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Androgen Receptor Promotes Tamoxifen Agonist Activity by Activation of EGFR in ER α -Positive Breast Cancer

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Abstract

Purpose—Tamoxifen (Tam) resistance represents a significant clinical problem in estrogen receptor (ER) -positive breast cancer. We previously showed that decreased expression of Rho guanine nucleotide dissociation inhibitor (Rho GDI), a negative regulator of the Rho GTPase pathway, is associated with Tam resistance. We now discover that androgen receptor (AR) is overexpressed in cells with decreased Rho GDI and seek to determine AR's contribution to resistance.

Methods—We engineered ER -positive cell lines with stable knock-down (KD) of Rho GDI (KD cells). Resistance mechanisms were examined using microarray profiling, protein-interaction studies, growth and reporter gene assays, and Western blot analysis combined with a specific AR antagonist and other signaling inhibitors.

Results—Tam-resistant tumors and cell lines with low Rho GDI levels exhibited upregulated AR expression. Microarray of Rho GDI KD cells indicated that activation of EGFR and ER was associated with Tam treatment. When AR levels were elevated interaction between AR and EGFR was detected. Constitutive and Tam-induced phosphorylation of EGFR and ERK1/2 was blocked by the AR antagonist Enzalutamide, suggesting that AR-mediated EGFR activation was a mechanism of resistance in these cells. Constitutive ER α phosphorylation and transcriptional activity was inhibited by Enzalutamide and the EGFR inhibitor gefitinib, demonstrating that AR-mediated EGFR signaling activated ER. Tam exhibited agonist activity in AR over-expressing

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Conflict of interest:

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cells, stimulating ER α transcriptional activity and proliferation, which was blocked by Enzalutamide and gefitinib.

Conclusions—We describe a novel model of AR-mediated Tam resistance through activation of EGFR signaling leading to ER activation in ER -positive cells with low expression of Rho GDI.

Keywords

androgen receptor; epidermal growth factor receptor; estrogen receptor; breast cancer; endocrine therapy resistance; tamoxifen

Introduction

Current endocrine therapies, such as Tam and aromatase inhibitors (AIs), are effective at delaying recurrence and increasing overall survival for patients with ER -positive breast cancer [15]. Despite overall effectiveness, about a third of tumors exhibit *de novo* resistance to therapy, and about half of patients' tumors will acquire resistance to first-line endocrine therapy [19]. Several mechanisms of Tam resistance (TamR) have been described, including mutation and altered post-translational modifications of ER, changes in ER co-activator and co-repressor expression, and altered expression of cell-cycle regulatory pathway members [14]. Activation of growth factor receptors or altered cytoplasmic signaling has been shown to promote resistance by acting as alternative survival pathways or through bi-directional cross-talk with ER [14]. Co-targeting these pathways along with endocrine therapy treatment prevents resistance in pre-clinical models, but has often failed to achieve the same efficacy in the clinic [21]. Identification of both novel resistance mechanisms as well as biomarkers of response will be required to develop targeted therapy for resistant disease.

We have previously shown that a decrease in expression of a negative regulator of the Rho GTPase pathway, Rho GDI, leads to TamR associated with Tam agonist activity in ER -positive breast cancer cells [1]. Known mainly for regulating rearrangement of the actin-cytoskeleton, the Rho GTPases play a role in cell membrane morphology, cell motility, phagocytosis, and formation of secretory vesicles [10]. High expression of Rho GTPases and low expression of Rho GDIs has been observed in breast tumors and is associated with higher grade tumors, local recurrence, and increased metastasis [20].

We have demonstrated that AR overexpression in ER -positive breast cancer cells also increases Tam agonist activity and resistance to Tam and aromatase inhibitor (AI) therapy [9, 31]. AR is a nuclear receptor that exerts its effects on cells through both classical genomic mechanisms and rapid non-genomic actions. AR acts as a ligand-dependent transcription factor via a classical genomic mechanism which involves homo-dimerization and translocation to the nucleus upon binding androgen hormones, binding to specific DNA sequences, and recruiting co-regulators to initiate transcriptional changes over time [17]. AR can also cause rapid initiation of cytoplasmic signaling cascades, including activation of protein kinase A, protein kinase C, and ERK, via a non-genomic mechanism involving binding cytoplasmic and membrane-bound proteins, such as c-Src [17]. AR is expressed in approximately 80% of breast tumors, and is co-expressed with ER α in about 65% of tumors [24]. Clinically, a high AR to ER α expression ratio in primary breast tumors is associated

with failure of Tam treatment within 5 years and decreased disease free survival during Tam treatment [4]. Higher AR activity has also been found in tumors that recur following AI therapy [12].

In this study we report that decreased Rho GDI expression in ER -positive breast cancer cells is associated with overexpression of both AR and EGFR. In these cells we observe a novel mechanism of enhanced Tam agonist activity through AR-mediated activation of EGFR signaling to ER. AR and EGFR inhibitors restored Tam sensitivity suggesting that targeting of both AR and EGFR may be effective in resistant cells with low Rho GDI expression.

