in an ovarian epithelium–restricted manner. However, a number of investigators have recently capitalized on the presence of the ovarian bursa in mice for localized delivery of Ad-Cre to achieve Cre-LoxP–mediated gene inactivation in the OSE.[179–183] Following are examples of the use of this approach to develop GEM models by conditional inactivation of genes thought to be directly involved in the development of EOC.

Conditional Inactivation of p53 and Rb

The importance of mutation or loss of function of *p53* in EOC is well established.[184,185] There is significant evidence that loss of heterozygosity (LOH) of *Rb* or alterations of the Rb pathway also play an important role in EOC development.[150,186–190] Direct evidence of the importance of *Rb* in EOC development was provided by the development of a mouse model in which *p53* and *Rb* were conditionally inactivated in the OSE.[182] In this study, Flesken-Nikitin and colleagues[182] were the first to describe the use of intrabursal injection of Ad-Cre for conditional inactivation of tumor suppressor genes in the OSE. Results from this study showed that while conditional inactivation of *p53* or *Rb* alone in the OSE rarely resulted in tumor formation, inactivation of both of these genes simultaneously resulted in ovarian tumor formation in 97% of mice. Of the ovarian tumors arising in $p53^{LoxP/LoxP};Rb^{LoxP/LoxP}$ mice, 39% were characterized as well-differentiated serous adenocarcinomas (Fig. 17.3), 45% were poorly differentiated cytokeratin-positive (epithelial) neoplasms, and 15% were undifferentiated neoplasms of the ovary.[182] Ovarian tumors in $p53^{LoxP/LoxP};Rb^{LoxP/LoxP}$ mice were accompanied by the presence of ascites in 24% of cases and peritoneal spreading in 27% of cases. The median latency with which mice succumbed to tumor formation in the Ad-Cre–injected $p53^{LoxP/LoxP};Rb^{LoxP/LoxP}$ mice was 227 days, suggesting that additional genetic alterations may be required for tumorigenesis in this model. Results from this study confirmed both the successful use of intrabursal Ad-Cre delivery for Cre-mediated recombination of floxed sequences in the OSE as well as the potential for *Rb* to cooperate with *p53* in the development of serous EOC.

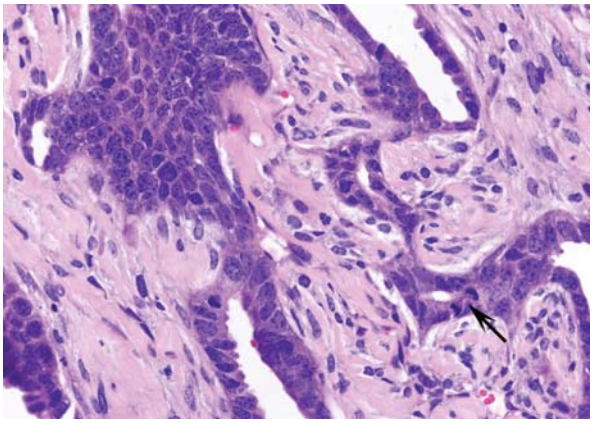

Fig. 17.3 Hematoxylin and eosin (H&E)-stained section of a murine ovarian serous adenocarcinoma arising in a $p53^{LoxP/LoxP};Rb^{LoxP/LoxP}$ mouse after intrabursal administration of adenovirus Cre-recombinase (Ad-Cre). The image is magnified 400×. (Image provided courtesy of Dr. Alexander Nikitin, Cornell University, Ithaca, NY.)

