$G\alpha_o$ potentiates estrogen receptor α activity via the ERK signaling pathway

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Abstract

The estrogen receptor α (ERα) is a transcription factor that mediates the biological effects of 17β-estradiol (E_2). ERα transcriptional activity is also regulated by cytoplasmic signaling cascades. Here, several Gα protein subunits were tested for their ability to regulate ERα activity. Reporter assays revealed that overexpression of a constitutively active $G\alpha_o$ protein subunit potentiated ERα activity in the absence and presence of E_2. Transient transfection of the human breast cancer cell line MCF-7 showed that $G\alpha_o$ augments the transcription of several ERα-regulated genes. Western blots of HEK293T cells transfected with ER ± $G\alpha_o$ revealed that $G\alpha_o$ stimulated phosphorylation of ERK 1/2 and subsequently increased the phosphorylation of ERα on serine 118. In summary, our results show that $G\alpha_o$, through activation of the MAPK pathway, plays a role in the regulation of ERα activity.
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Introduction

Various agents regulate estrogen receptor α (ERα) activity in addition to 17β-estradiol (E_2), including peptide growth factors (PGFs) such as epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF1; Ignar-Trowbridge *et al.* 1992, 1993, 1996, Aronica & Katzenellenbogen 1993, Curtis *et al.* 1996, Kenney & Dickson 1996, Lee *et al.* 2001, Klotz *et al.* 2002). PGFs and E_2 often operate in concert to potentiate/elevate ERα activity to a level above that observed with either factor alone (Ignar-Trowbridge *et al.* 1993, 1996). Mechanistic studies have revealed that cross talk between the PGF and E_2 signaling cascades occurs at the level of ERα, in that PGF activation of cytoplasmic signaling cascades such as the phosphatidylinositol 3-kinase (PI3K)–AKT and MAPK signaling cascades (Dufourny *et al.* 1997, Bartucci *et al.* 2001, Dunn *et al.* 2001, Kato 2001, Brazil *et al.* 2002, Cantley 2002) ultimately leads to activation of ERα (Martin *et al.* 2000, Campbell *et al.* 2001). The MAPKs, including ERK1/2, c-*jun* N-terminal kinase (JNK), and p38 kinase, are a family of related kinases stimulated by numerous extracellular stimuli including mitogens, peptide hormones, cytokines, and cellular stress (Ballif & Blenis 2001).

Activation of the ERα by cytoplasmic signaling cascades is not limited to PGFs. Recent evidence suggests that factors that activate G-protein-coupled receptors (GPCRs) may also regulate ERα function. Dopamine has been shown to activate ER-mediated transcriptional activity in the absence (Power *et al.* 1991) or presence (Smith *et al.* 1993) of E_2. Melatonin has been shown to inhibit breast cancer cell proliferation through modulation of ERα function (Ram *et al.* 1998, 2000*b*, Rato *et al.* 1999, Yuan *et al.* 2002, Girgert *et al.* 2003, del Rio *et al.* 2004). Indeed, melatonin may play a role in breast cancer (Sanchez-Barcelo *et al.* 2003), as high concentrations of melatonin in human breast cancer tissue have been correlated with good prognosis (Maestroni 1999). Also, the growth and proliferation of a number of breast cancers are influenced by β-adrenergic receptors (Cakir *et al.* 2002), and β-adrenergic stimulation has been shown to elicit classical 'estrogenic' responses, such as increases in uterine weight and ER levels (Re *et al.* 1993, Sukocheva *et al.* 2003).

Specificity of GPCR signaling is determined by the types of heterotrimeric GTP-binding proteins (G-proteins) that are recruited to, and activated by, an individual receptor (Rockman *et al.* 2002). The G-proteins consist of α-, β-, and γ-subunits, and they function as second messengers that

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initiate signaling cascades responsible for the regulation of numerous cellular events. The Gα subunit family includes four distinct subgroups, Gα$_s$, Gα$_{i/o}$, Gα$_q$, and Gα$_{12}$ (Downes & Gautam 1999, Radhika & Dhanasekaran 2001), and mutations in Gα subgroups have been linked to human diseases (Lania *et al.* 2001, Schulte & Fredholm 2003). Classically, Gα$_i$ inhibits and Gα$_s$ stimulates cAMP production. However, Gα signaling is not limited to the cAMP pathway, as all four Gα subgroups have been shown to regulate kinase-mediated signaling events, including the MAPK and PI3K–AKT pathways (Nava *et al.* 2002, Marinissen *et al.* 2003, Radeff-Huang *et al.* 2004). For example, sphingosine kinase type 1 (SPHK1), the enzyme that phosphorylates sphingosine to form sphingosine 1 phosphate, has been shown to regulate breast cancer cell proliferation and tumorigenesis, possibly through SPHK1's ability to regulate the level of phosphorylated AKT and ERK1/2 (Nava *et al.* 2002, Sukocheva *et al.* 2003).