Results

Low Levels of Rho GDI are Associated with AR Overexpression and Activation of the EGFR/MAPK/ERK Signaling Pathway in TamR Cells

To discover novel pathways involved in the development of Tam resistance, we analyzed differential gene expression in TamR ER α -positive metastatic tumors using microarray [8]. Expression in 5 metastatic tumors from Tam treated patients who had a recurrence while on therapy (within two years) was compared to 4 primary tumors from patients treated with Tam who did not have a recurrence during follow up. Rho GDI α was expressed at lower levels in the resistant tumors from this cohort [1]. We developed a cell line model of these tumors by stable knockdown of Rho GDI in MCF-7 breast cancer cells using specific shRNAs (KD cells); Tam acted as an agonist by increasing ER α transcriptional activity in this model [1]. We described a novel mechanism of resistance in which Rho GTPase pathway activation resulted in ligand-independent activation of ER α in these cells. AR was also overexpressed in a subset of the metastatic, resistant tumors with low Rho GDI α expression (Fig. 1A, B). Concomitant with Rho GDI α knockdown, endogenous levels of AR protein and mRNA were elevated in the MCF-7 KD cell line model of these tumors when compared to control cells expressing an empty vector plasmid (Vec cells) (Fig. 1C, D). Endogenous levels of AR were elevated in an additional knockdown model engineered using ZR-75-B cells with stable lentiviral Rho GDI α shRNA. We have previously demonstrated that AR overexpression can increase Tam agonist activity and promote hormone resistance in ER -positive breast cancer cells [9]. These observations imply that a reduction in Rho GDI α expression leads to increased expression of AR, a protein shown to increase Tam agonist activity and resistance.

Activation of growth factor and cytoplasmic signaling pathways, especially the ErbB receptor family and the MAPK/ERK kinases, has been associated with TamR [34, 36, 38]. To determine if these pathways are activated in response to Tam in our models, we treated ZR-75-B Vec and KD cells with Tam for 24 hours and performed microarray analysis. Our analysis identified 1,040 probes differentially expressed (>1.5 fold) in the KD cells with Tam treatment (Sup. Table 1), and we performed gene set enrichment analysis utilizing these genes. There was significant ($p < 0.001$) overlap with four oncogenic signatures in the MSigDB database (<http://www.broadinstitute.org/gsea/msigdb>). Sixty of the differentially expressed genes matched genes increased in expression and 58 matched genes decreased in expression with EGFR pathway activation (Table 1). In addition, 60 differentially expressed

genes matched genes whose expression increased and 68 matched genes whose expression decreased with MEK1 activation. This suggests that activated EGFR and MEK1 are associated with the Tam resistant phenotype observed with Rho GDI α knockdown.

Since we observed signatures of EGFR and MEK1 activation, we wanted to determine if EGFR and MEK1 expression was altered in our models. EGFR RNA levels were found to be increased 1.8 fold in the ZR-75-B KD cells following Tam treatment by microarray analysis (Table 2). Basal EGFR protein levels were elevated in both the ZR-75-B and MCF-7 KD cell line models (Fig. 1C). We also performed a reverse phase protein array (RPPA) and found increased MEK1 protein expression in the KD cells following Tam treatment (Fig. 1E, F). We conclude that a decrease in Rho GDI expression leads to increased EGFR and MEK1 expression that may also be associated with Tam agonist activity.

Since we previously observed that Tam increased ER transcriptional activity in the MCF-7 KD cells [1], we examined the microarray data for evidence of ER α transcriptional activation in the ZR-75-B KD model. The hallmark gene sets of the MSigDB database contain well-defined signatures of cellular processes developed by combining coordinate expression data from multiple related studies. We found significant ($p < 0.001$) overlap, with signatures of both early and late estrogen responses in the hallmark gene sets following Tam treatment (Table 1). In addition, genes associated with ER α transcriptional activation were upregulated following Tam treatment in the KD cells. For instance, a pioneer factor necessary for ER mediated transcription, forkhead box A3, was increased 2.9 fold, and the estrogen-regulated genes cathepsin D and trefoil factor 1 were increased by 2.5 and 10.3 fold, respectively (Table 3). These results indicate that Tam also acts as an agonist of ER transcriptional activity in the ZR-75-B KD model.

AR Interacts with EGFR and Promotes Activation of Downstream Pathway Members

It has been shown that AR expression promotes EGFR activation via non-genomic mechanisms, which involve protein complex formation between EGFR and AR [28]. AR non-genomic activity also enhances phosphorylation of EGFR and its downstream targets ERK1/2 in breast and prostate cancer cells [27, 28, 39]. We previously observed a constitutive increase in ERK1/2 phosphorylation in MCF-7 KD cells [1], so we addressed whether AR may be promoting constitutive activation of EGFR and downstream ERK1/2 in KD cells. Subcellular localization of AR and EGFR in ZR-75-B KD cells was examined by confocal microscopy. Co-localization of AR and EGFR was indicated by co-staining (Fig. 2A). Since AR and EGFR co-localized, we tested for interaction between the two. Interactions between AR and EGFR were observed with basal conditions using proximity ligation assays (Fig. 2B). Treatment with Enzalutamide, a third-generation AR antagonist used in the treatment of castration-resistant prostate cancer [33], decreased these interactions. In addition to interaction with AR, increased constitutive EGFR phosphorylation was seen in ZR-75-B KD cells (Fig. 3A), which was blocked by Enzalutamide (Fig. 3B). Enhanced constitutive EGFR phosphorylation that was sensitive to AR inhibition was also observed in MCF-7 KD cells (Sup. Fig. 1A, B). ZR-75-B KD cells exhibited increased basal ERK1/2 phosphorylation (Fig. 3C) that was also inhibited by

Enzalutamide (Fig. 3D). As expected, the EGFR inhibitor gefitinib also reduced ERK1/2 phosphorylation (Fig 3D), confirming that ERK1/2 was a downstream signaling target of EGFR. Enzalutamide and gefitinib treatment attenuated the constitutive ERK1/2 phosphorylation in MCF-7 KD cells as well (Sup. Fig. 1C, D). We conclude that AR forms a complex with EGFR in the KD cells, resulting in enhanced EGFR activation and downstream signaling to ERK1/2.