K-ras and Pten[181]

In an elegant example of the use of Cre-LoxP–mediated conditional gene expression, Dinulescu and colleagues developed the first mouse model of the endometrioid histologic subtype of EOC. This study evaluated the consequences of conditional expression of an activating mutation of *K-ras* and/or conditional inactivation of the *Pten* tumor suppressor gene in mouse OSE. Mice harboring a *LoxP*-Stop-*LoxP*-*K-ras*^{G12D} express a silenced mutant allele of *K-ras* that is activatable by Cre-mediated excision of the floxed-Stop sequence.[175,191] Expression of this mutant *K-ras* allele after intrabursal Ad-Cre administration in heterozygous *LoxP*-Stop-*LoxP*-*K-ras*$^{G12D/+}$ mice resulted in the development of benign endometriosis-like lesions within the ovary (e.g., endometrioid glandular morphology in the absence of endometrioid stroma) in all cases and peritoneal endometriosis (e.g., endometrioid glandular and stromal morphology) in approximately half of the mice.[181] Significantly, peritoneal endometriosis was not observed in mice receiving i.p. injection of Ad-Cre, suggesting that the lesions seen in mice injected intrabursally with Ad-Cre are a direct result of activation of K-ras in reproductive tract tissue rather than metaplastic differentiation of the pelvic peritoneum.[181] The anatomy of the mouse reproductive tract is such that one cannot exclude the possibility that intrabursal injection of Ad-Cre can result in localized infection of cells within the fallopian tube or the uterus at the utero-tubal junction; therefore, it is difficult to determine whether the origin of the peritoneal endometriosis is derived from the OSE or from other cells in the reproductive tract.

Based on the association of mutation and/or loss of heterozygosity (LOH) of the *PTEN* tumor suppressor with endometrioid ovarian cancer,[192–194] these investigators tested whether loss of *Pten* expression in combination with activation of *K-ras* resulted in this tumor type in mice. Results from this study showed that conditional activation of *K-ras* and inactivation of *Pten* by intrabursal injection of Ad-Cre in *LoxP*-Stop-*LoxP*-*K-ras*$^{G12D/+}$;*Pten*$^{LoxP/LoxP}$ mice resulted in the development of

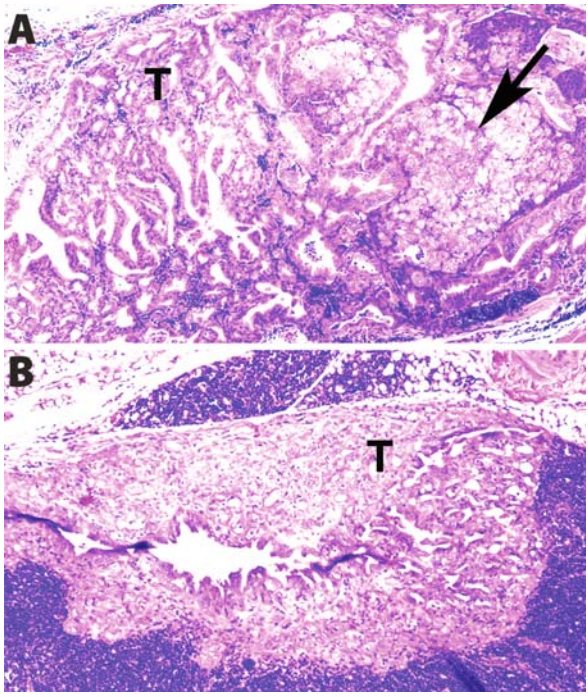

Fig. 17.4 Hematoxylin and eosin (H&E)-stained sections of ovarian tumors (T) arising in a *K-Ras* ^{G12D}*;Pten* $^{LoxP/LoxP}$ mouse after intrabursal administration of adenovirus Cre-recombinase (Ad-Cre). (**A**) The ovarian tumor has key features of endometrioid ovarian cancer including glandular histology with squamous differentiation (indicated by the *arrow*). (**B**) Endometrioid carcinomas in mice are invasive as evidenced by the presence of tumor cells in a regional lymph node. (Images provided courtesy of Dr. Daniela Dinulescu, Brigham and Women's Hospital, Boston, MA.)

invasive endometrioid ovarian carcinomas (Fig. 17.4 and Dinulescu et al.[181]). Ovarian tumors occurred in all mice with short latency (i.e., as early as 7 weeks postinjection) and were occasionally accompanied by peritoneal implants, invasion of regional lymph nodes (Fig. 17.4B), and ascites.[181] Histologically, tumors resembled human endometrioid ovarian carcinomas with regions of glandular and solid growth and some areas containing metaplastic squamous differentiation. Tumors expressed cytokeratin 8, evidence of their epithelial origin, and also expressed phosphorylated (activated) Akt, mTOR, and S6 kinase, indicative of activation of the phosphatidyl inositol 3 kinase (PI3K)-Akt-mTOR pathway as might be expected with loss of *PTEN* expression.

