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Green tea (−)-epigallocatechin-3-gallate down-regulates VASP expression and inhibits breast cancer cell migration and invasion by attenuating Rac1 activity

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ABSTRACT

(−)-Epigallocatechin-3-gallate (EGCG) is a polyphenolic compound from green tea that has been shown to have anti-tumor activities such as inhibiting adhesion, migration, and proliferation of tumor cells. However, the delicate mechanisms and signaling pathways underlying the potential anticancer effects of EGCG in breast cancer cells remain unclear. The goal of this study was to examine the effects of EGCG on the migration and invasion of MCF-7 cells and to identify the signaling pathway(s) underlying the cellular response to EGCG exposure. In a concentration-dependent manner, EGCG decreased the migratory and invasive potential of MCF-7 cells with a concomitant down-regulation of vasodilator-stimulated phosphoprotein (VASP) expression and Rac1 activity. Using specific siRNAs to block the expression of VASP and Rac1 in MCF-7 cells that were previously treated with epidermal growth factor (EGF), we demonstrated that the regulation of cell migration and invasion was associated with Rac1 activity and VASP expression. In addition, siRNA mediated knock-down of Rac1 decreased the amount of VASP expression at the mRNA level while VASP specific siRNA revealed no effect on the expression of Rac1 in MCF-7 cells. These findings suggest that the inhibitory effect of EGCG on MCF-7 cell migration and invasion may be produced by a down regulation of VASP expression *via* the Rac1 pathway.

1. Introduction

Breast cancer is the leading cause of death for women in many countries. Importantly, the ability of breast cancer cells to metastasize to distal organs contributes to the refractory and incurable nature of this disease (Lu and Kang, 2007). Therefore, strategies aimed at controlling the metastasis of breast cancer cells have received much attention in breast cancer therapy.

Green tea contains many polyphenols such as flavanols (catechins), flavonols, flavandiols, and phenolic acid. These polyphenols account for a third of the dry weight of the leaves. The major green tea catechins are (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (þ)-catechin (Graham, 1992). These compounds have been shown to exhibit cytostatic properties in several tumor models. Epidemiological studies have suggested that the low incidence of some cancers in Asian countries is linked to the regular consumption of green tea (Nakachi et al., 1998). EGCG is a major polypenolic compound in green tea and has been demonstrated to have anti-tumor activity (Mukhtar and Ahmad, 2000), and the preclinical research examining EGCG for cancer prevention has been promising. However, there is no agreement concerning the precise mechanisms and signaling pathways underlying the potential anticancer effects of EGCG in breast cancer cells. In this study, we examined the role of EGCG in the metastasis of a breast cancer cell line (MCF-7) and analyzed the possible mechanisms underlying EGCG functions in breast cancer metastasis.

One facet of the anti-tumor activity exerted by EGCG seems to be attributed to its ability to inhibit the epidermal growth factor (EGF) receptor (Sachinidis et al., 2000). Recent work has implicated the PI 3-kinase to serine/threonine kinase Akt/protein kinase B to NF-κB signaling pathway in control of growth and transformed phenotype of Her-2/neu-overexpressing cells (Ignatoski et al., 2000; Ozes et al., 1999; Pianetti et al., 2001). The Her-2/neu (or c-erbB-2) oncogene, which is the second member of the EGF receptor 4 family, encodes a transmembrane tyrosine receptor kinase (Pianetti et al., 2002). Pianetti et al. reported that treatment of MMTV-Her-2/neu mouse mammary tumor NF639 cells with EGCG slowed proliferation and reduced cell growth in soft agar *via* inhibiting the PI3K to Akt kinase to NF-κB pathway. EGCG reduced the levels of basal tyrosine phosphorylation in NF639 cells and two other Her-2/neu over-expressing cell lines (Pianetti et al., 2002).

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Rho GTPases act as molecular switches, coordinating and integrating multiple signal transduction pathways. Rearrangement of actin cytoskeletal proteins in response to Rho GTPase plays a significant role in the ability of tumor cells to metastasize (Takai et al., 2001). In addition, overexpression of Rho GTPase has been linked to the pathogenesis and metastasis of human breast cancer (Fritz et al., 1999). Rac1, a member of the Rho family of GTPases, controls multiple cellular functions including cell cycle progression, gene expression, apoptosis, actin organization, cell motility, and the invasive potential of human tumor cells (Chan et al., 2005; Guo et al., 2006). Indeed, the regulation of Rac1 signaling underlying the invasive migration behavior of tumor cells is of great clinical interest. Schnelzer et al. demonstrated that Rac1 was regulated by the extent of GTP binding in human breast cancer cells (Schnelzer et al., 2000). Van Aelst and D'Souza-Schorey showed that Rac1 transduced signals from both the EGF receptor and PI3K in numerous cell types (Van and Souza-Schorey, 1997). Furthermore, previous work has demonstrated that EGF stimulates the activation of Rac1 (Marcoux and Vuori, 2003). The aim of this study was to investigate how EGF stimulates the activation of Rac1 in breast cancer cells.

Ena/VASP (vasodilator-stimulated phosphoprotein) proteins are actin-binding proteins that also localize to actin stress fibers, the tips of filopodia, and the lamellipodial leading edge (Reinhard et al., 1992; Rottner et al., 1999; Gertler et al., 1996). In the past few years, a number of studies have focused on Ena/VASP functioning, pointing to roles for these proteins in the promotion of several actin-dependent processes (Krause et al., 2002). Deletion of the Ena/VASP-binding sites within the bacterial protein ActA, led to a decrease in actin-dependent intracellular motility of *Listeria monocytogenes* (Niebuhr et al., 1997). VASP regulation of the cytoskeleton is also mediated through interactions with or regulation of Rho GTPases. In the presence of N17 Rac1, which can suppress tumor progression, dominant negative GTPases were transfected into malignantly transformed cancer cells. Subsequently, VASP was down-regulated while vinculin and F-actin colocalizations were restored (Quinlan, 2004). In our previous studies, we found a positive effect on migration and invasion by VASP *via* the Rac1 pathway in human breast cancer cells (Han et al., 2008).

Based on these results, we were interested in determining whether EGCG affected the migration and invasion of breast cancer cells. In this study, we also observed the changes in Rac1 activation and VASP expression, as well as their interaction in EGCG treated breast cancer cells.

