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Estrogen and Resveratrol Regulate Rac and Cdc42 Signaling to the Actin Cytoskeleton of Metastatic Breast Cancer Cells[1]

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Abstract

Estrogen and structurally related molecules play critical roles in breast cancer. We reported that resveratrol (50 μM), an estrogen-like phytosterol from grapes, acts in an antiestrogenic manner in breast cancer cells to reduce cell migration and to induce a global and sustained extension of actin structures called filopodia. Herein, we report that resveratrol-induced filopodia formation is time-dependent and concentration-dependent. In contrast to resveratrol at 50 μM, resveratrol at 5 μM acts in a manner similar to estrogen by increasing lamellipodia, as well as cell migration and invasion. Because Rho GTPases regulate the extension of actin structures, we investigated a role for Rac and Cdc42 in estrogen and resveratrol signaling. Our results demonstrate that 50 μM resveratrol decreases Rac and Cdc42 activity, whereas estrogen and 5 μM resveratrol increase Rac activity in breast cancer cells. MDA-MB-231 cells expressing dominant-negative Cdc42 or dominant-negative Rac retain filopodia response to 50 μM resveratrol. Lamellipodia response to 5 μM resveratrol, estrogen, or epidermal growth factor is inhibited in cells expressing dominant-negative Rac, indicating that Rac regulates estrogen and resveratrol (5 μM) signaling to the actin cytoskeleton. These results indicate that signaling to the actin cytoskeleton by low and high concentrations of resveratrol may be differentially regulated by Rac and Cdc42.

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Keywords: Estrogen, resveratrol, breast cancer invasion, Rac, Cdc42.

Introduction

Estrogen and structurally related selective estrogen receptor modulators (SERMs) such as phytoestrogens are highly relevant to breast cancer promotion, prevention, and therapy. In addition to signaling by nuclear estrogen receptors (ERs) to facilitate transcriptional regulation, estrogen also induces rapid signaling through cell surface receptor networks. Numerous studies have demonstrated that plasma-membrane ERs are activated by estrogen to crossactivate tyrosine kinase–type cell surface receptors such as epidermal growth factor receptor (EGFR), as well as G proteins and G protein–coupled receptors [1]. Such rapid estrogen signaling has recently been implicated in the regulation of cytoskeletal dynamics and in the modulation of cell migration and invasion in endothelial, breast cancer, and endometrial cancer cells [2–5].

Resveratrol (*trans*-3,4′,5-trihydroxystilbene), a phytoestrogen present in grape skin and red wine, can bind to and activate ERα and ERβ and has mixed agonist/antagonist effects [6,7]. Resveratrol can exert biphasic effects where low concentrations are estrogenic and high concentrations are antiestrogenic [8–10]. High concentrations of resveratrol have antioxidant, proapoptotic, antigrowth, anti-inflammatory, antiangiogenic, and anti-invasive effects. Therefore, resveratrol is considered to be a cancer-preventive agent [11–13]. In addition to a preventive role in the initiation and promotion of cancers, resveratrol may have potential as a metastasis-preventive agent. Resveratrol has been demonstrated to reduce hepatoma cell invasion *in vitro* through hepatocyte growth factor signaling and to reduce *in vivo* hepatoma and Lewis lung carcinoma invasion in mice [14,15]. Moreover, oral administration of resveratrol decreased metastatic invasion in a mouse model of melanoma [16]. Another recent study indicated that resveratrol may act as a chemopreventive agent of spontaneous mammary tumor formation and may reduce metastasis in a HER2 transgenic mouse model [17].

Abbreviations: ECM, extracellular matrix; EGF, epidermal growth factor; ER, estrogen receptor

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In vitro studies, including our work, have shown that resveratrol at 50 μM significantly inhibits the migration and invasion of endometrial and breast cancer cells [2,18–21]. We reported that high concentrations of resveratrol can act in an antiestrogenic manner to inhibit cell migration and the formation of lamellipodia in the presence of estrogen [2]. These cellular effects of resveratrol may be exerted through modulation of ER-responsive gene transcription and rapid signaling by cell surface receptor crosstalk. Resveratrol has been shown to rapidly modulate mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK), and phosphoinositide 3-kinase (PI3-K)/Akt activity through ER-dependent and ER-independent pathways [2,9,10,22,23]. These signaling cascades are all relevant to the proliferation, motility, and survival processes that determine metastatic efficiency. The reported inhibition of matrix metalloproteinase (MMP) expression by resveratrol may also be a potential mode for resveratrol to regulate cancer cell invasion [20,24].

During metastasis, the actin cytoskeleton of invading cells is remodeled by molecular mechanisms common to all migrating cells, which involve the protrusion of cell surface actin structures such as filopodia and lamellipodia and the assembly of dynamic focal adhesions with the extracellular matrix (ECM) [25]. Filopodia are not essential for cell migration and are considered to function as environmental sensors that can contribute to cell migration by being converted to lamellipodia (actin structures central to forward migration) during growth factor receptor signaling [26,27]. We and others have shown that estrogen acts in a manner similar to EGF (a promoter of cell migration and invasion) in cancer cells to extend leading-edge lamellipodia with associated focal adhesions and to increase cell migration [2,3,28]. We also reported that resveratrol (50 μM) acts in a manner opposite to estrogen by the rapid modulation of the actin cytoskeleton to induce a global and sustained array of filopodia, decrease in focal adhesions, and inhibition of FAK activity resulting in reduced cell migration [2]. We have extended these results in the present study to analyze the role of key signaling intermediates of the Rho family of GTPases (Rac and Cdc42) in estrogen and resveratrol signaling to the actin cytoskeleton. Rho, Rac, and Cdc42 coordinately regulate the actin cytoskeleton and focal adhesion turnover during cell migration and may thus impact breast cancer metastasis [26,29]. Among Rho GTPases, Cdc42 has been specifically implicated in filopodia formation, whereas Rac regulates lamellipodia [26].

As a first step toward elucidating the molecular mechanisms of estrogen and resveratrol on the actin cytoskeleton, we investigated the role of Rac and Cdc42 in estrogen and resveratrol signaling. We report that resveratrol exerts a concentration-dependent effect on the actin cytoskeleton. Resveratrol at 5 μM acts in a manner similar to estrogen to promote Rac activity and lamellipodia formation, whereas resveratrol at 50 μM inhibits Rac and Cdc42 activity and lamellipodia formation. These data indicate that estrogen and low concentrations of resveratrol may promote breast cancer metastasis, whereas high concentrations of resveratrol may prevent breast cancer metastasis.

Materials and Methods

Cell Culture

MDA-MB-231 human breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Cells were made quiescent by starvation in serum-free phenol red–free DMEM for 24 or 48 hours.

Migration Assay

Migration assays were conducted as described in Baugher et al. [30]. Cells were serum-starved in phenol red–free DMEM for 24 hours and seeded at 1×10^5 cells/chamber in the upper well of Costar wells (BD Biosciences, Bedford, MA) containing membranes with 8-μm-diameter pores. Dimethyl sulfoxide (DMSO; 0.5%) in DMEM (control vehicle), 17β-estradiol (Sigma, St. Louis, MO), EGF (Upstate Biotechnology, Lake Placid, NY), or resveratrol (LKT Laboratories, St. Paul, MN) was added as a chemoattractant to the bottom well for 8 hours. Cells on the upper surface of the membrane were removed, and cells that had migrated to the underside of the membrane were fixed in cold methanol, stained with propidium iodide, and quantified.