Studies have suggested an association of endometriosis with the development of endometrioid and clear cell ovarian carcinomas and that endometriosis may be a precursor lesion of these tumors.[195–197] Although the *LoxP*-Stop-*LoxP*-*K-ras*$^{G12D/+}$;*Pten*$^{LoxP/LoxP}$ GEM model appears to establish a genetic relationship between activating *K-ras* mutations in both endometriosis and

endometrioid ovarian cancer in mice, such a relationship in humans has yet to be established. Molecular analyses of endometriotic lesions have failed to show activating mutations of K-Ras,[198–200] and such mutations are relatively rare (<10%) in human endometrioid ovarian carcinomas.[198–203] Further studies will be required to clarify the potential involvement of activation of K-Ras or the K-Ras pathway in these cancers.

Apc and Pten

In a recent study, molecular analyses of 72 clinical specimens of endometrioid EOC cases did not show an association of mutations of *PTEN* and *K-RAS* in endometrioid carcinomas.[183] However, this study did show a significant association of mutations of the Wnt/β-catenin and PI3K/PTEN pathways, particularly in low-grade endometrioid tumors.[183] Based on these results, these investigators sought to validate the hypothesis that activation of these two pathways can cooperate in the development of endometrioid EOC in a GEM model system. To test this directly, they used the Cre-LoxP–mediated approach for conditional gene inactivation as described above. Mice harboring floxed alleles of *Pten* ($Pten^{LoxP/LoxP}$) and *Apc* ($Apc^{LoxP/LoxP}$) were crossed to generate $Pten^{LoxP/LoxP}$;$Apc^{LoxP/LoxP}$ offspring, and conditional alleles of *Pten* and/or *Apc* were inactivated by unilateral intrabursal injection of ovaries with Ad-Cre.[183] Ovarian tumors occurred with 100% penetrance within 6 weeks of Ad-Cre injection of female $Pten^{LoxP/LoxP}$;$Apc^{LoxP/LoxP}$ mice, and tumor burden necessitated euthanasia between 7 and 19 weeks postinjection. Tumors were accompanied by ascites in 76% of cases, and peritoneal dissemination was observed in 21% of the mice.[183] Histologically, ovarian tumors had morphologic similarity to human endometrioid EOC (Fig. 17.5) with the formation of

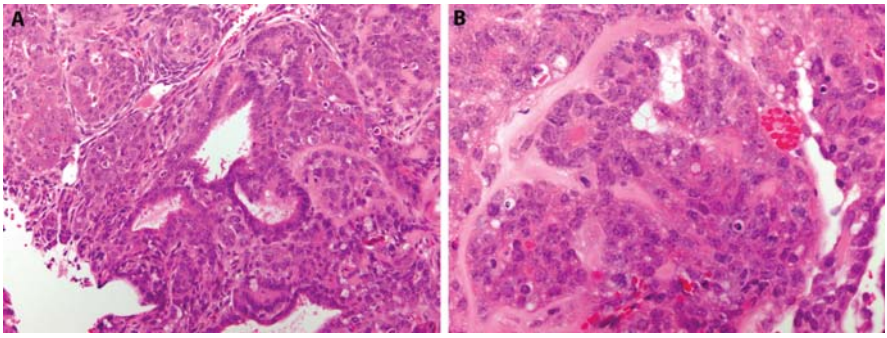

Fig. 17.5 Hematoxylin and eosin (H&E)-stained sections of an ovarian tumor arising in a $Pten^{LoxP/LoxP}$;$Apc^{LoxP/LoxP}$ mouse after intrabursal administration of adenovirus Cre-recombinase (Ad-Cre). The ovarian endometrioid tumor is characterized by glandular structures and occasional foci of squamous differentiation. The images are magnified (**A**) 200× and (**B**) 400×. (Images provided courtesy of Dr. Kathleen Cho, University of Michigan, Ann Arbor, MI.)

