Inhibition of the Rho GTPase, Rac1, decreases estrogen receptor levels and is a novel therapeutic strategy in breast cancer

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Abstract

Rac1, a Rho GTPase, modulates diverse cellular processes and is hyperactive in some cancers. Estrogen receptor-alpha (ERα) in concert with intracellular signaling pathways regulates genes associated with cell proliferation, tumor development, and breast cancer cell survival. Therefore, we examined the possibility of Rac1 and ERα crosstalk in breast cancer cells. We found that Rac1 enhanced ERα transcriptional activity in breast cancer cells. Vav3, a Rho guanine nucleotide exchange factor that activates Rac1, was an upstream mediator, and P21/Cdc42/Rac1 activating kinase-1 (Pak-1) was a downstream effector of Rac1 enhancement of ERα activity. These results suggest that Rac1 may prove to be a therapeutic target. To test this hypothesis, we used a small molecule Rac inhibitor, EHT 1864, and found that EHT 1864 inhibited ERα transcriptional activity. Furthermore, EHT 1864 inhibited estrogen-induced cell proliferation in breast cancer cells and decreased tamoxifen-resistant breast cancer cell growth. EHT 1864 decreased activity of the promoter of the ERα gene resulting in down-regulation of ERα mRNA and protein levels. Therefore, ERα down-regulation by EHT 1864 is the likely mechanism of EHT 1864-mediated inhibition of ERα activity and estrogen-stimulated breast cancer cell proliferation. Since ERα plays a critical role in the pathogenesis of breast cancer and the Rac inhibitor EHT 1864 down-regulates ERα expression and breast cancer cell proliferation, further investigation of the therapeutic potential of Rac1 targeting in the treatment of breast cancer is warranted.

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Introduction

Rho GTPases are a subgroup of the Ras superfamily and include the well-characterized members, RhoA, Rac1, and Cdc42. Rho proteins cycle between the active state (GTP-bound) and inactive form (GDP-bound) enabling them to act as molecular switches in numerous signaling pathways (Van Aelst & D’Souza-Schorey 1997, Hall 1998). Rac1 and other Rho proteins are critical components of key mitogenic pathways providing a link between the cell surface and transcriptional events (Bosco et al. 2009).

Substantial evidence supports roles for Rho GTPase signaling alterations in cancers (Sahai & Marshall 2002, Chan et al. 2005). Since mutations in Rho proteins are extremely rare, the mechanism for Rho action in cancer likely occurs through the overexpression or hyperactivity of these proteins (Schnelzer et al. 2000). Increased Rho GTPase activity may be mediated by deregulation of upstream Rho guanine nucleotide exchange factors (GEFs), such as Vav3. Rac1 activation has been implicated in breast cancer cell invasion and metastasis (Fritz et al. 2002,

The effects of Rac1 on estrogen receptor-alpha (ERα) transcriptional activity and cell proliferation have not been fully elucidated. One study described Rac1 activation of ERα-mediated transcription in ovarian cancer cells, but another study showed that Rac1 inhibits ERα transcriptional activity (Lee et al. 2000, Su et al. 2001). In addition, introduction of a constitutively active form of Rac1 resulted in resistance to the ‘pure’ ERα antagonist ICI 182 780 in an ERα-positive breast cancer cell line (Cai et al. 2003). This evidence suggests that Rac1 may play an important role in the pathogenesis of breast cancer. Both EGFR and Vav3, a growth factor-activated Rho GTPase GEF, enhance ERα transcriptional activity in breast cancer cells (Lee et al. 2001, 2008, Lange 2004). Moreover, Rac1 participates in both MAPK and PI3K pathways, which are involved in crosstalk between EGFR and ERα (Ali & Coombes 2002, Cai et al. 2003). Therefore, we speculated that inhibition of Rac1 may have an anti-proliferative effect in breast cancer cells. EHT 1864 is a Rac small molecule inhibitor that holds Rac in an inert and inactive state, and prevents downstream effector binding and activation without disturbing GEF–Rac interactions (Shutes et al. 2007, Onesto et al. 2008). EHT 1864 specifically inhibits Rac without interfering with other Rho GTPases such as Rho or Cdc42 (Shutes et al. 2007, Onesto et al. 2008).