Activation of HER receptor family members, including EGFR, is a common mechanism of escape from Tam inhibition in ER -positive cells [13, 26]. Our microarray results indicated that EGFR and downstream ERK activation were associated with Tam treatment, so we determined if Tam treatment altered phosphorylation of these proteins in our cells. EGFR and downstream ERK1/2 phosphorylation was increased by Tam in ZR-75-B KD cells compared to control conditions (Fig. 3E, F). Tam also induced phosphorylation of both proteins in MCF-7 KD cells (Sup. Fig. 1E, F). Since AR promotes constitutive activation of EGFR in our cells we next asked if it also contributed to Tam-induced EGFR activation. Tam-stimulated phosphorylation of EGFR and downstream ERK1/2 were blocked by Enzalutamide and gefitinib (Fig. 3E, F). AR and EGFR inhibitors had similar effects in MCF-7 KD cells (Sup. Fig. 1E, F). We conclude that AR enhances activation of EGFR in response to Tam treatment, and conclude that this activation is a potential mechanism of resistance in our models.

AR and EGFR Promote ER α Activation and Tam Agonist Activity

ER is phosphorylated at serine (Ser) 118 by ERK1/2 resulting in ligand-independent activation [3, 23]. Enhanced constitutive Ser118 phosphorylation was previously observed in MCF-7 Rho GDI α KD cells [1]. AR causes ERK1/2 activation through EGFR in these cells, so we asked whether this results in ER α phosphorylation at Ser118 and subsequent activation. Ser118 phosphorylation was constitutively elevated in ZR-75-B KD cells (Fig. 4A). EGF treatment stimulated Ser118 phosphorylation (Fig 4A). Enzalutamide and gefitinib reduced Ser118 phosphorylation levels in both ZR-75-B and MCF-7 KD (Fig. 4A, Sup. Fig. 2A). To examine the effect of AR on ER α transcriptional activity we treated our model cells with R1881, a potent synthetic ligand for AR that is non-aromatizable. R1881 increased AR and ER transcriptional activities by 1.4 fold and 3.0 fold respectively in ZR-75-B KD cells (Fig. 4B, C). Treatment produced a 1.6 fold increase in AR activity and a 2.8 fold enhancement of ER activity in MCF-7 KD cells (Sup. Fig. 4A, B). R1881 also increased interactions between AR and EGFR (Sup Fig. 3), indicating that ER activation by R1881 may be due to increased non-genomic signaling of AR to EGFR. A constitutive increase in ER α transcriptional activity was observed in both ZR-75-B and MCF-7 KD cells, which was inhibited by Enzalutamide and gefitinib (Fig. 4D, Sup. Fig. 4C). These results demonstrate that AR promotes ER α phosphorylation and activation through EGFR in cells with low Rho GDI expression.

In some cases, Tam treatment can induce Ser118 phosphorylation contributing to ER α activation and resistance [2, 30]. In MCF-7 KD cells we have previously shown that Tam acts as an agonist, stimulating ER α activity in MCF-7 KD cells [9]. Tam treatment enhanced Ser118 phosphorylation and increased ER α activation in ZR-75-B as well as MCF-7 KD

cells, suggesting that Tam agonist activity was due to induction of Ser118 phosphorylation (Fig. 4E, F, Sup. Fig. 2B, Sup. Fig. 4D). Since AR promoted constitutive Ser118 phosphorylation and activation of ER α in our models, we next asked whether Tam-induced ER α activation is through AR. Enzalutamide and gefitinib blocked Tam-induced ER α phosphorylation and Tam agonist activity in ZR-75-B KD cells (Fig. 4E, F). Similar results were observed in MCF-7 KD cells (Sup. Fig. 2B, Sup. Fig. 4D). We conclude that Tam agonist activity in the KD cell lines is due to enhancement of AR-mediated signaling via EGFR to ER α Ser118.

AR and EGFR Enhance Basal and Tam-Induced Proliferation

Activation of EGFR signaling stimulates proliferation in ER α expressing breast cancer cells [16, 32, 35]. MCF-7 Rho GDI α KD cells exhibit enhanced basal proliferation [1], so we tested if EGFR activation by AR was playing a role in the constitutive growth that was observed. R1881 treatment increased proliferation 2.8 fold in ZR-75-B KD cells and 1.3 fold MCF-7 KD cells (Fig. 5A). Constitutive proliferation of ZR-75-B KD cells was reduced to 60% and 40% of basal levels, respectively, by Enzalutamide and gefitinib treatments (Fig. 5B). The inhibitors were also able to reduce basal proliferation of MCF-7 KD by 20% (Fig. 5B). This suggests that activation of EGFR by AR increases KD cells' proliferative ability.