2. Materials and methods

2.1. Chemicals

The monoclonal mouse anti-VASP antibody (IE273) was obtained from ImmunoGlobe Company. Anti-Rac1 (23A8 clone) and the Rac1 Activation Assay Kit were purchased from Upstate Biotechnology. RPMI-1640 medium was from Hyclone Company. The DAB Kit was purchased from Beijing Zhongshan Biotechnology Co., Ltd. The 24-well Transwell system was from Costar Corporation. EGF was bought from PeproTech EC Ltd. EGCG was purchased from Shanghai Yousi Biotechnology Co., Ltd.

2.2. Cell culture and siRNA transfection

Human breast cancer MCF-7 cells were preserved by the Department of Pathophysiology, Medical College of Wuhan University. The study was approved by the Local Institutional Ethics Committee. Human breast cancer MCF-7 cells were maintained in RPMI-1640 medium with 10% calf serum at 37 °C in a humidified incubator supplemented with 5% CO_2. A day prior to transfection, cells were seeded in 6-well plates and grown overnight in RPMI-1640 medium supplemented without calf serum or antibiotics to reach approximately 60%–70% confluence. The RNA oligos containing 21 nucleotides were synthesized in sense and anti-sense directions corresponding to human Rac1 (Genbank accession no. AF498964) siRNA at nucleotides 439–459 bp (sense: 5′-GGAGATTGGTGCTGTAAAA-3′; anti-sense: 5′-UUUUACAGCACCAAUCUCC-3′) with dTdT overhangs at each 3′ terminus (Shanghai GenePharma Co., Ltd.). The RNA oligos containing 21 nucleotides were synthesized in sense and anti-sense directions corresponding to human VASP (Genbank accession no. BC038224) siRNA at nucleotides 942–960 bp (sense: 5′-CAACCUUGCCAAGGAUGAATT-3′; anti-sense: 5′-UUCAUCCUUGGCAAGGUUGTG-3′). The selected sequences were submitted to a BLAST search against the human genome to ensure that the only selected genes were targeted. One negative siRNA control (sense 5′-UUCUCCGAACGUGUCACGU-3′; anti-sense 5′-ACGUGACACGUUCGGAGAA-3′) was also supplied. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen Co., Ltd.). A day prior to transfection, cells were trypsinized, diluted with fresh medium and transferred to 6-well plates. Transfection of siRNAs were carried out using Lipofectamine 2000 reagent. Lipids and siRNAs were diluted into the RPMI-1640 medium; thereafter, the diluted lipids were mixed with the diluted siRNAs and the mixture was incubated for 20 min at room temperature for complex formation. After the addition of RPMI-1640 medium to each well containing cells to a level of 2 ml, the entire mixture was added to the cells in one well resulting in a final concentration of 100 pmol for the siRNAs. At 48 h after transfection, cells were fixed for RT-PCR analysis or scraped for Western blotting.

2.3. Western Blotting Analysis

Cells were washed twice with PBS and then lysed in ice-cold RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.25% deoxycholate (DOC), 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, 10 mM pervanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) on ice using 50 μl lysis buffer per well. After 10 min on ice, lysates were scraped into microcentrifuge tubes and centrifuged at 14,000 ×g for 15 min at 4 °C. The protein concentration was determined by a Coomassie Brilliant Blue assay. For direct immunoblotting, aliquots of lysate were mixed with 5× sample buffer containing 2-mercaptoethanol and boiled for 5 min before loading on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel with a 5% (v/v) stacking gel and a 12% (v/v) gradient gel. Following SDS-PAGE separation, proteins were transferred to a nitrocellulose membrane in 90 min for Rac1 and GAPDH, or in 120 min for VASP. After blocking with 5% (w/v) BSA in Tris-buffered saline (TBS) at 37 °C for 1 h, membrane strips were incubated with different dilutions of the various antibodies. After extensive washing, membrane strips were incubated with different dilutions of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG at 37 °C for 1 h with agitation and then extensively washed again. Detection was performed with DAB (3, 3-diaminobenzidine). The immuno-based enzymatic reaction was stopped by rinsing in distilled water. GAPDH was employed as an internal control.

2.4. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to determine the expression level of mRNA of the Rac1 and VASP target sequences in cells. Total RNA was purified using the Total RNA isolation system (Promega) and RT-PCR was performed using the Acess RT-PCR system (Promega). The upstream and downstream primers of Rac1 were 5′-ATGTGCTCTCCCGAGTAACC-3′ and 5′-GGAGGAAACTGAGGCAACAC-3′, respectively. The Rac1 PCR reaction parameters were 94 °C for 40 s, 54 °C for 30 s, and 72 °C for 30 s, with a total of 30 cycles. The upstream and downstream primers of VASP were 5′-CCACTGAACTTCTGATTCGC-3′ and 5′-GCGTGCTAGC TGGATGTCT T-3′, respectively. The VASP PCR reaction parameters were 94 °C for 40 s, 60 °C for 30 s, and 72 °C for 30 s with a total of 30 cylces. GAPDH was employed as an internal control for mRNA analyses.

2.5. Rac1 activation assay

The GTPase activity of Rac1 was measured as described in the instructions. Cells were rinsed with cold PBS and lysed in MLB buffer.

