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Ron Receptor Tyrosine Kinase Activation Confers Resistance to Tamoxifen in Breast Cancer Cell Lines[1]

Rebecca J. McClaine*,[2], Aaron M. Marshall†,[2], Purnima K. Wagh† and Susan E. Waltz†

*Department of Surgery, University of Cincinnati College of Medicine, Cincinnati, OH, USA; †Department of Cancer and Cell Biology, University of Cincinnati College of Medicine, Cincinnati, OH, USA

Abstract

Although tamoxifen treatment is associated with improved survival in patients with estrogen receptor (ER)–positive breast tumors, resistance remains an important clinical obstacle. Signaling through growth factor signaling pathways, in particular through receptor tyrosine kinases, has been demonstrated to confer tamoxifen resistance in an estradiol-independent manner. The Ron receptor tyrosine kinase, a member of the c-Met family of receptors, is expressed in a number of human epithelial tumors, and elevated expression of Ron is associated with poor prognosis in women with breast cancer. In this report, we evaluated the role of Ron receptor activation in conferring resistance to tamoxifen in human and murine breast cancer cell lines. Activation of Ron by its ligand, hepatocyte growth factor–like protein (HGFL) was associated with partial rescue from tamoxifen-induced growth inhibition in Ron-expressing cell lines. Western analysis revealed that treatment of the T47D human breast cancer cell line with tamoxifen and HGFL was associated with increased phosphorylation of mitogen-activated protein kinase (MAPK) 1/2 and phosphorylation of serine residue 118 of ER. Expression of ER-dependent genes was increased in cells treated with tamoxifen and HGFL by quantitative reverse transcription–polymerase chain reaction. All of these effects were inhibited by treatment with either a Ron-neutralizing antibody or a MEK1 inhibitor, suggesting the specificity of the effect to Ron, and the involvement of the MAPK 1/2 signaling pathway. In summary, these results illustrate a novel connection between the Ron receptor tyrosine kinase and an important mechanism of tamoxifen resistance in breast cancer.

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Introduction

Among women in the United States, breast cancer is the most common cancer diagnosed. It is second only to lung cancer in mortality, with 180,000 new cases each year and 40,000 deaths. Treatment with the selective estrogen receptor modulator tamoxifen has been shown to significantly reduce recurrence rates in women diagnosed with estrogen receptor alpha (ER)–positive breast tumors [1]. However, subsets of patients with ER-positive tumors do not respond to tamoxifen, or recur despite tamoxifen therapy, indicating tumor resistance to this treatment and a need to identify aggressive ER-positive tumors [2]. Several mechanisms have been implicated in tamoxifen resistance, including overexpression of coactivator proteins, such as amplified in breast cancer 1 [3], decreased expression of corepressor proteins, such as nuclear receptor corepressor [4], and activation of growth factor pathways leading to ligand-independent ER activation [5,6]. Interestingly, most tamoxifen-resistant tumors retain ER positivity [1].

Ligand-independent ER activation through growth factor signaling has been investigated as a mechanism of tamoxifen resistance. Phosphorylation of serine residue 118, located in the activation factor 1 (AF1) domain of ER, is known to result in the transcription of ER-dependent genes. This phosphorylation occurs not only because

Abbreviations: EGFR, epidermal growth factor receptor; ER, estrogen receptor alpha; HGFL, hepatocyte growth factor–like protein; MAPK, mitogen-activated protein kinase

Address all correspondence to: Susan E. Waltz, PhD, Department of Cancer and Cell Biology, Vontz Building for Molecular Studies, University of Cincinnati College of Medicine, 3125 Eden Ave, Cincinnati, OH 45267-0521. E-mail: susan.waltz@uc.edu

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[2]R.J.M. and A.M.M. contributed equally to this work and are primary coauthors.

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of estradiol binding to ER but also occurs by mitogen-activated protein kinase (MAPK) 1/2 activation, independent of estradiol [1]. When bound to ER, tamoxifen alters the binding and recruitment of coactivators to the AF2 domain of ER, but does not inhibit the activity of the AF1 domain, and upregulates transcriptional activity of ER through AF1 signaling in some breast cancer cell lines [7,8]. Activation of the receptor tyrosine kinases epidermal growth factor receptor (EGFR) and Her2 has been shown to result in the phosphorylation of serine 118 through MAPK1 signaling, leading to resistance to tamoxifen in human breast cancer cell lines [7,9]. Of seven genes identified that conferred tamoxifen resistance in a retroviral transfer of complementary DNA (cDNA) libraries into breast cancer cells, four were receptor tyrosine kinases, and the remaining three were ligands for these receptors, underscoring the potential importance of this mechanism of tamoxifen resistance in human disease [10].

Ron is a receptor tyrosine kinase related to the c-Met receptor that has been identified as an oncogene in the development and growth of human epithelial tumors [11]. In cell lines, wild-type Ron overexpression is associated with the induction of oncogenic properties, including malignant transformation, proliferation, and migration [12]. Overexpression of Ron in transgenic mouse models of both lung and breast cancers is associated with tumorigenesis in both organs [13,14]. In addition, Ron is known to be upregulated in a number of human epithelial cancers, including breast, lung, stomach, colon, pancreas, and prostate [15]. Whereas Ron is expressed at low levels in normal breast epithelium, it is highly expressed in approximately 50% of human breast cancers [16].

The expression of Ron in human breast tumors has been associated with poor prognosis in a number of analyses. In node-negative early stage breast cancers, Ron expression is associated with a two-fold increase in tumor recurrence compared with Ron-negative tumors [17]. Breast tumors expressing the genes for Ron, its ligand, hepatocyte growth factor–like protein (HGFL), and a membrane-bound serine protease that cleaves HGFL to its active form, are associated with worse disease-free and overall survival and increased metastases compared with tumors that do not express this three-gene combination [18]. Finally, in an examination of gene expression patterns from 189 breast tumors, Ron expression correlated with ER-positivity [19].