Recent studies have demonstrated pathological Gα signaling in several disease types, including immune system dysfunction, infectious diseases, endocrine disorders, heart disease, and cancer (Kimple *et al.* 2011, Vassart & Costagliola 2011, Kaur *et al.* 2012). In the endocrine system, altered Gα has been found in a number of disorders, including those involving the thyroid, hypothalamus, pituitary, pancreas, ovaries, prostate, and breast (Livingstone *et al.* 1998, Lania *et al.* 2001, D'Souza *et al.* 2004, Cotta-Grand *et al.* 2009, Ogawa *et al.* 2009, Zhao *et al.* 2010). In cancer, aberrant Gα$_o$ signaling has been found in teratocarcinoma, glioblastoma, neuroblastoma, colon, kidney, lung, gastrointestinal stromal-tumors, acute myeloid leukemia, ovarian, pancreas, thyroid, and breast cancers (Hurst & Hooks 2009, Lamba *et al.* 2009, Kimple *et al.* 2011). Recent findings have shed light on the role of Gα$_o$ signaling in breast carcinoma. Several somatic mutations in Gα$_o$ have been identified and characterized in patient breast biopsies (Ram *et al.* 2000*a*, Lamba *et al.* 2009, Kan *et al.* 2010, Garcia-Marcos *et al.* 2011). These mutations primarily result in a constitutively active Gα$_o$ phenotype in breast tumors, leading to increased Gα$_o$ signaling. However, the exact mechanism and consequence of enhanced Gα$_o$ signaling in breast cancer remains poorly characterized. A better understanding of the role of Gα$_o$ in ERα signaling would increase our understanding of the mechanisms of cancer biology. Given the ability of GPCRs to activate cytoplasmic signaling events that have previously been shown to potentiate ERα transcriptional activity, we hypothesized that G-protein-coupled signaling pathways may target and regulate the transcriptional activity of ERα. The recent identification of naturally occurring Gα$_o$ mutants led us to use a constitutively active Gα$_o$ vector to simulate the effects of the mutations found in clinical breast tumors. Given the relatively novel discovery of these mutations, *in vitro* and *in vivo* models of specific Gα$_o$ clinical mutations are currently unavailable. We demonstrated potentiation of ERα activity by Gα$_o$, which culminated in increased expression of the estrogen target genes *SDF1* (*CXCL12*), progesterone receptor (*PR* (*PGR*)), and *BCL2*. Mechanistic studies on Gα$_o$ revealed that this subunit activated the ERK1/2 signaling pathway, culminating in the phosphorylation of ERα.

There is emerging evidence linking the ER to nonclassical, rapid signaling events originating at the cell membrane followed by activation of downstream kinase cascades within the cytoplasm. This study focuses on the ability of the G-protein Gα$_o$ to modulate the activity of the ER and explores the cellular pathways through which this regulation takes place.

Materials and Methods

Reagents

DMEM, phenol red-free DMEM, fetal bovine serum (FBS), BME amino acids, MEM amino acids, L-glutamine, penicillin, streptomycin, and sodium pyruvate were obtained from GibcoBRL. Porcine insulin was purchased from Sigma, and charcoal-stripped (CS) FBS was obtained from HyClone (Logan, UT, USA). Effectene was purchased from Qiagen. E_2 was obtained from Sigma. BrightGlo luciferase assay substrate was purchased from Promega. The lysis buffer used was mammalian protein extraction reagent (M-PER) from Pierce (Rockford, IL, USA). The Berthold AutoLumat Plus luminometer was used for luciferase assays.

Plasmids

Expression constructs for constitutively active Gα subunits (GTPase-deficient mutants of Gα$_o$, Gα$_{12}$, Gα$_q$, Gα$_{12}$, Gα$_{13}$, and Gα$_{16}$) were generously provided by Dr Lynn Heasley (Heasley *et al.* 1996) or obtained through the UMR cDNA Resource Center (Rolla, MO, USA). We have previously described the use of dominant-negative mutant ERK2-DN, which was provided by Drs Melanie Cobb and Roger Davis (Frigo *et al.* 2004). The pcDNA3.1-ERα plasmids along with pERE-Luc plasmid, which contains three copies of the estrogen response element (ERE) linked to the luciferase gene, were purchased from Panomics (Fremont, CA, USA). ERα-S118A was provided by Benita Katzenellenbogen (Le Goff *et al.* 1994).

Cell culture

The two cell lines used were the ER-negative human embryonic kidney 293 (HEK293; Le Goff *et al.* 1994), and the ERα- and ERβ-positive human breast carcinoma (MCF-7) cell lines (Frigo *et al.* 2002). All were maintained in DMEM supplemented with 10% FBS, BME amino acids, MEM amino acids, L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, sodium pyruvate, and 1×10^{-10} M porcine insulin under mycoplasma-free conditions at 37 °C in humidified 5% CO_2. For described studies, cells were grown in phenol red-free DMEM supplemented with

5% CS FBS, and supplements as earlier, but without insulin (5% CS-DMEM) as described previously (Ignar-Trowbridge *et al.* 1993, Burow *et al.* 1999).

