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EGF-receptor regulation of matrix metalloproteinases in epithelial ovarian carcinoma

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Abstract

Ovarian carcinoma is most frequently detected when disease has already disseminated intra-abdominally, resulting in a 5-year survival rate of less than 20% owing to complications of metastasis. Peritoneal ascites is often present, establishing a unique microenvironmental niche comprised of tumor and inflammatory cells, along with a wide range of bioactive soluble factors, several of which stimulate the EGF-receptor (EGFR). Elevated EGFR is associated with less favorable disease outcome in ovarian cancer, related in part to EGFR activation of signaling cascades that lead to enhanced matrix metalloproteinase expression and/or function. The available data suggest that modulating the expression or activity of the EGFR and/or matrix metalloproteinases offers opportunity for targeted intervention in patients with metastatic disease.

Keywords

EGF-receptor; invasion; metastasis; MMP-9; MT1-MMP/MMP-14; ovarian carcinoma

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynecologic malignancy, resulting in 15,520 deaths in the USA in 2008 [201]. When disease is confined to the ovary, 5-year survival is greater than 90%. Unfortunately, the majority of women with EOC are diagnosed with already disseminated intra-abdominal disease and have a low 5-year survival rate of less than 20%. Thus, while early detection is a pressing clinical problem, analysis of factors that promote metastatic dissemination can ultimately improve the survival of women with ovarian cancer.

Ovarian tumor microenvironment

A dualistic model for ovarian tumorigenesis has been proposed that is characterized by specific genetic alterations and unique molecular signatures [1–3]. In this classification system, low-grade carcinomas are often confined to the ovary, arise from a recognized precursor lesion,

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and have an indolent clinical course, whereas high-grade tumors are more clinically aggressive, presenting with metastases, and are associated with poor clinical outcome. In addition to analysis of genetic alterations, further investigation of contributions of the unique ovarian carcinoma microenvironment to tumor progression and metastasis may provide novel insight into factors that regulate disease progression. Women with ovarian tumors often present with diffuse and multifocal intraperitoneal metastases [4]. Unlike most solid tumors, hematogenous metastasis of EOC is uncommon. Instead, dissemination occurs as a result of shedding of malignant cells as single cells and multicellular aggregates (MCAs) (Figure 1) [9–11]. These shed tumor cells may also block peritoneal lymphatics [12], contributing to the accumulation of peritoneal ascites, which can further facilitate metastatic implantation. Single cells and MCAs adhere to peritoneal mesothelium, where upon adhesive interactions and localized proteolysis enable anchoring of proliferative secondary lesions [9,10]. An exception is found in atypical proliferative serous tumors or micropapillary proliferative serous tumors (grouped as ‘borderline’ or ‘low malignant potential’), the prognosis of which requires evaluation of peritoneal implants that often accompany these tumors. While patients with ‘noninvasive implants’ have a 10-year survival of practically 100%, a 34% mortality within 7 years is observed for those patients with invasive implants [12].

An unique microenvironmental niche is thereby established in the peritoneal cavity, comprised of tumor cells, inflammatory cells and a plethora of soluble factors secreted by – or in response to – tumor cells, including growth factors, inflammatory mediators, bioactive lipids and proteolytic enzymes [13–20]. In addition, products of matrix invasion or partially resolved fibrosis, as well as shed cell surface components such as CA125 (MUC16) and E-cadherin ectodomain [16], also accumulate in ascites. Direct contact is maintained, via ascites, among primary tumor, suspended ascitic tumor cells and peritoneally anchored metastatic cells, establishing a unique mechanism for microenvironmental regulation of all stages of tumor progression (Figure 1).

EGF-receptor activation in ovarian cancer

There are many bioactive compounds within the ovarian tumor microenvironment, including those that stimulate cell-surface receptors such as the EGF-receptor (EGFR). The EGFR is a single membrane-spanning receptor tyrosine kinase (Figure 2A) and a therapeutic target for several human tumors (reviewed in [7,8,21–26]). Ligand binding promotes EGFR homo- and heterodimerization with related ErbB family members such as ErbB2/HER-2, activation of the catalytic 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, angiogenesis and tumor metastasis [7,8,21–26].

Overall, elevated EGFR is associated with less favorable disease outcomes in a number of human tumors [5,7,8,24,26–29]. In ovarian cancer, estimates of the frequency of EGFR overexpression range from 10 to 70%, with EGFR expression reported on average in 48% of ovarian tumors (reviewed in [5]). There is no uniform agreement regarding the relationship between EGFR expression and various disease parameters, but despite differences in results from individual studies, there is evidence that dysregulation of EGFR expression or activity is a factor in ovarian cancer outcome (reviewed in [5,13]). When the EGFR level in tumors was compared with that of metaplastic ovarian surface epithelium and normal tubal epithelium as a reference control, EGFR overexpression was significantly correlated with aggressive disease characteristics [30]. In a multivariable analysis, EGFR expression status has been reported to be the most significant prognostic factor for disease-free and overall survival [31], and a meta-analysis revealed a relationship between EGFR and decreased survival [32].

