The importance of endothelin axis in initiation, progression, and therapy of ovarian cancer

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Rosanò L, Spinella F, Bagnato A. The importance of endothelin axis in initiation, progression, and therapy of ovarian cancer. Am J Physiol Regul Integr Comp Physiol 299: R395–R404, 2010. First published June 10, 2010; doi:10.1152/ajpregu.00304.2010.—The endothelin-1 (ET-1)/ET A receptor (ET_{AR}) axis is involved in the pathobiology of different tumors, including ovarian carcinoma. Acting selectively on ET_{AR}, ET-1 regulates, through multiple signaling pathways, mitogenesis, cell survival, angiogenesis, lymphangiogenesis, invasion, and metastatic dissemination. Moreover, ET-1/ET_{AR} axis appears to be critical in epithelial-to-mesenchymal transition (EMT), providing a mechanism of escape to a new, less adverse niche, in which resistance to apoptosis ensures cell survival in conditions of stress in the primary tumor, and acquisition of “stemness” ensures generation of the critical mass required for tumor progression. Emerging experimental and preclinical data demonstrate that interfering with ET_{AR} pathways provides an opportunity for the development of new mechanism-based antitumor strategies by using ET_{AR} antagonists alone and in combination with cytotoxic drugs or molecular inhibitors. A specific ET_{AR} antagonist in combination with standard chemotherapy is currently evaluated in clinical and translational studies to provide us with new options to treat ovarian cancer and to predict response to therapy. Deeper understanding of molecular mechanism activated by ET_{AR} in ovarian cancer will be of paramount importance in the study of ET_{AR}-targeted therapy that, regulating EMT and other tumor-associated processes, represents an attractive but challenging approach to improve clinical management of ovarian cancer.

endothelin A receptor; ovarian cancer; targeted therapy

CANCER THAT ARISES from the ovarian surface epithelium accounts for ~90% of human ovarian cancer. This malignancy represents the leading cause of death from gynecological cancers and is a highly metastatic disease characterized by widespread peritoneal dissemination and ascites. Because treatment of patients in advanced stages is still penalized by low survival rates, the development of new treatment protocols depends on improved knowledge of the molecular mechanisms controlling tumor growth and progression (40). In the microecology of the tumor-host invasion field, growth factor exchange between the participating cells stimulates migration, invasiveness, neovascularization, and promotes proliferation and survival. Among these, endothelins are an example of such mediators. The endothelins (ETs), which include three 21-aa peptides, ET-1, ET-2, and ET-3, are ubiquitously expressed and are produced by endothelial cells and many epithelial cell types (38). ETs exert their effects by binding to two distinct cell surface ET receptors, ET_{A} and ET_{B}. While the ET_{B} receptor (ET_{BR}) has equal affinities for all three peptides, ET_{A} receptor (ET_{AR}) shows subnanomolar affinity for ET-1 and ET-2 and 100-fold lower affinity for ET-3. Both receptors belong to the G protein-coupled receptor (GPCR) family, mediate pleiotropic actions of ETs, and are distributed in a variety of cells and tissues in different proportions, suggesting a potentially opposite regulatory function (38).

ET-1 represents the most potent and long-lasting vasoconstrictor factor released by endothelial cells (79). ET-1, ET-2, and ET-3, characterized by a single α-helix and two disulfide bridges, are encoded by distinct genes and are regulated at transcriptional and post-transcriptional level. The primary translation product of the ET-1 gene is the 212-aa prepro-ET-1, which is cleaved by an endopeptidase to form the 38-aa big-ET-1. ET-1 is synthesized from big-ET-1 by an unusual hydrolysis of the Trp21-Val22 bond by the endothelin-converting enzyme (ECE) (78). The half-life of ET-1 in circulation is 7 min (58). Two pathways have been described for clearance of ET-1:ET_{B} receptor-mediated uptake followed by lysosomal degradation (15) and catabolism by extracellular neutral endopeptidase 24.11 (NEP, nephrilysin) (14, 75). ET-1 production is dynamically regulated throughout the reproductive cycle, fur-
ther supporting their physiological relevance (39). In particular, ET-1 inhibits premature luteinization of granulosa cells and promotes corpus luteum regression, whereas ET-2 affects follicular rupture and corpus luteum development, suggesting that these two closely related peptides, ET-1 and ET-2, are expressed by different types of cells, at specific stages of the reproductive cycle. Moreover, the corpus luteum, a highly vascular tissue constituted for 50% by endothelial cells, represents an important site for ET-1 synthesis, in which its level is hormonally regulated in the reproductive cycle, participating in structural luteolysis (39).

Regarding ET-1 receptors, it is well documented that human granulosa cells predominantly express the ETAR subtype, although both receptors are expressed in ovaries of many mammalian species, and there are species differences in the prevalence of ETAR vs. ETBR in ovarian cells (39).