Transient transfection and luciferase assay

HEK293T and MCF-7 cells were transfected as described previously (Burow *et al.* 1999). Briefly, cells were plated into 24-well plates at 1×10^4 cells/well in 5% CS-DMEM and allowed to attach overnight. The next day, the cells were transfected with plasmids for 5 h in the serum/supplement-free DMEM using Effectene according to the manufacturers' protocols. The following amounts of DNA were added to each well: for ERE-luc assays, 0·2 μg ERE-luciferase, 0·3 μg G-proteins, 0·2 μg ER (S118A), and 0·1 μg ERE-luc. After 5 h, the cells were treated with vehicle control or E_2 (10 pM). After 24 h, the treatment-containing medium was removed and 1× M-PER mammalian cell lysis buffer (Pierce; 150 μl) was added per well and gently shaken for 5 min at room temperature. Luciferase activity of 100 μl of cell extract was determined using BrightGlo luciferase assay substrate (Promega) in a Berthold luminometer. Data are presented as relative luminescence and are normalized as outlined in each figure. Each data point is the average of three separate measurements. Statistical analysis was performed using the two-sample *T* test in the Origin graphing program with a *P* value of 0·05.

Western blot analysis

HEK293 cells were maintained in 5% CS-DMEM and plated in 100×20 mm cell culture dishes at 50–80% confluency overnight. Cells were transfected with 1 μg ERα and either 1 μg empty vector or 1 μg $G\alpha_o$. For RT-PCR analyses, MCF-7 cells were transfected with either empty vector or 2·0 μg $G\alpha_o$. After 5 h, either steroid or vehicle was added. The cells were harvested after ~18 h using M-PER lysis buffer containing a mixture of protease inhibitors (Roche) and phosphatase inhibitors (Sigma) on ice for 10 min. Equal volumes of lysates were added to SDS–PAGE loading buffer (Invitrogen) containing 1% β-mercaptoethanol and then boiled for 5 min. The proteins were electrophoresed on a Bis–Tris 4–12% gradient polyacrylamide gel (Invitrogen) and were subsequently transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk solution in PBS and 0·1% Tween for 1 h at room temperature. The membrane was washed extensively and subsequently incubated overnight at 4 °C with rabbit antibodies to ERα (1:500 dilution; Santa Cruz Biotechnology); ERα-phoshpo-S118 (1:1000 dilution; Cell Signaling, Beverly, MA, USA); phosphorylated forms of ERK1/2, JNK, or p38 (1:1000; Cell Signaling Technology); or Rho-GDI (1:500 dilution; Santa Cruz Biotechnology). All antibodies were diluted in 5% BSA dissolved in 1× PBS+0·1% Tween. The next day, blots were washed in PBS+0·1% Tween and incubated with goat antirabbit antibodies conjugated to IRDye (1:10 000; Li-Cor, Lincoln, NE, USA) for 60 min at room temperature. Following three washes, immunoreactive proteins were scanned using the Li-Cor IR scanner set to 800 nm.

RT-PCR assays

Cell lysates were obtained as explained earlier. RNA was isolated using the RNAeasy miniprep kit (Qiagen). Second-strand cDNA synthesis was performed using 2 μg total RNA and the cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. RT-PCR assays were assembled in 96-well plates using 5 μl of 1:10 dilution of the synthesized cDNA, 0·1 μg of 1:1 mixture of forward and reverse primers, and 1× SYBR-Green solution (Bio-Rad).

Results

G-proteins potentiate ERα activity

Activation of G-protein signaling can occur in response to classic GPCRs, growth factor/cytokine receptors, and steroid hormones (Rockman *et al.* 2002, Radeff-Huang *et al.* 2004). There are four major families of Gα signaling proteins: $G\alpha_i$, $G\alpha_o$, $G\alpha_q$, and $G\alpha_{12/13}$. Therefore, we chose the primary members of these four major families to screen for effects on ERα transcriptional activity. To examine the role of specific