There is accumulating evidence that activated (tyrosine phosphorylated, pEGFR), rather than total EGFR may be a more pertinent parameter for drawing relationships between EGFR and prognostics in human tumors [33–38]. There are a limited number of studies on 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 or survival differences were noted [39]. In another study, 24 heavily pretreated patients with epithelial ovarian cancer all had detectable EGFR and pEGFR (Y1148), suggesting that EGFR activation might be more evident in advanced disease [40]. We conducted an immunohistochemical analysis of pEGFR in microarrayed ovarian tumor tissues and found evidence for pEGFR in approximately a third of the samples (Figure 2B–D). EGFR activation (pEGFR) was positively correlated with matrix metalloproteinase (MMP)-9 expression, a protein associated with tumor invasion and metastasis [41]. Further analysis of a panel of paired primary tumors and peritoneal metastases (from the same patient) showed that approximately a third (35%) of metastases displayed elevated EGFR activation (pEGFR staining) relative to the paired primary tumor. MMP-9 expression was high in 100% of pEGFR-positive metastases [41]. Together, these *in vivo* data indicate that activated EGFR is present in ovarian tumor specimens. This is significant because EGFR activation stimulates numerous signaling cascades known to drive tumor proliferation and metastasis.

Although the exact mechanisms leading to EGFR activation in ovarian tumors are unknown at this time, three activators of the EGFR present in ascites (heparin-binding EGF [HB-EGF], endothelin-1 [ET-1] and lysophosphatidic acid [LPA]) have been studied in some detail. 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 EGFR activation. There is accumulating evidence that the EGFR ligand HB-EGF is particularly important in ovarian cancer biology (reviewed in [42–44]). HB-EGF is elevated in advanced epithelial ovarian cancer tissues [45] and peritoneal fluid [46] when compared with ovarian cyst or normal controls. HB-EGF is present at higher levels than other EGFR ligands [45,46], and HB-EGF levels are significantly correlated with clinical outcome [45]. In addition to direct EGFR activation by ligands, transactivation can occur by stimulation of nonreceptor tyrosine kinases and/or G-protein coupled receptors (GPCRs) (reviewed in [5,24,44,47–50]). Activation of GPCRs by ligands such as ET-1, LPA and others can indirectly activate the EGFR through stimulation of the ADAM family of cell surface metalloproteinases, leading to cleavage of membrane-bound EGF family precursors such as HB-EGF [44,47–50]. An alternate mechanism for EGFR transactivation by GPCRs occurs by GPCR-dependent activation of nonreceptor tyrosine kinases such as c-Src [44,47–50]. These and other findings illustrate that pathophysiological levels of EGFR activators in ovarian peritoneal fluid and ascites regulate EGFR activity, providing a mechanism for modulation of metastasis-associated genes such as matrix metalloproteinases (MMPs).

EGFR regulation of MMP-9 & -14

MMPs are a family of zinc-dependent metallo-endopeptidases with activity directed against extracellular substrates, membrane-anchored receptors and soluble proteins and polypeptides (reviewed in [51,52]). MMP family proteinases share a common domain structure including a propeptide necessary for maintaining pro-enzyme latency, the catalytic domain containing the conserved zinc-binding consensus sequence HExxHxxGxxH, and a C-terminal hemopexin-like domain important for substrate recognition. MMP activity is linked to multiple physiologic and pathologic processes, including morphogenesis, angiogenesis, wound repair, arthritis and tumor invasion and metastasis.

Several MMPs, including MMP-1 (interstitial collagenase), -2 (gelatinase A), -7 (matrilysin), -9 (gelatinase B), -13 (collagenase-3) and -14 (membrane type 1 MMP, MT1-MMP) have been

implicated in ovarian cancer pathology (reviewed in [51–53]). The soluble gelatinases MMP-2 and -9 and the membrane-anchored collagenase MT1-MMP (MMP-14) are the most extensively studied in ovarian cancer (Figure 3). High expression of these three MMPs has been detected in epithelial and stromal compartments of ovarian tumors [54]. MMP-2 and MMP-9 are present in ovarian tumor ascites [55,56]. High MMP-9 expression was associated with decreased overall survival [57,58] and high epithelial and stromal expression of MMP-2, MMP-9 and MT1-MMP were each significantly associated with shorter disease-specific survival [54]. Interestingly, expression of MMP-9, but not MMP-2, in short-term primary cultures of EOC cells derived from primary ovarian tumors, intraperitoneal metastases or ascites decreased with time in culture, suggesting that MMP-9 levels in tumor cells are regulated by the tumor microenvironment [55]. This observation is consistent with the positive correlation between EGFR activation and MMP-9 expression in ovarian tumor tissues [41] and the presence of EGFR activators in ascites fluids.

Transcriptional mechanisms

MMP gene regulation is largely due to transcriptional mechanisms, although post-transcriptional and epigenetic mechanisms also contribute to total MMP levels [59]. Regulatory sequences in the promoters of *MMP* genes are responsive to exogenous stimuli including growth factors, cytokines or bioactive lipids, such as those found in the ovarian tumor microenvironment. Signaling pathways implicated in MMP expression converge on numerous transcription regulatory sequences including AP-1, ETS, Sp1, β -catenin/TCF-4 and NF- κ B sites (reviewed in [59–61]). Increased transactivation through AP-1 or PEA3 occurs largely through mitogen-activated protein kinases (MAPKs) [59]. EGF activates the ERK, JNK and p38 MAPKs in EGF-responsive ovarian tumor cells [62], and EGFR stimulation of the ERK/MAPK pathway has been reported to activate several *MMP* genes, including *MMP-1*, *MMP-3*, *MMP-7*, *MMP-9* and *MT1-MMP* [61]. EGF stimulation also promotes activation of AKT/PKB, a downstream target of PI3K [8,23,62].