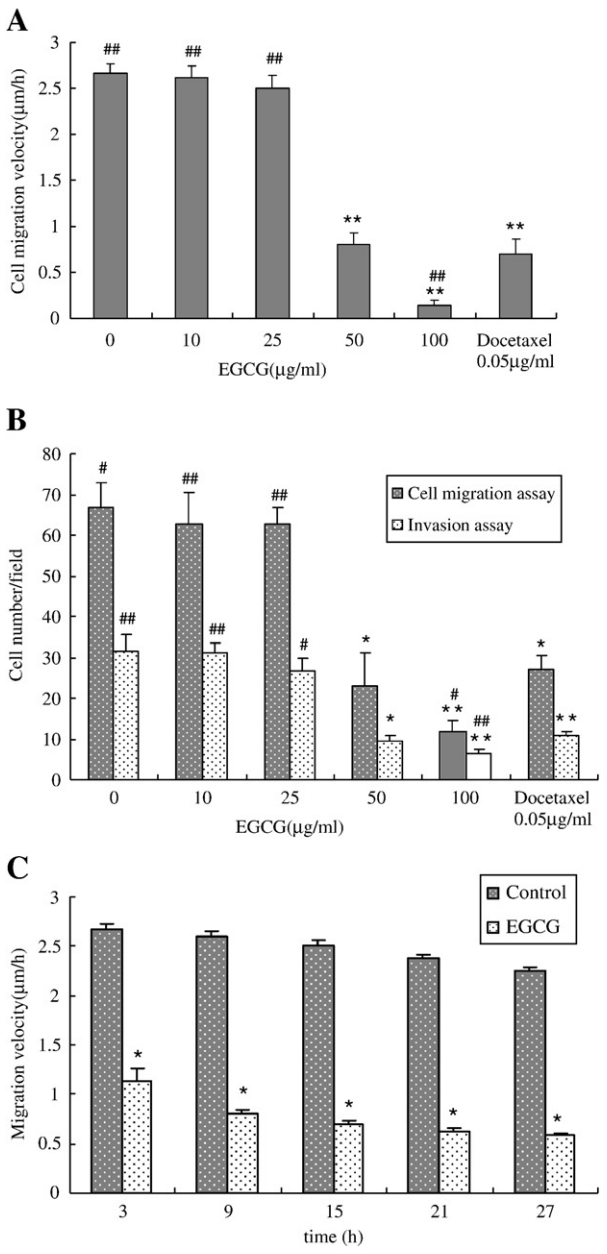

Fig. 1. EGCG decreased MCF-7 cell migration and invasion in a concentration- and time-dependent manner. (A) and (B) MCF-7 cells were treated with serum-free RPMI-1640 medium or EGCG (10, 25, 50, and 100 μg/ml) or docetaxel (0.05 μg/ml) for 24 h in wound healing assay and cell migration/invasion assay, respectively. The invasive migration ability was repressed significantly when MCF-7 cells were treated with EGCG (50 μg/ml). The cells treated with docetaxel were positive controls. Three independent experiments were performed. Results are presented as means ± S.D. of these independent experiments. *$P<0.05$, **$P<0.01$ *vs.* control group, and #$P<0.05$, ##$P<0.01$ *vs.* positive control group. (C) MCF-7 cells were treated with serum-free RPMI-1640 medium or EGCG (50 μg/ml) in a wound healing assay. Time-lapse images were captured at the time of 0 h, 6 h, 12 h, 18 h, 24 h, and 30 h after EGCG treatment and the transient cell migration velocities at 3 h, 9 h, 15 h, 21 h and 27 h were calculated to indicate the change of the migration ability of cells. Three independent experiments were performed. Results are presented as means ± S.D. of these independent experiments. *$P<0.05$ *vs.* control group.

Lysates were cleared by centrifugation and the protein concentration was measured. Equal amounts of protein (400–600 μg) were incubated with 50 μg Rac/cdc42 Assay Reagent (PAK-1 PBD, agarose) and then the reaction mixture was gently rocked at 4 °C for 60 min. The agarose beads were collected by pulsing for 5 s in the microcentrifuge at 14,000 ×g to remove and discard the supernatant. The beads were washed 3 times with MLB, resuspend the agarose beads in 40 μl of 2× Laemmli reducing sample buffer and boiled for 5 min. The bound proteins were eluted by boiling for 5 min and resolved in 12% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with the anti-Rac1 antibody as described above.

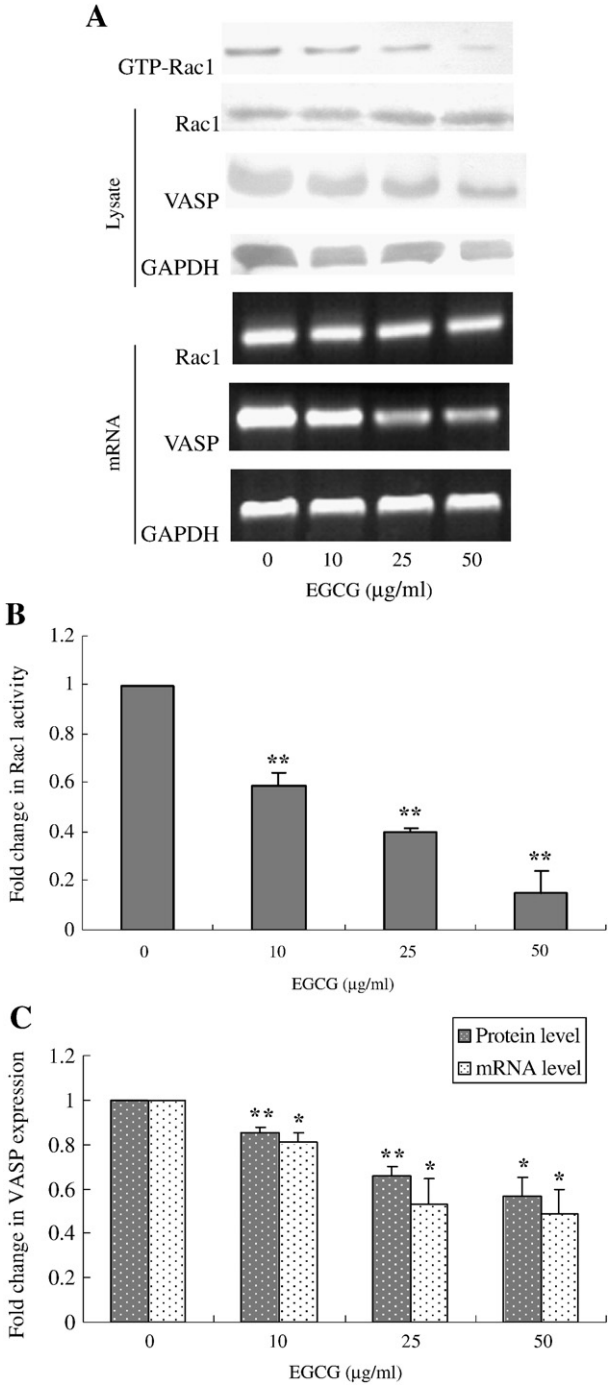

Fig. 2. EGCG revealed dose-dependent inhibition of VASP expression and Rac1 activity in MCF-7 cells. (A) Serum-starved MCF-7 cells were cultured in EGCG at different concentrations (0, 10, 25, and 50 μg/ml) for 24 h. The amount of GTP-bound Rac1 in cell lysates was determined by GST pull-down analysis, followed by Western blotting with anti-Rac1 antibody (top panel). The protein expression levels of Rac1, VASP, and GAPDH in cell lysates were determined by Western blotting (middle panels). The mRNA expression levels of Rac1, VASP, and GAPDH in cells were analyzed by RT-PCR (bottom panels). GAPDH was taken as an internal control. (B) The histogram shows the fold change of Rac1 activity in MCF-7 cells after treatment with different concentrations of EGCG relative to the control cells (treated with 0 μg/ml of EGCG). (C) The histogram shows the fold changes of VASP expression at the protein and mRNA levels after EGCG stimulation. The data shown are means ± S.D. of triplicate experiments. *$P<0.05$, **$P<0.01$ *vs.* control group.

