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Endothelin-1/Endothelin A receptor-mediated biased signaling is a new player in modulating human ovarian cancer cell tumorigenesis

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Abstract

The endothelin-1 (ET-1)/endothelin A receptor (ET_AR, a G protein-coupled receptor) axis confers pleiotropic effects on both tumor cells and the tumor microenvironment, modulating chemoresistance and other tumor-associated processes by activating Gα_q- and β-arrestin-mediated pathways. While the precise mechanisms by which these effects occur remain to be elucidated, interference with ET_AR signaling has emerged as a promising antitumor strategy in many cancers including ovarian cancer (OC). However, current clinical approaches using ET_AR antagonists in the absence of a detailed knowledge of downstream signaling have resulted in multiple adverse side effects and limited therapeutic efficacy. To maximize the safety and efficacy of ET_AR-targeted OC therapy, we investigated the role of other G protein subunits such as Gα_s in the ET_AR-mediated ovarian oncogenic signaling. In HEY (human metastatic OC) cells where the ET-1/ET_AR axis is well-characterized, Gα_s signaling inhibits ET_AR-mediated OC cell migration, wound healing, proliferation and colony formation on soft agar while inducing OC cell apoptosis. Mechanistically, ET-1/ET_AR is coupled to Gα_s/cAMP signaling in the same ovarian carcinoma-derived cell line. Gα_s/cAMP/PKA activation inhibits ET_AR-mediated β-arrestin activation of

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angiogenic/metastatic *Calcr1* and *Icam2* expression. Consistent with our findings, *Gas* overexpression is associated with improved survival in OC patients in the analysis of the Cancer Genome Atlas data. In conclusion, our results indicate a novel function for *Gas* signaling in ET-1/ET_AR-mediated OC oncogenesis and may provide a rationale for a biased signaling mechanism, which selectively activates *Gas*-coupled tumor suppressive pathways while blocking *Gαq*-/*β*-arrestin-mediated oncogenic pathways, to improve the targeting of the ET_AR axis in OC.

Keywords

β-arrestin; biased G protein-coupled receptor signaling; endothelin axis; metastatic genes

Introduction

Endothelin-1 (ET-1) is a peptide that has been implicated in various physiological and pathological conditions, including development, cell proliferation, differentiation, cardiac function and cancer [1, 2]. It is well recognized as a growth factor that is present in plasma and is produced by stromal and tumor cells. A recent study has also shown that elevated concentrations of ET-1 in plasma are associated with higher mortality in patients with certain malignancies [3]. ET-1 mediates cellular processes through its cell surface receptor by facilitating various signaling pathways including ILK, Akt, MAPK, *Gαq*/PKC, Src, EGFR, and GSK in an autocrine or paracrine fashion [2, 4, 5]. These pathways have been shown to regulate gene expression associated with cancer cell proliferation, adhesion, migration, invasion, angiogenesis and anti-apoptosis [6, 7]. In particular, phosphorylation of ILK and GSK via the ET-1 axis leads to downstream activation of *β*-arrestin1, which is a multifunctional adapter protein that regulates the invasive and metastatic behavior of ovarian cancer (OC) cells by activating multiple effector pathways independent of G protein-mediated second messenger accumulation [8, 9]. The ET-1 receptors, endothelin type A receptor (ET_AR) and endothelin type B receptor (ET_BR) are members of the G protein-coupled receptor (GPCR) family. These receptors are expressed in distinct cell types and are coupled to different *Gα* subunits. ET_BR coupled to *Gαq* and *Gαi*, is expressed mainly in endothelial cells, while ET_AR, which is coupled to *Gαq*, *Gαs* and *Gα_{12/13}*, is expressed in vascular smooth muscle cells and cardiomyocytes, and is also overexpressed in diverse solid tumors including OC [10–13].