Currently, patients with ERα-positive breast cancer are treated with either selective ERα modulators, such as tamoxifen, or aromatase inhibitors, such as anastrozole, which interfere with estrogen synthesis. These therapies are effective because they inhibit the action of ERα to promote expression of genes associated with cell proliferation, tumor development, and survival. However, both primary resistance and secondary resistance to these therapies are serious clinical problems, and thus it is important to develop new therapeutic strategies that will also target tamoxifen-resistant breast cancer (Schiff et al. 2003). Since the majority of breast cancers express ERα, and since Rac1 is a downstream mediator of EGFR, which activates ERα, we wanted to determine whether there is a crosstalk between Rac1 and ERα in breast cancer cells.

In this study, we found that Rac1 increased ERα transcriptional activity in breast cancer cells. Inhibition of Rac1 by EHT 1864 decreased ERα transcriptional activity as well as estrogen-induced breast cancer cell proliferation in ERα-positive as well as tamoxifen-resistant cells. Furthermore, Vav3 was an upstream activator, and P21/Cdc42/Rac1 activating kinase-1 (Pak-1) was a downstream effector of Rac1 enhancement of ERα transcriptional activity. We demonstrate that EHT 1864 inhibited ERα activity through down-regulation of ERα mRNA and protein.

Materials and methods

Cell culture and chemical reagents

The human breast cancer cell lines MDA MB 231, MCF-7, and T47D were kindly provided by Dr Catherine Welsh (University of Miami, Miami, FL, USA). The MCF-7 tamoxifen-sensitive and MCF-7 tamoxifen-resistant cells were kindly provided by Dr Rachel Schiff (Baylor College of Medicine, Houston, TX, USA). Cell culture media (RPMI-1640, DMEM/F12 50:50, DMEM, DMEM Glutamax) were obtained from Gibco-BRL. Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). T47D cells were cultured in RPMI supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (Gibco-BRL), and 10% FBS. MCF-7 cells and MDA MB 231 cells were cultured in DMEM/F12 50:50 supplemented with 5% FBS, 100 IU/ml penicillin, 1000 µg/ml streptomycin, and 2 mM L-glutamine. MCF-7 tamoxifen-sensitive cells were cultured in DMEM Glutamax supplemented with 10% FBS, 100 IU/ml penicillin, 1000 µg/ml streptomycin, 2 mM L-glutamine, and 15 µg/ml insulin. MCF-7 tamoxifen-resistant cells were cultured in phenol red-free DMEM supplemented with 5% charcoal-stripped serum supplemented with 100 IU/ml penicillin, 1000 µg/ml streptomycin, 2 mM L-glutamine, and 150 nM tamoxifen. EHT 1864 and tamoxifen were purchased from Sigma, and estradiol was purchased from Steraloids (Wilton, NW, USA). For estradiol depletion, cells were transferred to medium containing phenol red-free DMEM supplemented with 5% charcoal-stripped serum for 24 h. Assays of ERE luciferase and QPCR of ERα target genes were carried out at 24 h following readdition of estradiol 1 nM vehicle.

Plasmids

The 3X ERE-luciferase (ERE-Luc) reporter plasmid was provided by Dr Zafar Nawaz (University of Miami). Constitutively active (Ca) Vav3 (Ca Vav3) was constructed as previously described (Lyons & Burnstein 2006, Lyons et al. 2008). Ca Rac1 (CaRacQ61L) and Ca Rac1/PakEDM (CaRacQ61L/43D) were gifts from Dr Channing Der (University of North Carolina-Chapel Hill). The ER promoter reporter
plasmids (ER promoter wild-type luciferase (WT ER3500-210LUC) and ER promoter delta enhancer luciferase (delta Enh ER3500-135LUC)) were kindly provided by Dr Ronald Weigel (deConinck et al. 1995).

Retrovirus production and generation of stable cell lines

Constitutively active Rac1 Q61L was cloned into pQCXIN (BD Biosciences, Palo Alto, CA, USA) by PCR amplification of PCGN caRac1 vector and verified by sequencing. For viral production, GP2-293 cells at 60–80% confluency in 100 mm dishes were transfected with 7.5 μg VSV-G and 12.5 μg pQCXIN (encoding empty vector or caRac1) using CalPhos kit (Clontech). Forty-eight hours after transfection, media containing viral particles were collected and filtered through a 0.45 μm cellulose acetate filter and stored at –80 °C. Forselection, cells were infected overnight with appropriate constructs 24 h after seeding and cultured in 750 μg/ml G418 48 h after infection for 8–10 days.