glandular structures and occasional foci of squamous differentiation. Tumor cells stained positively for cytokeratin and showed nuclear accumulation of β-catenin.[183] Like tumors from *LoxP*-Stop-*LoxP*-*K-ras*$^{G12D/+}$;*Pten*$^{LoxP/LoxP}$ mice, tumor cells lacked detectable Pten expression and showed increased pS6K staining, suggestive of Akt pathway activation.[183] Also present in ovarian tumors arising in these mice were areas of less differentiated cytokeratin-negative mesenchymal-appearing cells with vague spindle-cell morphology. Unlike *K-Ras*$^{LoxP/LoxP}$; *Pten*$^{LoxP/LoxP}$ mice, endometriosis-like lesions were not identified in the ovaries of *Pten*$^{LoxP/LoxP}$;*Apc*$^{LoxP/LoxP}$ mice.[181,183] No tumors were observed in mice with inactivation of either *Pten* or *Apc* alone up to 30–42 weeks after Ad-Cre injection.

Comparison of gene expression profiles of human serous, endometrioid, mucinous, and clear cell EOCs with ovarian tumors isolated from *Pten*$^{LoxP/LoxP}$; *Apc*$^{LoxP/LoxP}$ mice showed greatest correlation between the human endometrioid and the murine ovarian tumors.[183] Significantly, some of the human tumors that were most highly correlated to murine tumors were known to have mutations of the Wnt/β-catenin and PI3K/PTEN pathways.[183] Taken together, these results suggest that ovarian tumors arising in mice with conditional inactivation of *Pten* and *Apc* exhibit similar histology, biology, and gene expression profiles as human endometrioid EOCs with alterations of the Wnt/β-catenin and PI3K/PTEN pathways.[183] These results are extremely encouraging with regard to the potential use of GEM models of EOC as preclinical models for the evaluation of molecular targeted therapeutic agents, particularly with specific subtypes of EOC.

Brca1

Approximately 10% of EOCs are inherited, and the majority of these hereditary cases are associated with germ-line mutations of the *BRCA1* tumor suppressor gene.[204,205] *BRCA1*-associated EOC is generally diagnosed at younger ages than sporadic EOC.[128] Whereas sporadic EOC is typically diagnosed in postmenopausal women, *BRCA1*-associated EOC is more commonly diagnosed in premenopausal women, suggesting that hormonal signaling from the ovary may play an important role in hereditary EOC.[128] Although EOC is widely thought to arise in the ovarian epithelium, the potential contribution of hormonal signaling in *BRCA1*-associated EOC led Chodankar and colleagues[179] to hypothesize that loss of expression of *BRCA1* in granulosa cells, the cells that are the primary source of steroid hormone synthesis within the ovary, might influence tumor ovarian development.[179] Because germ-line deletion of both copies of *Brca1* is embryonic lethal, a Cre-loxP–mediated strategy for conditional *Brca1* inactivation was employed. As intrabursal injection of Ad-Cre results primarily in the infection of the OSE, these investigators used a genetic approach. In transgenic mice expressing Cre-recombinase under transcriptional control of the follicle-stimulating hormone receptor (*FSHR*), gene promoter, Cre-recombinase is expressed exclusively in the granulosa cells of the ovary[179];

