β-Arrestin links endothelin A receptor to β-catenin signaling to induce ovarian cancer cell invasion and metastasis

Laura Rosanò[a], Roberta Cianfrocca[a], Stefano Masi[a], Francesca Spinella[a], Valeriana Di Castro[a], Annamaria Biroccio[b], Erica Salvati[b], Maria Rita Nicotra[c], Pier Giorgio Natali[a,d], and Anna Bagnato[a,1]

Laboratories of [a]Molecular Pathology, [b]Experimental Chemotherapy, and [d]Immunology, Regina Elena Cancer Institute, 00158 Rome, Italy; and [c]Molecular Biology and Pathology Institute, National Research Council, 00185 Rome, Italy

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The activation of endothelin-A receptor (ET_AR) by endothelin-1 (ET-1) has a critical role in ovarian tumorigenesis and progression. To define the molecular mechanism in ET-1-induced tumor invasion and metastasis, we focused on β-arrestins as scaffold and signaling proteins of G protein-coupled receptors. Here, we demonstrate that, in ovarian cancer cells, β-arrestin is recruited to ET_AR to form two trimeric complexes: one through the interaction with Src leading to epithelial growth factor receptor (EGFR) transactivation and β-catenin Tyr phosphorylation, and the second through the physical association with axin, contributing to release and inactivation of glycogen synthase kinase (GSK)-3β and β-catenin stabilization. The engagement of β-arrestin in these two signaling complexes concurs to activate β-catenin signaling pathways. We then demonstrate that silencing of both β-arrestin-1 and β-arrestin-2 inhibits ET_AR-driven signaling, causing suppression of Src, mitogen-activated protein kinase (MAPK), AKT activation, as well as EGFR transactivation and a complete inhibition of ET-1-induced β-catenin/TCF transcriptional activity and cell invasion. ET_AR blockade with the specific ET_AR antagonist ZD4054 abrogates the engagement of β-arrestin in the interplay between ET_AR and the β-catenin pathway in the invasive program. Finally, ET_AR is expressed in 85% of human ovarian cancers and is preferentially co-expressed with β-arrestin-1 in the advanced tumors. In a xenograft model of ovarian metastasis, HEY cancer cells expressing β-arrestin-1 mutant metastasize at a reduced rate, highlighting the importance of this molecule in promoting metastases. ZD4054 treatment significantly inhibits metastases, suggesting that specific ET_AR antagonists, by disabling multiple signaling activated by ET_AR/β-arrestin, may represent new therapeutic opportunities for ovarian cancer.

beta-arrestin | beta-catenin | endothelin A receptor | metastasis | ovarian cancer

Identification of critical signaling effectors of cancer cells is mandatory in defining mechanisms relevant to metastases that can be therapeutically targeted. Endothelin-1 (ET-1) has a relevant role on initiation and progression of a wide spectrum of malignancies, including ovarian carcinoma (1, 2). Our earlier studies have shown that ET-1 and the selective ET A-receptor (ET_AR) subtype, a G-protein-coupled receptor (GPCR), are overexpressed in primary and metastatic human ovarian carcinomas correlating with tumor grade (3), and that ET-1 is present at high levels in ovarian tumor effusions (4). In ovarian tumor cells, the autocrine ET-1/ET_AR axis triggers the activation of multiple signaling pathways, which concurrently drive cell proliferation, survival, angiogenesis and invasion (3–6). ET-1 is also capable of transactivating epithelial growth factor receptor (EGFR) through a Src-dependent mechanism, thus contributing to the ET_AR-dependent invasive and migratory capability of ovarian cancer cells (7, 8). The sustained autocrine ET_AR signaling drives inhibition of glycogen synthase kinase-3β (GSK-3β) to stabilize Snail and β-catenin proteins in a coordinated manner to engage transcriptional programs that modulate epithelial-to-mesenchymal transition and cell invasion (9). In this context, the ability of ET-1 to control the tumor-host interactions (6, 9–11), underlines its key role allowing close coordination in the cellular signaling network in ovarian cancer growth and progression. These findings complement and extend the analysis of gene expression profile of late-stage ovarian cancer whereby ET_AR has been identified as a metastasis-associated gene (12). To characterize downstream mediators in ET-1-induced ovarian cancer invasion and metastasis, we focused on the role of β-arrestins, as scaffold proteins of GPCR, in the β-catenin signaling pathway. β-Arrestins are adapter proteins that, through the formation of multiprotein complexes, play a central role in the interrelated processes of most GPCR desensitization, trafficking, and signaling (13). Comprehensive studies demonstrated that Src activation upon GPCR stimulation required β-arrestins (14, 15). Recently, a new signaling mechanism, that involves the activation of β-arrestins in routing signals from GPCR to Src and EGFR, has been identified (16, 17). Moreover, β-arrestin has been shown to be a necessary component in the Wnt/β-catenin pathway by forming a complex with axin and the cytoplasmic molecule dishevelled (18, 19). These observations require further attention in the light of the recent report that β-arrestin-1 plays a prominent role in the metastases of human colorectal cancer (17). Previous studies showed the involvement of β-arrestins in the regulation of the ET receptors, in terms of internalization and intracellular trafficking pathways, demonstrating that agonist-activated ET_AR is able to recruit with different affinities both β-arrestin-1 and -2, at the plasma membrane (20, 21). Moreover, ET-1 via the ET_AR forms a molecular complex with the Src family Tyr kinase Yes, and β-arrestin-1 in adipocytes (22). Few studies have so far examined the involvement of β-arrestin in mediating ET-1-stimulated signaling pathways, leaving unanswered the exact mechanisms by which ET-1 mediates its effects on tumor cells.