EGF induces MMP-9 in ovarian tumor cells [43,63–65], and EGF regulates MT1-MMP expression in ovarian cell lines [65] and in other cell types [66,67]. Inhibition of the MAPK or PI3K signaling cascades interfered with EGF-stimulated pro-MMP-9 production and ovarian tumor cell *in vitro* invasion, indicating that multiple EGFR-regulated kinase pathways converge to regulate *MMP-9* gene expression [62]. Interestingly, EGF does not induce MMP-9 in every ovarian tumor cell line expressing EGFR [63], suggesting that in some tumor cells signaling components downstream of the EGFR may be disrupted. Ultimately, cell- and disease-specific regulation of MMPs relies on integration of multiple signaling pathways and effective activation or recruitment of essential transcription factors.

A particular role for the ETS family transcription factor PEA-3 in EGF-dependent regulation of MMP-9 and MT1-MMP has been revealed [65]. PEA-3 is of interest because it is not detected in normal ovarian tissue, but is overexpressed in human ovarian tumors, and PEA-3 expression in ovarian tumors has been correlated with poor overall survival [68–71]. In one study, 92% of stage III and IV ovarian tumors (both primary lesions and metastases) expressed elevated PEA-3 [69], suggesting a relationship between PEA-3 and disease progression. In a variety of cell types, PEA-3 has been reported to regulate several MMPs that are implicated in cancer cell invasion, including *MMP-1*, *MMP-7*, *MMP-9*, *MMP-13* and *MT1-MMP* [61,72–77]. Notably, there is significant overlap of MMPs regulated by EGF [62,63,65] and PEA3 [73], suggesting that PEA-3 may be a downstream mediator of EGFR activation.

We find that EGFR regulates PEA-3 nuclear localization and binding to endogenous MMP-9 and MT1-MMP promoters, and disruption of PEA-3 by short interfering RNA (siRNA) disrupts EGFR-stimulated MMP expression and cell invasion. Furthermore, ectopic expression of PEA-3 alone is sufficient to induce MMP-9 and MT1-MMP, and promote an invasive

phenotype nearly equivalent to that detected in response to EGF [65]. Although EGFR regulates multiple ETS family transcription factors, there are distinctions in ETS protein activation and subsequent MMP targets in different model systems with selective regulation of MMP-9 and MT1-MMP by PEA-3 detected in ovarian tumor cells [65]. As tumor-specific changes in the transcription factor network (such as elevated PEA-3) may cooperate or synergize with EGFR signaling to regulate distinct cohorts of MMPs, further study of these potential inter-relationships in human tumors may reveal new avenues for therapeutic intervention.

EGFR regulation of MMP localization

Beyond transcriptional regulation of *MMP* gene expression, it is clear that localization of MMP activity to the cell surface contributes significantly to matrix remodeling events that occur during cellular migration and invasion. Although MMP-9 is a secreted protease, MMP-9 can also associate with the cell surface of diverse cell types ([78–84], reviewed in [85]). The association of pro-MMP-9 with the cell surface occurs by multiple mechanisms with distinct outcomes. Two proteins have been reported to mediate surface targeting ($\alpha 2$ [IV] and CD44), RECK inhibits MMP-9 and LRP directs the internalization of pro-MMP-9 [85]. In ovarian tumor cells, surface localization of pro-MMP-9 is stimulated by EGF and requires PI3K activity [62]. EGF promotes pro-MMP-9 binding to the cell surface through a mechanism that is independent of extracellular enzyme concentration. Interestingly, inhibition of PI3K activity abolishes EGF-induced cell surface association of pro-MMP-9, whereas inhibitors of MAPKs only partially block the response. These data suggest that EGF stimulation promotes the cell surface expression of an unidentified pro-MMP-9-binding component(s). As PI3K has been implicated in intracellular membrane and protein trafficking events, PI3K-mediated trafficking of pro-MMP-9 or its binding component to the cell surface may be a potential mechanism of gelatinase association.

While the function(s) of surface-localized MMP-9 in ovarian cancer remain unknown, surface localization may promote directed pericellular proteolysis. This is supported by data showing that MMP-9 is preferentially localized to the filopodial compartment in EGF-stimulated cells (Figure 4A & B), suggesting a contribution of MMP-9 to migratory and/or invasive activity. Furthermore, surface MMP-9 is associated with tumor cell migration and invasion in other model systems [80,81,83,84]. Thus, mechanisms that regulate MMP-9 expression and localization, such as EGFR activation, may play particularly important roles in the net MMP-9 activity present at the pericellular space.

