

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

Laura Rosanò, Francesca Spinella, and Anna Bagnato

Molecular Pathology Laboratory, Regina Elena National Cancer Institute, Rome, Italy

Submitted 6 May 2010; accepted in final form 4 June 2010

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, neprilysin) (14, 75). ET-1 production is stimulated by a variety of stimuli, including IL-1 β , tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), PDGF, vasopressin, hypoxia, and shear stress. Inhibitory factors include nitric oxide, prostacyclin, and atrial natriuretic peptide (41).

To date, several studies demonstrate that ETs play crucial roles in ovarian physiology and participate in diverse reproductive processes, in which members of the ET network are dynamically regulated throughout the reproductive cycle, fur-

Address for reprint requests and other correspondence: A. Bagnato, Molecular Pathology Laboratory, Regina Elena National Cancer Institute, Via delle Messi D'Oro 156, 00158 Rome, Italy (e-mail: bagnato@ifo.it).

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 ET_AR subtype, although both receptors are expressed in ovaries of many mammalian species, and there are species differences in the prevalence of ET_AR vs. ET_BR 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 receptor (4, 29, 41).

Expression of ET-1 Axis

The *in vivo* analysis of ET-1 axis expression levels demonstrated a higher expression of ET-1 and ET_AR in primary and metastatic tumors than in normal ovarian tissues. Interestingly, ET-1-producing cells also expressed functional ET_AR, but not ET_BR, indicating that in ovarian tumor cells, ET-1 acts as an autocrine factor selectively through the ET_AR (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 ET_AR 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 ET_AR 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 ET_AR results in p125 focal adhesion kinase (FAK) and paxillin activation, which are thought to transduce signals involved in tumor cell invasion. Furthermore, ET_AR activation triggers the antiapoptotic signaling through phosphatidylinositol 3-kinase (PI3-K)-mediated Akt pathways (20, 21), indicating the existence of multiple signal transduction pathways downstream to ET_AR activation in ET-1-stimulated ovarian cancer cells involved in tumorigenic events.

However, these ET_AR-generating pathways alone may not be able to explain the diversity of effects stimulated by ET-1 through ET_AR. 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 ET_AR (45, 46). In these cells upon ET-1 stimulation, β-arrestin is recruited to ET_AR to form a trimeric complex with Src. The association of ET_AR/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

ET-1 axis in tumor growth and survival. ET-1/ET_AR axis, through the activation of different kinases and rapid induction of early response genes, including *c-fos*, *c-jun*, and *c-myc*, induces mitogenic responses (3, 10).

In ovarian cancer cells, ET-1 stimulates DNA synthesis with the same efficacy as EGF, and at maximally effective concentrations, its action was additive to that of EGF. The findings that EGFR transactivation is, in part, responsible for the mitogenic effect of ET-1/ET_AR pathway and that ET-1 exerts additive proliferative effects in the presence of EGF, suggest that the coexistence of ET-1 and EGF autocrine circuits in tumor cells could provide maximal growth advantage (10, 74).

In addition to inducing proliferative effects, ET-1 acts as an antiapoptotic factor in different cell types, indicating that the peptide may also modulate cell survival pathways. In ovarian carcinoma cells, the addition of ET-1 markedly inhibited serum withdrawal and paclitaxel-induced apoptosis. Paclitaxel-induced apoptosis resulted in the phosphorylation of Bcl-2 that was suppressed by the addition of ET-1. Further analysis of the survival pathway demonstrated that ET-1-stimulated Akt activation that was dependent on PI3-K. Interestingly, the addition of a specific ET_AR antagonist blocked the ET-1-induced resistance to paclitaxel-mediated apoptosis, indicating that ET-1 contributes to paclitaxel resistance through ET_AR binding via activation of antiapoptotic signaling pathways, such as Akt (20, 21).