In this study, we tested whether β-arrestins could be recruited to the ET_AR to regulate molecular events involved in tumor progression, such as β-catenin signaling. The results obtained in *in vitro* and *in vivo* models of ovarian cancer establish the functional role of β-arrestin-1 or -2 in ET_AR-induced cross-talk with EGFR, β-catenin signaling, cell invasion, and metastasis.

Results

β-Arrestin-1 and -2 Associate with ET_AR in Ovarian Cancer Cells. Upon agonist stimulation, β-arrestin translocates from the cytosol to the

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[1]To whom correspondence should be addressed. E-mail: bagnato@ifo.it.

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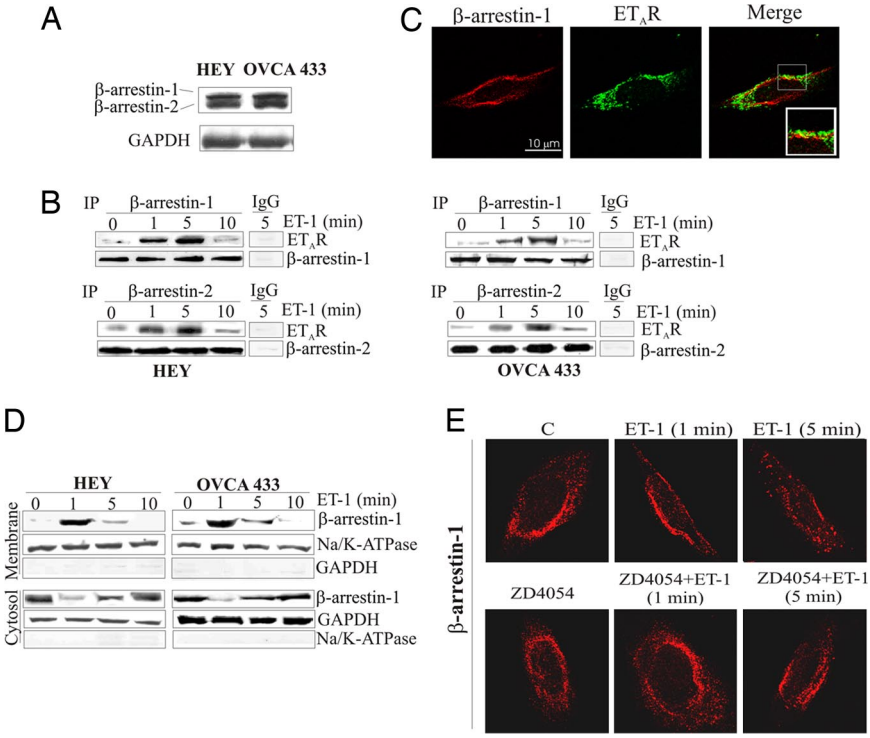

Fig. 1. ET-1 induces the association of β-arrestin-1 and -2 with ET$_A$R in ovarian cancer cells. (*A*) Expression of β-arrestin-1 and -2 in HEY and OVCA 433 cells by using anti-β-arrestin-1/-2. Anti-GAPDH was used for internal control. (*B*) HEY and OVCA 433 cells, treated with ET-1 (100 nM) for the indicated times, were immunoprecipitated (IP), with anti-β-arrestin-1 or -2-conjugated beads or IgG control beads and IB with anti-ET$_A$R, anti–β-arrestin-1, and -2. (*C*) Confocal microscopic analysis of HEY cells incubated with 100 nM ET-1 for 1 minute using anti-β-arrestin-1 (red) and anti-ET$_A$R (green). Co-localization of β-arrestin-1 and ET$_A$R is represented as yellow in the merge image. Box in the merged image indicates the location of the enlarged view. Similar results were observed in three separate experiments. (*D*) Cytosolic and pure plasma membrane fractions of HEY and OVCA 433 cells, incubated for indicated times with 100 nM ET-1, were analyzed by IB with anti-β-arrestin-1. Anti-GAPDH and anti-Na/K-ATPase were used as markers of cytosolic and plasma membrane compartments, respectively. (*E*) Confocal microscopy analysis of HEY cells untreated (*C*) or incubated for indicated times with 100 nM ET-1 and/or ZD4054 (1 μM) using anti-β-arrestin-1. Similar results were observed in three separated experiments.

membrane, where it associates with ET$_A$R (15, 21). Therefore, we analyzed the recruitment of β-arrestin to ET$_A$R in HEY and OVCA 433 ovarian cancer cell lines, in which the ET-1/ET$_A$R axis is well characterized (6). Cell lines co-expressed β-arrestin-1 and -2 (Fig. 1*A*), which associate with ET$_A$R in a time- and agonist-dependent manner as demonstrated by immunoprecipitation experiments (Fig. 1*B*). Consistent with their physical association, we demonstrated that β-arrestin-1 and ET$_A$R co-localized in the membrane of ET-1-stimulated HEY cells (Fig. 1*C*). Immunoblotting analyses clearly showed that β-arrestin-1, which in quiescent cells is localized in the cytosolic fraction, translocated to the plasma membrane compartment after ET-1 stimulation in a time-dependent manner (Fig. 1*D*). Confocal microscopy confirmed these results and revealed that in the absence of ET$_A$R activation, β-arrestin-1 was distributed in the cytosol. After ET-1 stimulation, β-arrestin-1 staining was evident at the plasma membrane and in vesicle-like structures (Fig. 1*E*). Of note, the translocation of β-arrestin-1 is specific for ET$_A$R activation, as it was completely abrogated by the treatment with ZD4054, a selective ET$_A$R antagonist (Fig. 1*E*).