With this background, we hypothesized that Ron activation by HGFL may confer tamoxifen resistance in breast cancer cells. We present evidence that treatment of Ron-expressing human and murine breast cancer cell lines with HGFL is associated with rescue from tamoxifen-mediated growth inhibition. This effect is associated with MAPK-mediated phosphorylation of serine 118 of ER and the increased expression of genes associated with ER activation in the presence of tamoxifen. Our findings identify a novel link between the Ron receptor tyrosine kinase and an important mechanism of tamoxifen resistance in breast cancer.

Materials and Methods

Reagents and Plasmids

Antibody to Ron (C-20, rabbit polyclonal) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to ERα (6F11, mouse monoclonal) was purchased from Vector Laboratories (Burlingame, CA). Antibodies to total MAPK1/2 (06-182, rabbit polyclonal) were purchased from Millipore (Billerica, MA). Antibodies to phosphorylated p42/44 MAPK 1/2 (9106, mouse monoclonal) and ERα (S118) were purchased from Cell Signaling (Danvers, MA). Antibodies to pRon (Y1238/Y1239) were purchased from R&D Systems (Minneapolis MN). Plasmid containing the gene for the full-length wild-type human Ron was cloned into PCI-Neo vector, purchased from Promega (Madison, WI). 4-Hydroxy-tamoxifen was purchased from Sigma (St Louis, MO) and resuspended in 100% ethanol as per the manufacturer's instructions. Activated HGFL protein (recombinant human MSP, Cys 672 Ala) and mouse and human Ron-neutralizing antibodies were purchased from R&D Systems and were resuspended in phospho-buffered saline with 0.1% bovine serum albumin as per the manufacturer's instructions. PD98059 was purchased from Cell Signaling, and PP2 was from Calbiochem (San Diego, CA); both were resuspended in dimethyl sulfoxide as per the manufacturer's instructions. Cell culture reagents, including media, serum, and antibiotic additives, were purchased from Invitrogen (Carlsbad, CA).

Cell Culture and Transfection

MCF-7 and T47D cells were purchased from American Type Culture Collection (Manassas, VA). The R7 cell line is a murine mammary epithelial tumor cell line established from a mammary tumor from a transgenic MMTV-Ron mouse. This transgenic mouse model has been described previously [13]. Cell lines were maintained in RPMI, supplemented by 10% fetal bovine serum and 1% penicillin-streptomycin. Transfection of PCI-Neo Ron, PCI-Neo empty vector, ERE-Firefly luciferase, and CMV-Renilla luciferase was carried out using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions.

Crystal Violet Measurement of Cell Number

Cells were trypsinized, counted, and plated in equal numbers in 24-well tissue culture plates in maintenance media. After 24 hours, cells were treated in quadruplicate with specified reagents or vehicle controls in a maintenance medium. After 120 hours of treatment, the medium was removed, and cells were rinsed with sterile phospho-buffered saline, fixed in 4% formaldehyde, and stained with 0.1% crystal violet solution in 25% methanol. Extra crystal violet solution was then removed by rinsing repeated with water. After drying, 0.2% Triton X-100 detergent was added to all wells and gently rocked for 30 minutes. The resulting supernatant underwent photometric analysis on a monochromator (SynergyMx plate reader; BioTek Instruments, Winooski, VT) at an absorbance of 590 nm.

Luciferase Reporter Assay

Twenty-four hours before transfection, T47D cells were switched to phenol red–free Dulbecco modified Eagle medium (DMEM) containing 5% charcoal-stripped serum. Transfection of plasmids was carried out with Lipofectamine 2000 reagent as per the manufacturer's protocol. Cells were treated with HGFL, estradiol, or the Ron neutralizing antibody 24 hours after transfection, and cell lysates were collected and assayed 18 hours later. Luciferase assay was performed using the Dual-Luciferase Assay System (Promega) as per the manufacturer's instructions. Each sample was run in duplicate and averaged, and renilla-luciferase was used to control for transfection efficiency between samples.

Real-time Polymerase Chain Reaction Analysis

Cells were plated in maintenance a medium for at least 24 hours. The medium was then changed to phenol red–free DMEM containing 5% charcoal-stripped fetal bovine serum for 24 hours. Cells were

then treated with tamoxifen, HGFL, the Ron-neutralizing antibody, or PD98059, along with vehicle controls, for an additional 24 hours in phenol red–free DMEM with 5% charcoal-stripped fetal bovine serum. Cells were rinsed in phospho-buffered saline and lysed with TRIzol reagent (Invitrogen). RNA was isolated from TRIzol reagent solutions by chloroform-isopropanol preparation. DNA was prepared from isolated RNA using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. Experimental plates consisted of cDNA samples from three independently treated and independently isolated RNA samples for each treatment group, plated in duplicate. The sequence for the forward and reverse primers for cyclin D1 is 5′-GTT CGT GGC CTC TAA GAT-3′ and 5′-ACT CTG GAA AGA AAG TGC-3′, respectively. The expression of these genes was normalized to the expression of β-glucuronidase (GUS: forward, 5′-TTG AGA ACT GGT ATA AGA CGC ATC AG-3′ and reverse, 5′-TCT GGT ACT CCT CAC TGA ACA TGC-3′). Real-time polymerase chain reaction was performed using SYBR green in 96-well plates read with 7900HT Fast Real-time PCR/Sequence Detection Systems (Applied Biosystems). Graphic results represent the relative change in the messenger RNA (mRNA) expression compared with vehicle for each treatment group.