OC is the leading cause of gynecological cancer death. While various targeted therapeutics have been explored, overall survival has not appreciably improved in more than three decades [14]. The ET axis appears to be involved in advanced stages of OC, where up-regulated expression of ET_AR and down-regulated expression of ET_BR are found [15]. Specifically, ET_AR overexpression is higher in late stage OC compared to early stage OC, suggesting that ET_AR overexpression may promote epithelial to mesenchymal transition (EMT) [16]. Moreover, gene expression profiling analyses suggest that ET_AR expression may be metastasis-associated [17, 18]. It has been proposed that ET-1 binds to ET_AR receptors, thereby inducing oncogenic signaling; this idea is supported by a number of preclinical studies using ET_AR antagonists that demonstrate a significant reduction in tumor growth and progression in multiple malignancies, including OC [17, 19]. Accordingly, a

range of specific and selective ET_AR antagonists has been evaluated in clinical trials of OC patients. However, these trials using ET_AR antagonists have been prone to poor efficacy and adverse side effects [20, 21], in part because the drugs have been developed without detailed understanding of signaling mechanisms and they have numerous off-target effects.

To improve the use of these antagonists in treatment of OC patients, this study focuses on the utility of a concept referred to as “biased GPCR signaling”, in which ligands have selectivity between G-protein- and β -arrestin-mediated signaling pathways [22, 23]. The ability of biased ligands to differentially activate a subset of signaling pathways raises the possibility of selective induction of favorable signaling pathways. Several ‘biased’ ligands, which have been discovered through cell-based assays, indeed have been shown to provide important therapeutic utilities by blocking undesired complications while maintaining desired effects in both cardiovascular and central nervous system tissues [24–28]. This ability to differentially activate disparate signaling pathways via the same receptor raises the possibility of manipulating existing ET_AR antagonists to achieve selective induction of tumor suppressive signaling pathways, while inhibiting oncogenic pathways.

Here, we investigated the roles of individual G α subunits such as G α_s in ET_AR-mediated OC progression to distinguish known oncogenic downstream effects (i.e. G α_q -coupled or β -arrestin-mediated signaling pathway) from potential tumor suppressive effects, both of which are mediated by ET-1/ET_AR axis. To test the hypothesis that G α_s -coupled ET_AR signaling is tumor suppressive, we selected the human metastatic OC-derived HEY cell line (derived from a metastatic lesion in a patient with a moderately differentiated cystadenocarcinoma of the ovary), because it expresses abundant ET_AR, secretes high levels of ET-1, and has unique phenotypes such as chemo-resistance, high invasiveness and the ability to grow xenografted tumors in 100% of immunologically deprived mice [16, 29, 30]. We then applied both pharmacological and genetic approaches to assess *in vitro* tumorigenesis. Interestingly, pre-treatment of cells with forskolin (adenylate cyclase activator inducing G α_s /cAMP/PKA signaling) before ET-1 stimulation resulted in a decrease in the number of migrating cells and colonies on soft agar, and an increase in apoptosis. These results suggest a potential tumor suppressive effect of G α_s /cAMP/PKA signaling on ET_AR-mediated ovarian tumorigenesis. Our loss-of-function genetic approach using shRNAs supported these findings on G α_s -mediated signaling in HEY cells. We further demonstrated that ET_AR-mediated β -arrestin signaling activates a subset of metastasis/angiogenesis-associated genes including *Calcr1* and *Icam2*, which are inhibited by G α_s /cAMP/PKA activation. By showing that stimulation of the ET-1/ET_AR axis can activate both tumor suppressive and oncogenic OC properties, our data provide the first evidence of biased signaling mechanisms in ET_AR-mediated OC progression, which may contribute to the development of improved ligand therapies for OC with more sustained efficacy by emphasizing G α_s -biased ET_AR signaling mechanisms.

Materials and Methods

Details of cell culture, OC cell migration in transwell chambers, scratch wound migration assay, TUNEL assay, soft agar assay, generation of HEY stable cell lines expressing lentivirus-mediated shRNAs, RNA isolation and quantitative real-time RT-PCR analysis,

immunoblotting and detection, cell growth curve analysis, OC patient survival analysis, cAMP reporter assay, siRNA experiments targeting β -arrestins and Taqman PCR array analysis, and statistical analysis are provided in online supplemental materials.