β-Arrestin and ET$_A$R Form a Molecular Signaling Complex with Src. Because previous studies have shown that Src activation by GPCR requires β-arrestin (16, 17), we determined by combinatorial immunoprecipitations whether ET$_A$R, β-arrestin-1 or -2 could form a molecular complex with Src. In both HEY and OVCA 433 cells, the association of β-arrestin-1 and -2 with the ET$_A$R and Src began 2 minutes after ET-1 exposure and declined after 10 minutes (Fig. 2*A* and [supporting information (SI) Fig. S1 *A*, *B*, and *D*]. ZD4054 inhibited this signalplex formation, indicating that ET$_A$R activation by ET-1 is necessary for this trimeric complex formation (Fig. 2*A*

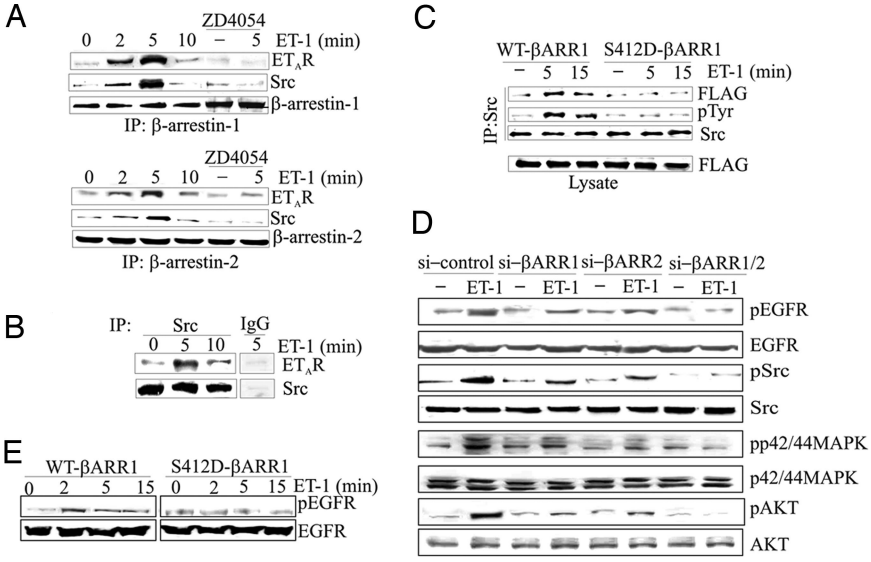

Fig. 2. ET-1 induces the formation of the ET$_A$R/β-arrestin/Src signaling complex to transactivate EGFR. (*A*) HEY cells, treated with ET-1 (100 nM) for the indicated times or in combination with ZD4054 (1 μM) for 5 minutes, were IP with anti-β-arrestin-1 or -2. The IP samples were analyzed by IB with anti-ET$_A$R, anti-Src, anti-β-arrestin-1 and -2. (*B*) HEY cells, treated with ET-1 (100 nM) for the indicated times, were IP with anti-Src-conjugated beads or IgG control beads and IB with anti-ET$_A$R and anti-Src. (*C*) IP with anti-Src of HEY cell lysates after knockdown with β-arrestin-1 siRNA, subsequent rescue with FLAG-tagged WT- or mutant S412D-β-arrestin-1, and treatment for indicated times with 100 nM ET-1. The IP samples and cell lysates were analyzed by IB with anti-FLAG, anti-phospho-Tyr, and anti-Src. (*D*) HEY cells, transfected with scrambled (si-control) or siRNA targeting either β-arrestin-1 or -2 or both, were treated with ET-1 (100 nM). Cell lysates were analyzed by IB with anti-phospho-EGFR, anti-EGFR, anti-phospho-Src, anti-Src, anti-phospho-p42/44 MAPK, anti-p42/44 MAPK, anti-phospho-AKT, and anti-AKT. (*E*) IB of HEY cell lysates treated as in (*C*), using anti-pEGFR and anti-EGFR.