All of these findings suggest that specific ET_AR antagonists may, therefore, provide an additional approach to the treatment of ovarian carcinoma, in which ET_AR 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 metalloproteases-2 (MMP-2), migrate, proliferate, and invade surrounding tissues to form capillaries. ET-1, mainly by ET_BR, 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_AR 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 α (HIF-1 α) transcription factor (66). Similar to hypoxia, ET-1 promotes VEGF production through HIF-1 α . After ET_AR activation by ET-1, HIF-1 α protein levels are increased and stabilized, leading to the formation of HIF-1 transcription complex that binds to the hypoxia-responsive element binding sites. Therefore, ET-1/ET_AR-induced HIF-1 α 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- β . In ovarian carcinoma cells, ET-1 significantly increases the expression of COX-1 and -2, COX-2 promoter activity, PGE₂ 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 PGE₂ 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_BR. In these cells, ET-1

promotes proliferation, MMP activation, invasiveness, vascular-like structure formation, and phosphorylation of AKT and p42/44 MAPK through ET_BR (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 α expression similarly to hypoxia. Moreover, HIF-1 α silencing desensitizes VEGF-C and VEGF-A production in response to ET-1 or hypoxia, implicating HIF-1 α /VEGF as downstream signaling molecules of ET-1 axis. Double immunofluorescence analysis of human lymph nodes reveals that lymphatic vessels express ET_BR together with the lymphatic marker podoplanin, indicating that ET_BR 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_BR blockade with the specific antagonist inhibits in vitro and in vivo ET-1-induced effects, demonstrating that ET_BR 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 α -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_AR consistently induced the activity of 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_AR 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 $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins. Interaction of β_1 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 β_1 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-3 β and Akt, the major downstream components of ET_AR-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 β_1 integrin by ECM, ILK is recruited into the β_1 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 β_1 -integrin, signaling amplifying ILK activity. The β_1 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 micro-environmental 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/ET_AR 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 ET_AR 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 ET_AR 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 ET_AR signaling caused by an autocrine ET-1/ET_AR loop is required for the maintenance of EMT 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 ET_AR antagonists or after ET_AR silencing, indicating that an ET-1/ET_AR 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 ET_AR 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-3 β (GSK-3 β) 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-3 β 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 ET_AR 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 ET_AR to interlink β -catenin path-

ways, we demonstrated that the association of ET_AR/ β -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_AR-promoted tyrosine phosphorylated β -catenin binds TCF4 in nuclear extracts promoting activation of target genes (45, 46).

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

Altogether, these results strongly imply that the ET_AR signals through β -arrestin as an integral component of at least two trimeric functional complexes involved in β -catenin signaling, one consisting of ET_AR, β -arrestin and Src that controls cross-talk with the EGFR, and another with axin, which mediates signaling to GSK-3 β . Moreover, ET_AR/ β -arrestin could also mimic the canonical Wnt signaling to inactivate GSK-3 β 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_AR-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_AR 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_AR 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_AR 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_AR 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