Results

Adenylate cyclase activator forskolin decreases ET-1-mediated OC cell migration

To identify the roles of G_{α_s} /PKA- and G_{α_q} /PKC-mediated signaling in ET-1/ ET_A R-mediated *in vitro* OC tumorigenesis, HEY (derived from a metastatic human OC tumor) cells which endogenously overexpress ET_A R were serum-starved and pre-treated with pharmacological agents including forskolin (Forsk; adenylate cyclase activator inducing PKA signaling), H-89 (PKA inhibitor), phorbol 12-myristate 13-acetate (PMA; PKC activator), and Ro-318425 (Ro-3; PKC inhibitor) followed by ET-1 stimulation. We then assessed cell migration in both transwell chamber- and scratch wound healing-based assays. Consistent with previous findings showing the oncogenic properties of ET-1 [31–33], we found that HEY cells treated with ET-1 for 6–36 hours displayed an increase in OC cell migration (Fig. 1A–B, Fig. 2A–B and Fig. S1). In our ET-1 time course experiments, 24 hours was the time point showing the maximum number of migrated cells (Fig. S1). This increase in ET-1-mediated migration was unchanged by H-89 or PMA pretreatment, but Forsk or Ro-3 pretreatment was able to block the ET-1-stimulated increase (Fig. 1 and Fig. 2). Collectively, these results indicate that activation of G_{α_s} /cAMP/PKA signaling and inhibition of G_{α_q} /PKC signaling can exert inhibitory effects on ET-1/ ET_A R-mediated migration of HEY cells.

G_{α_s} /cAMP/PKA activation reverses ET-1-mediated anti-apoptosis in human OC cells

Since Forsk inhibited HEY cell's migrative potential, we next investigated if G_{α_s} /cAMP/PKA signaling inhibits ET-1/ ET_A R-mediated anti-apoptosis of HEY cells, analogous to the effect exerted in classical tumor suppressive pathways. We used the same pharmacological approaches employed in the cell migration assays as above and performed TUNEL assays in treated HEY cells. About 0.35% of HEY cells underwent spontaneous cell death in serum-starved condition. ET-1-stimulated cells showed a significant reduction in apoptosis, but DNase treatment, as a positive control, resulted in over 98% apoptosis (Fig. 3A, B, G and H). ET-1-mediated anti-apoptosis was abrogated in HEY cells pretreated with Forsk or Ro-3, but not with H89 (Fig. 3), consistent with the migration data (Fig. 1 and Fig. 2). We also showed that PMA pretreatment enhanced ET-1-mediated anti-apoptosis (Fig. 3A, B, E and H), confirming the previous findings that G_{α_q} /PKC signaling is oncogenic in OC progression [2, 34]. These data suggest that G_{α_s} /cAMP/PKA-mediated signaling may be tumor suppressive whereas G_{α_q} /PKC-mediated signaling is oncogenic in ET-1/ ET_A R-stimulated OC cells.

G_{α_s} /cAMP/PKA activation reduces anchorage-independent growth of HEY cells on soft agar

To examine whether activation of G_{α_s} /cAMP/PKA signaling by Forsk treatment would inhibit ET-1-mediated anchorage-independent growth of the OC cells on soft agar, HEY cells were serum-starved and pretreated with pharmacological agents targeting PKA or PKC

as aforementioned, followed by ET-1 stimulation. The cells were then plated on soft agar for 2 weeks. PKA activation by Forsk pretreatment significantly reduced ET-1-mediated anchorage-independent growth, as evidenced by the significant decrease in the number of HEY cell colonies growing on soft agar (Fig. 3I). In contrast, pretreatment using H89, PMA or Ro-3 did not significantly affect the number of HEY cell colonies growing on soft agar although PMA or Ro-3 pretreatment appeared to result in an increase or a decrease of colony formation, respectively (Fig. 3I). These results suggest that activation of $G\alpha_s$ /cAMP/PKA signaling reduces the ET-1/ ET_A R-mediated oncogenic properties of the human ovarian carcinoma-derived cells by reducing their capacity to grow in an anchorage-independent manner. Altogether, our pharmacological approaches demonstrate that activation of $G\alpha_s$ /cAMP/PKA signaling can confer tumor suppressive effects in ET-1/ ET_A R-stimulated OC cells.