therefore Tg*Fshr-Cre* mice were crossed with *Brca1*$^{LoxP/LoxP}$ mice to generate compound *Brca1*$^{LoxP/LoxP}$;Tg*Fshr-Cre* transgenic mice. At 2 months of age, *Brca1*$^{LoxP/LoxP}$; Tg*Fshr-Cre* mice had histologically normal ovaries and were fertile.[179] Analysis of *Brca1*$^{LoxP/LoxP}$; Tg*Fshr-Cre* mice euthanized between 12 and 20 months of age showed benign cysts in the ovary (Fig. 17.6) and the uterine horn proximal to the ovary in the majority (40 of 59; 68%) of mice, whereas no abnormalities were observed in control *Brca1*$^{LoxP/LoxP}$ mice.[179] Histologically, these cysts resembled human serous cystadenomas, a putative precursor lesion of EOC.[58,206] The epithelial origin of the cysts was confirmed by detection of cytokeratin expression and the absence of Müllerian inhibiting substance (MIS), a marker of granulosa cell differentiation.[179] Molecular analysis of the *Brca1* allele by PCR amplification of genomic DNA showed deletion of the floxed sequences in the granulosa cells, but not in laser capture microdissected epithelial cells lining benign cysts.[179] These results led the investigators to conclude that loss of *Brca1* in the granulosa cells results in a cell nonautonomous effect on the epithelium, leading to the development of benign epithelial tumors in this model.

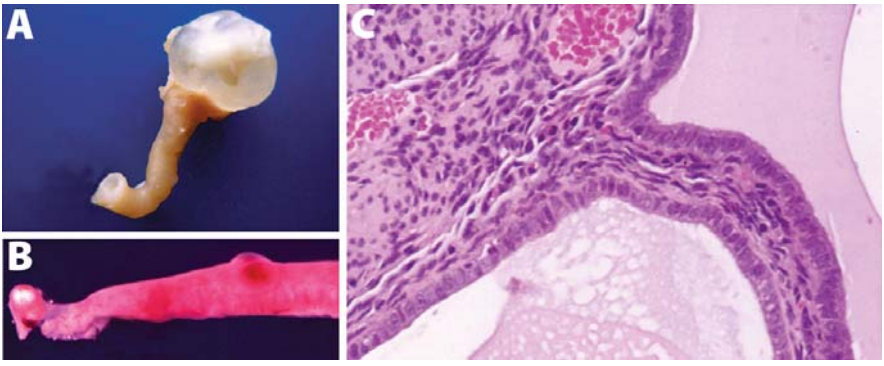

Fig. 17.6 Ovarian cystadenomas arising in *Brca1*$^{LoxP/LoxP}$*;TgFshr-Cre* mice. (**A**) Gross image an ovarian cystadenoma attached to left uterine horn. The right uterine horn has been removed. (**B**) Uterine horn showing a periuterine cystadenoma. (**C**) Hematoxylin and eosin (H&E)-stained section of a benign bilocular ovarian cystadenoma. (Images provided courtesy of Dr. Louis Dubeau, University of Southern California, Keck School of Medicine, Los Angeles, CA.)

As an alternative strategy, intrabursal administration of adenovirus Cre-recombinase was used to study the effects of conditional inactivation of *Brca1* in the ovarian epithelium in vivo.[180] This study showed that Cre-mediated deletion of floxed sequences in the OSE cells in *Brca1*$^{LoxP/LoxP}$ mice resulted in the earlier and more frequent development of preneoplastic changes in the ovaries compared with control mice with unexcised *Brca1*$^{LoxP/LoxP}$.[180] Preneoplastic changes observed in the ovaries included hyperplasia, epithelial invaginations, and the formation of epithelial inclusion cysts (Fig. 17.7); each of these morphologic alterations of the ovarian epithelium have been proposed as putative

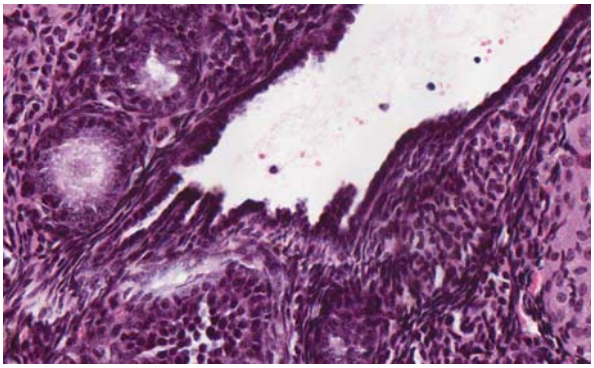

Fig. 17.7 Hematoxylin and eosin (H&E)-stained section of the ovary of a *Brca1*$^{LoxP/LoxP}$ mouse 180 days after intrabursal injection of adenovirus Cre-recombinase (Ad-Cre). The ovarian surface epithelium exhibits preneoplastic morphologic changes including the presence of papillary structures. The image is magnified 400×. (Image provided courtesy of Dr. Barbara Vanderhyden, Ottawa Health Research Institute, Ottawa, ON, Canada.)