Immunoblot Analysis

Cells were plated and grown to near confluence in maintenance media. The media was then changed to phenol red–free DMEM containing 5% charcoal-stripped fetal bovine serum for 24 hours. Cells were then treated with tamoxifen, the Ron-neutralizing antibody or PD98059, along with vehicle controls, for 20 minutes, followed by treatment with HGFL and estradiol for an additional 20 minutes. Whole-cell lysates were prepared in radioimmunoprecipitation assay buffer (20 mM Tris, 150 mM sodium chloride, 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, and 10% glycerol), supplemented with complete protease inhibitor tablets (Roche, Palo Alto, CA) and HALT phosphatase inhibitors (Pierce, Rockford, IL). Protein concentrations were determined by Micro BCA Protein Assays (Pierce). Lysates were then boiled for 10 minutes in buffer containing β-mercaptoethanol and were separated by SDS-PAGE. Immunoblot analysis was carried out according to standard procedures on polyvinylidene fluoride membrane, with enhanced chemiluminescence detection (GE Healthcare, Piscataway, NJ). Densitometry was performed on NIH Image J analysis software.

Statistical Analysis

Data shown are indicative of at least three independent experiments. Results are expressed as mean ± SEM. For multiple comparisons, one-way analysis of variance (ANOVA) was performed, followed by Tukey posttest (for parametric data). For Ron knockdown study, a two-way ANOVA was performed. For all analyses, $P < .05$ was considered significant.

Results

Stimulation of Endogenous Ron in Breast Cancer Cell Lines Enhances Survival in the Presence of Tamoxifen

Correlative evidence has demonstrated that Ron and ER are coexpressed in a subset of tamoxifen-resistant breast cancers. Therefore, to test whether Ron signaling is a factor in regulating the resistance of breast cancer cells to tamoxifen, the coordinate expression of Ron and ER was first examined by Western analysis in two ER-positive human breast cancer cell lines (MCF-7 and T47D), an ER-negative cell line (MDA-MB-231), as well as in murine mammary tumor cells (R7 cells) derived from transgenic mice selectively overexpressing Ron in the breast epithelium [13,20]. The Ron receptor was highly expressed in T47D cells with limited expression in MDA-MB-231 and low to undetectable levels of Ron observed in MCF-7 cells (Figure 1*A*). Similarly, high Ron expression was observed in the R7 cells (Figure 1*B*). The antibody used to detect Ron in these studies was generated against peptide sequences corresponding to human Ron, but cross-reaction with the murine Ron homolog was observed, although the detection of Ron in mice is less efficient than that observed for human Ron. Moreover, ER expression was also observed in the R7 murine breast cancer cells (Figure 1*B*).

Next, to examine the tamoxifen sensitivity of ER-positive cell lines, T47D, MCF-7, and R7 cells were plated in the presence or absence of increasing concentrations of tamoxifen. Crystal violet assays were used to measure the cytotoxic effect of tamoxifen in all three breast cancer cell lines. The cytotoxic effect of tamoxifen treatment occurred in a dose-dependent fashion (Figure 2, *A*–*C*). The median lethal dose for R7 cells and T47D cells was approximately 3.5 to 4 μM, whereas MCF-7 cells had a median lethal dose of 2.5 μM, which is similar to concentrations reported previously for MCF-7 cells [5,20,21]. To examine the outcome of endogenous Ron stimulation during tamoxifen treatment, T47D and R7 cells, both of which express high levels of endogenous Ron, were treated with tamoxifen alone, tamoxifen and Ron ligand (HGFL), or vehicle alone. HGFL treatment partially rescued cells from the cytotoxic effects of tamoxifen (Figure 2, *D* and *E*). Specifically, treatment of R7 cells with tamoxifen and a physiologic dose of HGFL (100 ng/ml) [22,23] was associated with a 400% increase in the number of R7 cells compared with tamoxifen treatment alone. Similarly, in T47D cells, a dose of 50 ng/ml of HGFL was associated with a 12.5% increase in the number of T47D cells; increases of up to 30% were observed with a dose of 100 ng/ml (data not shown). For both R7 and T47D cells, treatment with all doses of HGFL was associated with a significant increase in the number of cells compared with tamoxifen treatment alone. Conversely, HGFL treatment of MCF-7 cells, which demonstrate low levels of Ron expression, had no significant effect on tamoxifen-treated cells (Figure 2*F*). For all three cell lines, the total

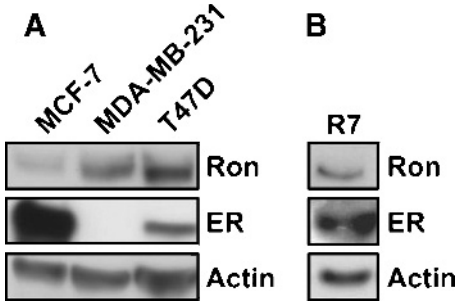

Figure 1. Ron and ER are expressed in human and murine breast cancer cell lines. Expression of ER and Ron was examined by Western analysis of cell lysates generated from the human breast cancer cell lines MCF-7, MDA-MB-231, and T47D (A) and the murine epithelial breast cancer line R7 (B). T47D cells express high levels of human Ron compared with MDA-MB-231 cells and MCF-7 cells. R7 cells express high levels of murine Ron.