ET-1 has been implicated in the pathobiology of a wide range of human tumors, including ovarian carcinoma, exerting pleiotropic effects, including mitogenic effects on tumor and stromal cells, cell survival, angiogenesis, and lymphangiogenesis, invasion and metastasis, modulation of tumor-infiltrating immune cells, bone formation, and stimulation of nociceptor pathways, such as agonist dose and structure, receptor clustering, and perhaps the prevalence of downstream signaling components, can switch the signal from a G protein-dependent to G protein-independent signaling (76). β-arrestins can work in opposition or in synergy with the G protein signal. In other cases, β-arrestins and G proteins can activate the same downstream enzyme, but through different mechanisms, leading to distinct cellular outcomes. The various functions of GPCR are often mediated by the ability of β-arrestin to serve as a signal transducer, bringing elements of diverse signaling pathways into proximity, thereby facilitating their activation, which lead to MAPK activation, DNA synthesis, protein translation, and cell migration (12). This new paradigm for understanding the unrecognized signaling properties of the β-arrestin has been recently explored in ovarian cancer cells, expressing endogenous levels of ETAR (45, 46). In these cells upon ET-1 stimulation, β-arrestin is recruited to ETAR to form a trimeric complex with Src. The association of ETAR/β-arrestin/c-Src signaling complex or "signalplex" leads to EGFR transactivation and downstream activation of Akt and MAPK.

ET-1 Axis in Ovarian Tumor Biology

The in vivo analysis of ET-1 axis expression levels demonstrated a higher expression of ET-1 and ETAR in primary and metastatic tumors than in normal ovarian tissues. Interestingly, ET-1-producing cells also expressed functional ETAR, but not ETBR, indicating that in ovarian tumor cells, ET-1 acts as an autocrine factor selectively through the ETAR (9). In human tumors, ET-1 axis expression is associated with ascites formation, malignant progression, advanced tumor stages, and degree of tumor angiogenesis (8).

Signaling Pathways Activated By ET-1

The intracellular signaling pathways activated by ET-1 after engagement of ETAR were originally identified in the activation of a pertussis toxin-insensitive G protein that stimulates phospholipase C activity and increases intracellular Ca2+ levels, activation of PKC, and MAPK.

Signaling pathways do not act in isolation, but there is cross-talk with each other, forming a complex signaling network regulating multiple pathobiological tumor functions. Recent findings clearly indicate that ETAR is also involved in cross-pathway events with the tyrosine kinase receptor, such as epidermal growth factor receptor (EGFR) transactivation, as the mechanism capable of expanding the cellular communication signaling network. In ovarian cancer cells, ET-1 causes EGFR transactivation that leads, through the formation of Shc/Grb-2 complexes, to activation of the ras/MAPK pathway (3, 11, 41, 74). In addition, ET-1 binding to the ETAR results in p125 focal adhesion kinase (FAK) and paxillin activation, which are thought to transduce signals involved in tumor cell invasion. Furthermore, ETAR activation triggers the apoptotic signaling through phosphatidylinositol 3-kinase (PI3-K)-mediated Akt pathways (20, 21), indicating the existence of multiple signal transduction pathways downstream to ETAR activation in ET-1-stimulated ovarian cancer cells involved in tumorigenic events.

However, these ETAR-generating pathways alone may not be able to explain the diversity of effects stimulated by ET-1 through ETAR. Recent studies suggest that a single GPCR can couple to multiple G proteins (G protein-dependent), as well as to other adaptor proteins (G protein-independent) (19). A primary transducer of G protein-independent signaling appears to be the β-arrestin family. These proteins, β-arrestin-1 and β-arrestin-2, were originally identified as terminators of heterotrimeric G protein coupling and mediators of endocytosis, but they were later shown to serve as scaffolds linking receptor activation to a variety of signaling cascades (37). There is now a large body of work demonstrating that various parameters, such as agonist dose and structure, receptor clustering, and perhaps the prevalence of downstream signaling components, can switch the signal from a G protein-dependent to G protein-independent signaling (76). β-arrestins can work in opposition or in synergy with the G protein signal. In other cases, β-arrestins and G proteins can activate the same downstream enzyme, but through different mechanisms, leading to distinct cellular outcomes. The various functions of GPCR are often mediated by the ability of β-arrestin to serve as a signal transducer, bringing elements of diverse signaling pathways into proximity, thereby facilitating their activation, which lead to MAPK activation, DNA synthesis, protein translation, and cell migration (12). This new paradigm for understanding the unrecognized signaling properties of the β-arrestin has been recently explored in ovarian cancer cells, expressing endogenous levels of ETAR (45, 46). In these cells upon ET-1 stimulation, β-arrestin is recruited to ETAR to form a trimeric complex with Src. The association of ETAR/β-arrestin/c-Src signaling complex or "signalplex" leads to EGFR transactivation and downstream activation of Akt and MAPK.
All of these findings suggest that specific ET\(_{\alpha}\)R antagonists may, therefore, provide an additional approach to the treatment of ovarian carcinoma, in which ET\(_{\alpha}\)R blockade could result in tumor inhibition by reducing tumor growth, as well as by inducing apoptosis.