Knockdown of $G\alpha_s$ expression causes increased survival and proliferative signaling in HEY cells

Based on our pharmacological data, we hypothesized that in the OC cells $G\alpha_s$ -coupled signaling may confer tumor suppressive effects on ET-1-mediated oncogenesis. We further tested this hypothesis by performing genetic loss-of-function studies. We first used lentiviral shRNA clones for $G\alpha_s$ along with $G\alpha_q$, β -arrestin1 and β -arrestin2, and made HEY cell lines stably expressing shRNAs for each gene. Knockdown efficiency was verified at both the mRNA and protein level (Fig. S2 and Fig. 4A–D). Using these novel stable cell lines, we performed TUNEL assays as described in Fig. 3 and cell growth curve analyses to investigate the roles of $G\alpha_s$, $G\alpha_q$ and β -arrestins in OC cell anti-apoptosis and proliferation with or without ET-1 stimulation. Loss of $G\alpha_s$ expression induced both basal anti-apoptosis and ET-1-mediated anti-apoptosis, whereas β -arrestin knockdown induced apoptosis in both basal and ET-1-stimulated cells. Interestingly, $G\alpha_q$ knockdown promoted apoptosis only following ET-1 treatment (Fig. 4E). Our cell growth analyses also demonstrated that $G\alpha_s$ knockdown resulted in increased OC cell proliferation in both baseline (Fig. 5A) and ET-1-treated cells (Fig. 5B), while knockdown of $G\alpha_q$ or β -arrestin resulted in decreased OC cell proliferation (Fig. 5). These data are consistent with the results of our pharmacological studies (Fig. 1–3), and together these studies suggest that $G\alpha_s$ signaling is tumor suppressive whereas $G\alpha_q$ -mediated signaling or β -arrestin promotes aspects of oncogenic signal in ET-1/ ET_A R-stimulated OC cells.

OC patients with $G\alpha_s$ overexpression exhibit better overall survival than those with normal $G\alpha_s$ expression

Given our observation that $G\alpha_s$ signaling may be tumor suppressive in human OC cells, we investigated whether $G\alpha_s$ overexpression is associated with survival in OC patients using publically available clinical and expression data from The Cancer Genome Atlas (TCGA) portal [35–37]. A majority of patients express normal levels of $GNAS$ ($G\alpha_s$, $N=409$) and ET_A R ($N=304$); about 15% overexpress $G\alpha_s$ ($N=66$), whereas fewer than 5% overexpress ET_A R ($N=11$). In general, patients who overexpress $G\alpha_s$ show better overall survival than those with normal expression levels with median overall survival of 60 months among patients with $G\alpha_s$ overexpression versus 40 months among those with normal $G\alpha_s$ expression. (Fig. 6A), indicating that $G\alpha_s$ signaling is tumor suppressive in OC. We further

show that ET_AR overexpression is associated with worse survival than those with normal ET_AR expression (Fig. 6B); this result is consistent with previous findings demonstrating that ET_AR overexpression tends to be more frequent in later stages of OC [8, 16].

ET-1/ET_AR axis is coupled to G_α_s/cAMP/PKA signaling in human OC cells

Because previous studies showed that ET_AR is coupled to G_α_s signaling in vascular smooth muscle, CHO and tracheal cells [38–40], we next tested whether ET-1/ET_AR stimulation can activate G_α_s signaling in OC-derived HEY cells. We performed luciferase assays by transfecting a cAMP biosensor into HEY cells; cells were then serum-starved and stimulated with ET-1. We observed that ET-1 induced a time-dependent activation of cAMP activity. Elevated cAMP levels were observed upon ET-1 stimulation for 3–36 hours (Fig. S3), suggesting that the ET-1/ET_AR axis is able to activate G_α_s/cAMP/PKA-mediated tumor suppressive signaling in OC cells.

G_α_s/cAMP/PKA activation inhibits β-arrestin-mediated ET_AR activation of angiogenic and metastatic gene expression