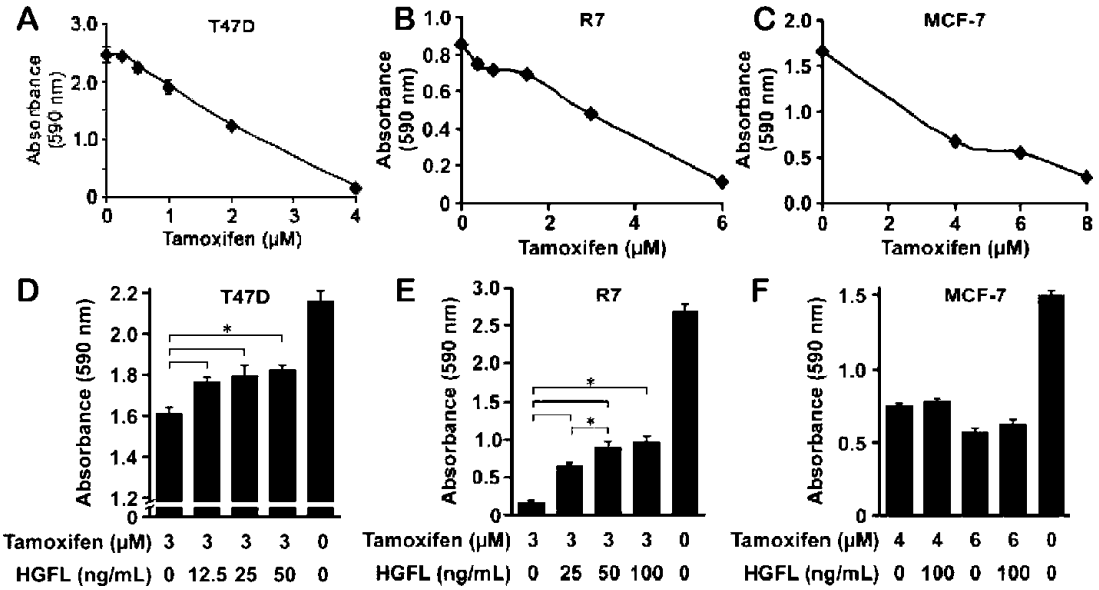

Figure 2. Stimulation of endogenous Ron in breast cancer cell lines enhances survival in the presence of tamoxifen. Establishment of the endogenous sensitivity of T47D (A), the murine breast cancer cell line R7 (B), and MCF-7 (C) cells to tamoxifen. Tamoxifen-induced growth inhibition is reversed by HGFL in T47D cells (D) and R7 cells (E); HGFL does not affect tamoxifen-induced growth arrest in MCF-7 cells (F). Columns represent mean values ± SEM with $*P < .05$. Each assay was performed in quadruplicate, and the graphs are representative of several independent experiments.

cell number was significantly decreased with tamoxifen and tamoxifen plus HGFL treatment, compared with vehicle treatment. Importantly, treatment with HGFL alone, at any dose, did not independently increase the number of T47D, MCF-7, or R7 cells during this experiment (data not shown).

Exogenous Ron Expression Confers Tamoxifen Resistance

To confirm the specific requirement for the Ron receptor to rescue cells from tamoxifen-induced cytotoxicity, we transiently transfected MCF-7 cells, which express low endogenous levels of this receptor, with a Ron expression plasmid or control vector. Our transient transfections resulted in approximately 40% of cells being transduced with exogenous expression of Ron that was maintained for at least 120 hours during these experiments (data not shown). Ron expression was confirmed by Western analyses. Mock-transfected (MT) MCF-7 cells and cells transfected with empty vector (PCI-Neo EV) served as controls. Robust Ron expression was observed after transfection with a Ron-expressing plasmid (PCI-Neo Ron) compared with controls (Figure 3*A*). Consistently, treatment of the MT MCF-7, PCI-Neo EV MCF-7, and PCI-Neo Ron MCF-7 cells with tamoxifen was associated with dose-dependent cytotoxicity similar to that observed in wild-type MCF-7 cells (Figure 3, *B–D*). Graphs represent relative absorbance values normalized to the vehicle-treated cells for each transfection group. Importantly, treatment with HGFL was associated with a 50% increase in the number of PCI-Neo Ron MCF-7 cells at both 3 and 4 μM concentrations of tamoxifen (Figure 3*D*). This effect was not observed in MT MCF-7 cells (Figure 3*C*) or in PCI-Neo EV MCF-7 cells (Figure 3*B*).

Knockdown of Ron in a Breast Cancer Cell Line Enhances Tamoxifen Sensitivity

Next, to determine whether knockdown of Ron could sensitize breast cancer cells to tamoxifen, we transduced T47D cells with short hairpin RNA constructs targeting Ron mRNA (T47D RonKD). The efficacy of the Ron knockdown on protein levels was approximately 50% to 70% (Figure 4*A*). These cells were then treated with a concentration range of tamoxifen in complete medium containing HGFL, and its effects on the cell number were assessed by crystal violet staining. T47D RonKD cells were more sensitive to tamoxifen over the entire concentration range given (0.5-8 μM; Figure 4*B*). The concentrations of tamoxifen metabolites detected in breast tissue from tamoxifen-treated patients are reported to fall between 0.67 and 14 μM, suggesting that our treatment doses fall within the physiologic range [1].

HGFL-Mediated Rescue of Ron-Expressing Breast Cancer Cells from Tamoxifen Treatment Is Attenuated by Ron or MAPK Inhibition

Having demonstrated the ability of HGFL treatment to partially reverse tamoxifen-induced cytotoxicity in T47D and R7 cells, we next aimed to determine the importance of Ron and Ron-dependent downstream signaling mechanisms for this protective effect. T47D and R7 cells were treated with tamoxifen and HGFL, as previously mentioned, and with mouse or human Ron-neutralizing antibodies or the MEK1 inhibitor PD98059, at doses that were not independently associated with altered cell growth (data not shown). Treatment of T47D cells with 4 μM tamoxifen alone was associated with approximately 50% reduction in cell number. Concurrent treatment with HGFL leads to an approximately 30% rescue in cell number, similar to the effect described previously in Figure 2. Addition of a Ron-neutralizing antibody to HGFL-treated cells reversed the rescue effect seen with HGFL alone, resulting in a cell number equivalent to that seen with tamoxifen treatment alone. Similarly, addition of PD98059 negated the HGFL-mediated rescue to that seen with tamoxifen treatment alone (Figure 5*A*). In R7 cells, treatment with 3 μM tamoxifen resulted in a 67% reduction in cell number, and