Reporter gene assays and transfections

All transfections were carried out using the cationic lipid reagent Lipofectamine (Invitrogen Life Technologies) according to the manufacturer’s instructions. Cells were plated in 35 mm dishes 16–20 h prior to transfection. Immediately prior to transfection, media were replaced with unsupplemented DMEM. Cells were transfected with 1.5 μg reporter (ER promoter wild-type luciferase (WT) or ER promoter delta enhancer luciferase (delta Enh)), or 1.5 μg ERE-luc, and 125 ng Ca Rac1, Ca Vav, or Ca Rac1/PakEDM. Following a 4–5 h incubation with DNA/lipid complexes, cells were transferred to estrogen-depleted media (phenol red-free media supplemented with 2% charcoal-stripped serum) for 24 h and then treated with vehicle or 1 nM estradiol in the absence or presence of 10 μM EHT 1864 and phenol red-free media supplemented with 2% charcoal-stripped serum. In total, 40 μg protein from each sample was resolved on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris base (pH 7.5) and 50 mM NaCl and 2.5 mM EDTA) containing 0.1% Tween 20 (TBS-T), and then probed with anti-ERα (HC-20; 1:200) or anti-actin (1:500) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies were diluted in 5% nonfat dry milk in TBS-T. After washing in TBS-T, membranes were incubated with their appropriate HRP-conjugated secondary antibodies (Santa Cruz) and developed using an enhanced chemiluminescence detection system (Amersham Biosciences) according to the instructions of the manufacturer.

Cell proliferation assay

Cells were plated at an initial density of 20 000 (MCF-7 cells), 10 000 (T47D cells), 10 000 (MDAMB231), and 10 000 (Ca Rac1 expressing and control T47D) cells per well in a 24-well dish containing media supplemented with 10% charcoal-stripped serum. The following day, cells were treated with vehicle, 1 nM estradiol, 10 μM EHT 1864, and/or 500 nM tamoxifen for various time periods. After the appropriate treatment period, adherent and floating cells were collected, and viable cells were counted by trypan blue exclusion using a hemocytometer. Experiments were performed in triplicate.

RNA extraction and real-time RT-PCR

RNA was harvested using the TRIzol method according to the manufacturer’s protocol (Invitrogen Life Technologies, Carlsbad, CA, USA). In total, 500 ng RNA was reverse transcribed using cDNA archive kit (Applied Biosystems). Real-time PCR was performed using ABI Prism 7700. Taqman probes from Applied Biosystems for 18S, Rac1, HPRT, ESR1 and pS2 were used. In total, 100 ng cDNA was used for qPCR (except 1 ng cDNA was used for 18S, the endogenous control). The comparative threshold cycle (Ct) method was used to determine the relative expression level of mRNA. This involved comparing the Ct values of the treated samples with a control sample. The Ct values of both the control and the samples of interest were normalized to 18S (an endogenous housekeeping gene). Relative mRNA levels were determined by the following equations (Schmittgen & Livak 2008):

1. Fold change = $2^{-\Delta\Delta C_t}$
2. $2^{-\Delta C_t} = \Delta C_t$, sample $- \Delta C_t$, control

Western blot analysis

Cells were grown to ~80% confluency in 60 mm plates. Twenty-four hours prior to harvest, cells were treated with vehicle or 1 nM estradiol in the presence or absence of 10 μM EHT 1864 and phenol red-free media supplemented with 2% charcoal-stripped serum. In total, 40 μg protein from each sample was resolved on 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris base (pH 7.5) and 50 mM NaCl and 2.5 mM EDTA) containing 0.1% Tween 20 (TBS-T), and then probed with anti-ERα (HC-20; 1:200) or anti-actin (1:500) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies were diluted in 5% nonfat dry milk in TBS-T. After washing in TBS-T, membranes were incubated with their appropriate HRP-conjugated secondary antibodies (Santa Cruz) and developed using an enhanced chemiluminescence detection system (Amersham Biosciences) according to the instructions of the manufacturer.