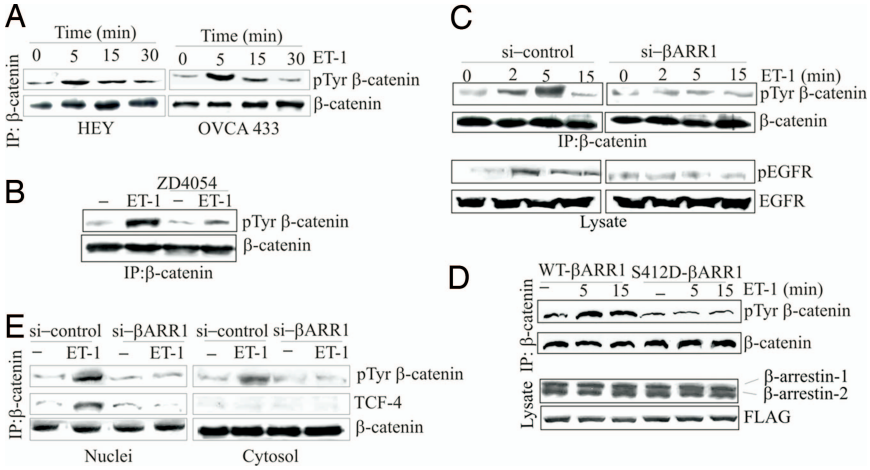

Fig. 3. ET-1 triggers tyrosine phosphorylation of β-catenin through signalplex and EGFR. (*A*) HEY and OVCA 433 cells were incubated for different times with 100 nM ET-1. IP were performed with anti-β-catenin and IB with anti-pTyr and anti-β-catenin. (*B*) Lysates of HEY cells, treated with 100 nM ET-1 and/or 1 μM ZD4054, were IP with anti-β-catenin and IB with anti-pTyr and anti-β-catenin. (*C*) Lysates of HEY cells transfected with scrambled (si-control) or β-arrestin-1 siRNA and incubated for the indicated times with ET-1 (100 nM), were IP with anti-β-catenin and IB with anti-pTyr and anti-β-catenin. The same lysates were IB with anti-pEGFR and anti-EGFR. (*D*) HEY cells, after knockdown with β-arrestin-1 siRNA and subsequent rescue with FLAG-tagged WT- or S412D-β-arrestin-1, were treated with 100 nM ET-1 for the indicated times. IP was performed with anti-β-catenin and IB with anti-pTyr and anti-β-catenin. The same lysates were IB with anti-β-arrestin-1/-2 and anti-FLAG. (*E*) Nuclear and cytosolic extracts of HEY cells, transfected with scrambled (si-control) or β-arrestin-1 siRNA and treated with ET-1 (100 nM), were IP with anti-β-catenin and IB with anti-pTyr, anti-TCF4, and anti-β-catenin.

and Fig. S1 *A*, *B*, and *D*). The presence of ET_AR in Src immunoprecipitates (Fig. 2*B* and Fig. S1 *C* and *E*) indicated that ET-1 induces the formation of an ET_AR/β-arrestin/Src signaling complex, or signalplex. In parallel with the ability to promote binding between β-arrestin-1 and Src, ET-1 induced a time-dependent dephosphorylation on serine-412 β-arrestin-1 in both cell lines (Fig. S1*F*), which causes reduced affinity for Src (14, 23). Then we performed siRNA knockdown of β-arrestin-1 followed by rescue with expression of FLAG-tagged WT or mutant S412D-β-arrestin-1. Specificity of siRNA oligos was confirmed by Western Blotting analysis, which showed an 80% knockdown of β-arrestin-1 (Fig. S2*A*), and by rescue (90%) of knockdown effects with expression of FLAG-tagged β-arrestin-1 (Fig. S2*B*). In cells expressing WT-, but not S412D-β-arrestin-1, ET-1 induced the association of β-arrestin-1 with Src and its Tyr phosphorylation (Fig. 2*C*), confirming that the association of β-arrestin-1 with Src is critical for its activation. To demonstrate that β-arrestin-1 is critical for the formation of the complex with ET_AR and Src, we silenced β-arrestin-1 in HEY cells, proving that ET_AR cannot bind Src independently of β-arrestin-1 (Fig. S2*C*).

ET_AR/β-Arrestin/Src Signaling Complex Is a Critical Event in EGFR Transactivation and Downstream Pathways. Because emerging evidence indicates that β-arrestins organize and scaffold an active signaling complex with Src, leading to EGFR transactivation (16, 17), we evaluated the potential functional role of β-arrestin-1 and β-arrestin-2 in ET-1-dependent multiple signaling pathways by specifically silencing either β-arrestin-1 or -2 or both (Fig. S2*A*). In HEY cells, ET-1 induced rapid Src and EGFR phosphorylation and an increase in the activation of p42/44 mitogen-activated protein kinase (MAPK) and AKT (Fig. 2*D*). Interestingly, knockdown of β-arrestin-1 or -2 inhibited the ET-1-induced Src and EGFR activation and their downstream pathways, which were completely blocked in the presence of siRNA targeting both β-arrestin-1 and -2 (Fig. 2*D*), indicating that both β-arrestins are required in ET_AR-induced signaling. ZD4054, gefitinib, an EGFR inhibitor, or PP2, a Src inhibitor, reduced the ET-1-induced Src and EGFR activation. However, gefitinib incompletely reduced the ET-1-mediated MAPK and AKT activation. A combination of ZD4054 plus gefitinib resulted in a greater inhibition of all these pathways (Fig. S3), indicating the critical role of ET_AR and EGFR interconnected signaling systems (24). Furthermore ET-1-induced EGFR phosphorylation was inhibited in HEY cells silenced for β-arrestin-1 and rescued with S412D-β-arrestin-1 mutant compared with WT-β-arrestin-1- expressing cells (Fig. 2*E*). Altogether these data demonstrate that silencing of both β-arrestin-1 and -2 inhibits ET_AR-driven signaling and that β-arrestin/Src complex formation is a critical event for activation of EGFR and related-pathways. The matrix metalloproteinases (MMP) inhibitor, GM6001, did not affect the ET-1-induced EGFR transactivation, demonstrating that this process is MMP independent (Fig. S4).

An ET_AR/β-Arrestin/Src Signaling Complex Is Required for ET-1-Induced β-Catenin Tyrosine Phosphorylation. Because recent studies have revealed that Tyr phosphorylation of β-catenin enhances β-catenin/TCF signaling (25–27), we tested whether ET-1 induces Tyr phosphorylation of β-catenin through the EGFR transactivation mediated by β-arrestin. In both HEY and OVCA 433 cells, ET-1 induced β-catenin Tyr phosphorylation, starting at 5 minutes and lasting for 15 to 30 minutes, indicating that its phosphorylation state was tightly regulated by ET-1 (Fig. 3*A*). This effect was also dose dependent, with a maximum at 100 nM of ET-1 in both cell lines (Fig. S5*A*). Moreover, in HEY cells, the ET-1-induced β-catenin Tyr phosphorylation mediated by the ET_AR/β-arrestin/Src complex was completely blocked by ZD4054 (Fig. 3*B*). The knockdown of EGFR with siRNA confirmed that EGFR transactivation is required for ET-1-induced Tyr phosphorylation of β-catenin (Fig. S5*B* and *C*). Interestingly, β-arrestin-1 siRNA markedly downregulated Tyr phosphorylation of EGFR and of β-catenin in ET-1-treated cells compared with control cells (Fig. 3*C*). The inhibition of β-catenin Tyr phosphorylation observed with β-arrestin-1 knockdown was rescued by the expression of WT- but not S412D-β-arrestin-1, indicating the critical role of β-arrestin-1-driven signalplex formation in EGFR-mediated β-catenin Tyr phosphorylation induced by ET-1 (Fig. 3*D*). It is noteworthy that ET-1 treatment promoted the binding between Tyr phosphorylated β-catenin and TCF-4 in nuclear extracts in scrambled but not in β-arrestin-1 siRNA-transfected HEY and OVCA 433 cells, suggesting that the ET-1-induced β-catenin Tyr phosphorylation can