We have previously shown that Tam exhibited agonist effects on proliferation in MCF-7 KD cells [1]. We wanted to determine if Tam effects on growth were also mediated by AR and EGFR. Tam acted as a growth agonist in both ZR-75-B and MCF-7 KD cells (Fig 5C). Enzalutamide blocked Tam-stimulated growth in ZR-75-B KD cells, bringing proliferation to untreated levels (Fig. 5C). Enzalutamide also attenuated Tam-induced proliferation of MCF-7 KD cells to 70% of basal levels (Fig. 5C). Gefitinib blocked Tam agonist growth as well, bringing proliferation to 40% of untreated levels in ZR-75-B KD cells and to untreated levels in MCF-7 KD cells (Fig. 5C). We conclude that Tam resistant growth is due to AR-mediated activation of EGFR in both cell lines with decreased Rho GDI α expression.

Discussion

About half of ER -positive breast tumors exhibit *de novo* resistance to endocrine therapies, such as Tam, and approximately a third of the remaining tumors will acquire resistance to first-line therapy [19]. Since 75% of breast tumors are ER -positive, resistance to endocrine therapy represents a major clinical problem affecting a significant portion of patients [23]. Signaling activation resulting in alternative survival pathways or activation of ER α through bi-directional crosstalk are common resistance mechanisms [14]. Co-targeting such pathways along with endocrine therapy often restores Tam sensitivity in pre-clinical models, but infrequently in clinical studies, indicating an urgent need for the identification of predictive biomarkers for targeted therapy response [21]. Here we demonstrate that Rho GDI α expression was correlated with expression of key nuclear and growth factor receptor pathways affecting response of breast cancer cells to tamoxifen. We also discovered a new mechanism by which AR acts through non-genomic activation of EGFR signaling to ER, leading to Tam-agonist activity and resistance following a decrease in Rho GDI expression.

Overexpression of Rho GTPases is common in breast tumors and Rho GTPase overexpression coupled with low expression of Rho GDIs is correlated with higher grade tumors, local recurrence, and increased metastasis [11, 20]. We have previously established that the Rho GTPase pathway is also a central mediator of tamoxifen response. A decrease in Rho GDI α expression and subsequent activation of Rho GTPases conferred resistance via activation of PAK1 and subsequent phosphorylation of ER α at a critical regulatory residue Ser305 [1]. In the current study, we show that a decrease in Rho GDI α expression increased expression of EGFR, AR, and MEK1, all of which participated in signaling to ER α and Tam resistance. Our work suggests that the Rho family of proteins act as drivers of endocrine therapy resistance both through Rho GTPase signaling indirectly to ER α , and via modulation of a key growth factor receptor (EGFR) and nuclear receptor (AR).

An increasing number of clinical and *in vitro* studies have linked increased AR expression and activity with endocrine therapy resistance [4, 9, 12, 31]. AR is expressed in 80% of breast tumors, often with ER α [24], so an understanding of how AR affects therapy response is of interest for a significant clinical population. We have previously demonstrated that activation of IGF-1R signaling through Akt is an escape pathway from AI therapy in AR-overexpressing ER α -positive breast cancer cells [31]. The current work describes a novel role for AR in Tam resistance via its endogenous up-regulation with reduced Rho GDI expression. In cells with Rho GDI α knockdown increased constitutive phosphorylation of EGFR and downstream ERK1/2 was blocked by Enzalutamide demonstrating that AR promotes activation of EGFR signaling. Constitutive ER α phosphorylation, activity, and proliferation were blocked by Enzalutamide and gefitinib suggesting that AR-mediated activation of EGFR signaling to downstream ERK1/2, and ultimately to ER α increased growth. R1881 increased ER α activity and proliferation confirming the role of AR. The observed effects of R1881 on ER α activity are in contrast to studies showing that androgens and AR can inhibit ER transcriptional activity and growth in hormone responsive cells [5, 29]. In these studies AR antagonizes ER α activity at the transcriptional level. The ability of R1881 to increase interactions between AR and EGFR suggests that in our model cells R1881 instead acts to increase ER activity and proliferation via non-genomic signaling of AR to EGFR. Tam acted as an agonist inducing both ER α transcriptional activity and cellular proliferation. Tam-induced phosphorylation of EGFR, ERK1/2, and ER α was also observed, suggesting that EGFR signaling to downstream ERK1/2, and ultimately to ER α was responsible for this Tam agonist activity. The role of AR and EGFR cooperation in this agonist activity was demonstrated by the ability of AR and EGFR inhibitors to block tam-induced phosphorylation, ER α activation, and growth.

Our work suggests that blockade of AR in combination with EGFR inhibition would be useful in the Tam-resistant setting. The AR inhibitor utilized in our study, Enzalutamide, is a third generation AR antagonist with a 5 to 8 fold higher binding affinity for the receptor that uniquely blocks nuclear transport, as well as binding to ligands, DNA, and co-activators [33]. A phase II clinical trial is ongoing to evaluate the safety of Enzalutamide in combination with the AI exemestane in ER α -positive breast cancer [37]. AR targeted therapy currently represents a promising avenue for management of resistant disease, and our study supports this avenue of clinical research.