GST protein purification

Rac/Cdc42-binding domain (p21-binding domain, PBD) of Pak-1 coupled to GST (PBD–GST) was
a gift from Dr Martin Schwartz (University of Virginia, Charlottesville, VA, USA). PBD–GST protein was purified according to the manufacturer’s protocol (Amersham). BL-21 competent cells were transformed with plasmids encoding GST proteins. Gene expression was induced with 100 mM isopropyl β-d-thiogalactosidase for 90 min. Cell lysates were added to glutathione columns and then eluted with reduced glutathione. Protein concentration was determined, and purified proteins were visualized by Coomassie stain.

**Rac GTPase activity ‘pull-down’ assay**

Rac1 activity was assessed by pull-down assay using PBD–GST as described previously (Knight-Krajewski et al. 2004). Briefly, cells were harvested and lysed in Mg2+ lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM MgCl2, 0.5% NP-40, 10% glycerol, and 10 μl/ml protease inhibitor) containing 60 μg/300 μl PBD–GST. Cell lysates containing 1 mg protein were added to 100 μl glutathione sepharose beads and rotated gently for 30 min at 4 °C. Sepharose beads were pelleted by centrifugation, and complexes were washed four times with lysis buffer. GTP-bound Rac1/Cdc42 was eluted and separated along with 5% inputs by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and analyzed using anti-Rac1 antibody (Millipore, Billerica, MA, USA).

**Analysis of ERα degradation**

T47D cells were estrogen depleted in 10% charcoal-stripped serum for 24 h followed by treatment with vehicle or 10 μM EHT 1864 for 24 h. After 24 h, cells were treated with vehicle or 1 nM estradiol for 1 h. Chromatin was then crosslinked with 1% formaldehyde for 10 min at room temperature. The crosslinked chromatin was then sonicated, diluted, and immunoprecipitated with ERα (HC-20) antibody (Santa Cruz Biotechnology) or normal rabbit IgG control at 4 °C overnight. Protein A agarose beads with salmon sperm DNA were added and then washed with a low salt buffer, followed by a high salt buffer, then LiCl buffer, and finally TE buffer. The protein DNA complexes were eluted, and the crosslinks were reversed. The DNA fragments were purified with the Qiagen PCR purification kit. The fragments were then analyzed by real-time PCR. Real-time PCR was performed using an iCycler iQ PCR detection system (Bio-Rad) with qSYBR Green supermix (Bio-Rad). Primers for pS2 and GREB1 promoters were described previously (Dhananjayan et al. 2006, Sun et al. 2007).

**Results**

**Rac1 enhances ERα transcriptional activity**

To determine whether there is a crosstalk between Rac1 and ERα, we created derivative cell lines of T47D and MCF-7 cells that express either constitutively active (Ca) Rac1 or vector (as a control) and performed ER reporter gene assays. As expected, Rac1 ‘pull-down’ activity assays showed elevated active Rac1 levels in the Ca Rac1-expressing cells compared to control (Fig. 1A). PC3, a prostate cancer cell line that has relatively high Rac1 activity, was used as a positive control (Knight-Krajewski et al. 2004). The Ca Rac1-expressing cells had active Rac1 levels comparable to PC3 cells, suggesting that Rac1 activity in the derivative breast cancer cells was in a range relevant to that observed in cancer cells (Fig. 1A). Reporter gene assays revealed that ERα transcriptional activity was increased in cells expressing Ca Rac1 compared to control cells in both T47D and MCF-7 cells (Fig. 1B and C). Ca Rac1-expressing cells depleted of estrogen for 24 h had increased ERα transcriptional activity in both the absence and presence of estradiol, suggesting that Rac1 enhances both ligand-dependent and ligand-independent receptor activity. Ca Rac1 enhancement of the ligand-independent activation of ERE-luciferase activity may be due to the persistence of very low levels of estrogens in the stripped serum and/or due to true ligand-independent activation of ERα. To confirm and extend the results of the reporter gene assays, we examined ERα regulation of a native ERα target gene (pS2) and found that pS2 mRNA levels were increased...
in cells expressing Ca Rac1 compared to control in both the presence and absence of estradiol (Fig. 1D).