2.6. Wound healing assay (two-dimensional cell migration assay)

After being serum-starved for 12 h in serum-free RPMI-1640 medium in 6-well plates, a confluent monolayer of MCF-7 cells was transfected with segments of siRNA for 48 h, or treated with EGCG (50 μg/ml), or docetaxel (0.05 μg/ml) for 24 h and the control cells were treated with serum-free RPMI-1640 medium. The monolayer was then scraped with a sterile 200 μl pipette tip into a 6-well-plate and washed with PBS. After this, time-lapse images were captured using an inverted phase-contrast microscope at 100× magnification for 48 h. The cell migration was evaluated by calculating the average cell migration velocity using the following formula:

The average cell migration velocity = migration distance(μm)/migration time(h), and migration index = migration velocity of experimental group/migration velocity of control group.

2.7. Cell migration and invasion assays

The cell migration assays were performed with the Transwell system, which allows cells to migrate through an 8-μm pore size polycarbonate membrane. The procedure for the invasion assay was the same as in the cell migration assay, except that the upper side of polycarbonate membrane was coated with a layer of 25 μg/cm^2 Matrigel®. The gels were allowed to polymerize for 2 h at 37 °C and had a minimum thickness of 20 μm. Serum-free RPMI-1640 medium was initially added to the 24-well plate well (the lower chamber of Transwell), and then to the Transwell insert (the upper chamber of Transwell), which then was equilibrated overnight to allow cell attachment at 37 °C in a incubator supplemented with 5% CO_2. Cells were trypsinized, washed, and resuspended in RPMI-1640 medium containing 10% calf serum (1×10^6 cells/ml). This suspension (100 μl) was added to the upper chamber of the Transwell. The lower chamber was filled with 600 μl RPMI-1640 medium with 20% calf serum. MCF-7 cells were adhered to the Transwell filters during a 2 h incubation at 37 °C in the presence of 5% CO_2. Then the RPMI-1640 medium containing 10% calf serum in the upper chamber was replaced with serum-free RPMI-1640 medium or EGCG (50 μg/ml) or EGF (15 ng/ml). After incubation for 18 h at 37 °C in the presence of 5% CO_2, the cells were fixed for 30 min in 4% formaldehyde and stained for 15 min with crystal violet. Filters were then rinsed thoroughly in distilled water and checked by bright-field microscopy to ensure that the cells were adherent and had migrated. The non-migrating cells were then carefully removed from the upper surface (inside) of the Transwell with a wet cotton swab. To quantify cell motility, cells that had migrated to the bottom surface of the filter were counted. Nine evenly spaced fields of cells were counted in each well, using an inverted phase-contrast microscope at 200× magnification.