Invasion Assay

Cells were handled in the same manner as a migration assay, except that quiescent cells were placed in Matrigel-coated invasion chambers (BD Biosciences). The bottom well contained DMSO (vehicle), estrogen, or resveratrol. The number of cells that migrated through the Matrigel matrix after 24 hours of incubation were fixed, stained with propidium iodide, and quantified relative to control.

Fluorescence Microscopy

Fluorescence microscopy was conducted as described by Azios and Dharmawardhane [2]. Quiescent cells were treated with vehicle (DMSO), estrogen, EGF, or resveratrol. Cells were immediately fixed (3.7% formaldehyde), permeabilized (0.2% Triton X), and stained with rhodamine phalloidin (Molecular Probes, Carlsbad, CA) to visualize F-actin. The actin structures of cells in 20 microscopic fields per treatment were counted for at least three separate experiments. Digital images of microscopic fields were enlarged with Adobe Photoshop (Adobe Systems, Inc., San Jose, CA) and counted by two independent investigators, where one investigator was uninformed of the treatments. Data are expressed as mean ± SEM. For images of cells invading Matrigel-coated Transwell membranes, Z-series of Tracker Green (Molecular Probes)–loaded cells were collected with a Leica TCS SP2 AOBS confocal microscope (Leica, Wetzlar, Germany) with an Ar laser at 488 nm. *XZ* sections were reconstructed using ImageJ (W. S. Rasband; US National Institutes of Health, Bethesda, MD).

Rac and Cdc42 Activity Assay

Rac and Cdc42 activities were determined as described by Baugher et al. [30]. Quiescent cells were lysed following vehicle (DMSO), EGF, estrogen, or resveratrol treatment,

and active Rac or active Cdc42 was pulled down using GST–p21-activated kinase (PAK)–Cdc42/Rac interactive binding (CRIB) or GST–Wiscott Aldrich Syndrome Protein (WASP)–CRIB fusion proteins (Cytoskeleton, Denver, CO), respectively. Proteins were subjected to Western blot analysis for Rac or Cdc42 with specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. Positive bands were imaged and quantified using VersaDoc system and Quantity One software (Bio-Rad, Hercules, CA). Rac or Cdc42 activity was determined as the amount bound to the PAK–CRIB domain or the WASP–CRIB domain as a function of total Rac or Cdc42 in a cell lysate.

Creation of Stable Transfectants

The construction of MDA-MB-231 cells stably expressing dominant-negative Rac or Cdc42 mutants was conducted as described by Baugher et al. [30]. Rac1(T17N) or Cdc42(T17N) myc-tagged constructs in pIRES.neo vector (Clontech, Palo Alto, CA) or control (vector alone) were transfected into MDA-MB-231 cells by standard methods. Stable transfectants were isolated by selection in 1 mg/ml G418 (Cellgro, Hendon, VA) and maintained in 0.1 to 0.5 mg/ml G418. Positive clones were determined according to myc expression by Western blot analysis using anti-myc antibody (Upstate Biotechnology) and were verified by anti-myc immunofluorescence microscopy.

Statistical Analysis

Data are expressed as mean ± SEM. *P* values were calculated with Student's unpaired *t* tests (Welch-corrected, when necessary) using Microsoft Excel (Microsoft, Redmond, WA) or with one-way analysis of variance using Tukey-Kramer multiple comparisons test and InStat3. *P* values were considered significant at $\leq .05$.

Results

Estrogen and Resveratrol Modulate Breast Cancer Cell Migration and Invasion

Because directed cell migration is a prerequisite for metastasis, we assessed the effect of estrogen or resveratrol on cell migration using Transwell migration assay. Previously, we have reported that, compared to control, estrogen and EGF treatments both significantly increased cell migration, whereas resveratrol (50 μM) significantly decreased cell migration [2]. Herein, we have extended this study to demonstrate a concentration-dependent effect of resveratrol on directed cell migration. As in our earlier report [2], 50 μM resveratrol inhibited cell migration by ~ 30% compared to control. Estrogen exerted an effect opposite to that of 50 μM resveratrol by increasing cell migration by two-fold compared to control. Interestingly, 5 μM resveratrol acted in a manner similar to estrogen by significantly increasing cell migration (Figure 1*A*).

Recent reports have demonstrated a 40% to 60% 50-μM resveratrol–induced inhibition of cervical cancer cell invasion through Matrigel and of MCF-7 breast cancer cells through fibronectin or collagen matrices [18,20,21]. We have confirmed these results through observations of a ~ 40% decrease in MDA-MB-231 cell invasion across a Matrigel matrix in response to 50 μM resveratrol and a ~ 1.6-fold increase in invasion in response to estrogen or 5 μM resveratrol. The response of invading cells to estrogen or resveratrol at 5 μM was significantly different from the response to resveratrol at 50 μM ($P < .01$), but not from each other (Figure 1*B*). Figure 1*C* is a representative confocal micrograph of interfaces between the Matrigel matrix and the