While there is an expansive body of literature outlining the central role of EGFR in the architecture of intracellular signaling networks, less is known about its ability to regulate transmembrane protein trafficking. EGFR signaling may modify the molecular landscape of the cell surface by regulating the trafficking, and consequently the function, of specific cell surface proteins, highlighting an underappreciated outcome of EGFR activation. Given the central role of MT1-MMP in cancer progression and metastasis, multiple mechanisms have evolved for transcriptional and post-translational regulation of MT1-MMP enzymatic activity. Using fluorescence resonance energy transfer imaging to visualize MT1-MMP activity in live cells, EGFR activation led to localization of active MT1-MMP at the leading edge of migrating cells [86].

Additional data support an EGF-dependent effect on MT1-MMP trafficking in ovarian cancer cells, as MT1-MMP is rapidly internalized from the cell surface following EGF stimulation (Figure 4C). In contrast to EGFR, which is endocytosed via clathrin-coated pits [87], internalized MT1-MMP is localized with caveosomal markers and is disrupted by pretreatment of cells with methyl- β -cyclodextrin, which disrupts caveolae [88]. The ability of EGFR activation to affect the trafficking of multiple transmembrane proteins that occupy caveolae (i.e., $\alpha 2$ integrin [87]; MT1-MMP, Figure 4C) suggests that EGF may regulate internalization

of the caveolar compartment, and thereby alter the surface profiles of proteins occupying these domains. EGFR-dependent alterations in cell surface localization of MMP-9 and MT1-MMP may have significant consequences for tumor cell behavior and represent functions that are distinct from those of the stromally derived MMPs in ovarian cancer.

Actions of MMPs in ovarian cancer

Growth of ovarian tumors involves the formation of a provisional fibrin–fibronectin matrix around the tumor, which is subsequently replaced by a mature tumor stroma [89]. Fibrin degradation products, resulting from proteolysis of the provisional stroma, are elevated in the sera of women with advanced ovarian cancer [90,91]. In addition, polypeptides resulting from degradation of both fibrin and collagen, as well as procollagen peptides indicative of new collagen biosynthesis, are prevalent in carcinomatous ascites, as well as sera from women with ovarian cancer [92–95]. These data implicate aberrant proteolysis as a contributing factor to ovarian cancer pathobiology.

Proteolytic activity is important at several stages in intraperitoneal metastasis, including dissolution of cell–cell and cell–matrix junctions to enable shedding from the primary tumor surface, disruption of mesothelial cell junctions to facilitate access to the submesothelial matrix, intraperitoneal anchoring in the submesothelial interstitial collagen (types I and III) stroma, and for tumor angiogenesis [14,54,96–99]. MMPs have been implicated in all of these processes, and thus play a key role in ovarian cancer pathobiology (reviewed in [53]). Activity of these proteinases has been linked to key events in ovarian cancer metastasis, including detachment of primary tumor cells from the ovarian surface [100], shedding of cell–cell adhesion molecules such as the E-cadherin ectodomain [41,56], attachment to peritoneal mesothelium [14], invasion of submesothelial collagen [9–11,101–103] and removal of matrix barriers that constrain proliferation of secondary lesions [99–104]. As EGFR regulation of MMP-9 and MT1-MMP expression and/or activity has been demonstrated in ovarian cancer cells and tissues, the remaining discussion will focus on these two proteinases.

MMP-9 in ovarian cancer

Gelatinolytic soluble MMPs (MMP-2 and MMP-9) are readily detectable in ascites fluid obtained from women with ovarian cancer [41,55,105], suggesting prevalence in the ovarian tumor microenvironment. Although the cellular source of soluble MMPs in ascites cannot be determined, analysis of a panel of established ovarian cancer cell lines revealed expression of MMP-9 that was not detected in normal ovarian epithelium [106]. Further examination of MMP expression by short-term primary cultures of ovarian cancer cells showed presence of both latent and activated MMP-2 (indicative of constitutive MT1-MMP activity), while MMP-9 was detectable only in very early passage cells (passage 1–3), suggesting regulation of expression by a factor(s) in the *in vivo* microenvironment [55]. In human ovarian tumors, MMP-9 immunoreactivity is detectable in both the epithelial and stromal compartments [54, 56,107], with epithelial MMP-9 immunoreactivity increased in malignant relative to borderline ovarian tumors [107]. Ovarian tumor-associated MMP-9 activity has been detected by gelatin zymography [58,108]. Three distinct studies have localized MMP-9 mRNA expression to the epithelial compartment of human ovarian tumors using *in situ* hybridization analysis [108–110]. High MMP-9 levels in both primary tumors and ascites correlate with more malignant tumors, disease recurrence and poor survival in univariate and multivariate analyses [57,107, 110,111].

Additional *in vitro* and *in vivo* studies have provided evidence that MMP-9 activity may contribute to ovarian tumor progression. Using immunohistochemical analysis, expression of MMP-9 was shown to be elevated in preneoplastic lesions relative to normal ovarian tissue [112]. Expression was associated with loss of basement membrane and morphological

transformation, suggesting a role for MMP-9 in early events in ovarian tumorigenesis. MMP-9 induces the release of biologically active VEGF from ovarian cancer cells [113]. A reciprocal relationship between VEGF and MMP-9 has been demonstrated, as VEGF-expressing tumor xenografts stimulate MMP-9 expression [114]. Further *in vivo* studies have demonstrated that both tumor and host MMP-9 contribute to tumor angiogenesis and progressive growth [109]. In addition to promoting angiogenesis, MMP-9 has also been shown to localize to the ovarian cancer cell surface and contribute to invasive activity, as function-blocking anti-MMP-9 antibodies block *in vitro* invasion [62,63].