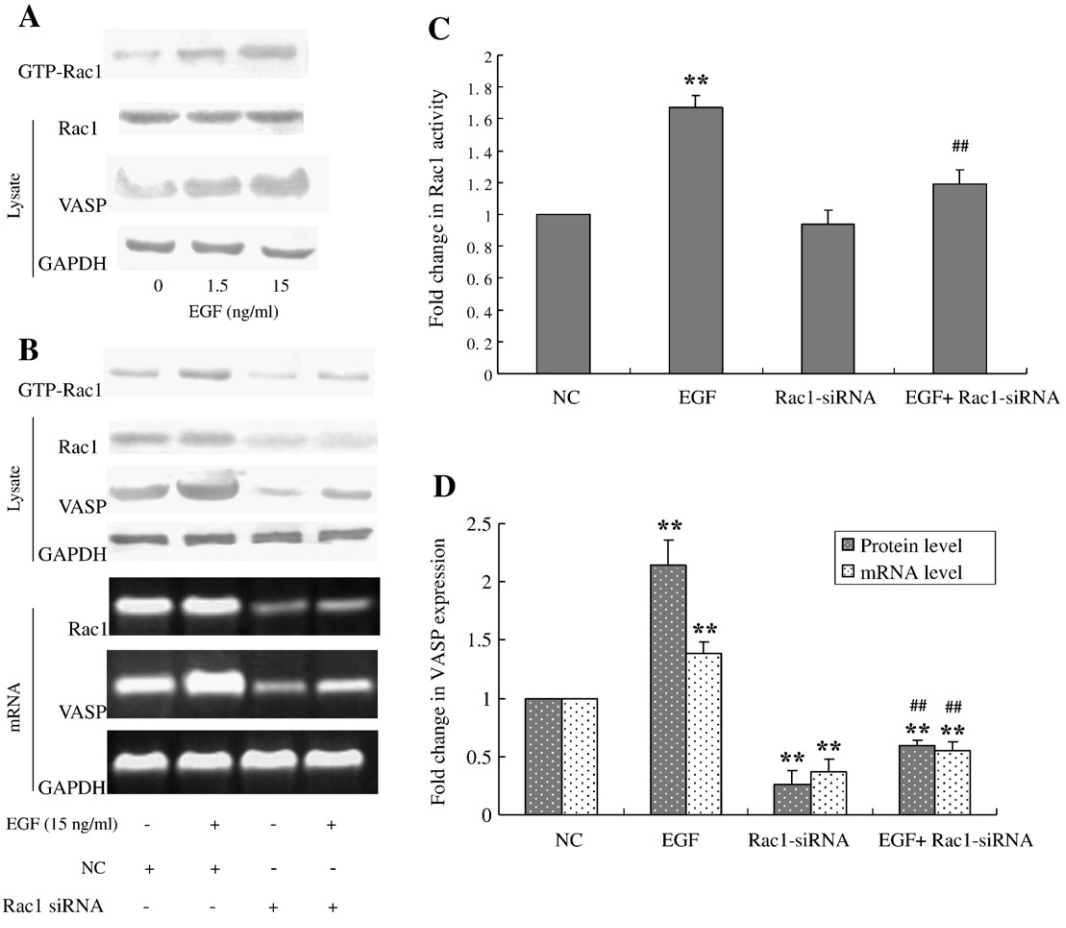

Fig. 3. VASP expression levels were up-regulated by EGF and were mediated *via* EGF-induced Rac1 activation. (A) Serum-starved MCF-7 cells were treated with EGF of 0, 1.5, and 15 ng/ml for 15 min and then lysed. The amount of GTP-Rac1 in cell lysates, which is shown in the top panel, was analyzed by GST pull-down assay, followed by Western blotting with an anti-Rac1 antibody. The expression levels of Rac1, VASP, and GAPDH in cell lysates were determined by Western blotting and are shown on the bottom. (B) MCF-7 cells transfected with Rac1 siRNA or negative control siRNA (NC) for 48 h were serum starved overnight and then treated with or without 15 ng/ml of EGF for 15 min as indicated. The amount of GTP-bound Rac1 in cell lysates was determined by GST pull-down analysis, followed by Western blotting with an anti-Rac1 antibody (top panel). The protein expression levels of Rac1, VASP, and GAPDH in cell lysates were determined by Western blotting (middle panels). The mRNA expression levels of Rac1, VASP, and GAPDH in cells were analyzed by RT-PCR (bottom panels). (C) The amounts of GTP-bound Rac1 were normalized by using those of total Rac1 protein present in cell lysates and are expressed as the fold induction relative to cells transfected with the negative control siRNA. (D) The protein and mRNA expression levels of VASP were standardized by using that of GAPDH in cells and were shown as the fold change relative to NC group. The data shown are means±S.D. of triplicate experiments. $^{**}P<0.01$ *vs.* NC group. $^{\#\#}P<0.01$ *vs.* EGF group.

2.8. Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M.) of multiple experiments. Paired Student's *t*-tests were used to compare two groups or one-way analysis of variance (ANOVA) with Student–Newman–Keul's test for multiple comparisons. A probability value of $P<0.05$ was considered statistically significant.

3. Results

3.1. EGCG decreased the migration and invasion of MCF-7 cells in a concentration- and time-dependent manner

To study the effect of EGCG on MCF-7 cell migration and invasion, we treated cells with EGCG (0, 10, 25, 50, and 100 μg/ml) or docetaxel (0.05 μg/ml) as the positive control, and then conducted wound healing assay and cell migration/invasion assay (Fig. 1). These results showed that the invasion and migration of MCF-7 cells were significantly reduced by EGCG at an initial concentration of 50 μg/ml ($P<0.01$ in the wound healing assay, Fig. 1A; $P<0.05$ in the cell migration/invasion assay, Fig. 1B), and its inhibitory effects were similar to that of the positive control (docetaxel 0.05 μg/ml) (Fig. 1A and B). To further investigate the inhibitory effect of EGCG on the migration behavior of MCF-7 cells over time, we cultured cells with EGCG (50 μg/ml) and captured time-lapse images during a time course of 0 h, 6 h, 12 h, 18 h, 24 h, and 30 h after EGCG treatment in the wound healing assay. These findings allowed the calculation of the transient cell migration velocity at 3 h, 9 h, 15 h, 21 h, and 27 h in order to reveal changes in the migration ability of treated cells. As shown in Fig. 1C, the migration velocity of MCF-7 cells diminished over time after EGCG treatment. These findings suggested that EGCG could inhibit the invasive migration ability of MCF-7 cells not only in a dose-dependent manner but also in a time-dependent manner.

3.2. EGCG revealed dose-dependent inhibition of VASP expression and Rac1 activity in MCF-7 cells

Because our previous studies had shown that the novel expression of VASP and activity of Rac1 in breast cancer cells promote migration and invasion, we investigated the expression of VASP following exposure to EGCG. From an initial concentration of 10 μg/ml, EGCG began to reduce the expression of VASP and the activity of Rac1, which were found to be reduced in a concentration-dependent manner (Fig. 2A). To determine whether the inhibition by EGCG in MCF-7 cells could be further regulated by the Rac1 signaling pathway, the activity of Rac1 was assessed by GST pull-down assays. As shown in Fig. 2B, EGCG at a concentration of 10 μg/ml decreased Rac1 activity to 59.1% compared to the control group. Accordingly, the expression of VASP was attenuated to 85.4% ($P<0.01$) at the protein level and 80.8% at the mRNA level ($P<0.05$) (Fig. 2C). However, the total Rac1 protein expression remained unchanged, even after treatment with 50 μg/ml of EGCG. These findings suggest that EGCG could inhibit cellular migration and invasion not only by the suppression of VASP expression but also by the down-regulation of the Rac1 signaling pathway.

3.3. VASP expression levels were up-regulated by EGF-induced Rac1 activation

In an attempt to identify the role of EGF on the expression level of VASP and activation of Rac1 in MCF-7 cells, serum-starved MCF-7 cells were stimulated by EGF (0, 1.5, 15 ng/ml) for 15 min. Then Western blotting was conducted to identify the amounts of VASP, Rac1, and GAPDH in cell lysates. These findings demonstrated that the protein expression level of VASP was up-regulated by EGF in a concentration-dependent manner (Fig. 3A). The activation of Rac1 also revealed a dose-dependent increase that was concomitant with the changes in VASP expression (Fig. 3A). To characterize the relationship between EGF-induced Rac1 activation and changes in VASP expression levels in MCF-7 cells, a loss-of-function approach was applied. We knocked down Rac1 in MCF-7 cells by using Rac1-specific siRNA, and then detected the expression of VASP mRNA and protein levels, as well as Rac1 activity after treatment with 15 ng/ml of EGF. These results revealed that Rac1-siRNA depressed Rac1 expression at both the mRNA and protein levels (Fig. 3B). The transcription and translation of VASP were reduced to 36.3% ($P<0.01$) and 25.9% ($P<0.01$), respectively (Fig. 3D). On the other hand, EGF stimulation of MCF-7 cells that had Rac1 silenced by siRNA, elevated Rac1 activation in these cells (Fig. 3B and C), and the expression of VASP was also up-regulated at both the mRNA and protein levels (Fig. 3B and D). These findings implied that EGF induced the transcription and translation of VASP, which was mediated by activation of Rac1 in MCF-7 cells.