HGFL treatment was associated with a partial reversal of this effect, as described previously in Figure 2. Treatment with a mouse Ron-neutralizing antibody essentially reversed the effect of HGFL treatment. Similar results were observed with treatment with the MEK1 inhibitor PD98059 (Figure 5*B*). Treatment with the MEK1 inhibitor alone at the doses used in this experiment had no effect on cell number (data not shown). To determine the possible importance of the non–receptor tyrosine kinase c-Src as a potential intermediary between Ron and MAPK activation, we repeated the experiments in the presence of PP2, a known c-Src inhibitor, at concentrations ranging from 0.1 to 10 μM. This inhibitor has been shown to decrease c-Src activation *in vitro* at 10 μM concentration [24]. Treatment of both cell lines with all concentrations of PP2 was associated with a decrease in overall cell growth in the presence of tamoxifen and HGFL, similar to the effects seen with a Ron-neutralizing antibody and MEK1 inhibitor PD98059 (data not shown). However, treatment of R7 and T47D cells with all concentrations of PP2 was independently associated with a decrease in cell number during the experiment, suggesting that, although c-Src is important for the growth of these cell lines, it may not play a role in the HGFL-mediated rescue from tamoxifen.

Treatment of Ron-Expressing Breast Cancer Cell Lines with Tamoxifen and HGFL Is Associated with Increased Phosphorylation of the Serine 118 Residue of ER and Activation of MAPK

To further evaluate the mechanism by which HGFL may be regulating resistance to tamoxifen, we performed Western analyses of T47D cells treated with HGFL, a Ron-neutralizing antibody, and/or PD98059. Cells were pretreated for 20 minutes with PD98059 (MEK inhibitor) or a Ron-neutralizing antibody, then treated for an additional 30 minutes with HGFL, and total cell lysates were generated. This time course is consistent with the previously published literature on the phosphorylation of ER and MAPK [5]. Figure 6 depicts a representative Western of whole-cell lysates after each treatment, which is representative of three independent experiments. HGFL stimulation induced Ron phosphorylation at tyrosine residues 1238/1239, which are responsible for its kinase activity. We also observed the activation (i.e., phosphorylation) of pMAPK (Figure 6) after HGFL. Ron activation was associated with an increase in the amount of phosphorylated ER (pER), which was specifically phosphorylated at serine 118, per total amount of ER (ER), compared with vehicle. Ron neutralization reduced the levels of tyrosine-phosphorylated Ron (pRon) and pMAPK, whereas PD98059 only reduced levels of pMAPK, suggesting that the actions of HGFL are Ron-specific. However, both the Ron-neutralizing antibody and PD98059 blocked the phosphorylation of ER at serine 118, suggesting that Ron activation and MAPK activity are required for this effect (Figure 6).

Treatment of Ron-Expressing Breast Cancer Cells with Tamoxifen and HGFL Is Associated with Increased Expression of ER-Dependent Genes

To further explore the mechanisms by which HGFL may be inducing resistance to tamoxifen, we evaluated the expression of ER-dependent target genes. To accomplish this, R7 cells were treated with tamoxifen

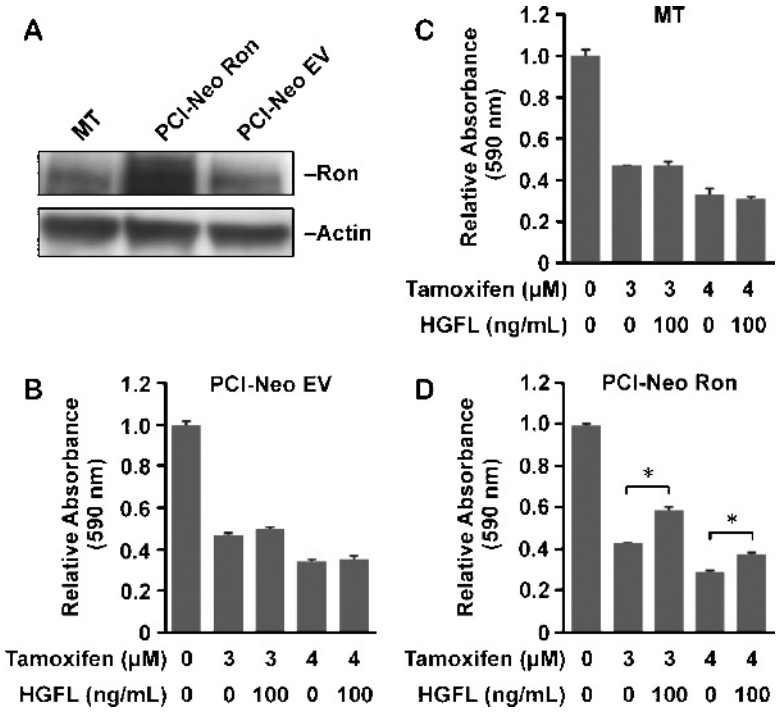

Figure 3. (A) Ron overexpression in MCF-7 cells leads to HGFL-induced tamoxifen resistance. MCF-7 cells were transfected with PCI-Neo Ron or with PCI-Neo EV. Increased levels of Ron expression in PCI-Neo Ron cells was confirmed by Western analysis compared with MT MCF-7 cells and PCI-Neo EV cells. Crystal violet cell growth assays were performed on all three cell lines, with cell growth normalized to vehicle-treated cells for graphic representation. The tamoxifen-induced growth inhibition of PCI-Neo Ron MCF-7 cells was reversed by treatment with HGFL (D); this effect was not seen in PCI-Neo EV cells (B) or in MT MCF-7 cells (C). Columns represent mean values ± SEM with $*P < .05$. Each assay was performed in quadruplicate, and the graphs are representative of several independent experiments, with mean values compared with vehicle-treated cells.