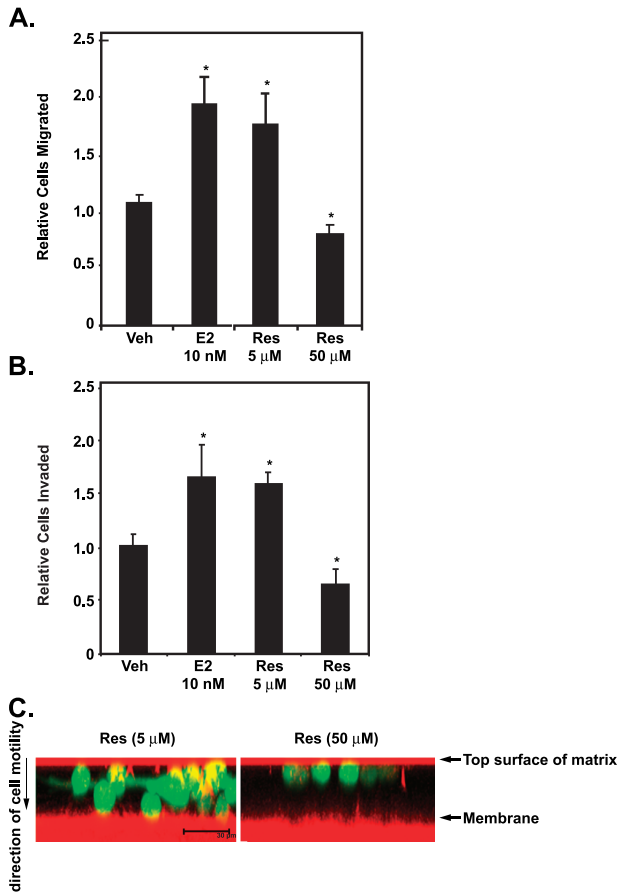


Figure 1. *Effects of estrogen or resveratrol on cell migration and on the invasion of MDA-MB-231 cells. (A) Cell migration. Quiescent cells were placed on the top well of Transwell chambers in a serum-free medium using the following as chemoattractants on the bottom well for 8 hours: DMSO control (Veh), 10 nM estrogen (E_2), or 5 or 50 μM resveratrol (Res). The number of cells that migrated through the membrane of the top well was quantified relative to control. Data are quantified from an analysis of 25 microscopic fields per treatment from six experiments and expressed as mean relative cells migrated ± SEM. *Statistical significance from control vehicle at $P < .05$. (B) Invasion. Quiescent cells were placed on the top well of Transwell chambers where the membrane was coated with Matrigel. The bottom well contained DMSO (Veh), 10 nM estrogen (E_2), or 5 or 50 μM resveratrol (Res). The number of cells that migrated through the Matrigel matrix after 24 hours of incubation was quantified and made relative to control. Data are quantified from an analysis of 25 microscopic fields per treatment from four experiments and expressed as mean relative cells migrated ± SEM. *Statistical significance from control vehicle at $P < .05$. (C) Cells invading through the Matrigel matrix. Representative images of MDA-MB-231 cells invading the interface (~ 30 μM) of the Matrigel matrix and the membrane of the top well in a Transwell chamber are shown. Left: Cells in response to 5 μM resveratrol (Res) on the bottom well. Right: Cells in response to 50 μM resveratrol (Res) on the bottom well.*

membranes of top wells of Matrigel-coated invasion chambers, where the bottom well contained either 5 or 50 μM resveratrol. This figure illustrates the chemotactic response of MDA-MB-231 cells to 5 μM, but not to 50 μM, resveratrol.

Estrogen and Resveratrol Modulate the Actin Cytoskeleton of Breast Cancer Cells

To investigate the structural mechanism for the effect of resveratrol on directed migration, we monitored the effect of estrogen or 5 or 50 μM resveratrol on the actin cytoskeleton. Our published data show that resveratrol (50 μM) treatment resulted in a dynamic and sustained global extension of filopodia [2]. Therein, we hypothesized that an unpolarized filopodia response to 50 μM resveratrol was responsible for resveratrol-induced inhibition of cell migration. We have observed that 50 μM resveratrol can induce unpolarized filopodia, regardless of whether MDA-MB-231 cells were in the serum (1% or 10%) or in the ECM environment, as observed by plating cells on glass, Matrigel (both in 2D and 3D cultures), laminin, or collagen IV (data not shown). In all environments, resveratrol-induced filopodia were sustained up to 8 hours, indicating that this response may be involved in the resveratrol-induced inhibition of migration. We have also analyzed the actin cytoskeleton of cells that were left behind on the Transwell membrane after an 8-hour migration assay and Matrigel invasion assay, and we have observed that those cells had the expected morphology of numerous unpolarized filopodia (data not shown).

To determine the concentration dependence of resveratrol effects on the actin cytoskeleton, we stimulated quiescent MDA-MB-231 cells with a range of resveratrol concentrations. As shown in Figure 2, *A* and *B*, the unpolarized filopodia response peaked at 50 μM resveratrol. Resveratrol at 5 and 10 μM did not induce a significantly different filopodia

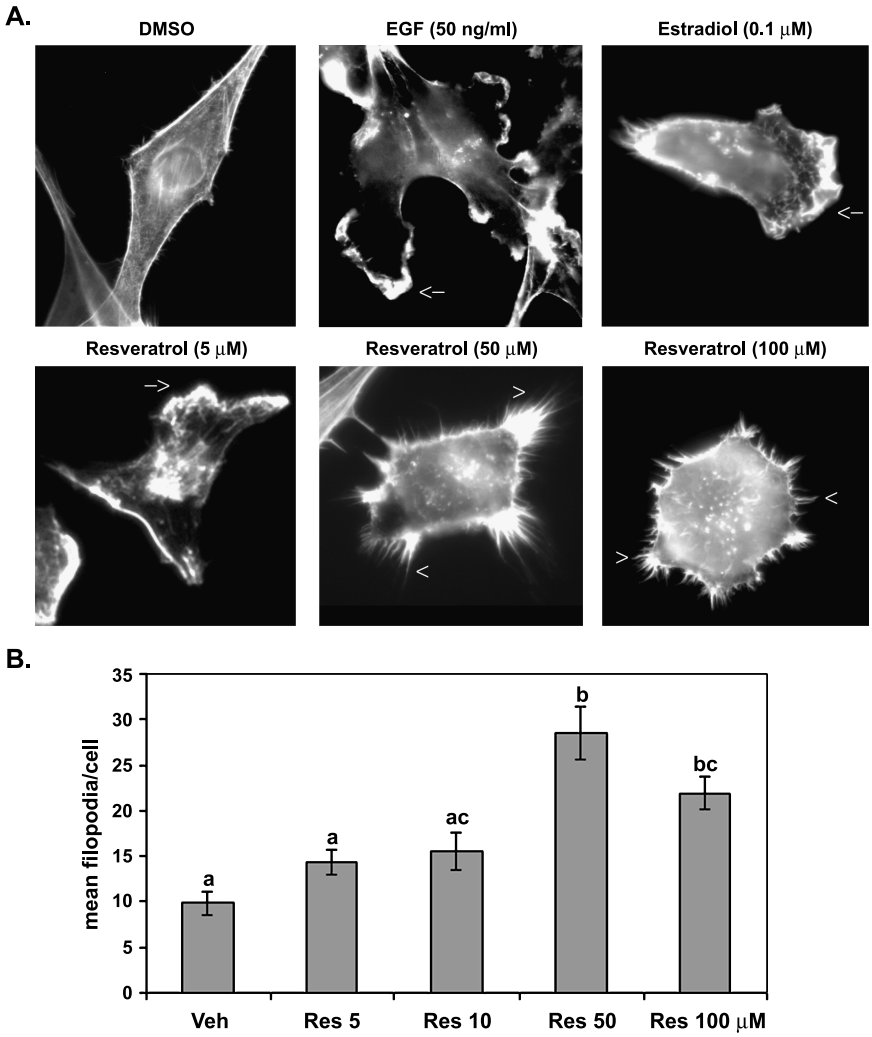


Figure 2. *Reorganization of the actin cytoskeleton in response to resveratrol. (A) Quiescent MDA-MB-231 cells were treated with vehicle (DMSO), EGF (50 ng/ml), estrogen (0.1 μM; top panel), or resveratrol at 5, 50, or 100 μM (bottom panel) for 10 minutes, then fixed and stained for F-actin. Results shown are representative of at least 100 cells/treatment (original magnification, ×600). Arrowheads indicate filopodia; arrows indicate lamellipodia. (B) The mean filopodia number per cell was quantified from at least 50 cells/treatment in response to vehicle (Veh) or various concentrations of resveratrol (Res). Treatments denoted by the same letter indicate no significant difference between those treatments. Treatments denoted by different letters indicate a significant difference between those treatments at $P < .05$. (C and D) Cytoskeletal structures in response to 5 or 50 μM resveratrol as a function of time. Quiescent MDA-MB-231 cells were treated with DMSO vehicle (Veh) or 5 or 50 μM resveratrol (Res), then fixed and stained for F-actin at various times following treatment. (C) Number of filopodia per cell. (D) Number of lamellipodia per cell.*