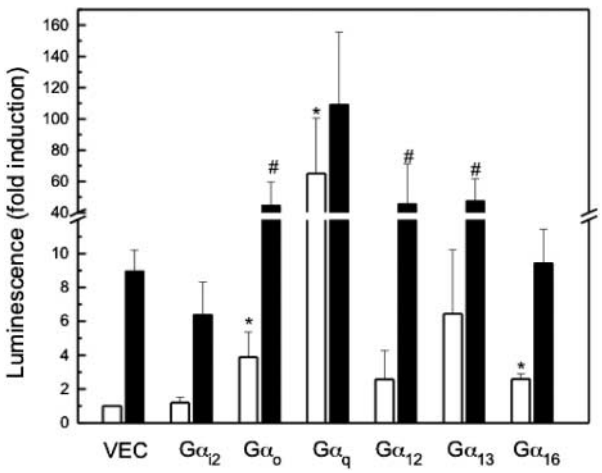


Figure 1 G-proteins enhance estrogen receptor activity. MCF-7 breast carcinoma cells were transfected with ERE-luciferase reporter plasmid (200 ng) along with 300 ng of empty vector (VEC), or constitutively active mutants of $G\alpha_{i2}$, $G\alpha_o$, $G\alpha_q$, $G\alpha_{12}$, $G\alpha_{13}$, or $G\alpha_{16}$. Cells were subsequently treated with vehicle control (white bars) or 1 nM E_2 (black bars) and were harvested 24 h later for luciferase assay. Data are represented as fold induction (±S.E.M.) with vehicle treatment of VEC cells set to 1·0. Each bar is an average of three experiments. *A statistical significance between the vector vehicle control and each G-protein vehicle control; #a statistically significant difference between the vehicle control and E_2 within each G-protein sample ($P<0·05$).

Gα subunits on ERα activity, expression vectors containing Gα subunits mutated to function in a constitutively active manner were used. MCF-7 breast carcinoma cells, which contain both the isoforms of ER, were transfected with empty vector (VEC), Gα$_{i2}$, Gα$_o$, Gα$_q$, Gα$_{12}$, Gα$_{13}$, and Gα$_{16}$, along with an estrogen-responsive luciferase reporter construct. The ability of 1 nM E_2 to activate the ER in VEC cells was normalized to 1·0. The expression of Gα$_o$, Gα$_q$, Gα$_{12}$, and Gα$_{13}$ potentiated estrogen-bound ERα activity (Fig. 1). Interestingly, only Gα$_o$ overexpression resulted in both a ligand-independent and a ligand-dependent increase in ERα activity.

Gα$_o$ potentiates ER-mediated gene transcription

We focused the remaining studies on Gα$_o$ because of its ability to augment ER activity, both in the presence and absence of estrogen. To determine whether Gα$_o$ overexpression affects transcription of endogenously ER-regulated genes, we transfected MCF-7 cells with either VEC or constitutively active Gα$_o$ in the absence or presence of 1 nM E_2 followed by real-time PCR. In the vector cells, the addition of 1 nM E_2 caused an expected increase in the transcription of ERα-regulated genes *pS2* (*TFF1*; fourfold), *SDF1* (fivefold), *PR* (33-fold), and *BCL2* (1·8-fold) (Fig. 2A). Transfection of Gα$_o$ resulted in an increase in the transcription of *PR* (2·7-fold), *SDF1* (1·9-fold), and *BCL2* (twofold) in the absence of estrogen; however, no change was seen in the expression of *pS2* after overexpression of Gα$_o$ (Fig. 2A). We believe that the latter effect is due, in part, to altered promoter contexts of each ER-mediated gene. Curiously, the addition of Gα$_o$ did not increase the transcription of *PR, pS2*, or *SDF1* beyond the level induced by estrogen. It is possible that, with these particular genes, treatment with E_2 resulted in a maximum transcriptional response in this cell context. It is important to note that in the cases of PR and SDF1, Gα$_o$ alone was able to induce transcription, although to a lesser level than that seen with E_2. Finally, we found that the Gα$_o$-mediated increase in expression of these ER-regulated genes was not due to an increase in ERα transcription levels in cells that were transfected with Gα$_o$ (Fig. 2B).

Gα$_o$ activates the ERK pathway

Several cell signaling cascades have been shown to cross talk with ERα (Dufourny *et al.* 1997, Martin *et al.* 2000, Bartucci *et al.* 2001, Campbell *et al.* 2001, Dunn *et al.* 2001, Kato 2001, Brazil *et al.* 2002, Cantley 2002). To determine whether Gα$_o$ activates ERK1/2, p38, or JNK kinases, western blot analysis was performed using phosphorylation-specific antibodies to detect the active forms of these kinases. Transfection of HEK293T cells with constitutively active Gα$_o$ resulted in an increase in the phosphorylation of ERK1/2 but not JNK or p38 (Fig. 3). Similar results were seen in MCF-7 cells (data not shown).