The role of AR in breast cancer is multifaceted, as androgens have been shown to both stimulate and inhibit proliferation of cancer cells [24–26]. AR expression in ER α -positive tumors is associated with indicators of good prognosis, including improved overall survival, lower grade and size of primary tumors, and decreased lymph node involvement [18, 29]. At the same time, a high expression of AR relative to ER α levels is associated with decreased response to endocrine therapy and recurrent lesions after AI therapy exhibit increased AR activity [4, 12]. Given the apparent paradoxical role of AR in ER-positive breast cancer, there is an urgent need to identify biomarkers of when AR may be acting as a driver of proliferation and resistance to endocrine therapy, or a potential tumor suppressor. Pre-clinical studies have shown that crosstalk of EGFR with ER is a major mechanism of endocrine therapy resistance and made co-targeting the receptor with endocrine therapy an attractive option for management of resistant disease [34, 36, 38]. However, a combined analysis of two phase II clinical trials co-targeting EGFR using gefitinib in combination with Tam or the AI anastrozole indicated benefit only in patients receiving no prior endocrine therapy [7], and a subsequent phase II trial using the EGFR inhibitor AZD8931 in endocrine-therapy naive patients found no significant improvement in progression-free survival [22]. Further evaluation of co-targeting EGFR with endocrine therapy would require biomarkers to determine which patients may best respond. Our work identifies candidate biomarkers for AR and EGFR targeted therapy. In our cell line models and TamR tumors from patients, Rho GDI α loss was associated with AR overexpression. Low Rho GDI α coupled with high AR may be a biomarker of resistance and this possibility needs to be examined clinically. Rho GDI α silencing increased expression of EGFR and downstream ERK pathway member MEK1. Resistance was also associated with phosphorylation and activation of EGFR and its downstream target ERK1/2. Since Enzalutamide and gefitinib both reversed resistance in these models, overexpression and phosphorylation of EGFR/ERK pathway members when coupled with AR overexpression may be useful biomarkers worthy of further clinical study as determinants for AR/EGFR-targeted therapy.

Our study has described a novel model of AR-mediated Tam resistance through activation of EGFR signaling to ER α in ER -positive cells with low expression of Rho GDI. The association of decreased Rho GDI expression, AR overexpression, and increased expression and activation of EGFR pathway members with resistance could potentially identify patients who would benefit from AR and EGFR targeted therapy in future clinical studies.

Materials and Methods

Cell Culture

All cell lines were maintained at 37°C in a 5% CO₂ environment. MCF-7 cells were grown in Eagle's Minimum Essential Media (EMEM) (Lonza, Allendale, NJ) and supplemented with 10 g/ml insulin, 200 g/ml penicillin, 200 g/ml streptomycin, 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), and 0.1mM non-essential amino acids (NEAA). ZR-75-B cells were grown in RPMI 1640 Media (Lonza, Allendale, NJ) supplemented with 200 g/ml penicillin, 200 g/ml streptomycin, 10% fetal bovine serum (FBS) (Gemini Bio-Products, West Sacramento, CA), and 0.1mM non-essential amino acids (NEAA).

Antibodies, Agonists, and Inhibitors

Tamoxifen was purchased from Sigma-Aldrich (St. Louis, MO). Enzalutamide and gefitinib were obtained from Selleckchem (Houston, TX). Methyltrienolone (R1881) was obtained from Sigma (St. Louis, MO). For immunoblot assays, AR, total and phosphorylated EGFR (Tyr 845), and phosphorylated ER (Ser118) antibodies were purchased from Cell Signaling Technology (Beverly, MA). The antibody to total ER (6F11) was purchased from Vector Laboratories (Burlingame, CA). Antibodies to GAPDH and Rho GDI were purchased from Santa Cruz Biotechnology (Dallas, TX). Horseradish peroxidase-linked mouse and rabbit secondary antibodies were purchased from GE Healthcare (Piscataway, NJ). For immunofluorescence experiments AR (441) antibody was purchased from Dako (Carpinteria, CA) and EGFR XP® antibody was purchased from Cell Signaling Technology (Beverly, MA). Alexa Fluor 546-conjugated goat-anti-mouse and Alexa Fluor 647-conjugated goat-anti-rabbit secondary antibodies were purchased from (Life Technologies, Carlsbad, CA).

Stable Transduction

Cell lines with stable Rho GDI silencing were engineered by viral transduction of a pGIPZ lentiviral plasmid containing a Rho GDI shRNA expression sequence (GE Dharmacon, Lafayette, CO) and control cell lines by transfection with an “empty” pGIPZ containing no shRNA expression sequence. Lentiviral particles were generated using the Trans-Lentiviral Packaging System (GE Dharmacon, Lafayette, CO) as per manufacturer instructions. Cell lines were incubated with viral particles in serum-free growth media overnight to transduce cell lines. Stable transductants were selected for with media containing 1 µg/ml puromycin for 2 weeks and were confirmed by western blot analysis of RhoGDI expression.