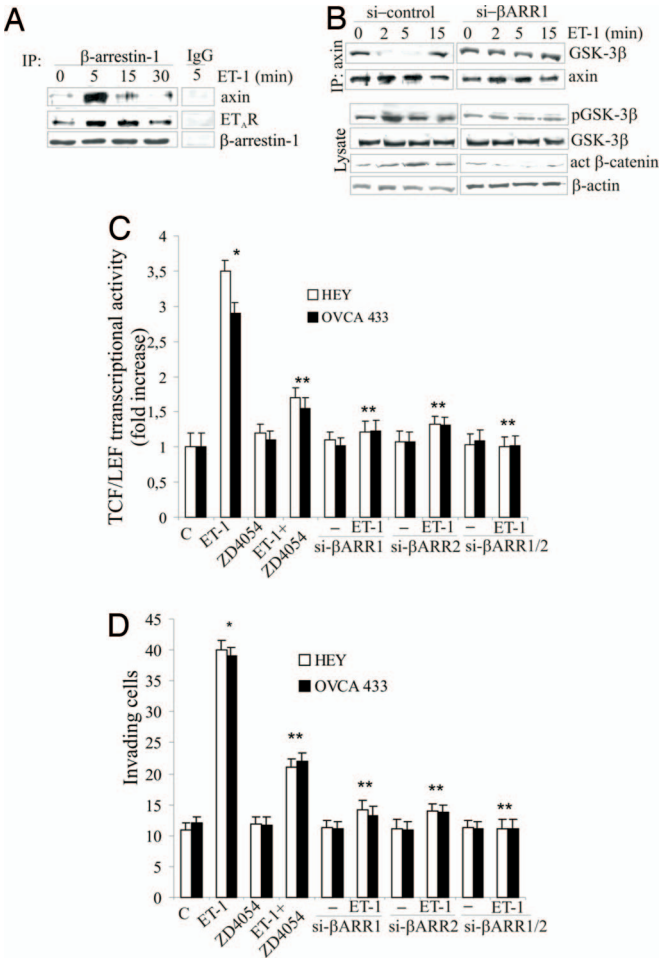

Fig. 4. β-Arrestin links ET_AR to axin in the activation of β-catenin signaling, its transcriptional activity, and cell invasion. (*A*) Lysates of HEY cells, stimulated with ET-1 (100 nM) for different times, were IP with anti-β-arrestin-1-conjugated beads or IgG control beads and IB with anti-axin, anti-ET_AR, and anti-β-arrestin-1. (*B*) HEY cells, transfected with scrambled (si-control) or β-arrestin-1 siRNA, were treated for the indicated times with 100 nM ET-1. IP was performed with anti-axin and IB with anti-GSK-3β and anti-axin. Lysates of the same cells were IB with anti-pGSK-3β (Ser 9), anti-GSK-3β, anti-active (act) β-catenin, and anti-β-actin for internal control. (*C*) β-catenin/TCF transcriptional activity was evaluated in HEY and OVCA 433 cells transfected siRNA targeting either β-arrestin-1 or -2 or both, incubated with ET-1 (100 nM) and/or ZD4054 (1 μM). Bars, ±SD. $*P < 0.001$ with control; $**P < 0.05$ compared with ET-1. (*D*) Invasion assay of HEY and OVCA 433 cells treated as in C. Bars, ± SD. $*P < 0.01$ compared with control; $**P < 0.05$ compared with ET-1.

represent a transcriptional active pool in a β-arrestin-1-dependent manner (Fig. 3*E* and Fig. S5*D*).

ET-1 Promotes Linking of β-Arrestin to Axin in the Activation of β-Catenin Signaling. Based on previous findings on the interaction between β-arrestin-1 and axin in Wnt signaling, we explored whether β-arrestin-1 may also provide a link between ET_AR and β-catenin signaling by its capacity to bind axin directly. In HEY cells, axin coimmunoprecipitated with ET_AR and β-arrestin-1 in an ET-1-dependent manner, with a peak at 5 minutes (Fig. 4*A*). Moreover, we demonstrated by coimmunoprecipitation experiments that in these cells axin and Src are not present in the same complex (Fig. S6). We also observed that ET-1 caused a reduction in the amounts of GSK-3β bound to axin, whereas this association was still present in β-arrestin-1-silenced cells, suggesting that the ET-1-induced binding of β-arrestin-1 to axin is required to induce the displacement of GSK-3β from an axin-containing complex (Fig. 4*B*). Because the inhibition of GSK-3β led to dephosphorylation and stabilization of β-catenin in response to ET-1 (9), we evaluated the role of β-arrestin-1 in the phosphorylation of GSK-3β, which renders it inactive. ET-1 treatment led to rapid phosphorylation of GSK-3β on serine 9, which was impaired after knockdown of β-arrestin-1, suggesting that the release of GSK-3β from axin-containing complexes regulated by β-arrestin-1 is associated with its functional inhibition (Fig. 4*B*). We observed that the ET-1-dependent pattern of β-catenin dephosphorylation, evaluated with an Ab against active β-catenin, was abolished in β-arrestin-1-silenced cells (Fig. 4*B*). Altogether, these results strongly imply that the association of β-arrestin-1 with ET_AR may signal through two parallel coordinated mechanisms that concur in the stabilization of β-catenin. One that is dependent on EGFR-mediated Tyr phosphorylation of β-catenin and another through axin and dephosphorylation of β-catenin in a manner similar to that of canonical Wnt signaling (Fig. S7).