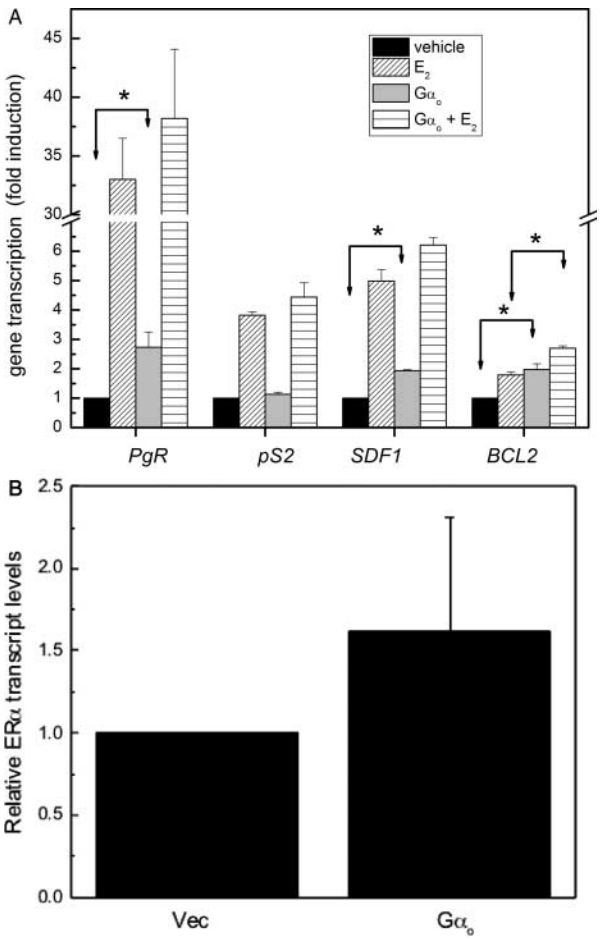


Figure 2 Gα$_o$ enhances ER-mediated gene expression. Real-time PCR was performed as described in the Materials and Methods section. (A) MCF-7 cells were transfected with either empty vector (vehicle or E_2) or constitutively active Gα$_o$. For each gene analyzed, the vehicle control was set to 1·0. (B) MCF-7 cells were transfected as in (A) with either empty vector (Vec) ora constitutive active Gap (Gao). ER transcript levels were set to 1.0 in vector samples for comparison. Each experimental condition is an average of three separate samples. *A statistically significant difference ($P<0·05$).

Gα$_o$ affects ERα's phosphorylation status

Our results indicate that Gα$_o$ activates the ERK1/2 pathway. Because ERα contains an ERK phosphorylation site at serine 118, we tested the ability of Gα$_o$ overexpression to increase phosphorylation of the ER at this site. We used western blot analysis of HEK293T cells that were transfected with ERα, with or without Gα$_o$. Using densitometry, we divided the overall increase in ER phosphorylation at serine 118 by the total amount of ER within the cell. Our results indicate that Gα$_o$ caused an increase in ERα phosphorylation at Ser118 of approximately twofold (Fig. 4A and B).

We validated these findings using a serine 118 to alanine point mutant of ERα in ERE reporter assays. Transfection of the mutant into HEK293T cells showed that alteration of this

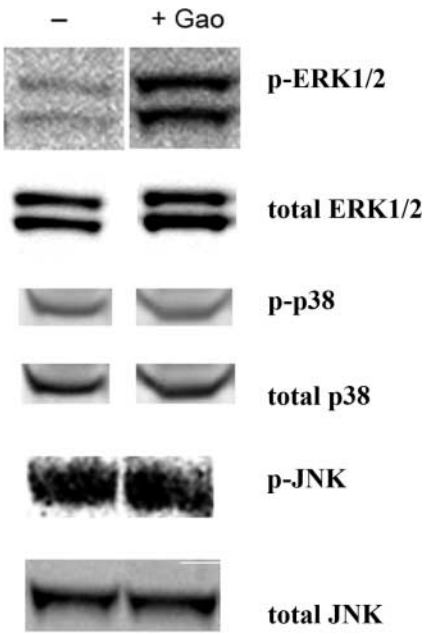


Figure 3 $G\alpha_o$ activates ERK1/2. HEK293T cells were transfected with either empty vector (−) or $G\alpha_o$ and the phosphorylated forms of JNK, p38, and ERK were probed using western blot analysis with phospho-specific antibodies. Total levels of each kinase were also evaluated as a loading control.

residue resulted in loss of the $G\alpha_o$ effect on ERα activity (Fig. 5A). We also used a dominant-negative mutant of ERK2 and found that this mutant was able to decrease $G\alpha_o$'s potentiation of ligand-bound ERα by ∼30% (Fig. 5B). Finally, we showed that when ERK was pharmacologically inhibited by UO126, $G\alpha_o$'s potentiation of ligand-bound ERα activity fell by ∼30% while the inhibitor had no effect on E_2-stimulated ERα activity (Fig. 5C). These results confirm that an intact ERK1/2 pathway is necessary for the maximal $G\alpha_o$ effect on ER activity.

Discussion

Rapid, 'nongenomic' signaling of estrogen has been the focus of intense research in recent years. E_2 has been shown to rapidly (seconds to minutes) activate many signaling molecules (Cheskis 2004, Shupnik 2004, Levin 2005), such as i) IGF1 receptor and EGF receptor, ii) p21ras and Raf, iii) MAPK and Akt, iv) protein kinase C, v) intracellular calcium transients, vi) nitric oxide and prolactin secretion, and vii) Maxi-K channels. However, the mechanisms of these activations remain unclear. Although several groups have proposed that alternative E_2-binding proteins exist within the plasma membrane, accumulating evidence supports the classical full-length ERα as the membrane E_2-binding protein. Current studies are underway to determine what accessory proteins are associated with and transmit the signal of ERα at the membrane to intracellular signaling cascades and ultimately to the nucleus.