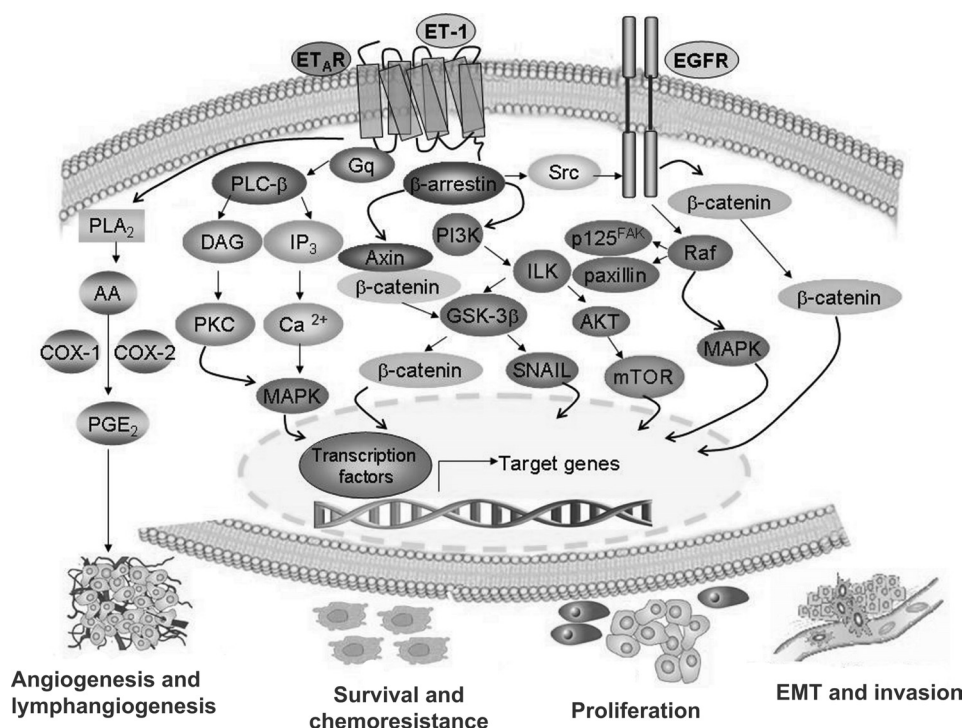


Fig. 1. Signaling pathways regulated by endothelin 1/endothelin A receptor (ET-1/ET_AR) axis controlling ovarian tumor growth and progression. A model proposed to illustrate that activation of ET_AR by ET-1 drives multiple coordinated signaling pathways to engage transcriptional programs, leading to cell proliferation, survival and chemoresistance, angiogenesis, lymphangiogenesis, and epithelial-to-mesenchymal transition (EMT). EGFR, endothelial growth factor receptor.

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 ET_AR-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/ET_AR signaling to undergo EMT and acquire a therapy-resistant phenotype.

To assess the role of ET_AR 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 ET_AR is overexpressed in the resistant tumors. Therefore, our results suggest that ET_AR expression can be considered a predictor of chemoresistance and that targeting ET_AR 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/ET_AR axis may mimic hypoxia acting through HIF-1 α 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

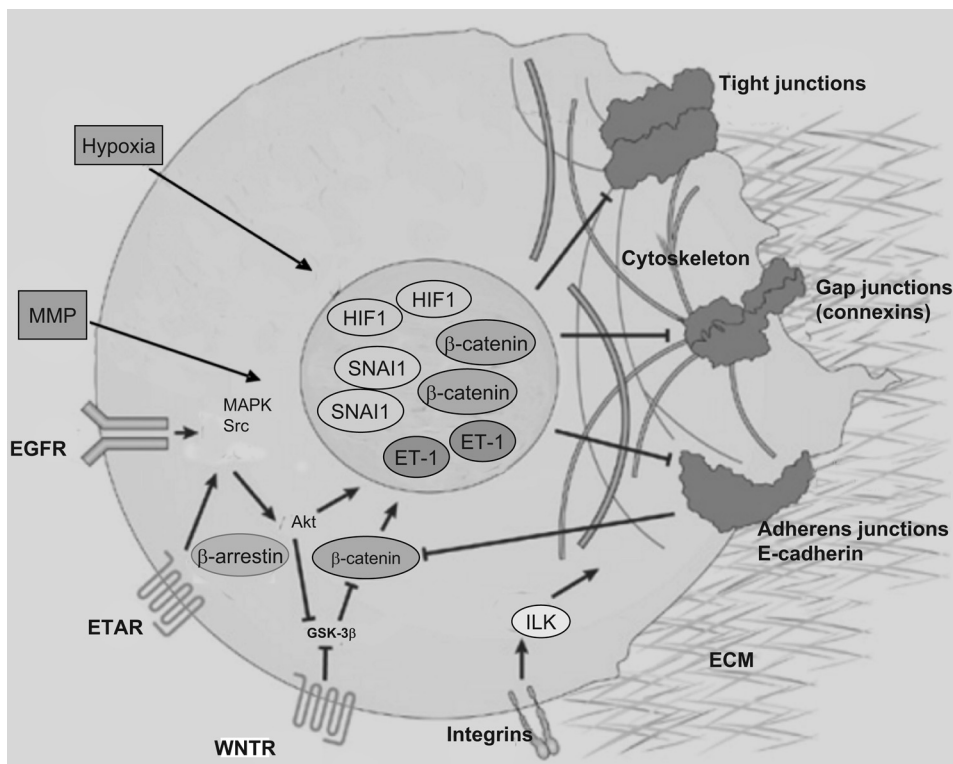
process is an attractive but challenging approach that is likely to improve clinical management of ovarian cancer.