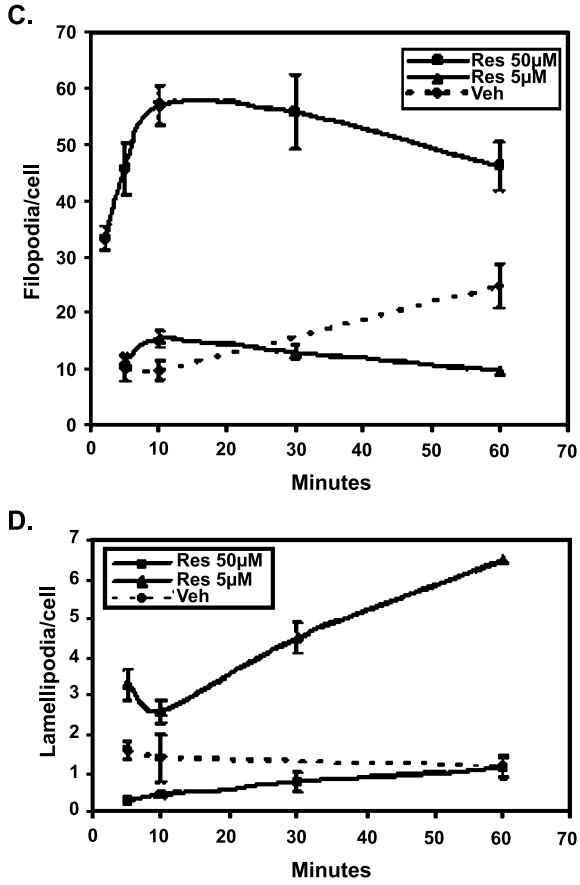


Figure 2. *(continued)*

response from vehicle at 10 minutes. Similar to estrogen and EGF, resveratrol at 5 μM initially induced filopodia at 1 to 2 minutes following treatment. However, these filopodia appeared to be reorganized to form lamellipodia in 5 minutes (data not shown). Resveratrol at 50 and 100 μM significantly induced filopodia compared to treatments with vehicle and 5 μM resveratrol (all $P < .01$). In contrast, 5 μM resveratrol acted in a manner similar to estrogen and EGF, and induced cell polarization and the formation of leading-edge lamellipodia—structures that have been implicated with directed cell migration in response to EGF [25].

Next, we monitored resveratrol effects on the actin cytoskeleton as a function of time. As expected with a rapid signaling response, 50 μM resveratrol induced filopodia at 2 minutes, with a peak response at 10 minutes. This response was sustained until 60 minutes following treatment (Figure 2*C*). Resveratrol at 5 μM or vehicle did not induce a filopodia response. However, 5 μM resveratrol increased lamellipodia at 5 minutes following treatment, and this response was linear from 10 to 60 minutes following treatment. Conversely, 50 μM resveratrol or vehicle did not induce lamellipodia at any time point (Figure 2*D*).

Estrogen and Resveratrol Modulate Cdc42 Signaling to the Actin Cytoskeleton

Our data demonstrate that ERβ(+) breast cancer cells extend filopodia in response to 50 μM resveratrol (Figure 2) [2]. The Rho family GTPase Cdc42 has been shown to regulate filopodia and cell polarization toward chemoattractants such as EGF during directed cell migration [31]. Therefore, we monitored the Cdc42 activity of breast cancer cells in response to estrogen or resveratrol by a pull-down assay using a GST fusion protein to the Cdc42–GTP binding domain of WASP, a downstream effector of Cdc42. As expected, bradykinin, a cytokine known to specifically induce filopodia through Cdc42 [32], activated Cdc42 in MDA-MB-231 cells at 5 and 10 minutes (Figure 3*A*). However, estrogen or resveratrol at 5 μM did not affect Cdc42 activity. Quantification of these data demonstrates that estrogen reduced Cdc42 activity, although it was not significantly different from control vehicle at any time point for resveratrol at 5 or 50 μM (Figure 3, *B* and *C*). Cdc42 activity in response to 5 μM resveratrol was not significantly different from control vehicle either. Conversely, 50 μM resveratrol at 10 minutes significantly inhibited Cdc42 activity by ~ 70% compared to control ($P < .004$). Resveratrol at 50 μM inhibited Cdc42 activity starting as early as 2 minutes following treatment and continued to inhibit until 30 minutes (Figure 3, *B* and *C*).

The Rho family GTPase Cdc42 is known to specifically regulate filopodia formation [26]; however, we found that 50 μM resveratrol, which induced a filopodia response, inhibited Cdc42 activity. To further investigate the role of Cdc42 in resveratrol action, MDA-MB-231 cells were transfected with a bicistronic vector containing myc-tagged dominant-negative Cdc42(T17N) cDNA and stable clones, which were selected according to neomycin resistance and myc-Cdc42 expression (Figure 4*A*).

When cells expressing a control vector were treated with bradykinin or resveratrol, they induced a ~ 2-fold ($P < .001$) and a ~ 2.5-fold ($P < .0001$) significant increase in filopodia, respectively. Vehicle-treated cells expressing Cdc42(T17N) produced ~ 44% less filopodia than vehicle-treated cells expressing vector alone ($P < .001$). These Cdc42(T17N) cells were completely unresponsive to bradykinin—a positive control for filopodia [32]. There was a significant difference in resveratrol-induced production of filopodia between the control vector and Cdc42(T17N) ($P < .0001$). However, Cdc42(T17N)–expressing cells still demonstrated a significant ~ 2-fold increase in filopodia in response to 50 μM resveratrol when compared to Cdc42(T17N) cells treated with vehicle ($P < .0001$; Figure 4, *B* and *C*). Comparing this two-fold response in resveratrol-treated Cdc42(T17N) cells to the 2.5-fold response in resveratrol-treated control vector cells indicates that resveratrol may not be signaling through Cdc42 alone to induce filopodia.

Estrogen and Resveratrol Modulate Rac Signaling to the Actin Cytoskeleton

The Rho GTPase Rac regulates the organization of polymerized actin into leading-edge lamellipodia during directed cell migration [31]. Because our data demonstrate that estrogen and resveratrol induce cell surface actin structures (Figure 2), we investigated a role for Rac in estrogen-mediated and resveratrol-mediated actin cytoskeletal rearrangement.

Endogenous Rac activity was analyzed in response to EGF (positive control), estrogen, or resveratrol at 5 or 50 μM. As shown in Figure 5, EGF activated Rac at 10 minutes following stimulation of MDA-MB-231 cells. Interestingly, estrogen activated Rac at 0.1 μM at 10 minutes following treatment at the same conditions that induce a lamellipodia response. Both EGF and estrogen significantly increased Rac activity by ~ 2-fold compared to control ($P < .01$ and .007, respectively). Similarly, 5 μM resveratrol significantly increased Rac activity by ~ 3-fold compared to vehicle ($P < .05$) at 10 minutes and was not significantly different from EGF or estrogen. This increase in Rac activity was observed starting at 5 minutes following resveratrol treatment and continued until 60 minutes. Resveratrol at 50 μM, however, significantly inhibited Rac activity compared to control at 10 minutes ($P < .007$) following addition. This inhibitory effect was observed from 5 to 60 minutes (Figure 5*B*). We have obtained similar results using ERα(+) ERβ(+) MCF-7 cells where estrogen and 5 μM resveratrol increased Rac activity (data not shown). These data indicate that in ER(+) breast cancer cells, a low concentration of resveratrol acts in a manner similar to estrogen and EGF (a known chemotactic ligand) by activating Rac, whereas a higher concentration of resveratrol is inhibitory.