Several laboratories have evidence suggesting that G-proteins provide this link between the ER at the plasma membrane and estrogen-induced rapid kinase activation within the cell. Wyckoff *et al.* (2001) showed that endothelial nitric oxide synthase (eNOS) stimulation was mediated by plasma membrane-tethered E_2-bound ERα and that this stimulation was dependent on ER coupling with $G\alpha_i$. Razandi *et al.* (2003) demonstrated that ER interaction with $G\alpha_i$ controls rapid signaling through Src–ERK. In addition, ER has been shown to interact with $G\alpha_{13}$, and this interaction stimulates the small GTPase RhoA and the downstream effector Rho-associated kinase (Simoncini *et al.* 2006).

Here, we investigated the role of the G-protein subunit $G\alpha_o$ as a mediator of ERα transcriptional activity. We have

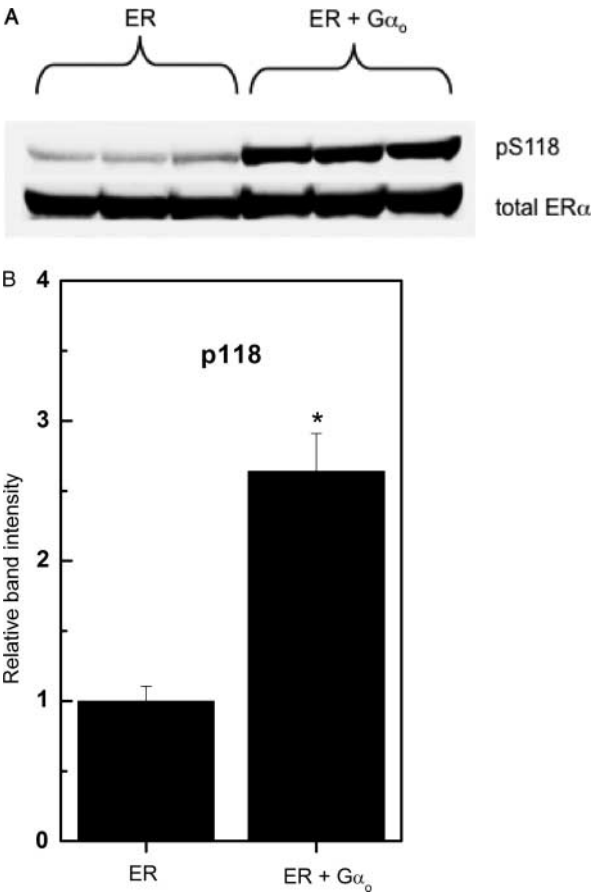
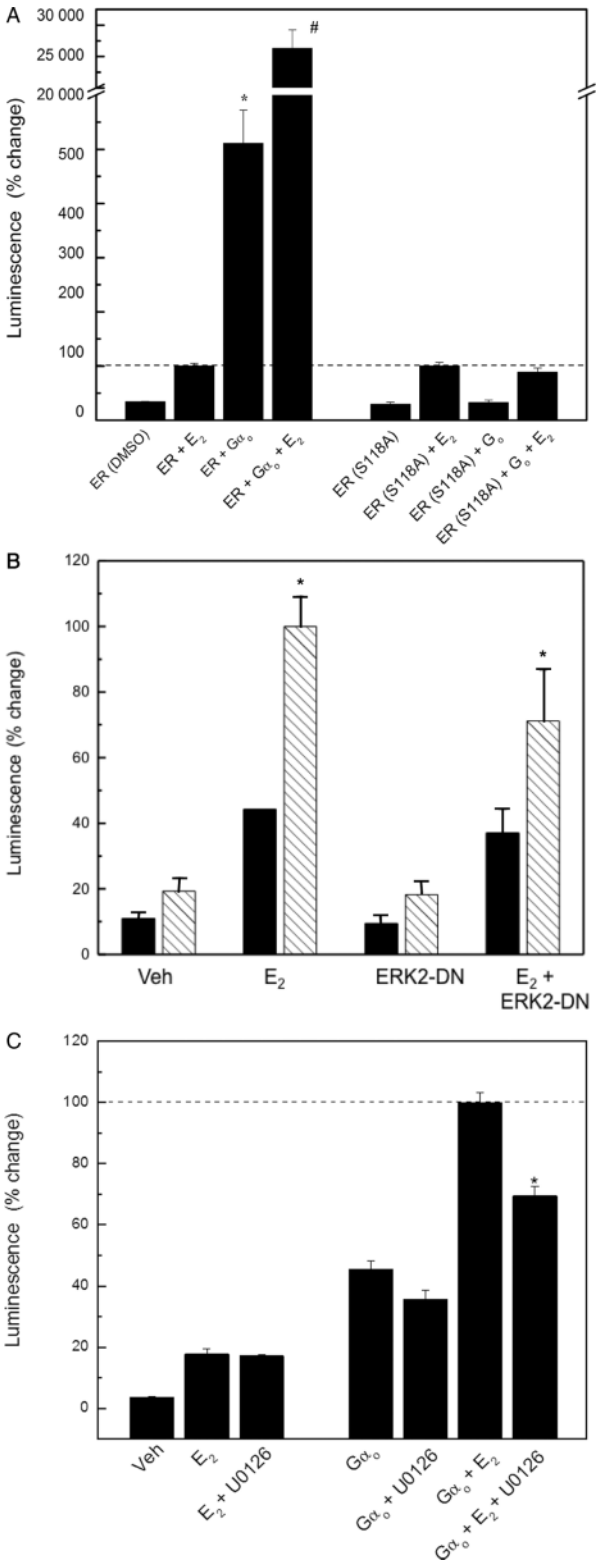