A recently described role for MMP-9 in ovarian tumor progression involves processing of the extracellular domain (ectodomain) of the cell–cell adhesion molecule E-cadherin. Interestingly, analysis of MMP-9 and E-cadherin levels in ovarian tumors show an increase in the MMP:E-cadherin ratio with increasing disease stage and a significantly higher death rate in women whose tumors showed a high MMP:E-cadherin ratio [115]. Both activation of the EGFR and engagement of collagen binding integrins have been shown to downregulate E-cadherin levels in ovarian cancer cells [41,56]. A common mechanism for E-cadherin downregulation downstream of integrin signaling or EGFR activation is via ectodomain shedding as a consequence of MMP-9 induction. Specific blocking of MMP-9 activity using an anticatalytic antibody or siRNA abolishes E-cadherin ectodomain shedding [41,56]. Furthermore, addition of exogenous activated MMP-9 to ovarian cancer cells at a concentration representing the average level found in human ovarian cancer ascites (90 ng/ml [41]) decreases junctional E-cadherin staining. The effect of MMP-9 on E-cadherin junctional integrity was confirmed by analysis of MMP-9 transfected ovarian cancer cells that exhibit loss of the epithelial phenotype and have no detectable E-cadherin mRNA [41]. This is supported by analysis of human ovarian tumors, showing that tumors with high MMP-9 expression exhibit low or absent E-cadherin staining [56]. The shed full-length E-cadherin ectodomain (sEcad) accumulates to high concentrations (12 µg/ml) in ascites from women with ovarian cancer, and is significantly elevated relative to ascites from women with benign gynecologic conditions [56]. In contrast to other tumors wherein shed E-cadherin ectodomain is released into the circulation, the unique microenvironment of ovarian tumors enables the primary tumor to maintain direct contact with sEcad-rich ascites, providing a physiologically relevant model to address sEcad function. Studies show that the sEcad ectodomain disrupts preformed junctions and increases cell dispersion. Together these data support a novel mechanism whereby MMP-9 may promote intraperitoneal metastatic dissemination by catalyzing E-cadherin ectodomain shedding, and sEcad-induced cellular disaggregation of cellular aggregates [41,56].

MT1-MMP in ovarian cancer

MT1-MMP (MMP-14) is a transmembrane collagenolytic MMP that is essential to matrix remodeling during physiological processes [116,117] and key to acquisition of a metastatic phenotype in a variety of tumor cells, including lung, colon, breast, cervical and ovarian carcinomas [118–121]. Ovarian carcinomas express abundant MT1-MMP [65,96,97,99,104, 110,122] and, consequently, high MT1-MMP levels correlate with poor survival of women with ovarian cancer [110]. Using both *in situ* hybridization and immunohistochemical analysis, MT1-MMP expression has been detected in both the stromal and epithelial compartments of ovarian tumors, and strong epithelial MT1-MMP is a significant predictor of poor disease-specific survival in univariate and multivariate analyses [54,104,111]. Similar results were obtained by analysis of human ovarian tumors grown in SCID mice, demonstrating enhanced human MT1-MMP in high-grade tumors with a minor contribution from the murine ovarian stroma [123]. In addition to functioning as a membrane-anchored collagenase, MT1-MMP also acts as a key physiologic activator of proMMP-2 (reviewed in [121]). Constitutive pro-MMP-2 activation has been observed in primary cultures of ovarian cancer cells, indicative of active cellular MT1-MMP [124]. This is supported by a clinical study showing that levels of active

MMP-2 are significantly higher in stage III/IV ovarian cancers relative to stage I/II tumors, and that active MMP-2 levels correlate with disease progression [125].

Numerous studies have demonstrated that acquisition of MT1-MMP expression alone promotes cell migration [126,127], invasion of 3D collagen [101,102,104,121,128,129] and growth within a matrix-constrained 3D microenvironment [100,129] – key cellular processes that drive ovarian pathology. Metastasizing ovarian cancer cells often encounter a collagen-rich environment, as the submesothelial matrix is comprised primarily of interstitial collagens (types I and III), and ovarian tumors induce a fibro-proliferative response characterized by increased synthesis of collagen in the peritoneal cavity [94,130,131]. Indeed, even the noninvasive peritoneal implants found in 30% of women with atypical proliferative serous tumors ('low malignant potential' or 'borderline') often have desmoplastic features with significant fibrosis [12]. Ovarian cancer cells adhere preferentially to interstitial collagens via cellular integrins, activating a src-dependent signaling pathway that leads to upregulation of MT1-MMP expression [104]. MT1-MMP activity is likely key for peritoneal anchoring of invasive metastatic lesions, as inhibition of this proteinase blocks penetration of 3D collagen gels [101,102,123,132]. Successful growth of invasive implants to seed intraperitoneal metastasis also requires proliferation within the confines of the interstitial collagen-rich submesothelial matrix [133–136], and the ability of ovarian cancer cells to survive long-term and proliferate in 3D collagen gels is enhanced by MT1-MMP expression [15]. This observation is supported by data showing that expression of MT1-MMP in paired peritoneal metastases is greater than or equal to that of the primary tumor in more than 80% of cases [15].