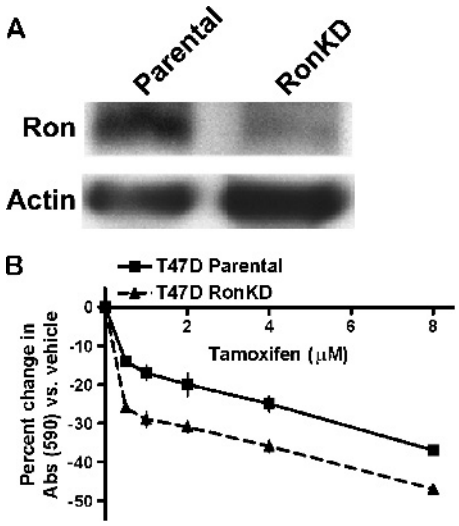

Figure 4. (A) Knockdown of Ron in breast cancer cell lines enhances tamoxifen sensitivity. Lentivirus-delivered short hairpin RNA to Ron was transduced into T47D cells. The efficacy of the knockdown on Ron protein in a polyclonal population of T47D cells is shown. Actin serves as a loading control. Both the parental and RonKD cells were treated with increasing concentrations of tamoxifen. (B) After 96 hours of treatment, the cells were fixed and stained with crystal violet, and cell number was assessed by photometric analysis at 590 nm of the supernatant after dye extraction from stained cells. Tamoxifen treatment results in a dose-dependent growth inhibition of parental and RonKD T47D cells. Note, however, that the RonKD cells were more sensitive to tamoxifen at every dose assessed ($n = 4$, $P < .001$, two-way ANOVA).

and HGFL, in combination with a mouse Ron-neutralizing antibody or the MEK1 inhibitor PD98059, in doses that alone did not alter cell number. HGFL and estradiol alone stimulated cyclin D1 expression over vehicle treatment, whereas tamoxifen treatment alone did not (Figure 7*A*). Cotreatment of HGFL and estradiol did not enhance cyclin D1 expression over estradiol alone. Treatment of cells with PD98059 or the Ron-neutralizing antibody alone had no effect on cyclin D1 expression (data not shown). Interestingly, combined treatment with tamoxifen and HGFL was associated with an expression of cyclin D1 mRNA similar to that induced by estradiol treatment (Figure 7*A*). This induction was approximately 3.5-fold higher than that observed with tamoxifen treatment alone and was inhibited by treatment with a Ron-neutralizing antibody or MEK1 inhibition (Figure 7*A*).

To better understand whether Ron activation can affect ER responsiveness and, therefore, partially explain changes in ER target gene expression, we used an estrogen response element–luciferase plasmid (ERE-luc). HGFL-stimulated luciferase expression in ERE-luc–transfected T47D cells was approximately 1.8-fold higher than vehicle-treated cells. Furthermore, this stimulation was inhibited by the Ron-neutralizing antibody, highlighting the specificity of this effect on Ron (Figure 7*B*).

Discussion

The selective estrogen receptor modulator tamoxifen has been used successfully to prevent recurrence of ER-positive breast cancer, significantly improving disease-free and overall survival. Despite this success, a subset of breast cancer patients will recur despite tamoxifen or other antiestrogen therapy, highlighting the clinical importance of elucidating mechanisms by which breast cancer cells become resistant to these therapies and devising strategies to overcome this resistance to further improve outcomes. The activation of ER through growth factor signaling, independent of estradiol, has been demonstrated to be an important mechanism of resistance. The receptor tyrosine kinase Ron is known to be associated with poor prognosis in breast cancer [17,18]. This association led us to hypothesize that activation of Ron in breast cancer cells may confer resistance to tamoxifen, leading to poorer clinical outcomes. Here, we demonstrate that 1) activation of Ron by its ligand, HGFL, partially reverses tamoxifen-induced cytotoxicity in human and mouse breast cancer cell lines, and that this effect is attenuated with Ron inhibition; 2) activation of Ron is associated with phosphorylation of the serine 118 residue of ER through

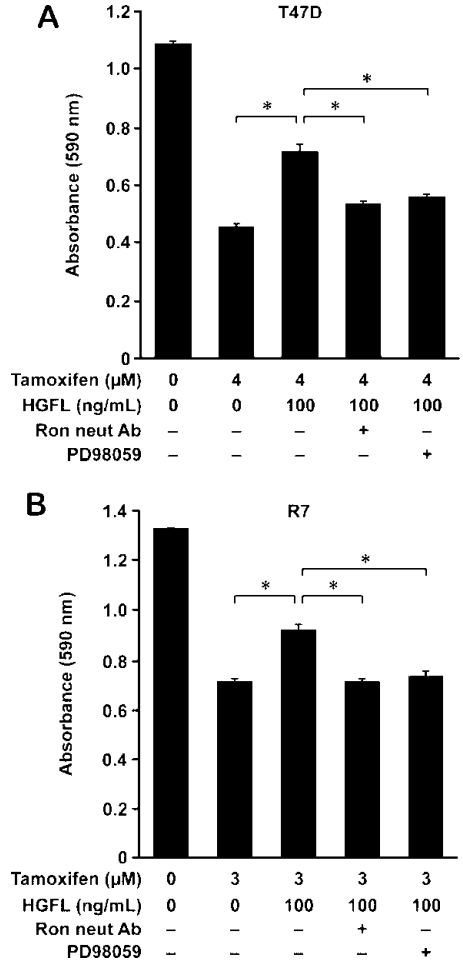

Figure 5. Ron receptor neutralization or the use of a MEK1 inhibitor restores tamoxifen sensitivity in breast cancer cell lines treated with HGFL. The human breast cancer cell line T47D and the murine epithelial breast cancer cell line R7 were treated with tamoxifen, HGFL, Ron-neutralizing antibody, and/or a MEK1 inhibitor (PD98059) for 120 hours. Cells were then fixed, and cell number was analyzed by crystal violet assays. HGFL-mediated resistance of T47D (A) and R7 (B) cells to tamoxifen exposure is inhibited with the inclusion of both a Ron-neutralizing antibody and a MEK1 inhibitor. Columns represent mean values ± SEM with $*P < .05$. Each assay was performed in quadruplicate, and the graphs are representative of several independent experiments.