Figure 4 $G\alpha_o$ overexpression results in hyperphosphorylation of S118 of ERα. (A) Triplicate samples of HEK293T cells were transfected with ERα and either pCDNA or $G\alpha_o$ followed by western blot analysis of total ER and ER that has been phosphorylated at serine 118. (B) Densitometric analysis of the above western blots was performed, and the densities of the phosphorylated ER bands were divided by those of the total ER for each individual lane. *A statistical difference ($P<0{\cdot}05$).



shown using ERE-luciferase assays that a constitutively active Gα_o is able to potentiate ERα activity in the absence and presence of E_2 (Fig. 1). Using RT-PCR analysis of MCF-7 cells transfected with the constitutively active Gα_o, we showed a Gα_o-mediated potentiation of transcription of the ERα-regulated genes *BCL2, SDF1*, and *PR* (Fig. 2). The increase in transcription of these genes was not the result of a Gα_o-mediated increase in ERα transcription.

Because several laboratories have previously shown that G-proteins mediate phosphorylation of cellular signaling kinases and that these phosphorylation events can impact ERα activity, we used western blot analysis of HEK293T cells transfected with either pCDNA or constitutively active Gα_o to probe the phosphorylation status of several major signaling kinases. We showed that overexpression of Gα_o resulted in increased phosphorylation and hence activation of ERK1/2 kinase but not p38 kinase or JNK kinase (Fig. 3). This result led us to explore the phosphorylation status of the ER in the presence and absence of constitutively active Gα_o. Again, we used western blot analysis of HEK293T cells that were transfected with ER$\alpha \pm$Gα_o. Our results indicated an increase in phosphorylation of ERα at serine 118, a known ERK1/2 site (Fig. 4A). Our densitometric analysis showed that serine 118 was in fact hyperphosphorylated as a result of Gα_o overexpression (Fig. 4B). In addition, ERE-luciferase assays using an ERK-DN construct, a specific ERK pharmacological inhibitor, or a serine 118 to alanine point mutant of ERα, we confirmed that serine 118 is necessary for a maximal Gα_o effect (Fig. 5A, B and C). Previous laboratories have shown a direct link between ERα phosphorylation at serine 118 and an increased ability of the receptor to dimerize and bind coactivators (Sheeler *et al.* 2003). Therefore, the increased activity of ERα in the presence of Gα_o can be partially explained by our evidence linking Gα_o to activation of ERK1/2, followed by hyperphosphorylation of ER at serine 118, and an increase in ER dimerization and coactivator recruitment.

Figure 5 Serine 118 of ERα is necessary for a maximal Gα_o effect. (A) HEK293T cells were transfected with either wild-type ERα or ER (S118A) and either pCDNA or Gα_o, along with an ERE-luciferase reporter plasmid. Cells were incubated with vehicle control or 10 pM E_2. Luminescence values are expressed as relative % change with ER or ER (S118A)+E_2 set to 100%. Each condition was performed in triplicate. *A statistical difference between ER and ER+Gα_o, #difference between ER+Gα_o and ER+Gα_o+E_2. (B) MCF-7 cells were transfected with ERE-luciferase reporter plasmid along with empty vector (vec) or constitutively active Gα_o and empty vector (vec) or dominant-negative ERK (ERK-DN). Cells were treated with vehicle control (veh) or 1 nM E_2. Data are represented as percent estrogen activity ($\pm$S.E.M.) relative to ER+Gα_o+E_2 treatment (100%). *A significant decrease in Gα_o-stimulated estrogen-bound ER activity as a result of ERK-DN ($P<0{\cdot}05$). (C) MCF-7 cells were transfected with either vector or Gα_o and were treated with vehicle, E_2, U0126 (ERK1/2 inhibitor), or E_2+U0126. *A significant decrease in Gα_o-stimulated estrogen-bound ER activity as a result of U0126. All experiments were performed in triplicate.