Since Ca Rac1 enhanced ERα activity in the presence and absence of estradiol, we wanted to verify that these effects were not due to global activation of transcription. HPRT encodes an enzyme involved in the purine synthesis pathway and is not regulated by ERα (de Kok et al. 2005). Ca Rac1 did not alter HPRT mRNA levels compared to control in either MCF-7 or T47D cells consistent with a selective effect of Rac1 on ERα-mediated transcription (Fig. 1E).

The Rac inhibitor, EHT 1864, decreases ERα transcriptional activity and ERα recruitment to target gene promoters

Since Rac1 enhanced ERα transcriptional activity in breast cancer cells, we examined the effect of Rac1 inhibition on ERα-mediated responses. EHT 1864 is a small molecule Rac inhibitor that blocks Rac GTPase activity and prevents Rac activation of downstream effectors (Shutes et al. 2007, Onesto et al. 2008). Reporter gene assays in MCF-7 and T47D cells showed that even in the absence of Rac1 overexpression, EHT 1864 inhibited estradiol-stimulated ERα transcriptional activity (Fig. 2A and B). EHT 1864 also modestly inhibited ERα activity in the estradiol-deprived cells. Both effects were more notable in T47D cells compared to MCF-7 cells. Similarly, EHT 1864 treatment decreased pS2 but not HPRT mRNA levels in T47D and MCF-7 cells, confirming our finding that EHT 1864 selectively impaired ERα transcriptional activity (Fig. 2C–E).

To determine whether EHT 1864 also disrupts ERα recruitment to target gene promoters, we performed chromatin immunoprecipitation. As expected, estradiol treatment increased ERα recruitment to the promoters of the ERα target genes pS2 and GREB1, while tamoxifen inhibited receptor recruitment (Fig. 2F and
EHT 1864 inhibits estrogen-induced breast cancer cell proliferation

Since EHT 1864 inhibited ERα activity, we wanted to determine whether Rac1 inhibition would also decrease breast cancer cell proliferation. EHT 1864 significantly inhibited the rate of T47D and MCF-7 cell proliferation in the presence or absence of estradiol (Fig. 3A and B). While EHT 1864 also inhibited MDA MB 231 cells, an ERα-negative breast cancer cell line, the extent of inhibition was substantially less than that observed in the ERα-positive, estradiol-stimulated MCF-7 and T47D cells (Fig. 3C). In the presence of estradiol, there was ~90% decrease in the T47D cell number, 80% in MCF-7 cells, and about 50% growth inhibition in MDA MB 231 following EHT 1864 treatment. Therefore, EHT 1864’s anti-proliferative effects appear to be at least partially mediated by inhibiting ERα activity.

Tamoxifen, a potent ERα-blocking drug, is of important therapeutic value in breast cancer. Since EHT 1864 inhibits estrogen-induced breast cancer cell proliferation, we tested whether the combination of tamoxifen and EHT 1864 would cause a greater inhibition of cell proliferation. However, there was no additive effect on cell proliferation from the combination of EHT 1864 and tamoxifen treatment in T47D cells (Fig. 3D) or MCF-7 cells (data not shown). There was also no additive inhibition of cell proliferation at lower (suboptimal) concentrations of each drug in either T47D or MCF-7 cells (data not shown). Together, these results reveal that EHT 1864 treatment not only diminished ERα-dependent gene transcription but also diminished breast cancer cell proliferation.

Vav3, a Rho GTPase GEF, is an upstream activator of Rac1 enhancement of ERα transcriptional activity

In addition to its well-recognized role as an activator of Rho GTPases, Vav3 has been shown to increase ERα transcriptional activity (Lee et al. 2008). Therefore, the role of Vav3 in Rac1 enhancement of ERα transcriptional activity needs to be further investigated.
transcriptional activity was examined. As expected, constitutively active (Ca) Vav3 increased ERα transcriptional activity in breast cancer cells (Fig. 4A). EHT 1864 prevented Ca Vav3-mediated stimulation of ERα activity in the absence and presence of estradiol; thus, Rac1 action appears to be required for Vav3-mediated stimulation of ERα activity (Fig. 4A).