**ET-1 axis in tumor neovascularization.** Angiogenesis is controlled by different regulators, including local hypoxia, which activate the expression of angiogenic factors that can stimulate endothelial cell growth (16). During the formation of new blood vessels, endothelial cells are stimulated to release protease, such as matrix metalloproteinases-2 (MMP-2), migrate, proliferate, and invade surrounding tissues to form capillaries. ET-1, mainly by ET\(_{\alpha}\)R, induces these angiogenic effects in vitro and in vivo and, in concert with vascular endothelial growth factor (VEGF), displays a potent additive effect on the different stages of the angiogenic process (6, 61). Although ET-1 directly modulates angiogenesis, it can also act indirectly through the induction of major angiogenic factors, such as VEGF. In ovarian cancers, elevated expression of ET-1 and its cognate receptor is significantly associated with expression of VEGF, its receptors (KDR and flt-1), and tumor-induced vascularization (59), indicating that ET-1 and VEGF might have complementary and coordinated role during neovascularization in this tumor. Thus, in ovarian carcinoma cells, ET-1 through ET\(_{\alpha}\)R increases VEGF expression and secretion and does so to a greater extent during hypoxia (64). Transcriptional upregulation has an important role in the induction of VEGF expression, and this has been linked to a critical mediator of hypoxia signaling, the hypoxia-inducible factor 1\(\alpha\) (HIF-1\(\alpha\)) transcription factor (66). Similar to hypoxia, ET-1 promotes VEGF production through HIF-1\(\alpha\). After ET\(_{\alpha}\)R activation by ET-1, HIF-1\(\alpha\) protein levels are increased and stabilized, leading to the formation of HIF-1\(\alpha\) transcription complex that binds to the hypoxia-responsive element binding sites. Therefore, ET-1/ET\(_{\alpha}\)R-induced HIF-1\(\alpha\) accumulation in ovarian carcinoma cells might be responsible for increasing VEGF-mediated angiogenesis (6, 66).

Different works using cell-specific gene knockout and transgenic animals elucidated specific temporal and spatial relationships between PGs and their rate-limiting enzymes cyclooxygenase (COX)-1 and -2 and ovarian cancer progression (22, 25, 26, 30) and defined the role of these enzymes also in tumor angiogenesis at multiple steps both directly and indirectly (72). First, COX upregulation leads to production of PGs that have distinct roles for angiogenesis. Second, overexpression of COX in tumor cells directly stimulates the production of angiogenic factors from these cells, such as VEGF, PDGF, basic fibroblast growth factor, and TGF-\(\beta\). In ovarian carcinoma cells, ET-1 significantly increases the expression of COX-1 and -2, COX-2 promoter activity, PGE\(_2\) and VEGF production. These effects depend on multiple MAPK signal pathways, including p42/44 MAPK, p38 MAPK, and transactivation of the EGFR (67, 69, 70). COX-2 and -1 inhibitors blocked ET-1-induced PGE\(_2\) and VEGF release, MMP activation and cell invasion, demonstrating that both enzymes function as downstream mediators of ET-induced angiogenic and invasive properties (67).

**ET-1 axis in lymphangiogenesis.** Expression of ET-1 axis has been correlated with increased lymphatic dissemination (77), suggesting that ET-1 axis may be involved also in lymphangiogenesis. Lymphatic endothelial cells (LEC) produce ET-1 and ET-3, and express ET\(_{\alpha}\)R. In these cells, ET-1 promotes proliferation, MMP activation, invasiveness, vascular-like structure formation, and phosphorylation of AKT and p42/44 MAPK through ET\(_{\alpha}\)R (62, 63). ET-1 axis regulates lymphangiogenesis also by an indirect mechanism, as demonstrated by the capacity of ET-1 to increase the expression of the selective lymphangiogenic factor VEGF-C and its receptor, VEGFR-3 and VEGF-A, and to stimulate HIF-1\(\alpha\) expression similarly to hypoxia. Moreover, HIF-1\(\alpha\) silencing desensitizes VEGF-C and VEGF-A production in response to ET-1 or hypoxia, implicating HIF-1\(\alpha\)/VEGF as downstream signaling molecules of ET-1 axis. Double immunofluorescence analysis of human lymph nodes reveals that lymphatic vessels express ET\(_{\alpha}\)R together with the lymphatic marker podoplanin, indicating that ET\(_{\alpha}\)R is expressed in lymphatic vessels in vivo. Furthermore, a Matrigel plug assay shows that ET-1 promotes the outgrowth of lymphatic vessels in vivo. Functional assays performed by using intradermal lymphangiography demonstrated that ET-1 promoted the formation of lymphatic vessels and that these vessels were capable of lymphatic flow (63). ET\(_{\alpha}\)R blockade with the specific antagonist inhibits in vitro and in vivo ET-1-induced effects, demonstrating that ET\(_{\alpha}\)R is involved in the regulation of the growth and in the formation of functional vessels upon activation by ET-1 and that interacting with the HIF-1\(\alpha\)-dependent machinery, can amplify the VEGF-mediated lymphatic vascularization (62, 63).