To further investigate a role for Rac in estrogen and resveratrol signaling to the actin cytoskeleton, we created MDA-MB-231 cells stably expressing a myc-tagged dominant-negative Rac1 (Rac1(T17N)). Stable clones were selected by neomycin resistance and expression of myc-Rac (Figure 6*A*). Our laboratory has previously demonstrated that expression of Rac(T17N) inhibits lamellipodia extension, invasion, and metastasis in metastatic breast cancer cells [30]. Quiescent cells expressing the control vector or Rac1(T17N) were stimulated with vehicle, EGF, estrogen, or 5 or 50 μM resveratrol. As expected, EGF treatment resulted in a dramatic increase in lamellipodia compared to vehicle in control cells ($P < .0001$), and estrogen induced a similar increase in lamellipodia in control cells ($P < .0001$). Resveratrol at 5 μM induced a slightly less but significant (~ 10-fold) increase in lamellipodia in control cells. Lamellipodia responses to EGF, estrogen, or resveratrol (5 μM) were completely blocked by the expression of a dominant-negative Rac1 (Figure 6, *B* and *C*).

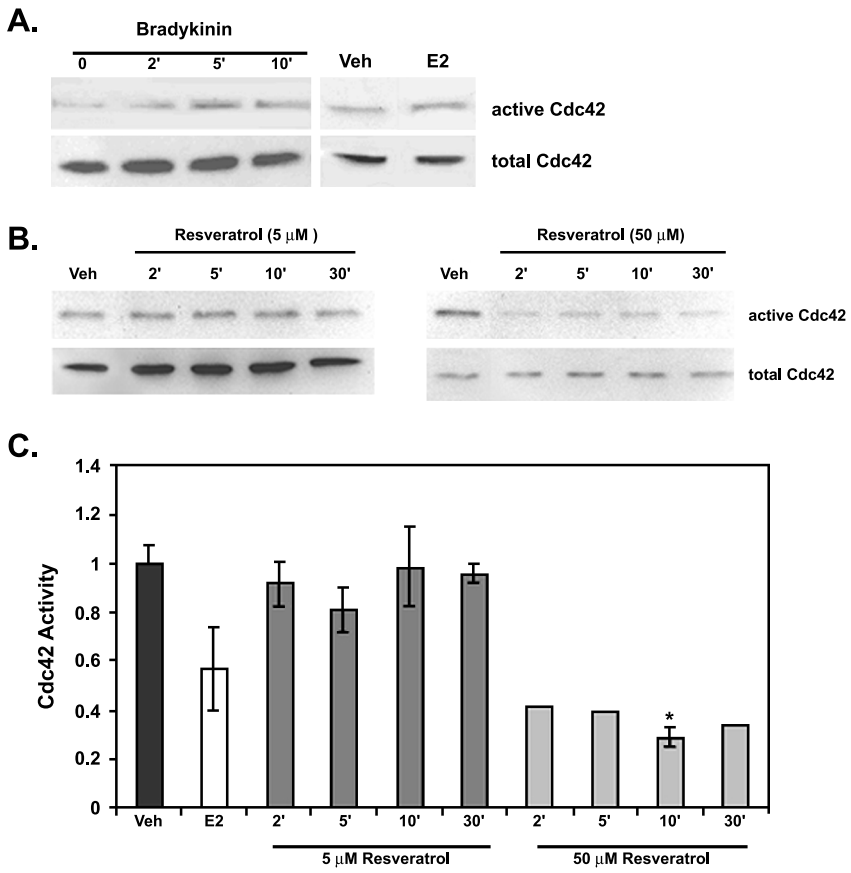


Figure 3. *Cdc42 activity of MDA-MB-231 cells in response to estrogen or resveratrol. Quiescent cells were lysed immediately after treatment with compounds for the indicated times, and WASP–PBD–GST beads were used to pull down active GTP-bound Cdc42. Active and total Cdc42 levels were detected by Western blot analysis with anti-Cdc42 antibody. (A) Quiescent MDA-MB-231 cells were treated for 10 minutes with DMSO (0) or for 2, 5, or 10 minutes with 400 ng/ml bradykinin or 0.1 μM estrogen (E_2) and used for pull-down assays to determine endogenous Cdc42 activity. Representative Western blot analyses of active and total Cdc42 are shown. (B) Cells were treated with DMSO (Veh) or resveratrol at 5 or 50 μM for 2, 5, 10, or 30 minutes. Cells were lysed immediately and used for pull-down assays to determine endogenous Cdc42 activity. Representative Western blot analyses of active and total Cdc42 levels from three separate experiments are shown. (C) Cdc42 activity (active Cdc42/total Cdc42) relative to vehicle (Veh) as quantified from densitometric scans of Western blot analyses. *Statistical significance from control at $P \leq .05$. Error bars represent SEMs from at least three experiments. n = 2 for 2, 5, or 30 minutes, and n = 6 for 10 minutes in treatments with 50 μM resveratrol.*

Treatment with 50 μM resveratrol did not affect lamellipodia extension in control or Rac1(T17N)–expressing cells, but instead showed a filopodia response in both cell lines. Cells expressing Rac1(T17N) responded to 50 μM resveratrol by filopodia extension similar to the control vector (Figure 6, *B* and *D*), and these responses were significant from control vehicles for both cell lines (both $P < .001$). Estrogen or EGF treatment of control cells expressing vector alone or a dominant-negative Rac1 did not cause extension of filopodia. Because 50 μM resveratrol inhibits Rac activity, this may contribute to the global and sustained induction of filopodia without subsequent conversion to lamellipodia.

Discussion

This investigation extends our studies on the disparate roles of estrogen and 50 μM resveratrol in cell migration and in the extension of cell surface actin structures in metastatic breast cancer cells. As we have shown previously, the data presented herein demonstrate that 50 μM resveratrol induces a unique actin morphology through the rapid extension of unpolarized filopodia [2]. Therein, we hypothesized that the observed decreased migration and invasion by 50 μM resveratrol are due to this phenotype of sustained filopodia. Subsequent studies have also shown inhibition of invasion by high concentrations of resveratrol (50–100 μM), with potential additional mechanisms for resveratrol-mediated inhibition of invasion. Resveratrol at high concentrations has been shown to inhibit MMP-2 and MMP-9 activity, to increase tensin expression, and to inhibit Jun kinase and protein kinase C to, in turn, inhibit cancer cell invasion [18,20,21,33].