precursor lesions in human EOC.[58] Further evaluation and comparison of cultured MOSE cells with excised and unexcised *Brca1*$^{LoxP/LoxP}$ showed that loss of functional *Brca1* results in a slightly decreased rate of proliferation and, like human EOC, increased sensitivity to cisplatin.[128,180,207,208]

In Vivo Imaging

Due to the relatively deep anatomic location of the ovaries and characteristically diffuse and miliary nature of peritoneal metastases, in vivo quantitation of tumor burden in animal models can be difficult using conventional techniques such as caliper measurements. The application and adaptation of noninvasive imaging technologies for small animals has vastly improved the ability to quantitate tumor burden in vivo (reviewed in Ref. 209). Imaging modalities such as magnetic resonance imaging (MRI), ultrasound (US), positron emission tomography (PET), computed tomography (CT), and single photon emission computed tomography (SPECT) have been used to image tumors in mice in vivo. In addition, optical imaging strategies have been developed to detect luminescent and/or fluorescent reporter gene expression and for molecular imaging of activatable fluorescent probes. Most imaging modalities can be adapted for use with xenograft, allograft, or spontaneous autochthonous ovarian tumor models (e.g., transgenic, conditional, or inducible GEM mouse models). Many considerations are involved in the selection of modality for in vivo imaging of mouse models of ovarian cancer including the institutional availability of the imaging equipment, imaging session time, throughput, and overall expense. Several examples of the use of noninvasive in vivo imaging in ovarian cancer models are highlighted below.

MRI, US, and PET

MRI has been successfully employed for imaging tumor burden in human tumor xenograft models[210–213] and in our own studies with Tg*MISIIR-TAg* transgenic mice.[151,153] Using T2-weighted images, tumor nodules as small as 2.4 mm^3 or with diameter of 0.5 mm could be detected in mice injected intraperitoneally with human ovarian cancer cells.[210,213] Auzenne et al.[210] used this strategy to monitor in vivo tumor growth and therapeutic response to hyaluranonic acid (HA)-conjugated paclitaxel and showed significantly enhanced survival by targeting the drug to the $CD44^+$ receptor with the HA ligand. In our own studies, we have found that MRI is an excellent modality for noninvasive tumor detection, acquisition of high-resolution images of anatomic structures, and accurate, serial measurement of ovarian tumors in mice over time. To image spontaneous tumors in Tg*MISIIR-TAg* transgenic mice, we use T1-weighted, contrast-enhanced images (Fig. 17.8 and Refs. 151, 153). Using these methods, we have successfully used MRI to monitor in vivo tumor growth rates and responsiveness to a standard cytotoxic chemotherapy regimen (i.e., cisplatin and paclitaxel) and a molecularly targeted agent (i.e., the mTOR inhibitor RAD001 [everolimus]).[151,153]

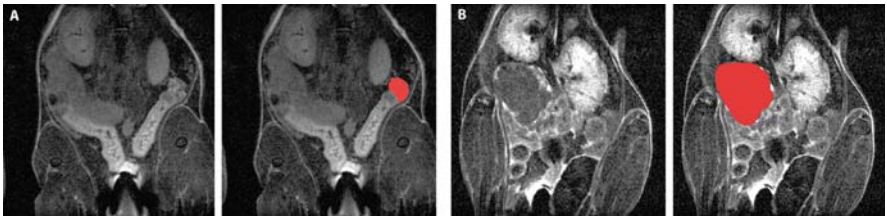

Fig. 17.8 T1-weighted, contrast-enhanced MRI images showing (**A**) the normal ovary of a wild-type C57Bl/6 mouse and (**B**) an ovarian tumor in a TgMISIIR-TAg-DR26 transgenic mouse. In each case, the region of the scan containing the ovary is highlighted in *red* in the image on the *right*