Targeting ET_AR 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-

Fig. 2. ET_AR-driven signaling networks regulating epithelial-to-mesenchymal transition. ET_AR, along with EGFR, integrins, Wnt, and matrix metalloproteinases (MMP), can induce EMT through multiple different signaling pathways (42). EMT is associated with dramatic changes in the cytoskeleton and extracellular matrix (ECM) composition, including the disruption of tight junctions, gap junctions, and adherent junctions, as well as upregulation of tumor proteases (such as MMP). Hypoxic tumor microenvironment leads also to activation of hypoxia-inducible factor 1 α (HIF-1 α) and EMT-transcriptional factors. Besides the interaction among the various signaling pathways, there is also extensive crosstalk among the EMT-inducing factors and the ET_AR-driven signaling, suggesting that ET-1 axis takes a relevant place in the complex cooperation between the intracellular signaling pathways and extracellular signals triggering EMT. WNT3R, Wnt receptor; HIF-1, hypoxia-inducible factor-1; SNAI1, Snail.



nance of malignant phenotype. In particular, the development of small molecules acting as specific ET_AR 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 ET_AR antagonists, atrasentan (ABT-627; Abbott Laboratories, Abbott Park, IL) and zibotentan (ZD4054, AstraZeneca, Macclesfield, UK) are orally bioavailable ET_AR antagonists that potently and specifically bind to the ET_AR, blocking signal transduction pathways implicated in cancer cell proliferation and other host-dependent processes that promote cancer growth (7, 48).

Treatment with ET_AR 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 ET_AR 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 ET_AR 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 cisplatin, 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 cisplatin and paclitaxel. Remarkably, in ovarian carcinoma xenografts, the coadministration of zibotentan with cisplatin 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 ET_AR 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/ET_AR pathways provides a rationale to combine EGFR inhibitors, such as gefitinib, with ET_AR 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 ET_AR.

Conclusions

Upon being activated, ET_AR activates a signaling network that regulates tumorigenesis, EMT, and progression of ovarian cancer (8). Therefore, short circuiting the ET_AR 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 chemotherapy. ET_AR blockade sensitized tumor cells to the apoptotic potential of chemotherapeutic agent, resulting in tumor regression. The cooperative antitumor effect of combination therapy, in which ET_AR 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 intratumoral 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 ET_AR 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 ET_AR 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-

tainly fuel the interest of basic and translational scientists to evaluate new treatment strategy that incorporate ET_AR 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, ET_AR 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 ET_AR antagonist therapy in the near future. Despite these advances, there are multiple goals to be achieved in the future studies of ET_AR-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.

ACKNOWLEDGMENTS

We thank V. Di Castro, R. Cianfrocca, A. Lupo, V. Caprara, and M. R. Nicotra for their contributions to in vitro and in vivo studies, P. G. Natali for critical reading and insightful comments, and M. V. Sarcone for secretarial assistance.

GRANTS

This work was supported by the Associazione Italiana Ricerca sul Cancro, Ministero della Salute and CNR-MIUR.