In view of the correlation between tumor expression of ET-1 axis and lymphatic metastasis (77) together with the recent gene expression profile identifying ET-1 as one of the significantly upregulated genes in LEC isolated from metastatic lymph node (17), these results raise the possibility that ET-1 contributes to tumor progression by promoting hypoxia-mediated lymphangiogenic signaling disclosing a yet unidentified regulatory mechanism, which relies on the involvement of tumor microenvironment. Therefore, targeting ET-1 axis represents a potential strategy in the treatment of lymphatic associated diseases, as well as metastasis, because the capacity to block lymphangiogenesis and angiogenesis that represent important routes for the metastatic spread of cancer cells.

**ET-1 axis in the regulation of tumor proteinases.** High levels of ET-1 are present in the majority of ascitic fluids of ovarian cancer patients, suggesting that ET-1 could participate in the progression and invasion of ovarian carcinoma (59). Moreover, in several ovarian carcinoma cells, ET-1 acting through the ET\(_{\alpha}\)R consistently induced the activation of the two families of metastasis-related proteinases, the MMPs, and the urokinase type plasminogen activator (uPA) system at several levels: mRNA transcription, zymogen secretion, and proenzyme activation. ET-1, in fact, activates MMP-2, MMP-9, MMP-3, MMP-7, and MMP-13. In addition to soluble MMPs, ET-1 enhances the activation of membrane type 1-MMP (MT1-MMP) and the secretion of tissue inhibitor of MMP (TIMP-1 and -2), increasing the net MMP/TIMP balance and gelatinolytic activity, which causes rapid degradation of the extracellular matrix (ECM). Moreover, coinduction of uPA system by the concomitant stimulation of production and secretion of uPA and uPAR, and MMPs by ET-1 caused the highest invasive potential of tumor cells (36, 37). ET\(_{\alpha}\)R antagonists inhibit cell migration, invasion, and possibly other FAK-associated processes, which also contribute to aggressiveness and metastasis of this tumor (10, 51, 53).
ET-1 axis in the regulation of integrins. ET-1 enhances the adhesion of ovarian cancer cells on the collagen via upregulation of α5β1 and α6β1 integrins. Interaction of β1 integrin with collagen, which represents the unique protein composition of the mesothelial ECM, increases integrin-linked kinase (ILK) activity, and ET-1 may mimic this signal and synergize with β1 integrin to activate ILK (31, 53). Furthermore, transfection with dominant-negative ILK and ILK small-molecule inhibitor have revealed the critical role of ILK in the stimulation of phosphorylation of GSK-3β and Akt, the major downstream components of ETAR-mediated ILK signaling pathways. ILK activation is implicated in ET-1-enhanced migratory and invasive ability of ovarian cancer cells, which correlates with the increased secretion and activation of tumor-associated MMP-2 and MMP-9. These findings clearly identify ET-1 as a critical upstream mediator of ILK activation through the capacity to upregulate its expression and activity at different levels. Recent studies on integrin outside-in signaling indicate that upon ligation of β1 integrin by ECM, ILK is recruited into the β1 integrin-associated focal adhesion complex, thereby activating Akt (31). Because the activity of ILK induced by ET-1 is more effective during ovarian cancer cell spread and adhesion on type I collagen, it is likely that ET-1-mediated stimulation of ILK potentiates β1-integrin, signaling amplifying ILK activity. The β1 integrin-mediated activation of ILK by ET-1, therefore, points to a complex mechanism through which integrins and growth factors could synergize to expand the cellular communication signaling network leading to metastatic dissemination of ovarian carcinoma cells.

ET-1 axis in the regulation of intercellular communications. Following malignant transformation, stepwise changes in intercellular communication enable tumor cells to escape microenvironmental control from the normal surrounding tissue, thus promoting local invasiveness and metastatic spread. Human ovarian surface epithelial cells exhibit extensive gap junction intercellular communications (GJIC) and expression of different types of connexin (Cx), predominantly Cx43. Defects in intercellular communication, including reduced or inappropriate expression of Cx43, have emerged as key factors in ovarian carcinoma progression (73). In ovarian carcinoma cells, ET-1/ETAR axis induces transient and a dose-dependent reduction of GJIC (50–75%) and phosphorylation of Cx43 through Src pathway, indicating that ET-1 promotes cellular uncoupling at the level of connexin maturation and subsequent degradation (68). The capacity of ET-1 to disrupt gap junctions could serve as a basis to further evaluate the cell-cell metabolic uncoupling and cell detachment that occurs during tumor progression, and underlines the overall relevance of ETAR in regulating the complex array of cell-cell or cell-matrix interactions that promote ovarian cancer cell spreading.