β-Arrestin-Driven Signalplexes Are Required in β-Catenin Transcriptional Activity and Cell Invasion Induced by ET-1. Because dephosphorylated β-catenin is stabilized and leads to activation of transcription in a TCF/Lef-dependent manner, we also investigated the role of β-arrestin-1 or -2 in ET-1-induced activation of the TOP/Flash luciferase reporter construct. As shown in Fig. 4*C*, although HEY and OVCA 433 cells strongly respond to ET-1 stimulation, the silencing of either β-arrestin-1 or -2 or both significantly inhibited the TCF/Lef reporter activity. Moreover, the knockdown of both β-arrestins caused complete inhibition of ET-1-induced invasion (Fig. 4*D*), highlighting a new role of β-arrestins in ET-1-induced β-catenin transcription and the invasive potential of ovarian cancer cells. The stimulatory effect of ET-1 on β-catenin transcriptional activity (Fig. 4*C*) and cell invasion (Fig. 4*D*) was also blocked by pretreatment with ZD4054. These results underline the relevant role of β-arrestin-dependent ET_AR-induced β-catenin signaling and invasiveness.

ET_AR Links β-Arrestin to Promote Metastasis in HEY Xenografts. To determine the role of β-arrestin-1 in ovarian cancer metastasis formation, we used HEY cells, as well as clonally derived HEY cell lines overexpressing WT- or S412D-β-arrestin-1, in an i.p. metastatic model. After 4 weeks of tumor cell injection, multiple metastatic seeding tumors were distributed on the peritoneal surface, omentum, small bowel, mesentery, and in both ovaries (Fig. 5*A*). Interestingly, HEY cells produced a number of metastases similar to those produced by clonally derived HEY cell lines that overexpress WT-β-arrestin-1 (Fig. 5*B*). Differentially, clonal HEY cells overexpressing S412D-β-arrestin-1 mutant showed reduced metastatic ability by nearly 50% (mean, 4.5 ± 0.7 lesions vs. 9 ± 1.9 of WT-β-arrestin-1-expressing cells; $P < 0.001$), suggesting that β-arrestin-1 may serve a pivotal role in ovarian carcinoma metastasis (Fig. 5*B*). To explore the therapeutic potential of ET_AR blockade in the control of ovarian peritoneal metastasis, we tested the efficacy of ZD4054. The number of intra-abdominal metastases was significantly decreased in ZD4054-treated mice (mean, 4.1 ± 0.7 lesions) when compared with controls (mean, 10.8 ± 1.9 lesions; Fig. 5*B*). The treatment at the dose and schedule tested was well tolerated, as evaluated by the absence of weight loss or other signs of acute or delayed toxicity. Because *in vitro* data indicate that β-arrestin is essential for β-catenin signaling and invasive properties of ovarian cancer cells, we sought to determine the status of β-catenin in metastases expressing WT or S412D-β-arrestin-1. We observed by immunoblotting a strong decrease in the active β-catenin in the metastatic nodules derived from clonal HEY cells overexpressing S412D-β-arrestin-1 mutant when compared with HEY cells overexpressing WT-β-arrestin-1 (Fig. 5*C*). Similar results were obtained in the metastatic lesions from ZD4054-treated mice when compared with control. These results, together with the

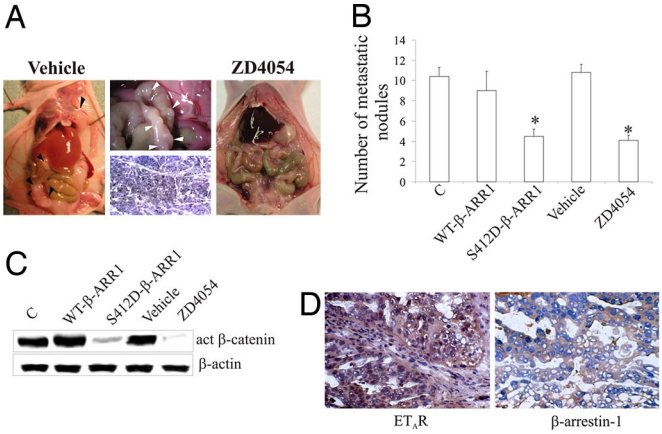

Fig. 5. ET$_A$R links β-arrestin-1 to promote metastasis in HEY ovarian cancer xenografts. (*A*) Representative views of the peritoneal cavity of mice treated with vehicle or ZD4054. Arrows point to metastatic nodules. *Inset*: Histological section of small-bowel implant. Original magnification, ×20. (*B*) HEY cells or clonally derived HEY cells expressing WT- or mutant S412D-β-arrestin-1 were injected i.p. into nude mice. One week after injection, one group of control mice injected with HEY cells was treated for 21 days with vehicle or with ZD4054 (10 mg/kg/day). Values represent the average ± SE of 10 mice from three independent experiments. *$P < 0.001$ compared with control or WT-β-arrestin-1-expressing cells. (*C*) Immunoblotting for anti-active (act) β-catenin and anti-β-actin expression of representative metastatic samples of HEY xenografts, as described in (*B*). We analyzed homogenized HEY metastasis specimens from 10 mice for each group. (*D*) Immunoistochemical staining of representative human primary ovarian carcinoma tissue samples for expression of ET$_A$R and β-arrestin-1 (original magnification, ×200).

reduced metastatic ability, further support that the ET$_A$R-dependent β-catenin pathway is involved *in vivo* in a β-arrestin-dependent manner.