Because our results suggested that activation of the ET-1/ET_AR axis can result in both tumor suppressive (G_α_s-coupled) and oncogenic signaling activated by β-arrestin, we next hypothesized that the G_α_s/cAMP signaling pathway might inhibit β-arrestin-mediated ET_AR activation of angiogenic/metastatic genes. We first performed PCR gene array analyses using HEY cells to identify potential angiogenic/metastatic gene signatures regulated by β-arrestin-mediated ET_AR signaling. We treated HEY cells with ET-1 or vehicle in the presence of siRNAs targeting β-arrestin1/2 and performed a custom designed-Taqman PCR array analysis for human angiogenesis/metastasis-associated genes [for details about the genes, see De Val et al., [41] and Table S1]. Among genes that were profiled, 12 genes are upregulated and 1 gene is downregulated upon stimulation with ET-1 (Table S2). Knockdown of β-arrestin1/2 abrogated this increase of 8 angiogenic/metastatic genes (*Calcr1*, *Ece1*, *Foxc1*, *Icam2*, *Igfbp1*, *Igfbp6*, *Itga5* and *Mfng*) (Fig. 7A), indicating that the expression of these genes is dependent on β-arrestin-mediated ET_AR signaling. Two of these β-arrestin-dependent angiogenic/metastatic genes (*Calcr1* and *Icam2*) are inhibited by Forsk pretreatment (Fig. 7B), suggesting that the G_α_s/cAMP/PKA signaling pathway may confer tumor suppressive effects during ET_AR-mediated OC progression, in part via inhibition of β-arrestin-mediated ET_AR activation of *Calcr1* and *Icam2*.

Discussion

In this study, we demonstrate tumor suppressive effects of G_α_s/cAMP/PKA signaling on ET-1/ET_AR-mediated OC cell tumorigenesis. We further demonstrate that these processes are associated with inhibition of β-arrestin-mediated ET_AR activation of angiogenic/metastatic *Calcr1* and *Icam2* (Fig. 7C). Our working hypothesis for the molecular mechanism by which G_α_s/PKA signaling inhibits β-arrestin-mediated activation of angiogenesis/metastasis-associated genes is that G_α_s signaling blocks β-arrestin recruitment to a selective group of promoters on genes in a nuclear complex with transcriptional co-activators such as p300. In support of this hypothesis, previous studies have shown that: (1) G protein-coupled receptor kinase5/6 (GRK5/6) phosphorylation of ET_AR mediates the

recruitment of β -arrestin to the ligand-occupied receptor, resulting in translocation of β -arrestin to the nucleus where it functions as an epigenetic regulator of ET-1-induced β -catenin signaling in OC [8, 33, 42] and (2) cAMP signaling decreases p300 protein levels in lung cancer cells [43]. Our QRT-PCR data, which show that Forsk pre-treatment inhibits the activation of two β -arrestin-dependent genes *Calcr1* and *Icam2* (Fig. 7B), provide further support for this hypothesis.

The notion that $G\alpha_s$ /cAMP/PKA signaling can confer tumor suppressive effects has been reported for several carcinoma-derived cell lines [44–48]. Although the role of $G\alpha_s$ signaling in OC progression has not been clear, our survival analysis of TCGA data for ovarian serous carcinomas agrees with our findings on human OC cells that $G\alpha_s$ signaling is tumor suppressive in OC. Interestingly, different genotypes of the SNP T393C in the $G\alpha_s$ (GNAS) gene have been shown to predict clinical outcome in patients with urothelial carcinoma, sporadic colorectal cancer, renal cell carcinoma, and chronic lymphocytic leukemia [49–52]. These cancer patients with homozygous CC patients had an increased risk for cancer progression, metastasis, and tumor-related death when compared to those with TT genotypes, who displayed the highest GNAS mRNA expression. Further studies are warranted to see if the SNP T393C and other known mutations of GNAS [53, 54] are associated with survival of OC patients.

Consistent with previous findings associating oncogenic properties of $G\alpha_q$ -mediated downstream signaling pathways [2, 17], here we are able to extend these findings by demonstrating that $G\alpha_q$ -coupled ET_A R pathways contribute to the oncogenic phenotype in OC cells (Fig. 1–5). In addition, we demonstrate that stimulation of β -arrestin-mediated ET_A R signaling is also oncogenic in OC cells (Fig. 4 and Fig. 5), as suggested for other cancers [55–58]. These observations complement a report, which demonstrated that β -arrestin-mediated ET_A R signaling pathway activates OC cell invasion via stabilization and nuclear translocation of β -catenin [8]. β -arrestin1 also has been shown to be co-expressed with ET_A R in advanced OC patients. This co-expression was more frequent in high-grade ovarian tumors [8]. We also showed for the first time that β -arrestin-mediated ET_A R signaling activates two angiogenic/metastatic genes (*Calcr1* and *Icam2*), which are inhibited by tumor suppressive $G\alpha_s$ /cAMP/PKA signaling. Notably, these two angiogenic/metastatic genes have been shown to be over-expressed in human vascular tumors [59], uterine leiomyoma [60], glioma [61] and B cell chronic lymphocytic leukemia [62]. Further investigation is warranted to investigate whether these genes are associated with OC progression.