In addition to its fairly well-described role in matrix invasion and expansive 3D growth, recent data support a novel role for MT1-MMP in ovarian cancer metastasis. An early event in the initial dissemination of ovarian cancer involves exfoliation of cells from the primary ovarian tumor into the peritoneal cavity as single cells or multicellular aggregates (MCAs) that range in size from 30 to 200 μm , survive anoikis, and form a free-floating population of highly malignant cells [9,10,13,137–139]. MCAs express higher levels of MT1-MMP relative to two-dimensional cultured ovarian cancer cells, and acquisition of MT1-MMP expression promotes cellular detachment and MCA formation [100]. Together these data suggest MT1-MMP activity is necessary to promote metastasis of primary ovarian tumor cells via enhanced tumor cell shedding as MCAs, as well as for subsequent intraperitoneal anchoring of malignant MCAs and expansive growth in the submesothelial matrix.

Conclusion & future perspective: opportunities for targeted intervention

As discussed in this review, the unique microenvironmental niche of ovarian cancer is rich in activators of the EGFR; EGFR activation stimulates signaling cascades that lead to increased expression and altered localization of certain MMPs such as MMP-9 and MT1-MMP, and MMPs contribute to ovarian tumor pathobiology. It then follows that modulating the expression or activity of the EGFR and/or MMPs offers an opportunity for targeted intervention in patients with metastatic disease (Figure 1). Metastatic processes have been proposed to offer new therapeutic targets based on blocking metastatic dissemination or disruption of reciprocal interactions between tumor cells and the microenvironment [140]. Interventions directed to the EGFR or MMPs fit these criteria (Figure 1). Based on the known consequences of EGFR activation in ovarian tumor cells and pre-clinical studies of EGFR antagonists, inhibition is predicted to confer benefit due to modulation of cell proliferation and survival, chemoresistance, epithelial–mesenchymal transition, migration and invasion (Figure 1) [13, 25]. Similarly, selective targeting of specific MMPs may reduce tumor cell shedding, decrease proliferation and angiogenesis through inhibition of receptor ligand shedding, alter cell:cell

and cell:matrix adhesive properties, inhibit epithelial–mesenchymal transition by reducing E-cadherin cleavage and decrease tumor cell invasion (Figure 1) [13,51,141–143].

Despite the recognized potential of these targets, the current status of MMP inhibitors and EGFR antagonists in cancer treatment is mixed. Preclinical trials of the broad-spectrum MMP inhibitor batimastat in mice harboring ovarian cancer xenografts led to solidification of the tumor, resolution of ascitic disease and increased survival [144]. In Phase I/II trials of a related compound, marimastat, ovarian cancer patients were monitored for a change in the rate of rise of the serum tumor marker CA-125 to assess drug effect. Although a promising decrease in the CA-125 detection rate was observed, a definitive correlation with drug efficacy can only be determined through Phase III trials [145–147]. Overall, clinical trials of broad-spectrum MMP inhibitors failed to provide a significant survival advantage in a variety of advanced stage cancers and were accompanied by significant side effects [142,143]. Several reasons have been offered to account for these clinical failures. One issue was a lack of sufficient information on the beneficial roles of specific MMPs in normal physiology, thereby contributing to the observed toxicities of broad-spectrum inhibitors [142]. New research suggests that MMPs have activities throughout cancer progression, so clinical trials focused solely on advanced disease may not have provided optimal benefit [51,142,143]. Furthermore, although MMPs play important roles in metastatic disease [51,148], MMPs also display protective effects in certain stages of tumor progression [142,143]. Many researchers now postulate that more selective targeting of specific MMPs based on validation of their substrates, coupled with a better understanding of the functions and contributions of individual MMPs to distinct stages of cancer progression, may result in more favorable outcomes with the next generations of MMP inhibitors.

Several EGFR-targeted therapeutics, including monoclonal antibodies (cetuximab and panitumumab), and small-molecule tyrosine kinase inhibitors (TKIs) (gefitinib, erlotinib and lapatinib) have been approved by the US FDA for cancer therapy. Additional agents are in development and being evaluated in clinical trials [25,26,149]. EGFR antagonists (TKI or monoclonal antibody) provide clear clinical benefit in certain cancers, but clinical trial results in ovarian tumor patients have been disappointing. The outcomes of ovarian cancer trials are summarized in a recent review [150], but briefly, results from Phase II trials of EGFR-targeted therapeutics suggest that their activity as single agents is modest in unselected women with advanced or recurrent ovarian cancer. In general, antitumor activity against platinum- and taxane-resistant ovarian cancers may be best characterized as cytostatic rather than cytotoxic [150].