Anchorage-Independent Growth Assays

In a 24 well plate, 2,500 cells per well, were plated in 1ml of a 0.35% solution of low melting point agarose (SeaPlaque Agarose, Lonza Allendale, NJ) in phenol-red-free media (EMEM for MCF-7 and RPMI for ZR-75-B) (Lonza, Allendale, NJ) that was supplemented with 5% charcoal-stripped FBS (J R Scientific, Inc., Woodland, CA). The cell layer was plated on top of a 350 µl 0.7% low melting point agarose base prepared in the same media as the cell layer. Cells were incubated at 37°C for 48 hours after plating to allow initial formation of colonies. Indicated treatments diluted in 1ml of the same media used to plate the assay were then added to the top of well. Media containing treatments was replaced every 4 days. At indicated time points colonies were counted using a GelCount™ scanner unit with provided software (Oxford Optronix, Abingdon, Oxfordshire, United Kingdom).

qRT-PCR

Total RNA was extracted from cells using the RNeasy micro kit (Qiagen, Valencia, CA) according to manufacturer's protocol. First strand cDNA synthesis was carried out using the SuperScript III Reverse Transcriptase kit (Life Technologies, Carlsbad, CA). cDNAs were amplified and detected using a SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA) and a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Relative gene expression levels were calculated using the Ct method with normalization to a reference gene (GAPDH). Primers for cDNA amplification were as follows for:

GAPDH (forward 5'-CCCACTCCTCCACCTTTGAC3' and reverse 5'-TGTTGCTGTAGCCAAATTCGTT-3'), AR (forward 5'-TCTAAGATGAAGGAGACCATC-3' and reverse 5'-GCGGTAGTAGGACAGGAAGTTGTT-3'), and ARHGDIA (forward 5'-CGAGGAGTGTGGCCCATTT-3' and reverse 5'-GGTCCATATGTGTCTTCCCATTC-3').

Protein Extraction and Immunoblot

Cells were washed with cold PBS prior to lysing with RIPA buffer. Lysates were then clarified by centrifugation for 10 minutes at 4°C at 20,000xg. Protein concentrations were determined using a BCA protein assay reagent kit (Pierce, Rockford, IL). Equal amounts of protein were loaded to a 10% SDS-PAGE gel for resolving. Proteins were then transferred to a nitrocellulose membrane (GE Healthcare, Piscataway, NJ). The membrane was then blocked in 5% BSA for 1 hour at room temperature, incubated with primary antibody (diluted in 5% BSA) overnight at 4°C, and lastly incubated with secondary antibodies (diluted in 5% milk) for 1 hour at room temperature. Membranes were visualized by addition of enhanced chemiluminescence reagent (Protein Simple, San Jose, CA).

Reporter Gene Assays

Following starvation for 48 hours in phenol-red-free media (EMEM for MCF-7 and RPMI for ZR-75-B) (Lonza, Allendale, NJ) that was supplemented with 5% charcoal-stripped FBS (J R Scientific, Inc., Woodland, CA), 30,000 cells per well were plated in 24 well plates. Cells were transfected with 0.25µg/well of luciferase reporter plasmid containing either an estrogen response element (ERE) or an androgen response element (ARE) in the promoter and 0.1µg/well of a -galactosidase expressing plasmid. Cells were treated as indicated following transfection. Transfection and luciferase assay were performed as described in [31].

Immunofluorescence

15,000 cells per well were plated in an 8 well chamber slide in phenol-red-containing media (EMEM for MCF-7 and RPMI for ZR-75-B) supplemented with 10% FBS and incubated at 37°C for 48 hours to allow attachment. The media was then changed to phenol-red-free media (EMEM for MCF-7 and RPMI for ZR-75-B) supplemented with 5% charcoal-stripped FBS for starvation for 48 hours and incubated with treatments for indicated times. Fixation, permeabilization, blocking, antibody incubation, and microscopy were carried out as described in [31]. Nuclei were stained using Vectashield® mounting media with DAPI purchased from Vector Laboratories (Burlingame, CA). All antibodies were used at a dilution of 1:100.

Proximity Ligation Assay (PLA)

15,000 cells per well were plated in an 8 well chamber slide in phenol-red-containing media (EMEM for MCF-7 and RPMI for ZR-75-B) supplemented with 10% FBS and incubated at 37°C for 48 hours to allow attachment. The media was then changed to phenol-red-free media (EMEM for MCF-7 and RPMI for ZR-75-B) supplemented with 5% charcoal-

stripped FBS for starvation for 48 hours. Cells were then incubated with treatments for indicated times before carrying out the PLA assay as described in [31].

Microarray

Microarray analysis of patient tumors has been previously described [8]. For cell line studies, 1,000,000 cells were plated in a 10cm³ tissue culture dish and incubated overnight at 37°C to allow attachment. Cells then underwent starvation for 48 hours in phenol-red-free media (EMEM for MCF-7 and RPMI for ZR-75-B) (Lonza, Allendale, NJ) that was supplemented with 5% charcoal-stripped FBS. Following starvation cells were treated with vehicle control or 100nM Tamoxifen for 24 hours. RNA extraction, cDNA preparation, and microarray were carried out as described in [31].