Implementation of the GPCR biased signaling concept has successfully been used for clinical benefit in the cardiovascular field, where biased ligands for several GPCRs have provided a novel therapeutic approach [24–28, 63]. To date, however, there have been no reports demonstrating the utility of GPCR biased signaling for the treatment of cancer. Here, we identify biased signaling mechanisms involved in the regulation of ET_A R-mediated OC cell tumorigenesis by demonstrating that the ET-1/ ET_A R axis can activate both tumor suppressive ($G\alpha_s$ -coupled) and oncogenic (β -arrestin/ $G\alpha_q$ -coupled) properties. We also show that G proteins activate differential signaling pathways (i.e. $G\alpha_s$ -coupled tumor suppressive vs $G\alpha_q$ -coupled oncogenic) via the same GPCR. Our results are further

supported by a recent study showing (1) pre-assembly of protease-activated receptor 2 (PAR2; a GPCR) with both $G\alpha_{i1}$ and $G\alpha_o$, and a rapid/transient activation of these G proteins upon receptor activation, but (2) no pre-assembly of PAR2 with $G\alpha_{12}$ and very slow/sustained kinetics similar to that of β -arrestin recruitment [64]. This study in real-time and live cells suggests that the kinetics of GPCR coupling to distinct G proteins is not only different, but that pre-assembly or ligand-induced G protein interaction depends on receptor-G protein pairs. Interestingly, our cAMP reporter assays show that ET-1/ ET_A R stimulation for 3–36 hours increases ~2 fold of cAMP levels in OC cells (Fig. S3), indicating that $G\alpha_s$ is not pre-assembled with ET_A R, but rather is slowly activated upon ET_A R stimulation in OC cells. Notably, it has been shown that ET-1/ ET_A R stimulation in OC cells induced >10 fold of $G\alpha_q$ -coupled signaling [7], suggesting that there is a disturbed balance on the oncogenic pathways (i.e. $G\alpha_q$ -coupled and β -arrestin-mediated) after ET-1/ ET_A R stimulation.

Altogether, our findings provide support for the development of $G\alpha_s$ -biased ligands for therapeutic benefit in the treatment of OC patients (Fig. 7C). Although our survival analysis using TCGA data showed that ET_A R overexpression is associated with poor survival of OC patients (Fig. 6B), and previous pre-clinical studies have stressed the importance of ET-1/ ET_A R axis in OC cell proliferation, invasion, survival and metastasis, recent therapeutic studies using specific ET_A R antagonists have been proven to be ineffective. One of the best examples is use of the drug zibotentan (ZD4054). Despite its ability to induce tumor suppressive effects *in vitro* and in animal models [9], zibotentan treatment has not shown significant efficacy in Phase II clinical trials when coupled with standard chemotherapy [65, 66]. Although it has been speculated that tumor heterogeneity may, in part, contribute to the discrepancy observed between these preclinical versus clinical studies [67–71], a more detailed understanding of crosstalk between ET-1/ ET_A R axis and other signaling pathways, particularly ET_A R-mediated downstream signaling, will be crucial to understand (and efficiently target) this signaling axis in OC patients. Based on our findings here, employing biased signaling approach to selectively activate $G\alpha_s$ /cAMP/PKA-mediated tumor suppressive signaling could be an effective therapeutic approach for targeted inhibition of the ET-1/ ET_A R axis in many cancers including OC.