ET-1 axis in epithelial-to-mesenchymal transition. One hallmark of epithelial cancer progression is epithelial-to-mesenchymal transition (EMT), in which tumor cells undergo loss of polarity and cell-cell junctions, acquire a mesenchymal phenotype, the ability to invade the extracellular matrix, and to migrate to distant sites (71). These changes, which enable tumor cells to overcome microenvironmental control from the host and to invade and metastasize, are characterized by disassembling of GJIC, tight junctions, and adherent junctions, reorganization of cell substrate adhesion complexes, loss of cell polarity, and significant remodeling of the cytoskeleton. A primary event that governs EMT is the disruption of the E-cadherin-mediated stable interactions between the cells (71).

E-cadherin is reduced in many advanced carcinomas, confirming the paradigm of EMT as an integral component of the acquisition of the invasive phenotype (1, 2, 24). Moreover, the higher immunoreactivity for E-cadherin and α-, β-, γ-catenin in the metastatic lesions compared with the respective primary ovarian tumors, indicates that E-cadherin downregulation is a dynamic event that is required during the initial invasion stage (33).

Loss of E-cadherin gene expression is mainly due to upregulation of the transcription factor Snail, a zinc finger protein that represses E-cadherin by binding the E-boxes present in its promoter. Increased expression of Snail has been correlated with loss of E-cadherin expression in vitro and in vivo (42).

In ovarian carcinoma cells, activation of the ETAR pathway by ET-1 contributes to disruption of normal host-tumor interactions by downregulating the expression of E-cadherin and associated β-catenin protein and concomitant upregulation of the mesenchymal N-cadherin. Sustained ETAR signaling caused by an autocrine ET-1/ETAR loop is required for the maintenance of E-cells in these cells, as shown by spindle-shaped and motile fibroblastoid phenotype. Interestingly, a large percentage of cells reverted to an epithelial phenotype in which the cells formed compact structures in association with repression of N-cadherin and vimentin, regained expression of endogenous E-cadherin, and β-catenin, and a significant decrease in the basal activity of cell invasion in the presence of ETAR antagonists or after ETAR silencing, indicating that an ET-1/ETAR autocrine loop in ovarian carcinoma cells has a critical role in inducing EMT (7, 52, 55). The mechanism responsible for ET-1-induced E-cadherin downregulation involves the regulation of the transcription factor Snail, at multiple levels. Thus, ET-1 through ETAR increases both Snail mRNA, protein stability, and transcriptional activity that closely correlate with downregulation of E-cadherin mRNA and transcriptional activity of E-cadherin promoter.

Another characteristic cellular event of EMT is an increase in the nuclear amount of β-catenin. In addition to its pivotal role in cadherin-based cell adhesion, β-catenin can act as a transcriptional activator through its interaction with T-cell-specific transcription factor/lymphoid enhancer factors (TCF/LEF). Activity of β-catenin/TCF complex is essential for the transcription of genes that direct cell fate, polarity, and proliferation of tumor cells (28). Cytosolic β-catenin is normally phosphorylated by glycogen synthase kinase-3β (GSK-3β) at serine and threonine residues in its amino (N)-terminal domain. This region is then recognized and ubiquitinated by a multi-protein complex containing the F-box protein β-TrCP, with resultant degradation of the polyubiquitinated β-catenin by the proteasome. Alternatively, the canonical Wnt signaling pathway can inhibit the ability of GSK-3β to phosphorylate target substrates, with resultant increases in β-catenin levels. The stabilization of β-catenin consequently leads to enhanced nuclear accumulation and its transcriptional activity through binding to TCF/LEF complex (28).

Recently, we describe the functional interaction between the ETAR and β-arrestin, leading to the activation of β-catenin pathways that occurred through several coordinated mechanisms (52, 53, 55). To underscore the complexity of the mechanisms available to ETAR to interlink β-catenin path-
ways, we demonstrated that the association of ET\textsubscript{A}R/\beta-arrestin/c-Src signaling complex leads to EGFR transactivation and downstream activation of β-catenin tyrosine phosphorylation, thereby mobilizing the fraction of β-catenin associated to E-cadherin and increasing its free cytosolic pool. ET\textsubscript{A}R-promoted tyrosine phosphorylated β-catenin binds TCF4 in nuclear extracts promoting activation of target genes (45, 46).