Statistical Analysis

Statistical analysis of microarray data was performed using Partek Genomics Suite software (<http://www.partek.com/>). Expressions were estimated using the RMA (Robust Multi-chip Average) method. ANOVA (Analysis of Variance) with contrasts was used to find differentially expressed genes and estimated FDR (False Discovery Rate) was determined using the Benjamini and Hochberg method to correct p-values for multiple comparisons. Data for all other assays were tested for significance using a student's t test (two-sided) with the p-value adjusted for multiple comparisons using the Holm method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Low Levels of Rho GDI Are Associated With AR Overexpression and Activation of EGFR and MAPK/ERK Pathways in TamR Cells

a. Heatmap of selected gene expression patterns from microarray analysis of primary breast cancer tissue from patients whose tumors were Tam-sensitive (TS, n=4) and metastatic breast cancer tissue from patients whose tumors were Tam-resistant (TR, n=5). **b.** Statistical analysis of ARHGDIA (Rho GDI) and AR expression from tumors in (A) (Columns=mean, Bars=SD, *p<0.05). **c.** Immunoblot analysis (left) of Rho GDIα, AR, EGFR, and GAPDH (loading control) in ZR-75-B and MCF-7 cells expressing Rho GDIα shRNA (KD) and empty vector control cells (Vec). Quantification (right) of relative pEGFR levels normalized to loading control (GAPDH). Data represents average of three independent experiments. (Columns=mean, Bars=SD) **d.** qRT-PCR analysis of ARHGDIA (Rho GDI) and AR mRNA expression in ZR-75-B and MCF-7 Vec and KD cells (Columns=mean, Bars=SD, *p<0.05). **e.** Heatmap of selected protein expression patterns as measured by RPPA in MCF-7 Vec and KD cells. Cells were treated with 100nM Tam for 24 hours prior to analysis. Three independent lysates were measured for each cell line and each lysate is represented by a column of the heatmap. **f.** Statistical analysis of AR and MEK1 protein expression from RPPA in (E) (Columns=mean, Bars=SD, *p<0.05)

Figure 2. Enhanced interaction between AR and EGFR in cells with Rho GDI loss

a. Immunocytochemistry assay showing localization of AR and EGFR in ZR-75-B Vec and KD cells after treatment with vehicle control (veh. ctrl.) (DMSO) or Enz (10 μ M) for 24 hours. **b.** Proximity ligation assay showing AR and EGFR complexes in ZR-75-B Vec and KD cells following 24 hour treatment with veh. ctrl. (DMSO) or the AR inhibitor Enzalutamide (Enz) (10 μ M). DAPI nuclear staining is indicated in blue and PLA reaction products are indicated as red dots.

Figure 3. AR activates EGFR signaling in cells with low Rho GDI α expression

a. Immunoblot analysis (left) of basal pEGFR (tyrosine 845) and GAPDH (loading control) expression in ZR-75-B Vec and KD cells. Corresponding total protein levels are also shown. Samples were run on a single gel; non-adjacent lanes are indicated by black line. Quantification (right) of relative pEGFR levels normalized to loading control (GAPDH) and total protein levels. Data represents average of three independent experiments. (Columns=mean, Bars=SD) **b.** Immunoblot analysis (left) of pEGFR (tyrosine 845) and GAPDH (loading control) expression in ZR-75-B Vec and KD cells following treatment with the EGFR inhibitor gefitinib (gef) (1 μ M), or Enz (10 μ M) for 30 minutes. Corresponding total protein levels are also shown. Samples were run on a single gel; non-adjacent lanes are indicated by black line. Quantification (right) of relative pEGFR levels normalized to loading control (GAPDH) and total protein levels. Data represents average of three independent experiments. (Columns=mean, Bars=SD) **c.** Immunoblot analysis (left) of basal p-P44/42 MAPK (threonine 202/tyrosine 204) levels in ZR-75-B Vec and KD cells. Corresponding total protein levels are also shown. Samples were run on a single gel; non-adjacent lanes are indicated by black line. Quantification (right) of relative p-P44/42 MAPK levels normalized to loading control (GAPDH) and total protein levels. Data represents average of three independent experiments. (Columns=mean, Bars=SD) **d.** Immunoblot analysis (left) of p-P44/42 MAPK (threonine 202/tyrosine 204) in ZR-75-B Vec and KD cells following treatment with EGF (200ng/ml), gef (1 μ M), or Enz (10 μ M) for 30 minutes. Corresponding total protein levels are also shown. Quantification (right) of relative p-P44/42 MAPK levels normalized to loading control (GAPDH) and total protein levels. Data represents average of three independent experiments. (Columns=mean, Bars=SD) **e.** Immunoblot assay (left) indicating pEGFR (tyrosine 845) levels of ZR-75-B KD cells

following 30 minutes treatment with Tam (100nM) or combination treatment with Tam and inhibitors of AR and EGFR (Enz (10μM) and gef (1μM) respectively). Corresponding total EGFR levels are also shown. Samples were run on a single gel; non-adjacent lanes are indicated by black line. Quantification (right) of relative pEGFR levels normalized to loading control (GAPDH) and total protein levels. Data represents average of three independent experiments. (Columns=mean, Bars=SD, *p<0.05) **f.** p-P44/42 MAPK (threonine 202/tyrosine 204) levels (left) in ZR-75-B KD cells following 24 hour treatment with Tam (100nM) or combination treatment with Tam and inhibitors of AR and EGFR (Enz (10μM) and gef (1μM) respectively). Samples were run on a single gel; non-adjacent lanes are indicated by black line. Quantification (right) of relative p-P44/42 MAPK levels normalized to loading control (GAPDH) and total protein levels. Data represents average of three independent experiments. (Columns=mean, Bars=SD, *p<0.05)