In conclusion, our study identifies $G\alpha_s$ signaling as an important tumor suppressive signal in OC, and provides new insights into our understanding of how biased signaling mechanisms for ET_A R may modulate OC progression. We postulate that the development of a novel class of ET_A R-biased ligands that have high-affinity for $G\alpha_s$ -mediated tumor suppressive signaling may open new opportunities for cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. The ET-1/ET_AR axis activates both tumor suppressive (G_{as}-coupled) and oncogenic (G_{αq}-coupled and β-arrestin-mediated) downstream signaling pathways in human metastatic ovarian cancer (OC) cells.
2. β-arrestin induces ET_AR-mediated oncogenic signals by activating a subset of angiogenesis/metastasis-associated genes.
3. G_{as}/cAMP/PKA activation inhibits ET_AR-mediated β-arrestin activation of angiogenic/metastatic *calcr1* and *Icam2*.
4. ET_AR overexpression is significantly associated with poor survival of OC patients.
5. G_{as} overexpression is associated with longer survival of OC patients.

Fig. 1. Forskolin (PKA activator) and Ro-3 (PKC inhibitor) decrease ET-1-mediated ovarian cancer cell migration

A–F, Migration of HEY cells treated with PKA/PKC activators or inhibitors with/without ET-1 by transwell chamber assays. HEY cells were serum-starved for overnight, pre-treated with 10µM of Forskolin (Forsk), H-89, phorbol 12-myristate 13-acetate (PMA) or Ro-318425 (Ro-3) for 4hrs, and stimulated with 100nM of ET-1 for 24hrs. Representative images of crystal violet-stained cells are shown. **G**, The number of migrated cells was determined by counting the number of crystal violet-stained cells. Significantly reduced number of migrated cells was observed when HEY cells were pre-treated with Forsk and Ro-3 compared to ET-1 alone. Values represent mean $\pm$ SEM from four independent experiments for each treatment. NS: no stimulation. *, $P < 0.05$ vs. NS; #, $P < 0.05$ vs ET-1.

Fig. 2. $G\alpha_s$ /cAMP/PKA activation and $G\alpha_q$ /PKC inhibition decrease ET-1-mediated ovarian cancer cell migration

A–F, Wound healing assays of HEY cells treated with PKA/PKC activators or inhibitors with/without ET-1. Confluent HEY cell monolayers were wounded by scratching with a pipette tip before pre-treatment with 10 μ M of Forskolin (Forsk), H-89, phorbol 12-myristate 13-acetate (PMA) or Ro-318425 (Ro-3) for 4hrs, followed by stimulation of 100nM of ET-1 for 24hrs. Wound closure was monitored by microscopy at the indicated time. Representative images are shown. Initial open areas (0 hr) were measured to serve as total open area, and the percentage of open area after 24 hrs of ET-1 stimulation was calculated to determine ovarian cancer cell's migrative potential. **G**, Confluent HEY cell monolayer treated with complete medium including serum was used as a positive control. **H**, Quantitative analysis of relative wound size. Forsk or Ro-3 pre-treatment significantly inhibited HEY cell's migration capabilities. Values represent mean $\pm$ SEM from four independent experiments for each treatment. NS: no stimulation. ***, $P < 0.001$ vs. NS; #, $P < 0.05$ vs ET-1; ##, $P < 0.01$ vs ET-1.

Fig. 3. G_{α_s} /cAMP/PKA activation inhibits ET-1-mediated ovarian cancer cell anti-apoptosis and the anchorage-independent growth of ovarian cancer cells on soft agar

A–G, HEY cells were pre-treated with 10 μ M of Forskolin (Forsk), H-89, phorbol 12-myristate 13-acetate (PMA) or Ro-318425 (Ro-3) for 4hrs and then stimulated with 100nM of ET-1 for 24 hrs (A–F). HEY cells treated for 1hr with DNase served as a positive control (G). Apoptotic cells were determined by TUNEL immunofluorescence staining (green). Cell nuclei were counterstained with DAPI (blue). **H**, The percentage of apoptotic cells was calculated by counting DAPI- and TUNEL-positive cells. Forsk or Ro-3 treatment resulted in significant inhibition of anti-apoptotic signaling mediated by ET-1, whereas PMA treatment induced anti-apoptotic signaling in HEY cells. **I**, Quantitative soft agar data. The HEY cells treated as A–F were trypsinized and plated on soft agar for 2 weeks. The colony formation was significantly reduced with Forsk pre-treatment. Data were shown as relative number of colonies formed on soft agar compared to NS. Values represent mean $\pm$ SEM from five independent experiments for each treatment. NS: no stimulation. *, $P < 0.05$ vs. NS; ***, $P < 0.001$ vs. NS; #, $P < 0.05$ vs ET-1; ##, $P < 0.01$ vs ET-1.