β-arrestin is also recruited to ET\textsubscript{A}R to form a complex through the physical association with axin, contributing to release and inactivate GSK-3β and to stabilize β-catenin. Thus, ET-1-induced binding of β-arrestin to axin is required to induce the displacement of GSK-3β from axin-containing complex and its functional inhibition (46).

Altogether, these results strongly imply that the ET\textsubscript{A}R signals through β-arrestin as an integral component of at least two trimeric functional complexes involved in β-catenin signaling, one consisting of ET\textsubscript{A}R, β-arrestin and Src that controls cross-talk with the EGFR, and another with axin, which mediates signaling to GSK-3β. Moreover, ET\textsubscript{A}R/β-arrestin could also mimic the canonical Wnt signaling to inactivate GSK-3β via AKT through PI3K/ILK, resulting in the stabilization and nuclear translocation of β-catenin (18). In the nucleus, β-catenin interacts with cofactor TCF and LEF to activate transcription of genes that promote ovarian cancer cell invasion. Interestingly, β-arrestin-silenced cells exhibit decreased cellular invasion and cells that overexpress mutant β-arrestin-1 show reduced metastatic ability compared with the control, implicating a functional role for β-arrestin as a mediator of cellular invasion and metastasis. In the metastatic nodules derived from cells expressing β-arrestin mutant, the expression of active β-catenin was strongly inhibited, further supporting that the ET\textsubscript{A}R-dependent β-catenin pathway in a β-arrestin-dependent manner is involved in the invasive and metastatic properties of ovarian cancer cells in vivo (46).

The present results, together with the observation that the coexpression of ET\textsubscript{A}R and β-arrestin may be indicative of a more aggressive phenotypes of primary human ovarian cancers, reveal a molecular map showing that the recruitment of β-arrestin to ET\textsubscript{A}R may represent a check-point controlling multiple pathways converging on β-catenin signaling to promote invasion and metastasis (Fig. 1).

**ET-1 Axis in Chemoresistance**

Drug resistance remains the major therapeutic barrier in epithelial ovarian cancer. An in-depth understanding of the mechanisms underlying the chemoresistance onset is likely to lead to improved therapeutic strategies for ovarian cancer (40). Emerging evidence suggests molecular and phenotypic associations between chemoresistance and the acquisition of EMT phenotype in cancer cells. EMT can generate multiple, distinct cellular subpopulations contributing to intratumoral heterogeneity. Some of these subpopulations have characteristics of stem cells that have a propensity to invade surrounding tissues and display resistance to therapy (43). As above outlined, ET-1/ET\textsubscript{A}R signaling is critical for promoting EMT by regulating the dynamic interactions of tumor/microenvironment in ovarian cancer (5, 43).

Analysis of a genome-wide expression profile of resistant ovarian carcinoma identified ET\textsubscript{A}R as a key gene related to chemoresistance (34). Interestingly, paclitaxel-resistant ovarian cancer cells showed phenotypic changes consistent with EMT (35), providing strong evidence linking chemoresistance to EMT. Moreover, a recent pathway analysis on nine published gene sets associated with platinum resistance in ovarian cancer revealed ET-1 signaling among the 48 canonical pathways significantly associated with resistance to platinum-based chemotherapy. Moreover several of these pathways are linked by 10.220.33.4 on June 29, 2017 http://ajpregu.physiology.org/ Downloaded from

![Fig. 1. Signaling pathways regulated by endothelin 1/endothelin A receptor (ET-1/ET\textsubscript{A}R) axis controlling ovarian tumor growth and progression. A model proposed to illustrate that activation of ET\textsubscript{A}R by ET-1 drives multiple coordinated signaling pathways to engage transcriptional programs, leading to cell proliferation, survival and chemoresistance, angiogenesis, lymphangiogenesis, and epithelial-to-mesenchymal transition (EMT). EGFR, endothelial growth factor receptor.](http://ajpregu.physiology.org/)

**ENDOTHELIN AXIS IN CANCER**

*Review*

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to EMT and stemness, reinforcing the relationship of both processes with therapy resistance (32). Given the documented role of EMT as ultimate adaptation of cancer cells following treatment, we can postulate that ETAR-mediated EMT signaling in ovarian cancer cells can represent a “salvage pathway” occurring during resistance development. On the basis of this rationale, we hypothesize that ovarian cancer cells rely on ET-1/ETAR signaling to undergo EMT and acquire a therapy-resistant phenotype.

To assess the role of ETAR as predictor of chemoresistance, we performed a screening of a series of ovarian cancers with all information regarding clinico-pathological characteristics, as well as the response to chemotherapy regimens. Immunohistochemical analysis of human ovarian cancer tissues, with different responses to chemotherapy, showed that ETAR is overexpressed in the resistant tumors. Therefore, our results suggest that ETAR expression can be considered a predictor of chemoresistance and that targeting ETAR can represent a therapeutic strategy to increase the sensitivity to chemotherapeutic agents (L. Rosanò, R. Cianfrocca, F. Spinella, V. Di Castro, M. R. Nicotra, A. Lucidi, G. Ferrandina, P. G. Natali, and A. Bagnato, unpublished observation).