DISCLOSURES

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

REFERENCES

- Ahmed N, Maines-Bandiera S, Quinn MA, Unger WG, Dedhar S, Auersperg N. Molecular pathways regulating EGF-induced epithelio-mesenchymal transition in human ovarian surface epithelium. *Am J Physiol Cell Physiol* 290: C1532–C1542, 2006.
- Auersperg N, Pan J, Grove BD, Peterson T, Fisher J, Maines-Bandiera S, Somasiri A, Roskelley CD. E-cadherin induces mesenchymal-to-epithelial transition in human ovarian surface epithelium. *Proc Natl Acad Sci USA* 96: 6249–6254, 1999.
- Bagnato A, Catt KJ. Endothelins as autocrine regulators of tumor cell growth. *Trends Endocrinol Metab* 9: 378–383, 1998.
- Bagnato A, Rosanò L. The endothelin axis in cancer. *Int J Biochem Cell Biol* 40: 1443–1451, 2008.
- Bagnato A, Rosanò L. Epithelial-mesenchymal transition in ovarian cancer progression: a crucial role for the endothelin axis. *Cells Tissues Organs* 185: 85–94, 2007.
- Bagnato A, Spinella F. Emerging role of endothelin-1 in tumor angiogenesis. *Trends Endocrinol Metab* 14: 44–50, 2003.
- Bagnato A, Spinella F, Rosanò L. Emerging role of the endothelin axis in ovarian tumor progression. *Endocr Relat Cancer* 12: 761–772, 2005.
- Bagnato A, Spinella F, Rosanò L. The endothelin axis in cancer: the promise and the challenges of molecularly targeted therapy. *Can J Physiol Pharmacol* 86: 473–484, 2008.
- Bagnato A, Salani D, Di Castro V, Wu-Wong JR, Tecce R, Nicotra MR, Venuti A, Natali PG. Expression of endothelin 1 and endothelin A receptor in ovarian carcinoma: evidence for an autocrine role in tumor growth. *Cancer Res* 59: 720–727, 1999.
- Bagnato A, Tecce R, Di Castro V, Catt KJ. Activation of mitogenic signaling by endothelin 1 in ovarian carcinoma cells. *Cancer Res* 57: 1306–1311, 1997.
- Bagnato A, Tecce R, Moretti C, Di Castro V, Spergel D, Catt KJ. Autocrine actions of endothelin-1 as a growth factor in human ovarian carcinoma cells. *Clin Cancer Res* 1: 1059–1066, 1995.
- Barki-Harrington L, Rockman HA. Beta-arrestins: multifunctional cellular mediators. *Physiology* 23: 17–22, 2008.
- Bast RC Jr, Hennessy B, Mills GB. The biology of ovarian cancer: new opportunities for translation. *Nat Rev Cancer* 9: 415–428, 2009.
- Battistini B, Chailier P, D'Orleans-Juste P, Briere N, Sirois P. Growth regulatory properties of endothelins. *Peptides* 14: 385–399, 1993.
- Burkhardt M, Barton M, Shaw SG. Receptor- and non-receptor-mediated clearance of big-endothelin and endothelin-1: differential effects of acute and chronic ETA receptor blockade. *J Hypertens* 18: 273–279, 2000.
- Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 407: 249–257, 2000.
- Clasper S, Royston D, Baban D, Cao Y, Ewers S, Butz S, Vestweber D, Jackson DG. A novel gene expression profile in lymphatics associated with tumor growth and nodal metastasis. *Cancer Res* 68: 7293–7303, 2008.
- Cianfrocca R, Rosanò L, Spinella F, Di Castro V, Natali PG, Bagnato A. β -arrestin-1 mediates the endothelin-1-induced activation of Akt and integrin-linked kinase. *Can J Physiol Pharmacol* In press.
- Defea K. Beta-arrestins and heterotrimeric G-proteins: collaborators and competitors in signal transduction. *Br J Pharmacol* 153 Suppl 1: S298–S309, 2008.
- Del Bufalo D, Di Castro V, Biroccio A, Salani D, Rosanò L, Spinella F, Bagnato A. Endothelin-1 acts as a survival factor in ovarian carcinoma cells. *Clin Sci (Lond)* 103 Suppl 48: 302S–305S, 2002.
- Del Bufalo D, Di Castro V, Biroccio A, Varmi M, Salani D, Rosanò L, Trisciuoglio D, Spinella F, Bagnato A. Endothelin-1 protects ovarian carcinoma cells against paclitaxel-induced apoptosis: requirement for Akt activation. *Mol Pharmacol* 61: 524–532, 2002.
- Denkert C, Kobel M, Pest S, Koch I, Berger S, Schwabe M, Siegert A, Reles A, Klosterhalfen B, Hauptmann S. Expression of cyclooxygenase 2 is an independent prognostic factor in human ovarian carcinoma. *Am J Pathol* 160: 893–903, 2002.
- Donninger H, Bonome T, Radonovich M, Pise-Masison CA, Brady J, Shih JH, Barrett JC, Birrer MJ. Whole genome expression profiling of advance stage papillary serous ovarian cancer reveals activated pathways. *Oncogene* 23: 8065–8077, 2004.
- Faleiro-Rodrigues C, Macedo-Pinto I, Pereira D, Ferreira VM, Lopes CS. Association of E-cadherin and beta-catenin immunorexpression with clinicopathologic features in primary ovarian carcinomas. *Hum Pathol* 35: 663–669, 2004.
- Ferrandina G, Lauriola L, Zannoni GF, Fagotti A, Fanfani F, Legge F, Maggiano N, Gessi M, Mancuso S, Ranelletti FO, Scambia G. Increased cyclooxygenase-2 (COX-2) expression is associated with chemotherapy resistance and outcome in ovarian cancer patients. *Ann Oncol* 13: 1205–1211, 2002.
- Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol* 16: 4604–4613, 1996.
- Fosslien E. Molecular pathology of cyclooxygenase-2 in cancer-induced angiogenesis. *Ann Clin Lab Sci* 31: 325–348, 2001.
- Gavert N, and Ben-Ze'ev A. β -Catenin signaling in biological control and cancer. *J Cell Biochem* 102: 820–828, 2007.
- Guisse TA, Yin JJ, Mohammad KS. Role of endothelin-1 in osteoblastic bone metastases. *Cancer* 97: 779–784, 2003.
- Gupta RA, Tejada LV, Tong BJ, Das SK, Morrow JD, Dey SK, DuBois RN. Cyclooxygenase-1 is overexpressed and promotes angiogenic growth factor production in ovarian cancer. *Cancer Res* 63: 906–911, 2003.
- Hannigan G, Troussard AA, Dedhar S. Integrin-linked kinase: a cancer therapeutic target unique among its ILK. *Nat Rev Cancer* 5: 51–63, 2005.