We report an interesting effect of a low concentration and a high concentration of resveratrol on cell migration in ERα(−) ERβ(+) MDA-MB-231 cells. At a low concentration, 5 μM resveratrol increases cell migration and invasion, whereas 50 μM resveratrol inhibits cell migration and invasion. This kind of concentration-dependent biphasic effect of resveratrol has been shown in numerous models and may be attributed to the mixed ER agonist/antagonist properties of this structurally similar phytosterol. It is often the case that lower concentrations (0.01–10 μM) of resveratrol act in an estrogenic manner to activate pathways such as MAPK or PI3-K/Akt, whereas higher concentrations (50–100 μM) inhibit them [8–10,34–36]. We have not observed any changes in cell cycle progression at 8 hours following 50 μM resveratrol; however, MDA-MB-231 cells undergo apoptosis at 24 to 48 hours following treatment with 50 μM resveratrol (data not shown). Therefore, the well-established effects of resveratrol on apoptotic pathways [8,10–12,21] may also contribute to the inhibition of cell migration and invasion by 50 μM resveratrol.

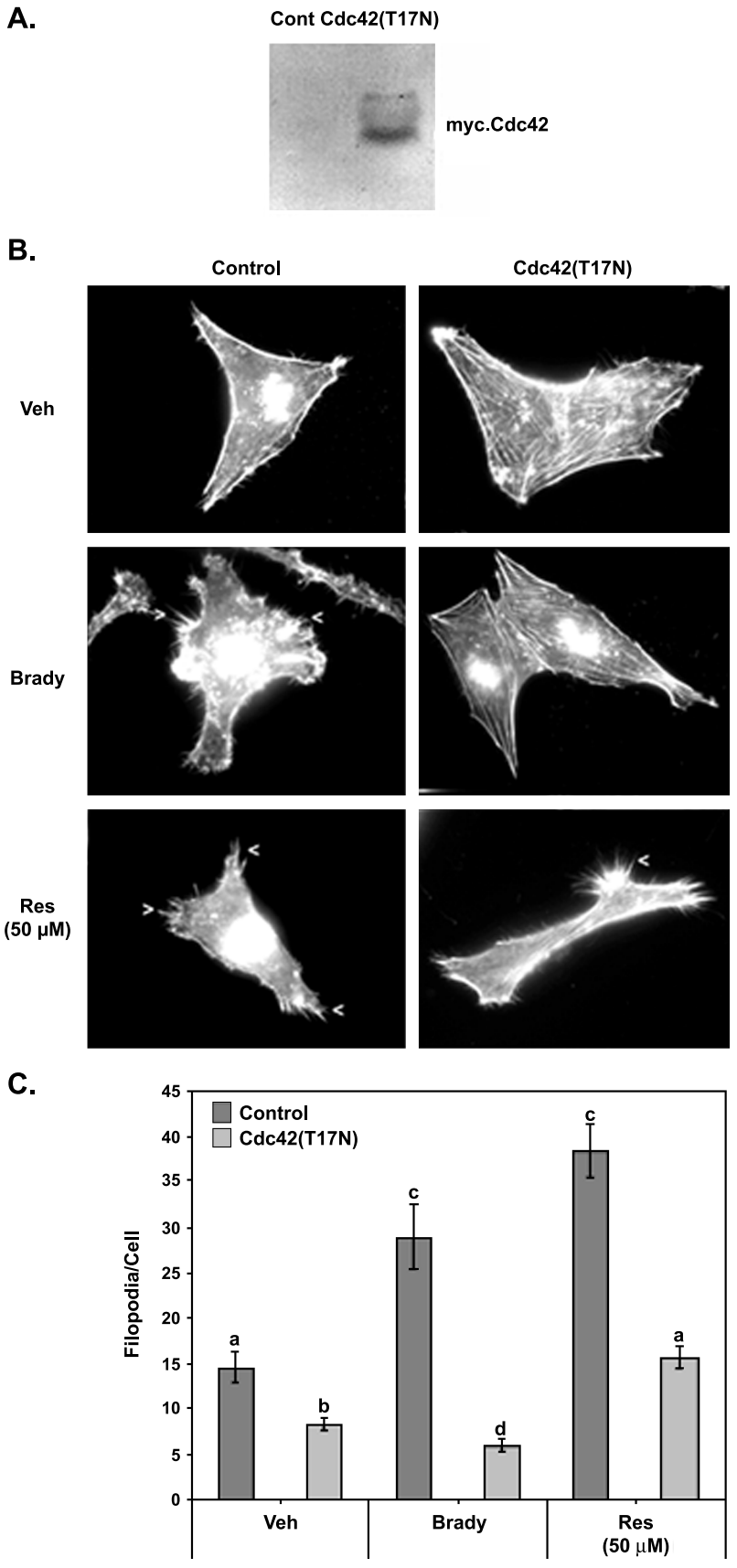
We show that resveratrol has opposing concentration-dependent effects on actin cytoskeletal morphology, which is hypothesized to contribute directly to differential effects on migration. Resveratrol at 5 μM induces a rapid, sustained, leading-edge lamellipodia in the same manner as estrogen and EGF. MDA-MB-231 cells stimulated with EGF, estrogen, or 5 μM resveratrol first form filopodia, which are rapidly converted to lamellipodia. Conversely, resveratrol at 50 μM induces a rapid, unpolarized, and sustained filopodia response even up to 8 hours in serum or ECM matrices. These data indicate that the maintenance of a nonmigratory phenotype by 50 μM resveratrol may be directly correlated with 50-μM resveratrol–induced filopodia that do not get converted to lamellipodia, which are useful for cell migration.

Directed cell migration involves the modulation of the actin cytoskeleton by Rho GTPases—a family of signaling proteins with 22 members [37]. The most characterized Rho GTPases Rho, Rac, and Cdc42 have been classically implicated in the reorganization of the actin cytoskeleton to form stress fibers, lamellipodia, and filopodia, respectively, and these three proteins are thought to act coordinately to regulate cell migration and, thus, invasion and metastasis [26,29,31,38]. Recent studies have implicated Rho proteins specifically in the promotion of breast cancer metastasis [29,39–41]. Studies have shown the importance of Rho isoforms A and C in breast cancer [29,39,42]. We and others have shown that Rac and Cdc42 are also important regulators of breast cancer metastasis [30,40,43]. Cdc42 activation by growth factor receptor signaling often leads to Rac activation and, thus, the reorganization of filopodia into lamellipodia [26,44,45]. Rac signaling to extend lamellipodia at the leading edge and subsequent cell migration away from the primary tumor have been specifically linked to the regulation of breast cancer metastasis [29,41,45].

Because filopodia and lamellipodia have been shown to be primarily under Rac and Cdc42 regulation, we focused the present investigation on the role of estrogen and resveratrol signaling to Rac and Cdc42. Our novel data reveal that resveratrol affects Rac and Cdc42 activity in MDA-MB-231 metastatic breast cancer cells differently, depending on concentration. The peak filopodia response to 50 μM resveratrol (10 minutes) can be correlated with the time of 50-μM resveratrol–induced inhibition of Rac and Cdc42 activity. Cdc42 activation generally leads to a transient polarized filopodia extension, followed by Rac activation and the subsequent conversion of filopodia into motile lamellipodia [31]. The 50-μM resveratrol–induced decrease in Cdc42 activity may indicate that Cdc42 may be primarily responsible for the polarization of MDA-MB-231 cells, and resveratrol-mediated inhibition of Cdc42 may contribute to global and sustained filopodia response to 50 μM resveratrol by inhibiting subsequent Rac activation that would lead to the conversion of filopodia into leading-edge lamellipodia. Contrary to our results with breast cancer cells, a study using leukemia cells showed that 20 μM resveratrol activated Cdc42 at 30 and 60 minutes to induce apoptosis through a Jun N-terminal/FasL signaling pathway [46]. This result may be a cell type–specific effect of resveratrol on leukemia cells.