An important contributor to EMT and chemoresistance onset is the interaction between tumor cells and the hypoxic microenvironment. Therefore, we can hypothesize that ET-1/ETAR axis may mimic hypoxia acting through HIF-1α and/or serve as a critical intermediate in conveying the hypoxic response into EMT transcriptional program. Interestingly, ET-1 is also a HIF-α target gene, suggesting that ET-1 axis could establish a complex cooperation between the intracellular signaling pathways and extracellular signals triggering EMT (Fig. 2). Because EMT represents a mechanism of escape to a new, less adverse niche, targeting the signaling network that triggers this process is an attractive but challenging approach that is likely to improve clinical management of ovarian cancer.

**Targeting ETAR As a Novel Approach in Ovarian Carcinoma Treatment**

The demonstration that ET-1 sustains many of the “hallmarks of cancer” identifies the ET-1 axis as a potential therapeutic target. This has propelled the development of several approaches targeting ET-1 axis in cancer therapy. In ovarian carcinoma, one approach is represented by the inhibition of the ETs biosynthesis with natural agents or compounds, such as green tea bioactive polyphenols (64, 65), or blocking ET production from big ETs with ECE inhibitors. In this regard, it has been demonstrated that siRNA targeting ECE-1 significantly suppressed ECE-1 expression and ET-1 biosynthesis and signaling in ovarian cancer cell lines, accompanied by reduced tumorigenic features of the cells, such as invasiveness, basement membrane adhesion, and expression of adhesion molecules (44), indicating that ECE-1 silencing may represent an effective tool for manipulating ECE-1 and ET-1 expression. A different approach is represented by transfection of NEP, a cell surface aminopeptidase capable of degrading a number of bioactive peptides, including ET-1. In ovarian carcinoma cells overexpressing NEP, there was a significant decrease in cell proliferation, survival, and invasiveness with a reduction of ET-1. Furthermore, tumorigenesis was reduced in vivo with the overexpression of NEP. This evidence suggests that NEP functionally suppresses the progression of ovarian carcinoma targeting ET-1 (39).

To date, endothelin receptor blockade represents the most rationale targeted approach in controlling the pleiotropic activities of ET-1, which are all pivotal in the gain and mainte-
nance of malignant phenotype. In particular, the development of small molecules acting as specific ETAR antagonists has contributed to an understanding of the physiopathological relevance of the ET-1 axis and its signaling circuitry in ovarian tumor progression and metastasis, paving their evaluation in clinical trials. Among various ETAR antagonists, atrasentan (ABT-627; Abbott Laboratories, Abbott Park, IL) and zibotentan (ZD4054, AstraZeneca, Macclesfield, UK) are orally bioavailable ETAR antagonists that potently and specifically bind to the ETAR, blocking signal transduction pathways implicated in cancer cell proliferation and other host-dependent processes that promote cancer growth (7, 48).

Treatment with ETAR antagonist produces tumor growth inhibition in ovarian cancer xenografts. This treatment, which is generally well tolerated, with no detectable signs of acute or delayed toxicity is long lasting and comparable to that achieved by paclitaxel. More marked and prolonged tumor growth inhibition is obtained by combined treatment of ETAR antagonist with paclitaxel, with no toxicity and with tumor regressions in 40% of treated animals. Analysis of the tumor tissues from xenografts reveals a marked reduction in the percentage of COX-2, VEGF, and MMP-2 in treated mice. Almost complete inhibition of VEGF, MMP-2 expression, and tumor neovascularization, as well as an increase in apoptosis, were observed following combined treatment of ETAR antagonist with paclitaxel (49, 54, 56).

Because clinical trial results defined the combination of platinum and taxane as standard of care in the management of ovarian cancer, we explored the therapeutic efficacy of the integration of zibotentan with cytotoxic drugs having a different mode of action. The combination of zibotentan and cisplatinum, as observed with zibotentan and paclitaxel, is more effective in the inhibition of ovarian cancer cell proliferation induced by endogenous ET-1, compared with a single agent. Interestingly, a significant enhanced efficacy was observed when zibotentan was combined with cisplatinum and paclitaxel. Remarkably, in ovarian carcinoma xenografts, the coadministration of zibotentan with cisplatinum plus taxol was very effective in inhibiting tumor growth, neovascularization, and cell proliferation, representing a preclinical endpoint to guide combination therapy in clinical trials (47).