Pak-1 is a downstream mediator of Rac1-dependent ERα transcriptional activation

Pak-1, a well-characterized downstream effector of Rac1, has been shown to mediate growth factor effects on motility and invasiveness in breast cancer cells (Manser et al. 1994, Adam et al. 1998, Vadlamudi et al. 2000). Therefore, we investigated the role of Pak-1 on Rac1 and ERα crosstalk. To determine whether Pak-1 is a downstream effector, a Ca Rac1 effector domain mutant (EDM) that is selectively unable to bind and activate Pak-1 (Ca Rac1/PakEDM) was transfected into T47D cells (Westwick et al. 1997). In contrast to Ca Rac1, the Ca Rac1/PakEDM construct did not enhance ERα transcriptional activity, indicating that Pak-1 may be a key mediator of Rac1 stimulation of ERα activity via direct Rac1–Pak-1 interaction (Fig. 4B).

EHT 1864 down-regulates ERα mRNA and protein levels

We next addressed the molecular mechanism whereby EHT 1864 inhibits ERα activity. One possible mechanism is through down-regulation of ERα levels. As expected, estradiol treatment resulted in decreased ERα protein levels. Following EHT 1864 treatment in both the presence and absence of estradiol, there was a significant decrease in ERα protein levels in both MCF-7 and T47D cells (Fig. 5A). RT-qPCR analysis revealed that EHT 1864 treatment reduced ERα mRNA levels in both the presence and absence of estradiol in MCF-7 and T47D cells without any evidence of global transcriptional inhibition since HPRT levels were unchanged (Fig. 5B and C). Estradiol treatment did not change ERα mRNA levels (Fig. 5B and C).

To determine whether decreased ERα protein levels were due to transcriptional down-regulation of the ER gene (ESR1) or due to decreased ERα protein stability, we first tested the drug’s effect on the promoter for ESR1. The construct for the promoter of ESR1 contains the requisite regions for ERα expression in breast cancer cells but does not contain ERα-binding sites (deConinck et al. 1995). When estrogen-deprived cells were treated with EHT 1864 in the presence or absence of estradiol for 24 h, EHT 1864 inhibited...
activity of the wild-type ESR1 promoter but did not affect activity of a reporter plasmid that lacked the enhancer region of the ESR1 promoter (Fig. 6A).

We next examined whether EHT 1864-mediated ERα loss might be due to destabilization of the ERα protein. ERα protein stability was assayed in estrogen-deprived cells following 24 h of EHT 1864 treatment. It is noteworthy that when ERα-positive MCF-7 breast cancer cells are estrogen deprived, ERα protein is highly stable, with an ER-α t1/2 in excess of 10 h, as previously demonstrated (Chu et al. 2007). Our cycloheximide chase showed that while EHT 1864 treatment dramatically reduced ERα steady-state levels, there was no significant effect of EHT 1864 on ERα protein degradation rate (Fig. 6B and C). Thus, EHT 1864-mediated down-regulation of ERα appears to be primarily through decreased transcription of ESR1.

**EHT 1864 inhibits tamoxifen-resistant breast cancer cell proliferation**

Breast cancer cells, following extended treatment with tamoxifen, can overcome the antagonistic effects of tamoxifen on ERα resulting in drug resistance.
(Schiff et al. 2005). Therefore, it is important to identify novel drug targets that may be effective in the treatment of tamoxifen-resistant breast cancer. Rac1 inhibition may be a useful strategy to inhibit growth of tamoxifen-resistant cells. To test this, we determined whether EHT 1864 inhibits the growth of a tamoxifen-resistant cell line, MCF-7 TAMR. EHT 1864 inhibited the proliferation of these cells, whereas, as expected, tamoxifen did not (Fig. 7A and B). Furthermore, T47D cells expressing Ca Rac1 were more resistant to tamoxifen treatment compared to control cells (Fig. 7C). These results suggest that inhibition of Rac1 may be a potential therapeutic strategy for ERα-positive, tamoxifen-resistant breast cancer.

Discussion

Rac1 is an important mediator of signaling pathways including those that promote cell proliferation, migration, and cancer cell invasiveness (Fritz et al. 2002, Baugher et al. 2005, Chan et al. 2005). We identified a novel crosstalk between Rac1 and ERα signaling in breast cancer cells, in which Vav3 served as an upstream activator and Pak-1 served as a downstream effector of Rac1 enhancement of ERα activity. Furthermore, inhibition of this crosstalk with a Rac inhibitor, EHT 1864, decreased ERα transcriptional activity through a mechanism involving transcriptional repression of the ESR1 promoter. In line with the possibility that Rac1 may be an effective therapeutic target in breast cancer, EHT 1864 significantly down-regulated ERα mRNA and protein levels leading to decreased ERα transcriptional activity as well as estradiol-stimulated breast cancer cell proliferation. The effect of EHT 1864 on our novel model of crosstalk between Rac1 and ERα is illustrated in Fig. 8.

