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Activated Epidermal Growth Factor Receptor in Ovarian Cancer

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I. Background: The EGF 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) [reviewed in 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 (Figure 1). Ligand binding promotes EGF receptor homo- and hetero-dimerization 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 (reviewed in [1–10].

Numerous ligands interact with the ErbB receptor family [reviewed in 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 cells at more distant sites following systemic distribution. In some instances, receptor activation by the precursor (membrane bound ligand) may occur as a consequence 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 homologue 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

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activity is common in solid tumors due to receptor overexpression, activating mutations or autocrine/paracrine stimulation by ligand and other mechanisms [reviewed in 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. Additional 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] may be found in a number of recent reviews.

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.

II. 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 post-ovulatory repair [23–25]. It has been proposed that EGF stimulation of the OSE contributes to its rapid post-ovulatory proliferation and to epithelial-mesenchymal transition (EMT) of OSE cells within the ruptured follicle. Malfunctions in post-ovulatory 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 10–70% of human epithelial ovarian cancer cases with an average of reported EGF receptor expression in 48% of ovarian tumors [reviewed in 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 ~10–20% 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 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 [reviewed in 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, siRNA knockdown of HB-EGF, inhibits EGF receptor tyrosine phosphorylation and 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 inhibits 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.

III. 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, histological subtype, response to treatment or overall survival [reviewed in 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 [reviewed in 5]. Technical factors inherent in immunohistochemical studies hamper efforts to identify relationships between EGF receptor expression levels and specific clinical endpoints. Notably, many studies classify tumors based simply on EGF receptor positive staining rather than compare EGF receptor expression in tumors to 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 three 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 endpoint 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 clinicopathological parameter or survival differences were noted [51]. In another study, twenty-four 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 1/3 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 endpoints are warranted.

IV. 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 including the ERK/MAPK, PI(3)K/Akt, STAT, PLC γ , STAT and other pathways [reviewed in 1–4, 8–10]. EGF increases the growth potential of primary ovarian surface epithelial (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 following 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 to tumor growth in 75% (8/12) of xenografts in sialoadenecomized 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 [reviewed in 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 three-fold 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 taxol enhanced cell death [99]. The authors hypothesize that the EGF and TKI combination downregulate 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 [reviewed in 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 post-ovulatory 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 metalloproteinases (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 to 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 [reviewed in 108,111,133].

V. EGF receptor in the ovarian tumor microenvironment

The dissemination of ovarian cancer is largely contained within the peritoneal cavity, establishing a unique microenvironmental niche comprised 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. While 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 over 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 below. 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 [reviewed in 137–139]. HB-EGF is elevated in advanced epithelial ovarian cancer tissues [41] and peritoneal fluid [43] when compared to ovarian cyst or normal controls. HB-EGF is present at higher levels than 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 non-cleavable forms of HB-EGF [42,43]. These findings illustrate that pathophysiological 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 non-receptor tyrosine kinases and/or G-protein coupled receptors (GPCRs) [reviewed in 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 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 non-receptor 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 since 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 [reviewed in 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 a ~80 kDa E-cadherin ectodomain fragment in ovarian tumor cells [62]. EGF-dependent down-regulation 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.

VI. 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 following 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 to a decrease in topoisomerase II expression levels [181]. In another study, extended treatment TGF- α promoted sequential conversion of mature astrocytes into neural progenitors and stem cells [182]. In each of these examples, the cellular responses observed following 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 1]. Other consequences include decreased expression of additional adhesion molecules (Table 1) and increased trafficking of integrin $\alpha 2$ [118,190]. Interestingly, similar changes were detected following extended ligand stimulation of the wild type EGF receptor (Figure 2). We compared short term (24–48h) with long term (36 days) EGF exposure of OVCA 433 cells. Distinct differences in response were observed for the mesenchymal markers vimentin and N-cadherin. Vimentin expression was elevated within 72h 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 (Figure 2). The migratory and fibroblastic phenotype also reverted to the epithelial morphology within 24h of EGF withdrawal following short term EGF exposure (Figure 3). After 36 days of continuous EGF exposure, both vimentin and N-cadherin were elevated and remained elevated following removal of EGF from the growth medium (Figure 2). These findings suggest that although acute signaling and downstream consequences of EGF receptor activation are largely 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* since 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, but 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.

VII. Summary and Conclusions

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 non-ligand 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 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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Figure 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.

Figure 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 72h (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 as indicated. Protein lysates were resolved by SDS-polyacrylamide gel electrophoresis and the mesenchymal markers N-cadherin and vimentin were detected by immunoblot analysis. GAPDH was used as a loading control.

Figure 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 h 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 24h, rinsed twice in phosphate-buffered saline, then returned to serum free medium. Cell phenotype was documented by phase contrast microscopy and digital imaging.

Table 10.1

Consequences of mutationally activated EGF receptor (EGFRvIII) expression in ovarian epithelial carcinoma cells.

Gene name	Function	Fold change in EGFRvIII-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 α 2	Adhesion	↓ 2.1	0.050	IB, IF [118]
Integrin β 4	Adhesion	↓ 7.8	0.001	IF
Integrin β 6	Adhesion	↓ 2.5	0.014	IF
Integrin β 8	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 Genetics, 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.