ET$_A$R Is Co-Expressed with β-Arrestin-1 in Advanced Ovarian Carcinomas. To explore the pathophysiological function of ET$_A$R in human ovarian cancer, a cohort of 35 primary ovarian tumors were assayed by immunohistochemistry for ET$_A$R and β-arrestin-1 expression. Approximately 83% of the ovarian cancer specimens were positive for ET$_A$R whereas about 20% of them were positive for β-arrestin-1. Notably, the expression of ET$_A$R as well as of β-arrestin-1 and their co-expression increased in grade 3–4 compared with grade 2 tumors. Moreover, more than 72% of β-arrestin-1-positive grade 3–4 tumors were positive for ET$_A$R (5/7), whereas no grade 2 tumors co-expressed β-arrestin-1 and ET$_A$R (0/8). A representative case of ET$_A$R and β-arrestin-1 expression is shown in Fig. 5*C* and Fig. S8.

Discussion

A detailed understanding of the molecular mechanisms that control ovarian cancer metastasis is a crucial step in identifying new effective therapies (28). Here, we provide an overall molecular outline showing that ET$_A$R, a critical GPCR involved in ovarian cancer progression, is a relevant receptor in modulating the invasiveness of ovarian cancer cells and metastatic spread. In this model, we identify an ET$_A$R-driven invasive signal pathway which is dependent on the scaffold protein β-arrestins as crucial point in the activation of β-catenin signaling and in the induction of a metastatic phenotype. We show that the ET$_A$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$_A$R, β-arrestin, and Src that controls cross-talk with the EGFR, and another with axin, which mediates signaling to GSK-3β (Fig. S7). These results may have important implications for the role of the ET-1 axis in tumor progression, because they indicate that the recruitment of β-arrestin to the activated ET$_A$R may represent a checkpoint controlling multiple pathways and promoting the stabilization and nuclear translocation of β-catenin, thereby stimulating invasion.

The findings that ET$_A$R-dependent EGFR transactivation requires activation of Src in a β-arrestin-dependent fashion (16, 17) raise the possibility that β-arrestin can direct ET$_A$R signaling toward alternative pathways. Thus, upon ET-1 stimulus, β-arrestin is recruited to the membrane, where it can functionally interact with the ET$_A$R and the downstream effector Src, resulting in the dephosphorylation of β-arrestin-1, a posttranslational modification necessary for activation of Src and signalplex formation leading to EGFR transactivation. These results elucidate an additional role of β-arrestin in GPCR signaling, in which ET-1-dependent β-arrestin recruitment acts as a signaling initiator in the ET$_A$R/EGFR cross-talk signaling to Tyr phosphorylated β-catenin, thereby promoting the formation of a nuclear complex β-catenin/TCF-4 that, in turn, increases its transcriptional activity.

Studies aimed at identifying the modulation of Wnt/β-catenin signaling indicated that β-arrestin-1 interacts with axin to regulate β-catenin transcriptional activity (18, 19). In this study, we demonstrated that β-arrestin is fully engaged in the interplay between ET-1/ET$_A$R and components of the β-catenin signaling cascade. The ET$_A$R/β-arrestin complex binds directly to axin, contributing to destabilize the degradation complex and resulting in the stabilization of β-catenin, thus indicating that ET-1 can mimic the Wnt pathway in a β-arrestin-dependent manner. Ultimately, the two β-arrestin-mediated, coordinated signalplexes result in the stabilization and nuclear translocation of β-catenin, thereafter stimulating Lef transcription and cell invasion, indicating that β-arrestins modulate finely tuned, interconnected signals induced by ET-1/ET$_A$R to promote β-catenin signaling in tumor cells. These findings, along with those previously reported (29), demonstrate that β-arrestin affects tumor progression by modulating multiple factors to provide a suitable microenvironment. Moreover, the recently identified nuclear functions for β-arrestin provide new insights into the complexity of this multifunctional protein (30). The present results also suggest that co-espression of ET$_A$R and β-arrestin may be indicative of the malignant phenotypes of primary human ovarian cancers. Likewise, the present results highlighting the activation of ET$_A$R/β-arrestin-dependent "signalosome" in gaining malignant potential, shed new light on the molecular mechanism implicated in the metastatic behavior of ovarian cancer cells.

The demonstration that ET$_A$R-driven signaling pathways (including EGFR transactivation, the β-catenin transcriptional activity, and cell invasiveness) require both β-arrestin-1 and -2 might reflect distinct roles in these process played by each isoform or the need for heterodimerization of β-arrestin-1 and -2, as recently demonstrated (31). It will hence be interesting to investigate the exact role of each β-arrestin in the progress toward ovarian cancer metastasis.

In conclusion, our study provides a detailed molecular dissection as to how ET$_A$R expression and its β-arrestin-mediated signaling can be linked to the invasiveness of ovarian cancer cells and to their metastatic activities. The recent preclinical demonstration of tumor growth inhibition (24), together with reduced metastatic potential in response to ZD4054, suggest that this treatment, by simultaneously disabling multiple signaling circuits activated by ET$_A$R in a β-arrestin-dependent manner, may allow the development of pathway-specific therapeutics in the control of ovarian cancer.

Materials and Methods

Materials. Clinical grade ZD4054, N-(3-methoxy-5-methylpyrazin-2-yl)-2-(4-[1,3,4-oxadiazol-2-yl]phenyl) pyridine-3-sulfonamide, was kindly provided by AstraZeneca. ET-1 was purchased from Peninsula Laboratories. Other materials are listed in *SI Materials and Methods*.