Interestingly, when MDA-MB-231 cells stably expressing dominant-negative Cdc42 were treated with 50 μM resveratrol, there was partial reduction of filopodia. Therefore, a transient increase in Cdc42 activity may be responsible for an initial extension of filopodia. However, as shown by our results, resveratrol (50 μM) decreased Cdc42 activity at times as low as 2 minutes following addition. Therefore, the

observed induction of filopodia by resveratrol may be largely independent of Cdc42 and may be under the regulation of alternative filopodia-associated Rho GTPases such as Rif and/or Wrch-1 [47,48]. It is also possible that the 50-μM resveratrol–mediated signaling pathway bypasses Cdc42 and directly signals to a downstream effector such as WASP



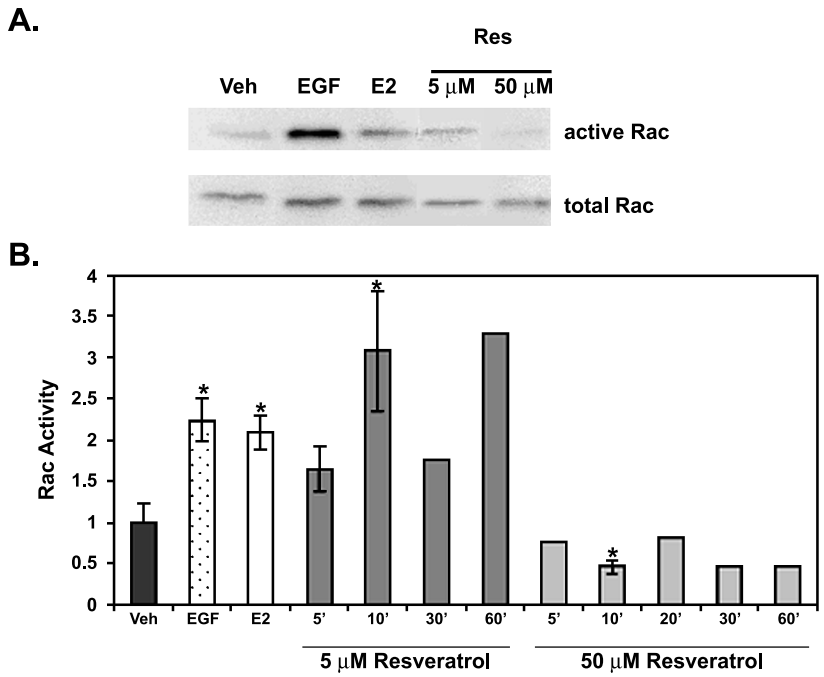


Figure 5. *Rac activity of breast cancer cells in response to estrogen or resveratrol. Quiescent MDA-MB-231 cells were treated with DMSO (Veh), 50 ng/ml EGF, 0.1 μM estrogen (E_2), or resveratrol (Res). PAK–PBD–GST beads were used to pull down active GTP-bound Rac from cell lysates. Active and total Rac levels were detected by Western blot analysis with anti-Rac antibody. (A) Representative Western blot analysis of the Rac activity of MDA-MB-231 cells in response to vehicle, EGF, E_2, or resveratrol at 5 or 50 μM following a 10-minute incubation. Results shown are representative of at least three experiments. (B) Rac activity (active Rac/total Rac) relative to vehicle-alone control as quantified from densitometric scans of Western blot analyses. *Statistical significance from control. Error bars represent SEMs from at least three experiments. n = 2 for 30 or 60 minutes in 5 μM resveratrol, and n = 2 for 5, 20, 30, or 60 minutes in 50 μM resveratrol.*

[49]. Thus, these results indicate that resveratrol modulation of the actin cytoskeleton may involve novel Cdc42-independent mechanisms that implicate other Rho GTPases or the direct activation of downstream effectors of Cdc42.

We have shown that treatment of MDA-MB-231 cells with EGF or estrogen resulted in a motile phenotype of increased lamellipodia and associated focal adhesions at the leading edge [2]. This estrogen-induced effect on lamellipodia formation has also been observed in endothelial, breast cancer, and endometrial cancer cells [2–5]. Such estrogen-induced membrane ruffles in ERαβ(+) MCF-7 breast cancer cells have been shown to colocalize with membrane ERα [28]. Recently, estrogen-induced lamellipodia formation and focal adhesion assembly of endothelial cells correlated with a G protein–coupled mechanism that signaled to Rho and Rho kinase [5].

Our data indicate a strong role for Rac in the extension of lamellipodia and in subsequent increases in breast cancer cell migration and invasion in response to estrogen and low concentrations of resveratrol. We demonstrate that low concentrations of resveratrol (5 μM) act in a manner similar to EGF and estrogen and induce Rac activity and lamellipodia extension at 10 minutes. Moreover, lamellipodia response to EGF, estrogen, or 5 μM resveratrol can be completely abolished by inhibition of Rac activity using cells expressing dominant-negative Rac.

The reported increase in Rac activity and migratory phenotype in response to estrogen and a low concentration of resveratrol elucidates a novel mechanism by which estrogen and resveratrol can promote breast cancer progression. An important finding is that resveratrol at low concentrations elicits signaling that may promote cell migration/invasion, whereas resveratrol at high concentrations may inhibit these signaling pathways. We hypothesize that inhibition of Rac and Cdc42 activities leading to sustained and unpolarized filopodia formation and subsequent inhibition of cell migration and invasion by 50 μM resveratrol may lead to reduced breast cancer metastasis. Overall, these data indicate that resveratrol can act as a breast cancer metastasis–preventive agent but should be used with caution with regard to concentration. Even though such high concentrations may not be

Figure 4. *Effect of a stable expression of a dominant-negative Cdc42(T17N) on resveratrol-mediated filopodia extension. (A) Representative Western blot analysis of MDA-MB-231 cells stably expressing vector or myc-tagged Cdc42. (B) Filopodia extension in response to resveratrol in cells expressing vector alone or Cdc42(T17N). Quiescent MDA-MB-231 cells expressing a control vector (control) or myc-tagged Cdc42(T17N) were treated with vehicle (Veh), 400 ng/ml bradykinin (Brady), or 50 μM resveratrol (Res), then fixed and stained for F-actin with rhodamine phalloidin. Representative cells of at least 100 cells/treatment are shown. Arrowheads indicate filopodia. (C) Quantification of the effect of the stable expression of a dominant-negative Cdc42(T17N) on resveratrol-mediated filopodia extension. Quiescent MDA-MB-231 cells expressing a control vector (dark-grey columns) or Cdc42(T17N) (light-grey columns) were treated with vehicle, 400 ng/ml bradykinin, or 50 μM resveratrol, then fixed and stained for F-actin with rhodamine phalloidin. The relative number of filopodia per cell (± SEM) was quantified from micrographs for at least 100 cells/treatment. Treatments denoted by the same letter indicate no significant difference between those treatments. Treatments denoted by different letters indicate a significant difference between those treatments at $P \leq .05$.*