In conjunction with MRI, Sallinen et al.[213] used ultrasound to quantitate in vivo tumor burden. A subsequent study[214] comparing the use of high-resolution two-dimensional ultrasonography (2DUS) and three-dimensional ultrasonography (3DUS) to measure ovarian volume in normal mice showed that 3DUS was superior and provides a reliable method for noninvasive in vivo imaging of ovarian volume in mice over time.

Growth of ovarian tumors in mouse models can be imaged by PET scanning with the use of radioactive tracers of glucose metabolism or proliferation; for example, [^{18}F] fluorodeoxyglucose (FDG) or [^{18}F] fluorothymidine (FLT), respectively. Using a xenograft model, Leyton and colleagues[215] used FLT-PET in conjunction with bioluminescent imaging to monitor the activity and therapeutic efficacy of oncolytic viral therapy for the treatment of ovarian cancer. Kim et al.[211] used FDG-PET in conjunction with MRI to monitor the

effects of the vascular disrupting agent AVE8062 showing that treatment with AVE8062 resulted in diminished metabolic activity and regression of tumors and that AVE8062 in combination with docetaxel resulted in potent inhibition of tumor growth in an i.p. xenograft model.

FLI and BLI

Fluorescent imaging (FLI) or bioluminescent imaging (BLI) have become important optical imaging techniques for the detection of light-emitting reporter genes to monitor tumor growth in animal models of EOC. For the use of FLI or BLI with human or murine ovarian carcinoma cell lines grown as tumor xenografts or allografts, cell lines can be engineered to express luminescent or fluorescent reporter genes (e.g., firefly luciferase or green or red fluorescent protein) by standard methods for transfection or retroviral transduction. In GEM mice in which tumor formation is accompanied by expression of a light-emitting reporter such as firefly luciferase or green fluorescent protein (GFP), BLI or FLI may also be employed for longitudinal in vivo imaging of tumor burden.

In 2001, Chaudhuri and colleagues[216] first described the application of FLI techniques to monitor in vivo tumor growth in a xenograft model. Using an adenoviral expression vector (Ad-GFP), these investigators showed that ovarian carcinoma cells could be infected at high efficiency and that the therapeutic efficacy of an agent such as adriamycin could be quantitated by in vivo fluorescent imaging.[216] By using FLI to monitor tumor growth in mice injected intraperitoneally with human ovarian cancer cells stably expressing DsRed2 fluorescent protein, Subramanian and colleagues[217] demonstrated that adeno-associated virus (AAV)-mediated delivery of a mutant human endostatin (AAV-P125A-endostatin) resulted in long-term survival in a third of mice. Moreover, the combination of AAV-P125A-endostatin and carboplatin resulted in significantly delayed tumor incidence and a reduction in overall size of tumors and metastasis in the animals that did develop tumors.[217]

Similarly, ovarian cancer cell lines have been modified to express luminescent reporter genes (e.g., firefly luciferase) and BLI used for longitudinal monitoring and quantitation of tumor growth in vivo to assess the efficacy of viral and vaccine based therapies for EOC.[218–222] As BLI does not require the subtraction of the autofluorescence background signal from the animal,[223] it may provide superior sensitivity relative to FLI. In our own unpublished studies, we have used bioluminescent imaging to monitor the growth of orthotopically implanted tumor cells. Using this strategy, we can monitor and quantitate the growth and spread of tumor cells implanted within the intrabursal space over time (Fig. 17.9), thus circumventing the issues associated with quantitation of tumor burden in deep organs.