Cell Culture, DNA Transfection, and siRNA Transfection Experiments. Human ovarian carcinoma cell lines, HEY and OVCA 433, generously provided by Gio-

vanni Scambia (Catholic University School of Medicine, Rome, Italy), were cultured as previously described (6). For the silencing of β-arrestin-1 or β-arrestin-2, cells were transiently transfected with duplex siRNAs (30 nM) targeting human β-arrestin-1 or -2 (Hs_ARRB1_11 and Hs_ARRB2_10 HP Validated siRNA, Qiagen, respectively), negative control (scrambled sequence) or no-RNA (MOCK), using RNAiFect transfection reagent (Qiagen). The specificity of the siRNA sequences for β-arrestin-1 and -2 have previously been validated (32). After 48 hours of incubation, cells were divided into six-well plates for further experiments and for β-arrestin immunoblotting. Each knockdown experiment described herein was detected for specific reduced expression of β-arrestins (75–90%) with A1CT Ab, a rabbit polyclonal Ab to β-arrestin-1/-2 kindly provided by Robert Lefkowitz (Howard Hughes Medical Institute, Duke University). In the rescue experiments, we performed transient transfection of pcDNA3 plasmid or 2 μg FLAG epitope-tagged WT or -S412D β-arrrestin-1 expression plasmids, kindly provided by Robert Lefkowitz, two ''wobble'' mutant constructs encoding rat β-arrestin-1 sequences resistant to siRNA targeting, using LipofectAMINE reagent (Invitrogen). Further details are available in *SI Materials and Methods*.

Confocal Fluorescence Microscopy. HEY cells were stimulated as described, fixed in 2% formaldehyde, permeabilized in 0.25% Triton X-100 in phosphate-buffered saline solution (PBS), and then immunostained with the primary Ab to β-arrestin-1 (Santa Cruz Biotechnology Inc.), anti-ET_AR (BD Transduction Laboratories). The TRITC conjugated donkey anti-goat and the FITC conjugated goat anti-mouse (Jackson Immunoresearch) were used as secondary Abs. Fluorescence signals were analyzed in confocal vertical (x-z) sections captured with a Zeiss Confocal Laser Scanning Microscope. For each image the entire thickness of the cells has been sectioned into optical slices of 7 μm and the focal plane corresponding to the basal level of the cells has been chosen to highlight the membrane staining.

Luciferase Reporter Gene Assay. To measure the transcriptional activity of β-catenin, cells were transiently cotransfected using LipofectAMINE reagent (Invitrogen) with 1 μg pTOP/Flash (Upstate Biotech) and 100 ng pCMV-β-galactosidase (Promega) vectors. Reporter activity was measured using the Luciferase assay system (Promega) and normalized to β-galactosidase activity. The mean of three independent experiments performed in sextuplicate was reported.

Immunoblotting and Immunoprecipitation. For Western blotting analysis, cells were detached by scraping, collected by centrifugation, and lysed in lysis buffer [250 mM NaCl, 50 mM HEPES (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40, protease inhibitors]. Whole-cell lysates or homogenized HEY metastases specimens or separated fractions were resolved by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS/PAGE), followed by immunoblotting (IB) using Abs to: anti-phosphoTyr (PY-20) (BD Transduction Laboratories), EGFR, phospho-EGFR (Tyr-845), phospho-GSK-3β (pSer9), GSK-3β, phospho-β-arrestin-1 (Ser-412), p42/44MAPK, phospho-p42/44MAPK, phospho-Akt (Ser-473), AKT, GAPDH, Na, K-ATPase and FLAG (Cell Signaling Technology), active-β-catenin (an Ab against non-serine-threonine phosphorylated, nonubiquitinated) and TCF-4 (Upstate), β-catenin, β-arrestin-1, β-arrestin-2, phospho-Src (Tyr-416) and Src (Santa Cruz Biotechology), ET_AR (Abnova GmbH), β-actin (Oncogene), and axin (Zymed Laboratories). Further details are available in *SI Materials and Methods*.

Chemoinvasion Assay. Chemoinvasion assay was performed as previously described (6). The filters were coated with an even layer of 10 mg/ml Cultrex Basement Membrane Extract Matrigel (Trevigen). After 6 hours of incubation at 37 °C, the filters were removed, stained with Diff-Quick (Merz-Dade), and the migrated cells in 10 high-power fields were counted. Each experimental point was analyzed in triplicate.

Metastasis Assay. HEY cells or clonally derived HEY cells, stably transfected with WT- or S412D-β-arrestin-1 (1.8×10^6), were i.p. injected into female athymic nude mice (Charles River Laboratories), following the guidelines for animal experimentation of the Italian Ministry of Health. In all experiments, each group consisted of 10 mice. In the treatment experiments, one week after injection of cancer cells, one group was treated i.p. for 21 days with ZD4054 (diluted in PBS) (10 mg/kg/day), and one group received the same volume saline solution (vehicle). At the end of the treatment, mice were killed; the number of metastases was counted and the removed tumors were weighed, carefully dissected, and snap-frozen for immunohistochemical and immunoblot analysis. For the immunohistochemical analysis of human tissue samples see *SI Materials and Methods*.

Statistical Analysis. Densitometric quantifications and normalizations were performed using National Institutes of Health Scion Image 1.63 software. Statistical analysis was done using the Student *t* test, Fisher's exact test, or one-way analysis of variance (ANOVA) to correct for multiple comparisons, as appropriated. All statistical tests were two-sided and were done using SPSS software (SPSS Inc., Chicago, IL).

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