Rac1 stimulates migration and invasion in breast cancer cells due to its well-established effects on cytoskeleton remodeling. One study showed that Rac1 participates in estrogen-stimulated MCF-7 cell growth and that these proliferative effects were dependent on the composition of the extracellular matrix (Xie & Haslam 2008). Estrogen-induced MCF-7 cell proliferation was significantly increased when cells were cultured on collagen I compared to laminin, and this was attributed to collagen I activation of Rac1 and Jun N-terminal kinase signaling (Xie & Haslam 2008). These effects on cell proliferation were attributed to Rac1 regulation of cyclin D1 mRNA levels independent of ERα (Xie & Haslam 2008).

We showed that EHT 1864 inhibited estrogen-stimulated ERα-positive breast cancer cells to a greater extent than an ERα-negative cell line, which expresses comparable Rac1 levels (data not shown), suggesting that inhibition of Rac1 and its resultant suppression of ERα levels contributed to EHT 1864 anti-proliferative effects. The mechanisms of EHT 1864-mediated

Figure 6 EHT 1864 inhibits activity of the promoter of ESR1 but does not disrupt ERα stability. (A) T47D cells were deprived of estrogen for 24 h and then transfected with a WT ESR1 promoter luciferase or the ESR1 promoter lacking the enhancer (delta enhancer) reporter plasmid. Cells were treated with either vehicle or 1 nM estradiol in the presence or absence of 10 μM EHT 1864. Luciferase activity was determined 48 h after transfection and normalized for protein. Results are reported as values relative to ER promoter luciferase WT set at 100%. Data represent the mean of three experiments performed in triplicate ± S.E.M. (B) T47D cells were deprived of estradiol for 24 h and then treated with vehicle or 10 μM EHT 1864 for 24 h. ERα protein levels were determined by western blot, and actin was used as a loading control. (C) T47D cells were deprived of estradiol for 24 h and then pretreated with vehicle or 10 μM EHT 1864 for 1 h followed by the addition of cycloheximide. Cells were harvested, and protein lysates were obtained at the indicated times after cycloheximide treatment. ERα protein levels were determined by western blot, and actin was used as a loading control. This is a representative experiment of two experiments. *P < 0.05.
growth inhibition of breast cancer cells require further work. There was no additive inhibition of estrogen-stimulated cell proliferation using a combination of EHT 1864 and tamoxifen perhaps because EHT 1864 down-regulation of ERα decreased the target for tamoxifen (ERα) thereby compromising the effectiveness of this drug. Furthermore, tamoxifen and EHT 1864 may not additively decrease cell proliferation because they both inhibit ERα. Since Rac1 is a well-known mediator of cell migration and invasion, Rac1 inhibition is likely to have a broader effect than tamoxifen beyond reducing ERα levels. EHT 1864 inhibits membrane lamellipodia formation in fibroblasts and prevents Ras- and Rac1-dependent transformation (Shutes et al. 2007, Onesto et al. 2008). Rac1 may integrate ERα signaling and other mitogenic pathways with cytoskeleton remodeling to increase breast cancer aggressiveness. Thus, by targeting multiple critical pathways involved in the pathogenesis and progression of breast cancer, Rac1 inhibition may be an effective therapeutic option.

Our findings also suggest that Rac1 may mediate crosstalk between growth factors and ERα signaling in breast cancer, since Vav3, a growth factor-activated GEF, stimulated Rac1 enhancement of ERα activity. While ligand-independent activation of ERα by growth factors can occur through phosphorylation of ERα by downstream kinases such as MAPK, glycogen synthase kinase-3, protein kinase A, and PI3K/AKT (Rayala et al. 2006a), we identified an alternative mechanism by which Vav3 and Rac1 may activate ERα. Vav3 is a Rho GTPase GEF that is activated by a variety of growth factor receptors including EGFR and stimulates Rac1 activity. Vav3 is up-regulated in breast cancer compared to benign mammary tissue (Lee et al. 2008). One study showed that Vav3 enhances ERα transcriptional activity; however, the authors proposed that Vav3 mediates its effects through PI3K signaling (Lee et al. 2008). Here, we showed that inhibition of Rac1 blocked Vav3 enhancement of ERα transcriptional activity, implicating Rac1 as a key mediator of growth factors.