Advances are also being made in the arena of target-specific activatable fluorescently tagged reporter probes for in vivo imaging.[224] This technology

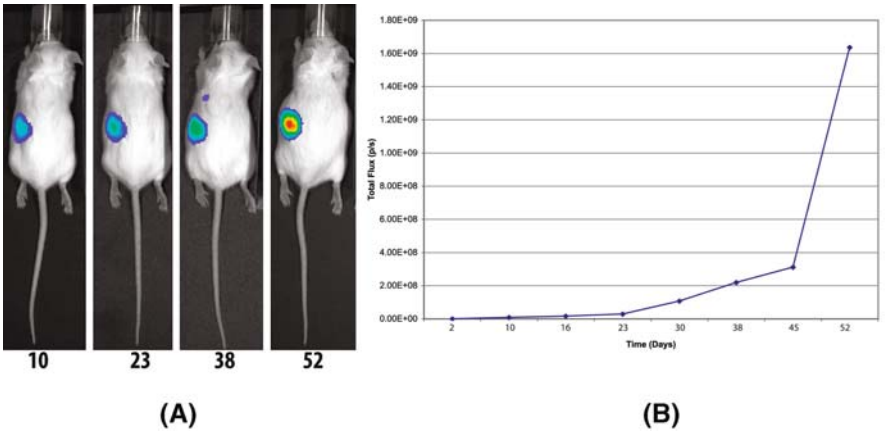

Fig. 17.9 Orthotopic tumor development monitored by bioluminescent imaging in a SCID mouse in which 4×10^5 MOVCAR 5009-luciferase cells were injected intrabursally in the left ovary. (**A**) BLI images were acquired weekly; images are shown for days 10, 23, 38, and 52 postinjection. (**B**) Tumor growth was monitored by quantitating the amount of light or *total flux* (photons/second) emitted in each scan

involves molecular imaging and detection using strong near-infrared fluorophores that are highly specific and subject to very little background fluorescence. In their inactive state, the probes remain quenched but on activation by the target are "dequenched" and become brightly fluorescent. Various target-specific probes that are recognized by proteases commonly expressed by tumors (e.g., cathepsin D or MMPs[224,225]) have been used for in vivo imaging in mice. To circumvent the potential for probe activation by proteases (or other targets) expressed by cells other than tumor cells in the surrounding microenvironment, a recent adaptation of this technology was the development of a self-quenched avidin-rhodamine conjugated probe that is activated only after internalization and degradation within the lysosomes in the target cell.[226] This probe was successfully used for ex vivo imaging of micrometastases occurring in an i.p. ovarian carcinoma xenograft model.[226] Further development of these technologies hold significant promise for improved in vivo molecular imaging in mouse models of EOC.

Conclusion

Although we have seen an improvement in the 5-year survival rates in patients over the past several years, the overall incidence and mortality of EOC has changed very little. Significant impact on the clinical outcome of EOC patients will require advances in early detection, treatment, and prevention. The use of animal models of EOC will be invaluable tools in these pursuits. Recent

successes in the development of the "first-generation" GEM models of EOC not only provide additional models to study ovarian tumor biology and therapeutic strategies, but will also likely lead to the development the next generation of genetically relevant EOC models. The ability to study spontaneously arising ovarian cancers in immune competent animals will likely allow the identification of morphologic and molecular alterations that contribute to disease initiation. Such models can also be used for the identification and validation of candidate biomarkers for early detection. As there are currently no effective strategies for prevention or early detection of EOC, an important focus remains the identification of better strategies for disease treatment, including treatment of drug refractory disease recurrence, which is the unfortunate outcome for most patients. The combination of GEM, syngeneic and human xenograft models coupled with the improved capacities for in vivo imaging will allow for better preclinical study design (e.g., the use of spontaneous or i.p. and orthotopic models vs. s.c. xenografts). Moreover, longitudinal imaging and quantitation of tumors allows for the evaluation and quantitation response to therapy rather than end-point assessments. Comparisons of results generated in different animal model systems may result in better predictions of novel therapeutic agents, including combination therapies that may be effective for the treatment of EOC in patients.

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Index

Note: The page numbers with letter 'f' denote figures and page numbers with letter 't' denote tables.

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