3.4. siRNA mediated knock-down of Rac1 decreased the amount of VASP mRNA, but VASP siRNA revealed no effect on the expression level of Rac1

Specific siRNA for Rac1 was sufficient to knockdown Rac1 expression levels and depress VASP gene transcription and translation (data not shown). To test if the gene transcription and translation of VASP were affected by the expression level of Rac1 within breast cancer cells, a loss-of-function approach was applied. The Rac1-siRNA construct effectively inhibited Rac1 gene transcription and translation in MCF-7 cells, which resulted in reduced expression levels of Rac1 mRNA by 57.1% (Fig. 4B, $P<0.01$) in contrast to the negative control group (NC) after transfection. Furthermore, we found that the Rac1-siRNA transfection also diminished VASP expression at the mRNA level by 59.6% ($P<0.01$) (Fig. 4B) compared to the NC group. In order to

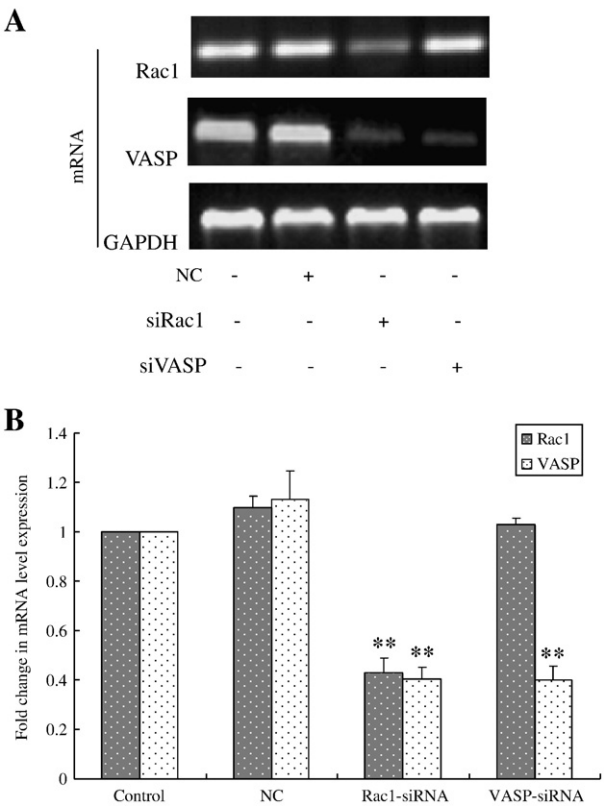

Fig. 4. siRNA mediated knock-down of Rac1 decreased the amount of VASP mRNA, but VASP siRNA revealed no effect on the expression level of Rac1. (A) RT-PCR analyses of mRNA extracted from MCF-7 cells which were treated by Rac1-siRNA, VASP-siRNA, or NC at 48 h after transfection. GAPDH was used as an internal control. (B) Semi-quantification of RT-PCR analysis of the mRNA expression levels of VASP and Rac1 in three independent experiments was shown. These results are presented as means ± S.D. of three independent experiments. $^{**}P<0.01$ *vs.* NC group.

demonstrate the interaction between VASP and Rac1 more rigorously, we examined the mRNA expression of Rac1 in VASP-siRNA transfected MCF-7 cells using RT-PCR. These results showed that the mRNA expression level of Rac1 was not reduced by the VASP-siRNA compared with the NC group ($P>0.05$) (Fig. 4A and B). In addition, it revealed that a change in VASP expression level does not affect the transcription of Rac1.

3.5. EGCG inhibited EGF-induced MCF-7 cells migration and invasion

We have previously shown that EGF up-regulated VASP expression *via* Rac1 activation. To further investigate the inhibitory function of EGCG on the migration and invasion of EGF-induced MCF-7 cells, we have examined the migration and invasion potential of these cells. Cells were cultured in four groups as indicated in Fig. 5A and B. The migration velocity of EGF treated cells was higher than that of the control group in a wound healing assay, while EGCG and EGF co-incubated cells migrated much slower than the cells that had been only incubated with EGF ($P<0.01$) (Fig. 5A). Similar results were also observed in cell migration/invasion assay ($P<0.01$) (Fig. 5B). These findings demonstrated that EGCG inhibited the invasive migration potential of EGF-induced MCF-7 cells.

3.6. Knocked down of Rac1 or VASP by siRNA diminished EGF-induced MCF-7 cells migration and invasion

To further investigate the role of Rac1 and VASP in EGF-induced cells invasion and migration, specific siRNAs for Rac1 or VASP were transfected into MCF-7 cells to knock down the expression of Rac1 or VASP before the cells were treated with EGF (15 ng/ml). Then we

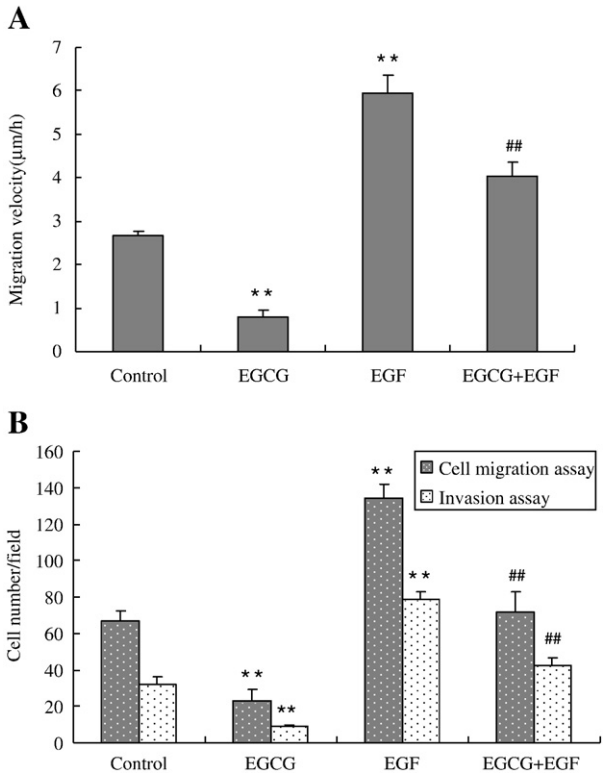

Fig. 5. EGCG inhibited EGF-induced MCF-7 cells invasive migration. (A) and (B) MCF-7 cells were treated with serum-free RPMI-1640 medium or EGCG (50 μg/ml) or EGF (15 ng/ml) or EGCG (50 μg/ml) combined with EGF (15 ng/ml) in wound healing assay and cell migration/invasion assay, respectively. More aggressive migration and invasive abilities in MCF-7 cells were induced by EGF and the effect was significantly inhibited by EGCG. Three independent experiments were performed. Results are presented as means±S.D. of these independent experiments. $^{**}P<0.01$ *vs.* control group, and $^{\#\#}P<0.01$ *vs.* EGF group.