Fig. 4. Genetic loss-of-function of Gαs inhibits apoptosis of HEY ovarian cancer cells
A–D, QRT-PCR and Western Blot analyses in stable cell lines expressing lentiviral shRNAs for Gαs, Gαq, β-arrestin1 and β-arrestin2. The best lentiviral shRNA clones were chosen based on transient infection data in Fig. S2 and infected for 2 weeks with puromycin-containing medium. 10 puromycin-resistant colonies for each gene were screened and the best knockdown clones are shown in A–D. **E**, Control and 4 stable HEY cells were stimulated with 100nM of ET-1 for 24hrs and apoptotic cells were determined by TUNEL immunofluorescence staining as described in Fig. 3A–H. Data are shown as relative fold induction compared to control NS (no stimulation). Note that ~ 0.35% of control NS cells underwent apoptosis, similar as Fig. 3A–H. Depletion of Gαs decreases basal HEY cell apoptosis and promotes ET-1-mediated anti-apoptosis, whereas loss of Gαq, β-arrestin1 and β-arrestin2 increases ovarian cancer cell apoptosis. Values represent mean ± SEM from five independent experiments for each treatment. KD: knockdown. *, $P < 0.05$ or **, $P < 0.01$ or ***, $P < 0.001$ vs. Control, or Control/NS; #, $P < 0.05$ or ##, $P < 0.01$ or ###, $P < 0.001$ vs Control/ET-1.

Fig. 5. Gas depletion promotes ovarian cancer cell growth

Control and 4 stable HEY cells were serum-starved for 16hrs and then either untreated (**A**) or stimulated with 100nM of ET-1 for indicated days (**B**). The numbers of viable cells at indicated days were counted. Proliferative capability of HEY cells was significantly attenuated in loss of Gαq, β-arrestin1 and β-arrestin2, whereas Gas loss induced cell proliferation compared to control shRNA stable cells. Values represent mean ± SEM from four independent experiments for each treatment. *, $P < 0.05$ vs. control; **, $P < 0.01$ vs. control; ***, $P < 0.001$ vs. control.

Fig. 6. Gas overexpression is associated with improved survival of patients with ovarian cancer Overall survival of patients with ovarian tumors with GNAS (Gas) overexpression (A) or ET_AR overexpression (B) compared normal expression. Data were retrieved from TCGA data portal as described in Methods. N=409 for GNAS normal and N=66 for GNAS overexpressed ($P=0.07$); N=304 for ET_AR normal and N=11 for ET_AR overexpressed ($P<0.01$).

Fig. 7. Gas signaling inhibits ET-1/ET_AR-mediated β -arrestin activation of *Calcr1* and *Icam2* in human ovarian cancer cells

A, Human angiogenic/metastatic gene signatures regulated by ET_AR-mediated β -arrestin signaling pathways. HEY cells were transfected with either si-control or si- β -arrestin1/2, stimulated with ET-1, and taqman PCR array analyses were performed. Only 8 genes were activated via β -arrestin-dependent pathways after ET-1 stimulation. Note that β -arrestin1 is a major isoform in HEY cells as shown by IB. **B**, HEY cells were pre-treated with Forsk and stimulated with ET-1. Only *Calcr1* and *Icam2* were inhibited by G_{α_s} -mediated tumor suppressive signaling. Values represent mean $\pm$ SEM from five independent experiments. NS: no stimulation. *, $P < 0.05$ or †, $P < 0.01$ or ‡, $P < 0.001$ vs. NS; #, $P < 0.05$ or \$, $P < 0.01$ or ¶, $P < 0.001$ vs ET-1/Si-control or ET-1. **C**, A model summarizing ET_AR biased signaling-mediated human OC cell tumorigenesis. ET-1/ET_AR stimulation activates both G_{α_s} -mediated tumor suppressive signaling and G_{α_q} -coupled or β -arrestin-mediated oncogenic signaling in human OC cells. With the novel concept that ET-1/ET_AR axis activates both beneficial and detrimental signaling, we postulate that ET_AR biased signaling is a plausible strategy to refine existing ET_AR antagonists for OC and development of G_{α_s} -biased ligands is imperative.