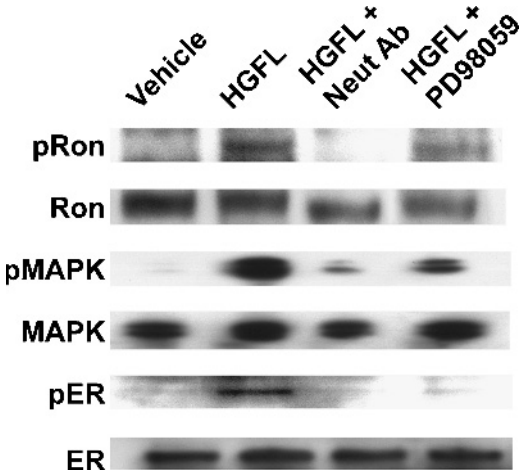

Figure 6. HGFL stimulation of breast cancer cells leads to MAPK and ER phosphorylation. T47D cells were treated with a Ron-neutralizing antibody or MEK1 inhibitor PD98059 for 20 minutes and subsequently treated with HGFL for 30 minutes. Cells were then lysed, and Western analysis was performed. Treatment with HGFL is associated with increased phosphorylation Ron and of serine 118 of ER and MAPK phosphorylation; both the MAPK and ER phosphorylation events are inhibited by treatment with a Ron-neutralizing antibody or with a MEK1 inhibitor. However, phosphorylation of Ron is only inhibited by the Ron-neutralizing antibody. Pictures depicted are representative of three independent experiments.

activation of MAPK, a pathway that has been implicated in tamoxifen resistance in other *in vitro* and *in vivo* studies; and 3) Ron activation by HGFL alters the expression of ER-dependent genes in the presence of tamoxifen.

The validation of Ron as an oncogene in human cancer has been the focus of many recent investigations [12–15,25]. *In vitro*, Ron has been shown to induce malignant transformation, proliferation, and migration [12]. *In vivo*, treatment with a specific Ron inhibitor decreases the growth rate of human tumor cell xenografts in nude mice [15]. Importantly, a mammary-directed expression of Ron causes breast tumor formation in 100% of mice [13]. Ron mediates its effects through a number of intracellular second messengers and signaling pathways, including Ras-RAF-MAPK 1/2, phosphoinositide-3 kinase–Akt, β-catenin, and nuclear factor-κB [26]. Cross talk between Ron and other tyrosine kinase receptors such as EGFR has also been described; this cross talk is associated with increased motility and malignant transformation in cell lines expressing more than one receptor [25]. Further work has demonstrated differences in downstream effects associated with ligand-dependent *versus* ligand-independent Ron activation [27]. Specifically, MCF-10A cells transfected with human Ron required HGFL only for proliferation; cells were able to migrate, spread, and survive in the absence of HGFL, presumably because of other growth factor signaling or heterodimerization with other receptors [27]. In our model, HGFL did not induce additional growth in T47D cells and in the murine breast cancer cell line, R7 cells, in the presence of serum; however, less cytotoxicity was seen with HGFL treatment in the presence of tamoxifen, suggesting that ligand-dependent Ron activation is necessary for the tamoxifen resistance that we observed. Furthermore, this effect was largely inhibited by treatment with a Ron-neutralizing antibody, suggesting that the actions of HGFL are limited to a specific interaction with Ron. Feres et al. [27] also described the importance of the non–receptor tyrosine kinase c-Src in downstream Ron signaling, for both ligand-dependent and ligand-independent effects. Treatment of R7 and T47D cells in our experiments with the c-Src inhibitor PP2 was associated with growth inhibition, even at very low doses (0.1 μM), which was similar to previously published results, and underscored the importance of c-Src signaling to cell proliferation in Ron-expressing cell lines [27]. However, by Western analyses, changes in phosphorylated c-Src were

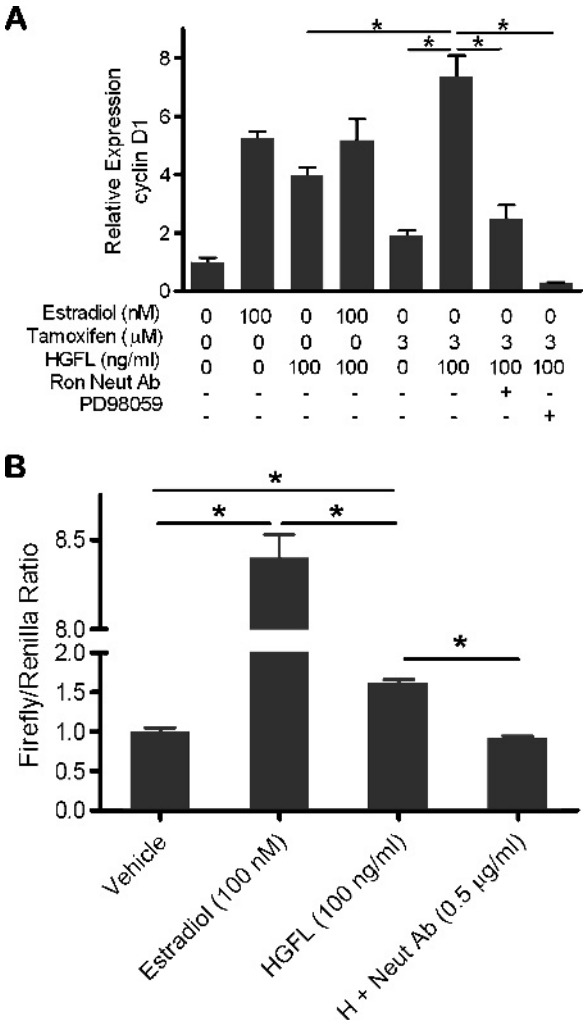

Figure 7. Transcriptional changes in ER and ER-dependent genes on HGFL treatment. Real-time reverse transcription–polymerase chain reaction analysis of ER-dependent mRNA expression was performed on RNA isolated from murine breast cancer cell lines after treatment with the indicated agent for 24 hours. (A) Expression values were normalized to GUS as an internal control, and the relative expression level of cyclin D1 is illustrated. Treatment with HGFL (H) in combination with tamoxifen (T) is associated with changes in expression of cyclin D1. Experiments were performed in duplicate on three independently treated samples, and relative expression levels were compared with those of vehicle-treated cells. All treatments achieve statistical significance ($P < .05$) compared with vehicle except for tamoxifen alone, H + T + PD98059, and H + T + neutralizing antibody (Neut Ab). ERE-luciferase plasmid was transiently transfected into T47D cells (phenol red–free medium) and treated with the listed compounds for 16 to 18 hours. (B) Luciferase expression from four independent replicates was averaged to create the graph. Columns represent mean values ± SEM with $*P < .05$.