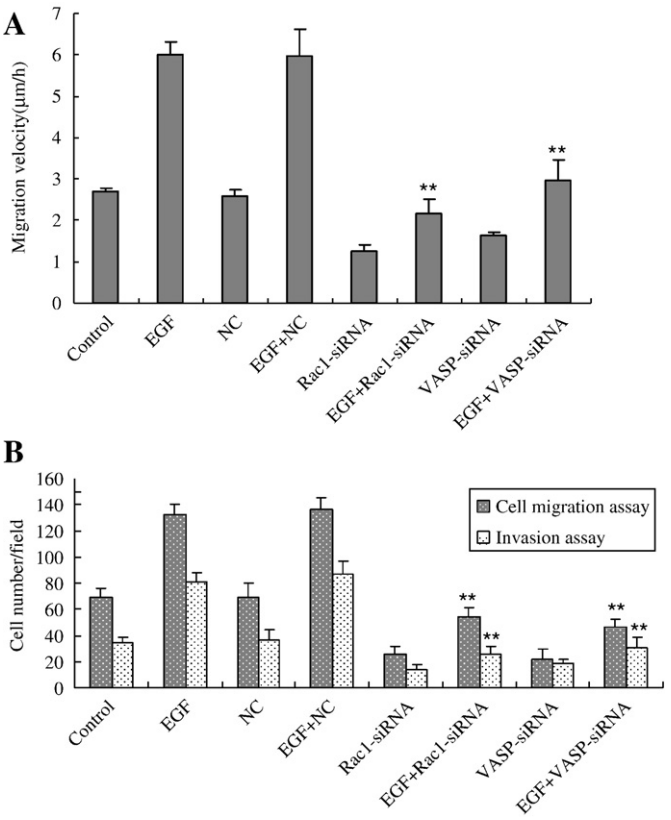

Fig. 6. Knocked down of Rac1 or VASP by siRNA diminished EGF-induced MCF-7 cell migration and invasion. The MCF-7 cells were transfected with Rac1-siRNA, VASP-siRNA, or NC siRNA for 24 h, and then treated with or without EGF (15 ng/ml) for 1 h. (A) The Rac1-siRNA or VASP-siRNA segment depressed the two-dimensional migration capacity of MCF-7 cells induced by EGF. (B) The Rac1-siRNA or VASP-siRNA segment depressed the three-dimensional migration and invasion capacities of MCF-7 cells induced by EGF. Three independent experiments were performed. Results are presented as means±S.D. of these independent experiments. $^{**}P<0.01$ *vs.* EGF+NC group.

assessed the migration and invasion capacities in wound healing assay as well as cell migration/invasion assay. The experimental data demonstrated that both Rac1-siRNA and VASP-siRNA segments inhibited the migration and invasion of EGF-induced MCF-7 cells. Rac1-siRNA and VASP-siRNA transfection reduced the migration velocity of EGF treated MCF-7 cells to 36.5% ($P<0.01$) and 49.7% ($P<0.01$), respectively (Fig. 6A). As for the cell migration and invasion assays, the cell number in each field of Rac1-siRNA or VASP-siRNA group induced by EGF was much less than that of the EGF+NC group (40.2%, $P<0.01$ or 33.8%, $P<0.01$ of the EGF+NC group in the cell migration assay; 29.7%, $P<0.01$ or 34.7%, $P<0.01$ of EGF+NC group in the invasion assay) (Fig. 6B). These results revealed that the loss-of-function of Rac1 or VASP reduced EGF-induced migration and invasion of MCF-7 cells.

3.7. EGCG antagonized the EGF-induced up-regulation of VASP expression by depressing the activation of Rac1

To identify whether the reduction of VASP expression and the inhibition of Rac1 activation caused by EGCG was important for the EGF signaling pathway, we treated serum-starved MCF-7 cells with 50 μg/ml of EGCG for 24 h and then stimulated with 15 ng/ml of EGF for 15 min. Western blotting was performed to detect the expression levels of VASP, Rac1, and GAPDH in cell lysates, and GST pull-down assays were conducted to assess the activation of Rac1. These results demonstrated that EGF up-regulated VASP expression (2.1 fold compared to the control group, $P<0.01$, Fig. 7C) and Rac1 activation (2.2 fold compared to the control group, $P<0.01$, Fig. 7B), which had been

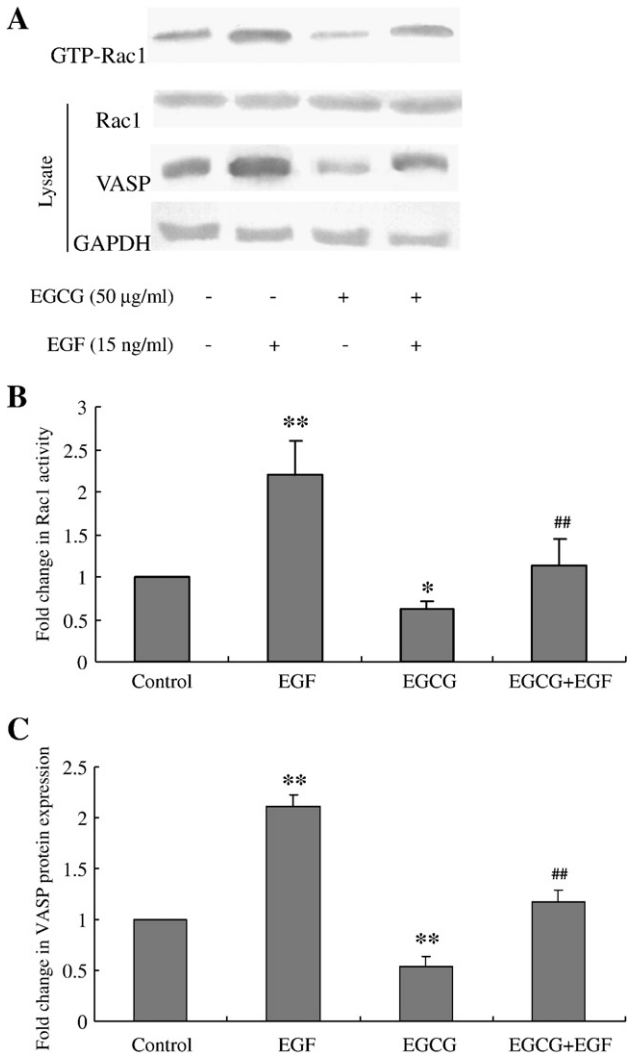

Fig. 7. EGCG antagonized the EGF-induced activation of Rac1, as well as the consequent up-regulation of VASP expression. (A) MCF-7 cells that had been serum starved for 12 h previously were treated with or without 50 μg/ml of EGCG for 24 h and then were stimulated with 15 ng/ml of EGF or not for 15 min as indicated. The amount of GTP-bound Rac1 in cell lysates was determined by GST pull-down analysis, followed by Western blotting with the anti-Rac1 antibody. The expression levels of Rac1, VASP, and GAPDH in cell lysates were determined by Western blotting. (B) The amounts of GTP-bound Rac1 were normalized by using those of total Rac1 protein present in cell lysates and were expressed as the fold induction relative to control cells which were treated with neither EGCG nor EGF to represent the change of Rac1 activity. (C) The quantity of VASP was standardized by using levels of GAPDH in cells and was shown as the fold change relative to control cells. The data shown are means ± S.D. of triplicate experiments. *$P<0.05$ or **$P<0.01$ *vs.* control group. ##$P<0.01$ *vs.* EGF group.