Figure 4. AR and EGFR promote increased constitutive and Tam induced ER transcriptional activity in cells with Rho GDI loss

a. Immunoblot analysis (left) indicating levels of pERα (Ser 118) in ZR-75-B Vec and KD cells following 1 min of treatment with EGF (200ng/ml), gef (1μM), or Enz (10μM). Total ERα protein levels are also shown. Samples were run on a single gel; non-adjacent lanes are indicated by black line. Quantification (right) of relative pERα (Ser 118) levels normalized to loading control (GAPDH) and total protein levels. Data represents average of three independent experiments. (Columns=mean, Bars=SD, *p<0.05) **b.** ARE-Luciferase reporter assay looking at AR mediated transcriptional activity following 24 hour treatment with the synthetic AR agonist R1881 (10nM) in ZR-75-B Vec and KD cells. Raw luciferase activity was normalized to expression from a co-transfected β-galactosidase (β-gal) reporter plasmid. (*p<0.05, N.S. = non-significant). **c.** ERE-Luciferase reporter assay looking at ER activity in ZR-75-B Vec and KD cells following 24hr treatment with 10nM R1881. Raw luciferase activity was normalized to expression from a co-transfected β-gal reporter plasmid. (*p<0.05, N.S. = non-significant). **d.** ERE-Luciferase reporter assay of ER activity in ZR-75-B Vec and KD cells following 24hr treatment with Enz (10μM) or gef (1μM). Raw luciferase activity was normalized to expression from a co-transfected β-gal reporter

plasmid. (* $p < 0.05$, N.S. = non-significant). **e.** Immunoblot analysis (left) of pER α (Ser 118) and total ER α protein levels in ZR-75-B KD cells following treatment with Tam (100nM), or combination treatment with Tam and inhibitors of AR and EGFR (Enz (10 μ M) and gef (1 μ M) respectively) for 30 minutes. Samples were run on a single gel; non-adjacent lanes are indicated by black line. Quantification (right) of relative pER α (Ser 118) levels normalized to loading control (GAPDH) and total protein levels. Data represents average of three independent experiments. (Columns=mean, Bars=SD, * $p < 0.05$) **f.** ERE-Luciferase reporter assay looking at ER activity following 24hr treatment with 100nM Tam, 10 μ M Enz, 1 μ M gef, or combinations as indicated. Raw luciferase activity was normalized to expression from a co-transfected β -gal reporter plasmid. (* $p < 0.05$, N.S. = non-significant).

Figure 5. AR and EGFR mediate growth and Tam-stimulated proliferation in ZR-75-B and MCF-7 cells with low Rho GDI α Expression

a. Soft agar growth assay looking at anchorage independent growth in ZR-75-B and MCF-7 Vec and KD cells after 2 weeks of treatment with R1881 (10nM) (* $p < 0.05$, N.S. = non-significant). **b.** Soft agar growth assay in ZR-75-B and MCF-7 Vec and KD cells analyzing anchorage independent growth following 2 weeks of treatment with 10 μ M Enz or 1 μ M gef. (* $p < 0.05$, N.S. = non-significant). **c.** Soft agar growth assay in ZR-75-B and MCF-7 Vec and KD cells looking at anchorage independent growth following 2 weeks of treatment with Tam (100nM), Enz (10 μ M), gef (1 μ M), and combinations as indicated. (* $p < 0.05$, N.S. = non-significant).

Table 1

Gene set enrichment analysis of differentially-expressed genes following 24 hour Tam treatment in ZR-75-B KD cells

Database	Gene Set	Gene Set Description	Genes Overlapped/ Total in Signature	P Value
Oncogenic Signatures	EGFR_UP.V1_UP [25]	Genes up regulated in MCF-7 cells over-expressing ligand inducible EGFR	60/193	<0.001
Oncogenic Signatures	EGFR_UP.V1_DN [25]	Genes down regulated in MCF-7 cells over-expressing ligand inducible EGFR	58/196	<0.001
Oncogenic Signatures	MEK_UP.V1_UP [6]	Genes up regulated in MCF-7 over-expressing constitutively active MAP2K1	60/196	<0.001
Oncogenic Signatures	MEK_UP.V1_DN [6]	Genes down regulated in MCF-7 over-expressing constitutively active MAP2K1	68/196	<0.001
Hallmark Gene Sets	HALLMARK_ESTROGEN_RESPONSE_EARLY	Genes defining early response to estrogen	30/200	<0.001
Hallmark Gene Sets	HALLMARK_ESTROGEN_RESPONSE_LATE	Genes defining late response to estrogen	26/200	<0.001

Gene set enrichment analysis of differentially-expressed genes following 24 hour Tam treatment in ZR-75-B KD cells. Gene set enrichment analysis performed using MSigDB (<http://www.broadinstitute.org/gsea/msigdb>). HALLMARK_ESTROGEN_RESPONSE_EARLY and HALLMARK_ESTROGEN_RESPONSE_LATE available from <http://www.broadinstitute.org/gsea/msigdb/collections.jsp>.

Table 2

Selected Genes Differentially Expressed Following Tam Treatment In KD Cells Compared To Vec Cells

Gene Symbol	Gene Name	False Discovery Rate	Fold Change
AR	Androgen receptor	1.57E-06	1.9
EGFR	Epidermal growth factor receptor	0.00761	1.8
CTSD	Cathepsin D	7.56E-08	2.5
TFF1	Trefoil factor 1	1.27E-28	10.3
FOXA3	Forkhead box A3	2.87E-08	2.9

Selected genes differentially expressed in ZR-75-B KD cells compared to Vec cells. Cells were treated with Tam for 24 hours and analyzed using gene expression microarray.