32. Helleman J, Smid M, Jansen MP, van der Burg ME, Berns EM. Pathway analysis of gene lists associated with platinum-based chemotherapy resistance in ovarian cancer: the big picture. *Gynecol Oncol* 117: 170–176, 2010.
33. Imai T, Horiuchi A, Shiozawa T, Osada R, Kikuchi N, Ohira S, Oka K, Konishi I. Elevated expression of E-cadherin and alpha-, beta-, and gamma-catenins in metastatic lesions compared with primary epithelial ovarian carcinomas. *Hum Pathol* 35: 1469–1476, 2004.
34. Jazaeri AA, Awtrey CS, Chandramouli GV, Chuang YE, Khan J, Sotiriou C, Aprelikova O, Yee CJ, Zorn KK, Birrer MJ, Barrett JC, Boyd J. Gene expression profiles associated with response to chemotherapy in epithelial ovarian cancers. *Clin Cancer Res* 11: 6300–6310, 2005.
35. Kajiyama H, Shibata K, Terauchi M, Morita T, Ino K, Mizutani S, Kikkawa F. Neutral endopeptidase 2411/CD10 suppresses progressive potential in ovarian carcinoma in vitro and in vivo. *Clin Cancer Res* 11: 1798–1808, 2005.
36. Kajiyama H, Shibata K, Terauchi M, Yamashita M, Ino K, Nawa A, Kikkawa F. Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells. *Int J Oncol* 31: 277–283, 2007.
37. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* 115: 455–65, 2002.
38. Masaki T. Historical review: Endothelin. *Trends Pharmacol Sci* 25: 219–224, 2004.
39. Meidan R, Nitzan L. The ovarian endothelin network: an evolving story. *Trends Endocrinol Metab* 18: 379–385, 2007.
40. Naora H, Montell DJ. Ovarian cancer metastasis: integrating insights from disparate model organisms. *Nat Rev Cancer* 5: 355–366, 2005.
41. Nelson J, Bagnato A, Battistini B, Nisen P. The endothelin axis: emerging role in cancer. *Nat Rev Cancer* 3: 110–116, 2003.
42. Peinado H, Olmeda D, Cano A. Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* 7: 415–428, 2007.
43. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 9: 265–273, 2009.
44. Rayhman O, Klipper E, Muller L, Davidson B, Reich R, Meidan R. Small interfering RNA molecules targeting endothelin-converting enzyme-1 inhibit endothelin-1 synthesis and the invasive phenotype of ovarian carcinoma cells. *Cancer Res* 68: 9265–78, 2008.
45. Rosanò L, Bagnato A. Convergent pathways link the endothelin A receptor to the beta-catenin: the β -arrestin connection. *Cell Cycle* 8: 1462–1463, 2009.
46. Rosanò L, Cianfrocca R, Masi S, Spinella F, Di Castro V, Biroccio A, Salvati E, Nicotra MR, Natali PG, Bagnato A. Beta-arrestin links endothelin A receptor to beta-catenin signaling to induce ovarian cancer cell invasion and metastasis. *Proc Natl Acad Sci USA* 106: 2806–2811, 2009.
47. Rosanò L, Cianfrocca R, Spinella F, Di Castro V, Natali PG, Bagnato A. Combination therapy of zibotentan with cisplatin and taxol is an effective regimen for epithelial ovarian cancer. *Can J Physiol Pharmacol* In press.
48. Rosanò L, Di Castro V, Spinella F, Decandia S, Natali PG, Bagnato A. ZD4054, a potent endothelin receptor A antagonist, inhibits ovarian carcinoma cell proliferation. *Exp Biol Med (Maywood)* 231: 1132–1135, 2006.
49. Rosanò L, Di Castro V, Spinella F, Nicotra MR, Natali PG, Bagnato A. ZD4054, a specific antagonist of the endothelin A receptor, inhibits tumor growth and enhances paclitaxel activity in human ovarian carcinoma in vitro and in vivo. *Mol Cancer Ther* 6: 2003–2011, 2007.