treated with EGCG and had attenuated Rac1 activity to 51.1% ($P<0.01$, *vs.* EGF group, Fig. 7B) and VASP expression to 55.7% ($P<0.01$, *vs.* EGF group, Fig. 7C).

4. Discussion

Green tea is one of the most widely consumed beverages in the world. It has been reported to provide potential protection against numerous cancers and has been shown to inhibit bladder tumors in different animal models (Kemberling et al., 2003; Chen et al., 2004; Kamat and Lamm, 2002; Sato and Matsushima, 2003). Epidemiological studies have suggested that the low incidence of some cancers in Asian countries is linked to the regular consumption of green tea (Nakachi et al., 1998; Imai et al., 1997; Galanis et al., 1997).

EGCG is a polyphenolic compound from green tea and has been shown to have anti-tumor activities such as inhibiting adhesion, migration, and proliferation of tumor cells (Hung et al., 2005). Indeed, many laboratory studies have demonstrated the inhibitory effects of tea polyphenols on tumor formation and growth. For example, Rieger-Christ et al. demonstrated that EGCG inhibited bladder carcinoma cell growth and suppressed the *in vitro* migration capacity of cells *via* down-regulation of N-cadherin and inactivation of Akt signaling (Rieger-Christ et al., 2007). Pezzato et al. found that EGCG, which inhibited prostate-specific antigen activities in a dose-dependent manner, was a natural inhibitor of prostate carcinoma aggressiveness (Pezzato et al., 2004). However, there is no conclusive verdict about the mechanisms and signaling pathways underlying the potential anticancer effects of EGCG in breast cancer cells. Therefore, we examined whether EGCG could inhibit MCF-7 cell migration and invasion, and investigated the mechanisms underlying EGCG's effects on MCF-7 cell migration and invasion. Many signaling molecules and signal transduction pathways have been demonstrated or proposed to be involved in the EGF-induced activation of Rac1, as well as the accompanying lamellipodia formation and membrane ruffling, and some examples include Vav2, Ras, Abl-Sos-1, PLC, and PI 3-kinase (Marcoux and Vuori, 2003; Sini et al., 2004; Bar-Sagi and Hall, 2000; Tamas et al., 2003; Scita et al., 2000). As a major member of the Rho GTPase family, Rac1 has been implicated in tumorigenesis (Qiu et al., 1995), tumor angiogenesis (Soga et al., 2001), invasion and metastasis (Zhuge and Xu, 2001), cell-cycle control, and apoptosis (Sherr and Roberts, 1999). During cell migration, activated Rac and Cdc42 induce reorganization of the actin cytoskeleton at the leading edge (Small et al., 2002). The vasodilator-stimulated phosphoprotein (VASP), which was first described in human platelets, is one member of the Ena/VASP family of cytoskeletal regulatory proteins (O'Connor et al., 2000). Therefore, it appears that multiple pathways elicited by EGF converge upon Rac1 activation, thereby promoting cell migration and invasion. In previous studies, we have shown that the migration and invasion of human breast cancer cells are positively regulated by VASP *via* the Rac1 pathway. Interestingly, this was consistent with our findings that the activity of Rac1 and VASP expression were stimulated by EGF in MCF-7 cells.

To further study the interaction between EGF-induced Rac1 activation and the change in VASP expression, a loss-of-function approach using siRNAs for VASP and Rac1 was applied. The blockage of Rac1 expression by Rac1-siRNA significantly inhibited EGF-induced VASP expression, while VASP siRNA did not affect the expression level of Rac1. These studies implied that VASP expression levels were up-regulated by EGF and were mediated by Rac1 activation, which was consistent with the results of the functional wound healing assay and cell migration/invasion assay. The wound healing assay and cell migration/invasion assay revealed that EGF treated MCF-7 cells exhibited clearly stronger migration and invasion abilities. While either Rac1 or VASP was silenced by siRNA, the EGF-induced invasive migration ability was sharply reduced.

Finally, we evaluated the effect of EGCG on EGF-induced Rac1 activation, VASP expression, and MCF-7 cell invasion and migration. These results showed that EGCG antagonized EGF-induced activation of Rac1 together with the concomitant up-regulation of VASP expression. In wound healing assay and cell migration/invasion assay, EGCG inhibited EGF-induced MCF-7 cell migration and invasion. Therefore, we conclued that the attenuation of VASP expression *via* the Rac1 pathway contributed to the additional mechanisms involved in EGCG's effects on the migration and invasion of MCF-7 cells.

In conclusion, our results demonstrated that EGCG could attenuate the activity of Rac1 and, consequently, down-regulated VASP expression in MCF-7 cells. In turn, this contributed to the inhibitory effect of EGCG on the invasion and migration of MCF-7 breast cancer cells. Furthermore, the effective concentration of EGCG for inhibiting cell invasion and migration started at 50 μg/ml, while the expression of Rac1 and VASP was effected at 10–25 μg/ml. These findings were congruent with the related studies concerning the effective concentration

of EGCG on invasion and migration of breast cancer cells, as well as the corresponding signaling pathway(s) (Punathil et al., 2008; Huang et al., 2008; Thangapazham et al., 2007). Moreover, these findings help substantiate the value of EGCG as an anti-cancer factor *in vitro*. Nevertheless, further animal and clinical studies should be conducted to confirm the role of EGCG as therapeutic against breast cancer, as well as the corresponding mechanisms. In addition, the optimal concentration and administration route of EGCG when functioning in the human body as an anti-cancer drug should also be investigated. After all, the factors influencing therapeutic benefit are much more complex *in vivo*, and might involve a series of steps related to the absorption, digestion, distribution, and metabolism of this drug.

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