As with MMP inhibitors, there are many likely reasons for these findings. First, EGFR inhibitors are effective in only a subset of patients for the best-studied tumors. Approximately 10–15% of patients with non-small-cell lung cancer have tumors that express activating *EGFR* mutations, and these patients are often highly responsive to treatment with erlotinib or gefitinib [25,26,149]. Interestingly, in one study of gefitinib in patients with recurrent or persistent epithelial ovarian cancer, the only objective response occurred in a patient with an *EGFR* mutation similar to those detected in non-small-cell lung cancer [151]. Another factor predictive of positive response is increased *EGFR* gene copy number [26,149]. Although estimates of increased EGFR protein expression in ovarian cancer exceed 50% on average [5], recent studies of *EGFR* gene copy number are significantly lower, ranging from 10–22% of ovarian cancer cases [30,152,153], suggesting that anti-EGFR therapies may be effective in a more select number of ovarian cancer cases. As with non-small-cell lung cancer, it will be important to validate predictive markers of patient response and identify ovarian cancer patients with tumors most likely to respond to EGFR antagonists [25,150,154]. This type of preselection is standard in breast cancer, for example, where the estrogen receptor status of a tumor plays a major role in the therapeutic decision-making strategy.

A second concern is that the doses of EGFR-based therapeutics may not optimally block EGFR signaling. In one study of gefitinib-treated patients with recurrent epithelial ovarian cancer, analysis of pre- and post-treatment tumor biopsies revealed that although a decrease in EGFR and pEGFR was evident in somewhat more than 50% of patients, detectable levels of pEGFR were present in all tumor biopsies regardless of treatment [40]. Especially under circumstances of elevated EGFR, partial inhibition may result in sufficient residual receptor activity to stimulate key signal transduction pathways associated with tumor growth, survival and metastasis. The development of imaging technologies for activated forms of the EGFR may help to facilitate patient selection and monitoring of patient response to EGFR signaling inhibitors, leading to more successful treatment choices [155]. Thirdly, in advanced cancers, other mutations that bypass or compensate for the EGFR may be present and have a negative impact on the success of EGFR-targeted therapies [26,145,150].

Despite the current limitations acknowledged previously, as we increase our understanding of the EGFR and/or MMPs in ovarian cancer, additional opportunities for targeted therapy will likely emerge. The presence of EGFR activators in the ovarian tumor microenvironment predicts that dual targeting of EGFR and EGFR activators such as HB-EGF or GPCRs would lead to more complete inhibition of EGFR signaling compared with monoclonal antibodies or TKIs alone. Targeting HB-EGF in preclinical *in vivo* models decreases peritoneal dissemination of ovarian tumor cell xenografts [42,156], suggesting that combinations of HB-EGF inhibitors and EGFR antagonists may improve outcomes. Similarly, ET-1 binding to its receptor transactivates the EGFR, and coadministration of an endothelin A receptor antagonist enhanced the antitumor effects of gefitinib in an ovarian tumor xenograft model [157]. Combined targeting of EGFR and specific MMPs also represent plausible approaches. The ADAM family of metalloproteinases is responsible for the ectodomain shedding of the ligands that are involved in direct (HB-EGF) and trans-EGFR activation, supporting the potential of these proteinases as novel antitumor targets [141]. EGFR activation promotes angiogenesis, and MMP-9 is a critical proangiogenic molecule [51,142], suggesting that dual inhibition of EGFR and select MMPs could be beneficial in advanced disease. Indeed, therapies targeted to EGFR and angiogenesis based on combining two agents highly selective against VEGF and EGFR, respectively, or a single dual targeted agent are under active investigation in many cancers [158]. The selection of optimal therapeutic combinations based on sound research holds potential to target metastatic disease and ultimately improve the efficacy of current treatment protocols [140]. Identification and validation of effective targets for management of metastatic disease would extend the future options for women with ovarian cancer [140,159].

Executive summary

Ovarian tumor microenvironment

- The peritoneal cavity provides a unique microenvironmental niche that facilitates direct contact between primary tumor, suspended ascitic tumor cells and anchored metastatic cells via carcinomatous ascites.

EGFR activation in ovarian cancer

- EGF-receptor (EGFR) is expressed in 48% of ovarian tumors; activated EGFR is present in 10–35% of ovarian tumors.
- Multiple EGFR ligands are present in ascites.
- In addition to ligand activation, EGFR transactivation is stimulated by ascites components.

EGFR regulation of MMP-9 & -14

- EGFR activation regulates matrix metalloproteinase (MMP) expression through transcriptional activation.
- The ETS family transcription factor PEA-3 is implicated in EGF-dependent MMP regulation, and PEA-3 expression is correlated with disease progression.
- Nontranscriptional mechanisms of MMP regulation occur via modulation of pericellular localization of transmembrane (MT1-MMP) or membrane-associated (MMP-9) proteinases.

Actions of MMPs in ovarian cancer

- MMPs participate in degradation of the provisional tumor stroma, dissolution of cell–cell and cell–matrix junctions to promote shedding of cells from primary tumor, intraperitoneal anchoring of metastatic cells and tumor angiogenesis.
- MMP-9 releases biologically active components, including VEGF and E-cadherin ectodomain, into ascites.
- MT1-MMP participates in initial shedding, intraperitoneal anchoring and regulation of expansive growth in the submesothelial matrix.