50. Rosanò L, Di Castro V, Spinella F, Tortora G, Nicotra MR, Natali PG, Bagnato A. Combined targeting of endothelin A receptor and epidermal growth factor receptor in ovarian cancer shows enhanced antitumor activity. *Cancer Res* 67: 6351–6359, 2007.
51. Rosanò L, Salani D, Di Castro V, Spinella F, Natali PG, Bagnato A. Endothelin-1 promotes proteolytic activity of ovarian carcinoma. *Clin Sci (Lond)* 103 Suppl 48: 306S–309S, 2002.
52. Rosanò L, Spinella F, Di Castro V, Decandia S, Nicotra MR, Natali PG, Bagnato A. Endothelin-1 is required during epithelial to mesenchymal transition in ovarian cancer progression. *Exp Biol Med (Maywood)* 231: 1128–1131, 2006.
53. Rosanò L, Spinella F, Di Castro V, Dedhar S, Nicotra MR, Natali PG, Bagnato A. Integrin-linked kinase functions as a downstream mediator of endothelin-1 to promote invasive behavior in ovarian carcinoma. *Mol Cancer Ther* 5: 833–842, 2006.
54. Rosanò L, Spinella F, Di Castro V, Natali PG, Bagnato A. Therapeutic targeting of the endothelin-A receptor in human ovarian carcinoma: efficacy of cytotoxic agents is markedly enhanced by co-administration with atrasentan. *J Cardiovasc Pharmacol* 44 Suppl 1: S132–S135, 2004.
55. Rosanò L, Spinella F, Di Castro V, Nicotra MR, Dedhar S, de Herrerros AG, Natali PG, Bagnato A. Endothelin-1 promotes epithelial-to-mesenchymal transition in human ovarian cancer cells. *Cancer Res* 65: 11649–11657, 2005.
56. Rosanò L, Spinella F, Salani D, Di Castro V, Venuti A, Nicotra MR, Natali PG, Bagnato A. Therapeutic targeting of the endothelin A receptor in human ovarian carcinoma. *Cancer Res* 63: 2447–2453, 2003.
57. Rosanò L, Varmi M, Salani D, Di Castro V, Spinella F, Natali PG, Bagnato A. Endothelin-1 induces tumor proteinase activation and invasiveness of ovarian carcinoma cells. *Cancer Res* 61: 8340–8346, 2001.
58. Rubin SA, Levin ER. Clinical review 53: The endocrinology of vasoactive peptides: synthesis to function. *J Clin Endocrinol Metab* 78: 6–10, 1994.
59. Salani D, Di Castro V, Nicotra MR, Rosanò L, Tecce R, Venuti A, Natali PG, Bagnato A. Role of endothelin-1 in neovascularization of ovarian carcinoma. *Am J Pathol* 157: 1537–1547, 2000.
60. Salani D, Rosanò L, Di Castro V, Spinella F, Venuti A, Padley RJ, Nicotra MR, Natali PG, Bagnato A. ABT-627, a potent endothelin receptor A antagonist, inhibits ovarian carcinoma growth in vitro. *Clin Sci (Lond)* 103 Suppl 48: 318S–321S, 2002.
61. Salani D, Taraboletti G, Rosanò L, Di Castro V, Borsotti P, Giavazzi R, Bagnato A. Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Am J Pathol* 157: 1703–1711, 2000.
62. Spinella F, Caprara V, Garrafa E, Di Castro V, Rosanò L, Natali PG, Bagnato A. Endothelin axis induces metalloproteinase activation and invasiveness in human lymphatic endothelial cells. *Can J Physiol Pharmacol* In press.
63. Spinella F, Garrafa E, Di Castro V, Rosanò L, Nicotra MR, Caruso A, Natali PG, Bagnato A. Endothelin-1 stimulates lymphatic endothelial cells and lymphatic vessels to grow and invade. *Cancer Res* 69: 2669–2676, 2009.