Given that there are multiple physiological mutations resulting in increased $G\alpha_o$ activity, we chose to use a constitutively active $G\alpha_o$ variant to mimic increased $G\alpha_o$ activity in our cell systems. The $G\alpha_o$ mutant used in our study is GTPase deficient, resulting in a constitutively active $G\alpha_o$ phenotype, similar to those found in human tumors. There are several known downstream effectors of $G\alpha_o$, including protein kinase C, Src, Stat3, and c-mos. $G\alpha_o$ uses these various effectors based on initiating signaling event and tissue type (Kroll *et al.* 1991, Diverse-Pierluissi *et al.* 1997, Ram *et al.* 2000*a*). While $G\alpha_o$ has been shown to use MAPKs to exert its effects, the role of MAPKs in $G\alpha_o$ signaling is less clear. There are varying reports in the literature regarding $G\alpha_o$ activation of MAPKs. For example, some studies have reported that $G\alpha_o$ can activate p38 and Erk1/2 signaling while others have shown that $G\alpha_o$ inhibits Erk1/2 activation (Yamauchi *et al.* 1997, Liu *et al.* 2002). Conversely, several studies have demonstrated a relationship between $G\alpha_o$ and JNK and Akt activity (Goel *et al.* 2004, He *et al.* 2006). To date, the majority of these studies have excluded breast tissue in their analysis and ability of $G\alpha_o$ to regulate MAPK activity in ER-positive cells has not been well characterized.

The vast majority of breast cancers are ERα positive, and pathological ERα signaling is the primary growth cascade in these cancers. Therefore, we chose to investigate the relationship between $G\alpha_o$ and ER pathways. Our findings demonstrate that $G\alpha_o$ can alter ERα signaling. Furthermore, we report a relationship between MAPK signaling and $G\alpha_o$. MAPKs are known to promote hormone-independent tumor growth and endocrine therapy resistance (Musgrove & Sutherland 2009). Furthermore, ligand-independent tumor growth is a hallmark of breast cancer progression (Musgrove & Sutherland 2009). Several mechanisms of ligand-independent activation of the ER have been identified, including increased endogenous activation of the ER through MAPK-specific phosphorylation (Kato *et al.* 1995). In breast cancer, ERK1/2 is known to activate the ER at the S118 phosphorylation site, which results in enhanced proliferation and tumorigenesis (Kato *et al.* 1995, Chen *et al.* 2000). Furthermore, phosphorylation of the ER by ERK1/2 promotes hormone-independent tumor growth and endocrine therapy resistance (Campbell *et al.* 2001). In the clinic, increased S118 phosphorylation is associated with decreased survival and the efficacy of therapeutic agents may be correlated with activation at this site (Yamashita *et al.* 2008, Zoubir *et al.* 2008). Our findings that $G\alpha_o$ activates ERK1/2, but not p38 or JNK, are similar to previously published studies in prostate but contrast those found in pituitary, kidney, and neuronal cells (Yamauchi *et al.* 1997, Liu *et al.* 2002, 2011, He *et al.* 2006). The fact that mutation of S118 to alanine blocks the $G\alpha_o$ effect entirely while pharmacological or molecular inhibition of ERK1/2 only partially does so implies that $G\alpha_o$ may influence other kinases within the cell that ultimately affect phosphorylation of ER at serine 118. These results provide evidence for a tissue-type-specific role of $G\alpha_o$ activation of MAPK in ER-positive cells. Identification of $G\alpha_o$-induced ER activity mediated by ERK1/2 could provide a novel target for ligand-independent tumors, as well as endocrine therapy-resistant breast cancer. Although further study is needed to determine the therapeutic potential of this pathway, identification and characterization of secondary ER growth pathways are necessary for the development of novel anticancer agents. Our findings that $G\alpha_o$ promotes ER signaling through the ERK signaling cascade may account for the previously published findings of increased tumorigenesis and tumor growth associated with $G\alpha_o$ (Nguyen *et al.* 2002, Prevost *et al.* 2006, Garcia-Marcos *et al.* 2011, Lin *et al.* 2011). Increased understanding of the interplay between $G\alpha_o$ and the ER may also identify novel breast cancer treatments that target ER signaling. For example, one recent study used a Gα inhibitor as an anticancer agent across several cancer types, including ER-positive breast cancer (Prevost *et al.* 2006). Given the high rate of endocrine therapy resistance, anticancer therapies directed against $G\alpha_o$, and indirectly targeting the ER, may be an effective therapeutic strategy. In conclusion, we have shown that $G\alpha_o$ plays a significant role in the regulation of ERα activity, both in the presence and absence of E_2. Specifically, $G\alpha_o$ can enhance ERK1/2 phosphorylation. This activation leads to specific phosphorylation of the ER at serine 118, culminating in a marked increase in ER-mediated transcriptional activity. Our results also indicated that $G\alpha_o$ can induce ER phosphorylation in the absence of E_2, which is especially relevant considering the clinical phenomenon of estrogen-independent proliferation of breast cancer cells. It is important to note that because of the constitutively active nature of the $G\alpha_o$ construct, we are circumventing the involvement of the GPCR, which is endogenously associated with $G\alpha_o$. Further studies using wild-type $G\alpha_o$ overexpression will explore the role, if any, of a GPCR in the $G\alpha_o$-ER mechanism. In any case, our results showing a direct link between an activated G-protein- and estrogen-independent phosphorylation and activation of the ER could open a new avenue of research regarding treatment of estrogen-independent and/or tamoxifen-resistant breast cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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