not observed with HGFL or tamoxifen treatment, suggesting that Ron's ability to confer tamoxifen resistance is not dependent on c-Src activation.

The ability of activated receptor tyrosine kinases, other than Ron, to overcome tamoxifen-induced cytotoxicity in breast cancer cells has been previously described [1,5,7,11,28]. Serine residue 118, located in AF1 of ER, is known to be a key target of phosphorylation, in carrying out the activities of ligand-independent and tamoxifen-dependent actions of ER [29,30]. Mutation of this residue to alanine, rendering it unable to be phosphorylated, is associated with altered gene expression patterns in response to both tamoxifen treatment and activation of K-*ras*, a downstream regulator of receptor tyrosine kinase activity. However, transcription patterns associated with estradiol treatment are largely unaffected by this mutation [7,30]. Treatment with tamoxifen alone is associated with phosphorylation of serine 118 on ER in breast cancer cell lines that are both sensitive and resistant to tamoxifen [5,29,31]. Multiple studies have confirmed the ability of EGFR and Her2, when activated by ligand, to phosphorylate MAPK and serine 118 of ER. This pathway has been shown to be the mechanism of resistance in tamoxifen-resistant cell lines expressing Her2 [5], and this resistance can be partially overcome by treatment with inhibitors of activation, or antibodies to, Her2 or EGFR [5,9]. Ron, like many other receptor tyrosine kinases, is known to signal through the MAPK pathway when activated. Our study also reveals the association between activated Ron and phosphorylated MAPK and ER in breast cancer cells. The rescue effect observed with HGFL in the presence of tamoxifen is attenuated with inhibition of the MAPK pathway, reinforcing the importance of this mechanism in our experimental system.

The activation of transcription by ER in the nucleus occurs through multiple mechanisms, including direct binding of the estradiol-ER complex to EREs in DNA and tethering of the estradiol-ER complex to DNA by other proteins associated with transcription factor binding sites such as those of specificity protein 1 or activator protein 1 [30]. The diverse effects of ER activation in various tissues are due to the complexity of its interactions with DNA through these sites and the presence or absence of various corepressors or coactivators [1]. To evaluate the activation of ER-mediated transcription in our model, we evaluated the mRNA expression of *cyclin D1*. The expression of *cyclin D1*, a coactivator in the ER signaling complex, in breast tumors is associated with poor prognosis in women receiving tamoxifen as adjuvant therapy [31,32]. Transcription of the *cyclin D1* gene is known to be activated by ER through tethering to an activator protein 1 site. Tamoxifen can induce transcription of *cyclin D1*, although at lower levels than those achieved with estradiol stimulation [30,33], which is similar to the effect we observed in the R7 murine breast cancer cell line. Treatment with Her2 agonist alone induces *cyclin D1* expression in MCF-7 cells, again similar to the effect we observed with HGFL treatment [5]. Cheng et al. [30] also reported that treatment with a MAPK pathway inhibitor in the presence of tamoxifen decreased transcription of *cyclin D1*, again similar to our observations. We further observed that treatment with both tamoxifen and HGFL had an additive effect on *cyclin D1* gene expression. Evaluation of another gene, *Wnt1-induced signaling protein-2* (*WISP-2*), also demonstrated differential regulation in response to HGFL and tamoxifen [34] (data not shown).

To examine the activation of ER-mediated transcription in our model, we evaluated ERE-driven luciferase expression after Ron activation. Although HGFL increased the accumulation of the luciferase protein during the experiment, this result, in itself, does not provide evidence of a direct Ron-ER connection. However, these data, in combination with the MAPK-dependent phosphorylation of ER, strengthen the hypothesis that Ron may be directly affecting ER and, by association, tamoxifen resistance. Whereas the mechanism of tamoxifen-, HGFL-, or other growth factor–induced *cyclin D1* expression has not been completely elucidated [30], our results provide further evidence that this gene is upregulated in *in vitro* models of tamoxifen resistance in breast cancer cells.

In addition to growth factor signaling through MAPK, several other pathways have been implicated in tamoxifen resistance. Inhibition of the phosphoinositide-3 kinase–Akt-mTOR signaling pathway, downstream of growth factor activation, has been associated with restoration of sensitivity in tamoxifen-resistant cells [35,36]. Inhibition of c-Src has been associated with restoration of tamoxifen sensitivity, potentially through facilitating cross talk between receptor tyrosine kinases and ER in breast cancer cells [37]. Finally, our study did not evaluate the role of Ron- or MAPK-mediated phosphorylation of AIB1 or other coactivators of ER signaling, which have also been identified as important mechanisms of tamoxifen resistance [38]. The involvement of these pathways in the Ron-mediated tamoxifen resistance that we observed is beyond the scope of our current study but merits future investigation.

In summary, we have described for the first time the ability of the activated Ron receptor tyrosine kinase to confer resistance to tamoxifen in breast cancer cells through a well-characterized pathway. Our findings illustrate a novel role for Ron in cancer pathology and may partially explain the poor prognosis associated with Ron expression in human breast cancer.

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