64. Spinella F, Rosanò L, Decandia S, Di Castro V, Albini A, Elia G, Natali PG, Bagnato A. Antitumor effect of green tea polyphenol epigallocatechin-3-gallate in ovarian carcinoma cells: evidence for the endothelin-1 as a potential target. *Exp Biol Med (Maywood)* 231: 1123–1127, 2006.
65. Spinella F, Rosanò L, Di Castro V, Decandia S, Albini A, Nicotra MR, Natali PG, Bagnato A. Green tea polyphenol epigallocatechin-3-gallate inhibits the endothelin axis and downstream signaling pathways in ovarian carcinoma. *Mol Cancer Ther* 5: 1483–1492, 2006.
66. Spinella F, Rosanò L, Di Castro V, Natali PG, Bagnato A. Endothelin-1 induces vascular endothelial growth factor by increasing hypoxia-inducible factor-1 α in ovarian carcinoma cells. *J Biol Chem* 277: 27850–27855, 2002.
67. Spinella F, Rosanò L, Di Castro V, Natali PG, Bagnato A. Endothelin-1-induced prostaglandin E2-EP2, EP4 signaling regulates vascular endothelial growth factor production and ovarian carcinoma cell invasion. *J Biol Chem* 279: 46700–46705, 2004.
68. Spinella F, Rosanò L, Di Castro V, Nicotra MR, Natali PG, Bagnato A. Endothelin-1 decreases gap junctional intercellular communication by inducing phosphorylation of connexin 43 in human ovarian carcinoma cells. *J Biol Chem* 278: 41294–41301, 2003.
69. Spinella F, Rosanò L, Di Castro V, Nicotra MR, Natali PG, Bagnato A. Inhibition of cyclooxygenase-1 and -2 expression by targeting the endothelin A receptor in human ovarian carcinoma cells. *Clin Cancer Res* 10: 4670–4679, 2004.
70. Spinella F, Rosanò L, Elia G, Di Castro V, Natali PG, Bagnato A. Endothelin-1 stimulates cyclooxygenase-2 expression in ovarian cancer cells through multiple signaling pathways: evidence for involvement of transactivation of the epidermal growth factor receptor. *J Cardiovasc Pharmacol* 44 Suppl 1: S140–S143, 2004.
71. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2: 442–454, 2002.
72. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* 93: 705–716, 1998.

73. Umhauer S, Ruch RJ, Fanning J. Gap junctional intercellular communication and connexin 43 expression in ovarian carcinoma. *Am J Obstet Gynecol* 182: 999–1000, 2000.
74. Vacca F, Bagnato A, Catt KJ, Tece R. Transactivation of the epidermal growth factor receptor in endothelin-1-induced mitogenic signaling in human ovarian carcinoma cells. *Cancer Res* 60: 5310–5317, 2000.
75. Vijayaraghavan J, Scicli AG, Carretero OA, Slaughter C, Moomaw C, Hersh LB. The hydrolysis of endothelins by neutral endopeptidase 24.11 (enkephalinase). *J Biol Chem* 265: 14150–14155, 1990.
76. Violin JD, Lefkowitz RJ. Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol Sci* 28: 416–22, 2007.
77. Wulfig P, Diallo R, Kersting C, Wulfig C, Poremba C, Rody A, Greb RR, Bocker W, Kiesel L. Expression of endothelin-1, endothelin-A, and endothelin-B receptor in human breast cancer and correlation with long-term follow-up. *Clin Cancer Res* 9: 4125–4131, 2003.
78. Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D, Yanagisawa M. ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* 78: 473–485, 1994.
79. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332: 411–415, 1988.