Figure 7 EHT 1864 inhibits tamoxifen-resistant breast cancer cell proliferation and active Rac1 confers partial tamoxifen resistance. MCF-7 tamoxifen-sensitive (TAMs) (A) and MCF-7 tamoxifen-resistant (TAMr) (B) cells were treated with either vehicle, 100 nM tamoxifen, 10 μM EHT 1864, or 100 nM tamoxifen plus 10 μM EHT 1864. Cells were counted following 5-day treatment. P values were determined by Student’s t-test. **P<0.01. (C) T47D cells stably expressing either vector or Ca Rac1 were treated with either vehicle or 100 nM tamoxifen. Cells were counted after 6 days of treatment. This is a representative experiment from three experiments performed in triplicate. Data are plotted as percent of vehicle-treated cells. P value was determined by comparing Ca Rac1-expressing cells to control using Student’s t-test. *P<0.05.

Figure 8 Effects of Rac1 inhibition on ERα levels. Vav3 activates Rac1 by promoting GTP binding. However, in the presence of EHT 1864, Rac1 fails to bind and activate downstream effectors resulting in ERα mRNA down-regulation. The decreased ERα levels result in lowered ERα transcriptional activity and ultimately inhibits breast cancer cell proliferation.
Vav3 and possibly growth factor-induced ERα activity. Further studies are necessary to test how Rac1 may mediate growth factor enhancement of ERα activity. PI3K signaling may also independently play a role in mediating Vav3 effects on ERα; however, this is complicated by the fact that Rac1 regulates PI3K as well as MAPK signaling (Bosco et al. 2009). Therefore, Vav3 activation of Rac1 may stimulate different signaling pathways, including PI3K, to ultimately increase ERα levels and ERα transcriptional activity.

One signaling protein that appears to be required for Rac1 and ERα crosstalk is Pak-1, a well-characterized downstream effector of Rac1. We found that Rac1 activation of Pak-1 was necessary for Rac1-mediated increase in ERα transcriptional activity. Therefore, Pak-1 may be responsible for regulating ERα mRNA expression downstream of Rac1. Pak-1 regulates gene expression in part through phosphorylation of factors such as FOXO1, a forkhead box transcription factor (Mazumdar & Kumar 2003, Guo & Sonenshein 2004). However, Pak-1 has not been shown to affect FOXO3A, which stimulates ERα expression (Masiello et al. 2002, Guo & Sonenshein 2004). Therefore, it is unclear whether forkhead box transcription factors play a role in Rac1 regulation of ESR1 expression.

Pak-1 interacts with and phosphorylates ERα at serine 305 leading to phosphorylation at serine 118, which is required for full ERα transcriptional activity (Wang et al. 2002, Balasenthil et al. 2004). While phosphorylation of ERα by Pak-1 may contribute to Rac1 regulation of ERα, we have identified a possible novel mechanism by which Rac1, via Pak-1, affects ERα levels. Importantly, this mechanism can be therapeutically targeted.

Pak-1 is implicated in breast cancer progression to tamoxifen resistance by stimulating ERα transactivation (Rayala et al. 2006b). We found that EHT 1864 inhibition of Rac1 (and likely the downstream effector, Pak-1) decreased the proliferation of tamoxifen-resistant breast cancer cells. We also showed that overexpression of Ca Rac1 in breast cancer cells leads to decreased tamoxifen-mediated growth inhibition consistent with a role for Rac1 in tamoxifen resistance.

Inhibition of Rac1 is recognized as a therapeutic target in cancer due to its effects on cell migration and invasion. Furthermore, we found that inhibiting Rac1 also had anti-proliferative effects, which were in part mediated by decreasing ERα levels in ERα-positive breast cancer cells that are either tamoxifen sensitive or resistant. Thus, inhibition of Rac1 may represent a new therapeutic opportunity in the treatment of ERα-positive breast cancer even in the setting of tamoxifen resistance.

Declaration of interest
Dr Laurent Désiré is an employee of ExonHit Therapeutics.

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