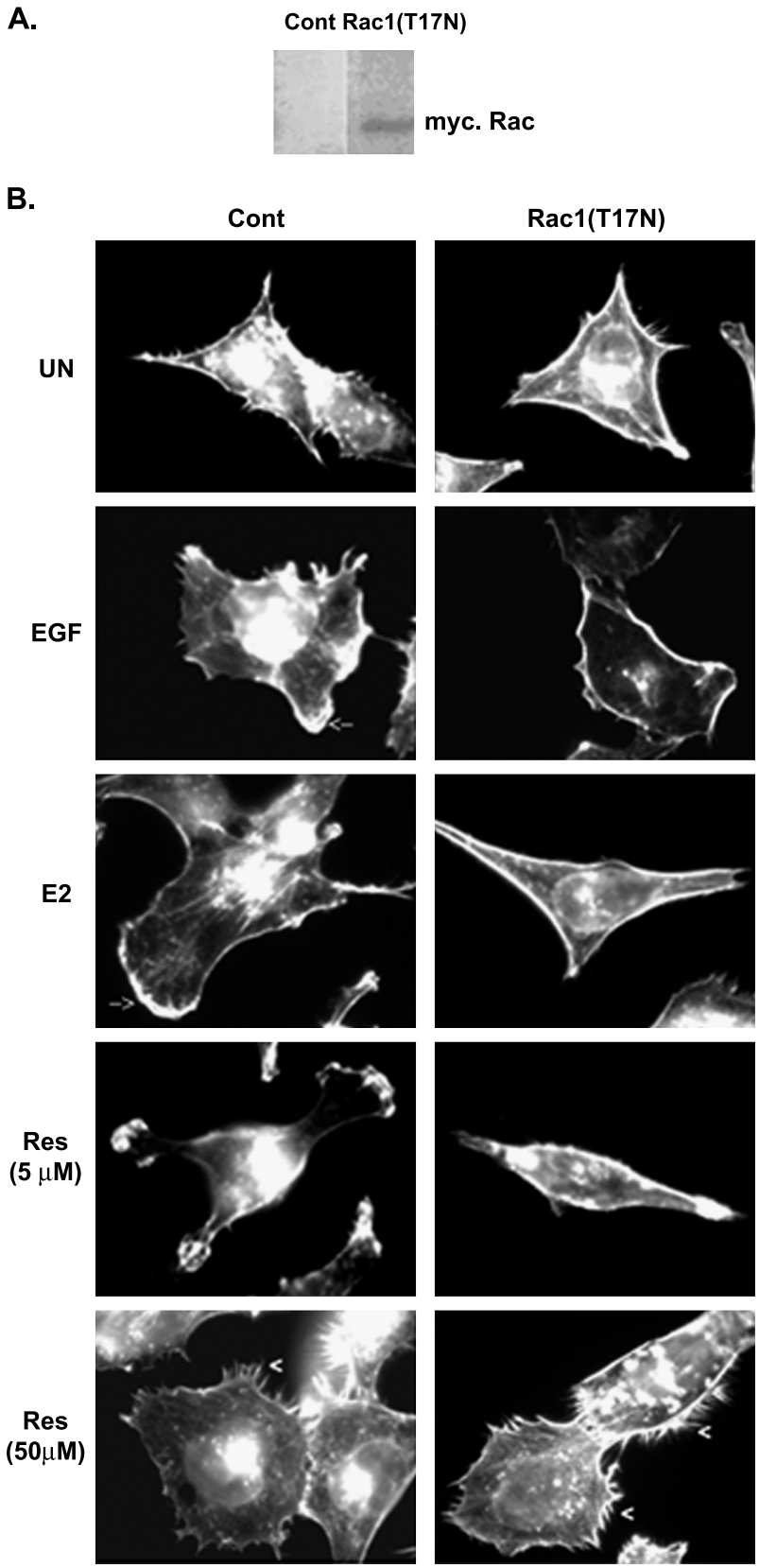


Figure 6. *Effect of a stable expression of a dominant-negative Rac on estrogen-mediated actin structures. (A) Representative Western blot analysis of MDA-MB-231 cells stably expressing vector (control) or myc-tagged Rac. (B) Actin structures in response to resveratrol in cells expressing Rac1(T17N). Quiescent MDA-MB-231 cells expressing vector (control) or myc-tagged Rac1(T17N) were treated with vehicle (UN), 50 ng/ml EGF, 0.1 μM estrogen (E_2), 5 or 50 μM resveratrol (Res), then fixed and stained for F-actin with rhodamine phalloidin. Representative cells of at least 100 cells/treatment are shown. Arrows indicate lamellipodia; arrowheads indicate filopodia. (C and D) Quiescent MDA-MB-231 cells expressing a control vector (dark-grey columns) or Rac1(T17N) (light-grey columns) were treated with vehicle (Veh), 50 ng/ml EGF, 0.1 μM estrogen (E_2), or 5 or 50 μM resveratrol (Res), then fixed and stained for F-actin with rhodamine phalloidin. The relative number of lamellipodia per cell (± SEM) quantified from micrographs for at least 100 cells/treatment (C). The relative number of filopodia per cell (± SEM) quantified from micrographs for at least 100 cells/treatment (D). Treatments denoted by the same letter indicate no significant difference between those treatments. Treatments denoted by different letters indicate a significant difference between those treatments at $P \leq .05$.*

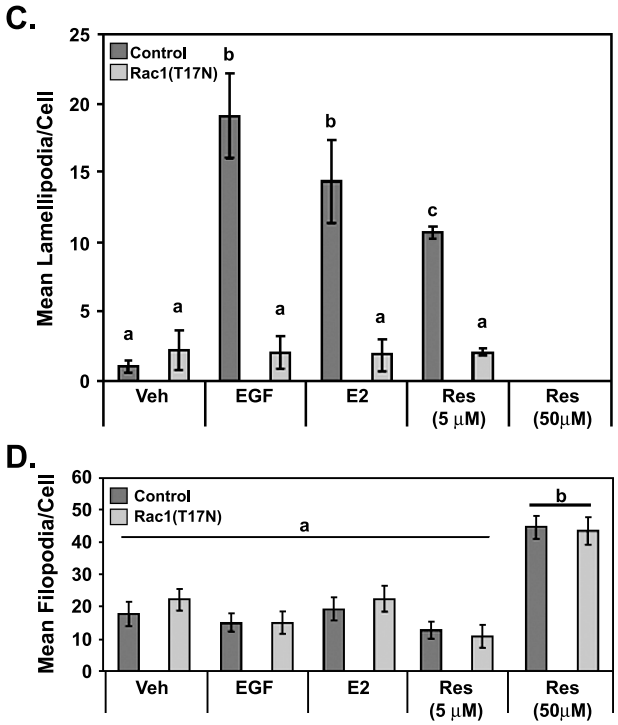


Figure 6. *(continued)*

achieved by consumption of foods containing resveratrol, pharmacological concentrations of resveratrol may have breast cancer–preventive/therapeutic potential. Therefore, this disparate unique mechanism of action of a common dietary compound at low and high concentrations directly impacts the use of resveratrol in breast cancer prevention and therapy.

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