A detailed understanding of the molecular mechanisms that control ovarian cancer metastasis is a crucial step in identifying new effective therapies (40). The recent preclinical demonstration of tumor growth inhibition (49, 54, 56), together with reduced metastatic potential in response to zibotentan (46), suggest that this treatment, by simultaneously disabling multiple intertwined circuits activated by ETAR in a β-arrestin-dependent manner, provide a molecular framework for the development of pathway-specific therapeutics for ovarian cancer.

The cross-signaling between the EGFR/ETAR pathways provides a rationale to combine EGFR inhibitors, such as gefitinib, with ETAR antagonists, identifying new effective therapeutic opportunities for ovarian cancer.

In ovarian carcinoma xenografts, the coadministration of zibotentan enhanced the efficacy of gefitinib, leading to partial or complete tumor regression. Antitumor effects were paralleled by biochemical and immunohistologic evidence of decreased vascularization, reduced Ki-67, MMP-2, VEGF, MAPK, and EGFR protein expression levels, and enhanced E-cadherin expression (50).

The findings demonstrating the antitumor, antiangiogenic, apoptotic activities of zibotentan in vivo provide a rationale for the clinical evaluation of this small molecule in patients with ovarian tumors and potentially in other epithelial tumors that overexpress functional ETAR.

Conclusions

Upon being activated, ETAR activates a signaling network that regulates tumorigenesis, EMT, and progression of ovarian cancer (8). Therefore, short circuiting the ETAR signaling pathways may offer effective strategies to manage or achieve cures in this disease.

Resistance to apoptosis is a principal mechanism whereby tumors are able to overcome cytotoxicity induced by chemotheraphy. ETAR blockade sensitized tumor cells to the apoptotic potential of chemotherapeutic agent, resulting in tumor regression. The cooperative antitumor effect of combination therapy, in which ETAR antagonist, by increasing the commitment of tumor cells toward apoptosis, potentiates the therapeutic efficacy of conventional cytotoxic drugs, offers a rationale for its clinical evaluation in combination with chemotherapeutic drugs in ovarian cancer (8). On the basis of this approach, a phase II multicenter randomized study is evaluating zibotentan vs. placebo when given in addition to the standard background therapy of carboplatin and paclitaxel, as a second-line treatment in platinum-sensitive patients with advanced ovarian cancer.

Ovarian cancers show high levels of intertumoral and intratumoural heterogeneity and possess distinct genetic abnormalities and treatment responses, indicating that predictive biomarkers that correlate with a positive response to targeted therapy are needed.

As zibotentan has not previously been evaluated in patients with ovarian cancer, it was felt appropriate to explore the potential relationships between zibotentan and a range of biomarkers. These relationships may help to identify a panel of useful biomarkers as surrogate markers for response in further clinical evaluation of zibotentan.

Therefore, the aim of our exploratory research is to investigate, emerging and as yet unidentified, biomarkers that correlate with a positive response to therapy. The success of this translational research has required a multidisciplinary approach, which will establish a seamless link from basic science to clinical trial evaluation and to quicker regulatory approval of novel therapeutics. For the time being, the completion of clinical trials using ETAR antagonist in combination with cytotoxic drugs, could open a novel therapeutic area for this promising class of drugs.

A novel approach to cancer therapy has emerged by the treatment with multiple selective inhibitors to different growth factor receptors or to key postreceptor signaling pathways. Engagement of ETAR by ET-1 induces pleiotropic tumor-promoting effects that are mediated by different downstream effectors, such as EGFR, which could become the preferentially escaping pathways used by tumor cells. In this context, the improved knowledge of the interconnected molecular mechanism promoted by ET-1 axis to trigger EMT (Fig. 2), which is linked to therapy resistance and stemness, will cer-
tainty fuel the interest of basic and translational scientists to evaluate new treatment strategy that incorporate ETA,R blockade in combination with other molecularly targeted drugs to find compensatory mechanisms of escape that can be eliminated therapeutically.

Perspectives and Significance

Targeted therapies appear to be a promising platform for clinical development in ovarian cancer. The future of cancer therapeutics will likely include tailored, individualized treatments, designed on the basis of an even deeper understanding of the critical alterations in ovarian carcinogenesis. As outlined in this review, ETA,R pathway is critical in forming and maintaining the malignant ovarian phenotype. Experimental and preclinical studies strongly suggest that this malignancy could become a new indication for ETA,R antagonist therapy in the near future. Despite these advances, there are multiple goals to be achieved in the future studies of ETA,R-targeted therapy, which include 1) a better understanding of the redundant pathways that exist in cell signaling, 2) combination targeting of horizontal and/or vertical pathways, 3) identification of other predictive markers to better identify a targeted subpopulation of patients that will respond, and 4) an understanding of the mechanisms of resistance/EMT/stemness.

Therefore, well-designed clinical studies are warranted to test and verify the therapeutic potential of this new class of drugs in combination with cytotoxic drugs or molecular inhibitors that revert EMT and can increase the sensitization to therapy, improving the clinical management of ovarian carcinoma.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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