Opportunities for targeted intervention

- EGFR activation leads to increased expression and altered localization of MMPs, and MMPs contribute to ovarian tumor pathobiology.
- Modulation of the expression or activity of EGFR and/or MMPs offers opportunities for targeted intervention in metastatic disease processes.
- Inhibition of EGFR and/or MMP activity may impact cell shedding, cell proliferation and survival, chemoresistance, epithelial–mesenchymal transition, migration/invasion and angiogenesis.
- More selective MMP inhibitors may promote more favorable outcomes.
- Patient preselection, to identify women with activated EGFR and/or high MMP levels, may enhance therapeutic efficacy.
- Currently used doses of EGFR-based therapeutics may be insufficient to block EGFR signaling.
- Optimal therapeutic combinations hold potential to target metastatic disease.

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Figure 1. Ovarian tumor microenvironment

(A) Malignant transformation and proliferation of OSE cells forms the primary ovarian tumor. **(B)** Ovarian cancer metastasis occurs by direct extension, via shedding of malignant cells into the peritoneal cavity. Exfoliated cells exist as MCAs or individual cells. Malignant ascites is common and is believed to promote dissemination of tumor cells intraperitoneally. **(C)** Intraperitoneal adhesion and localized invasion anchors secondary lesions in the peritoneum and omentum, followed by proliferation to establish disseminated intraperitoneal metastases. EMT: Epithelial–mesenchymal transition; MCA: Multicellular aggregates; OSE: Ovarian surface epithelium.

Figure 2. Activated EGF-receptor in ovarian tumors

(A) Model of the EGFR. 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 that contains the autophosphorylation sites. The EGFR dimerizes with other ErbB receptors. **(B–E)** Immunohistochemical staining for activated (phospho-)EGFR in ovarian tumors. Immunohistochemical analysis was performed retrospectively as described in [41], using antibodies to phospho-EGFR (1:400, Zymed®, CA, USA) according to standard procedures. EGFR activation (phospho-EGFR) was evident in 35% of the specimens analyzed (n = 146) and MMP-9 expression was statistically positively correlated with EGFR activation [41]. **(B)** Ovarian clear cell carcinoma; **(C)** mucinous ovarian carcinoma; **(D)** endometrioid ovarian carcinoma; **(E)** serous ovarian carcinoma. EGFR: EGF-receptor.

Figure 3. MMP domain structure

(A) MT1-MMP (MMP-14) is a transmembrane protease comprised of a pro-peptide (processed intracellularly by furin in the secretory pathway; not shown) and a catalytic domain containing the Zn^{2+} -binding consensus sequence HExxHxxGxxH . A flexible hinge region connects the catalytic domain to the hemopexin-like domain. The hemopexin-like domain may contain a dimerization interface and is important in substrate recognition. Following this region, a short stalk connects the transmembrane domain to the short cytoplasmic tail. In addition to degrading protein substrates such as interstitial (type I) collagen, MT1-MMP also catalyzes activation of pro-MMP-2. This reaction proceeds via an unusual mechanism requiring a trimeric complex among MT1-MMP, TIMP-2 and pro-MMP-2. Anchoring of pro-MMP-2 via trimeric complex formation enables cleavage of the pro-peptide domain of pro-MMP-2 by a second molecule of MT1-MMP, releasing soluble active MMP-2. (B) MMP-9 is initially secreted in zymogen form. Proteolytic processing of the pro-peptide region, catalyzed by numerous proteinases in the extracellular milieu, exposes the active Zn^{2+} -binding catalytic domain. The active site of MMP-9 is also connected via a flexible hinge to a hemopexin-domain that imparts substrate specificity.

MMP: Matrix metalloproteinase; MT1: Membrane type 1; TIMP-2: Tissue inhibitor of metalloproteinase-2.

Figure 4. EGF-receptor activation regulates matrix metalloproteinase localization

(A & B) Analysis of MMP-9 in filopodia of EGF-stimulated cells. OVCA429 cells (1×10^6) were serum-starved overnight and plated onto six-well plates containing 1.0 μ m pore size membrane inserts. Each bottom chamber contained 2.5 ml of serum-free medium containing EGF. Following a 12-h incubation (empirically determined based on the lack of visible nuclei on the filter bottom), lysates were individually collected from the top chamber (cellular fraction) or the bottom chamber (filopodial fraction). Lysates were pooled from replicate chambers, normalized for protein concentration, and an equal amount (40 μ g) of filopodial or total cellular protein was evaluated by gelatin zymography or western blotting, as indicated. Note that MMP-9 protein and activity are enriched in the filopodial pool following EGF treatment. β 1 integrin is also concentrated in filopodia, while GAPDH is evenly distributed.

(C) Cells expressing GFP-tagged MT1-MMP were seeded onto 35 mm Becton glass bottom dishes and serum-starved overnight prior to treatment with EGF (25 nM). Images were acquired at the indicated time points using a Nikon TE2000 inverted microscope (Nikon Inc., NY, USA) with a 60× oil immersion objective with numerical aperture 1.40 and analyzed using Metamorph Software (Molecular Devices, PA, USA). Note that EGF induces rapid internalization of MT1-MMP.

C: Cellular fraction; F: Filopodial fraction; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GFP: Green fluorescent protein; MMP: Matrix metalloproteinase; MT1: Membrane type 1.