Positive regulation of migration and invasion by vasodilator-stimulated phosphoprotein via Rac1 pathway in human breast cancer cells

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Abstract. This study aimed to investigate the role of the cytoskeleton-associated protein vasodilator-stimulated phosphoprotein (VASP) on the migration and invasion of human breast cancer cells and its relationship to Rac1 which is a member of the Rho family and has been found to be implicated in tumorigenesis, invasion and metastasis. We detected the mRNA and protein expression levels of VASP and Rac1 of the non-invasive breast cancer cell line MCF-7 as well as the invasive cell line MDA-MB-231 by RT-PCR and Western blotting. GST pull-down assay was used to examine the activitiy of Rac1. Accordingly, the cell invasive migration ability was analyzed in a wound-healing assay (2D) and transwell assays (3D migration and invasion). We then used VASP-siRNA to inhibit the expression of VASP in breast cancer cells in order to study the relationship between the VASP expression level and the invasive migration ability of breast cancer cells. Rac1-siRNA was used to decrease the expression of Rac1, and observe its effect on the VASP expression level together with the migration and invasion ability of MCF-7 and MDA-MB-231 cells. Our results revealed that the invasive breast cancer cell line MDA-MB-231 showed a higher Rac1 activity and VASP expression level compared with the non-invasive MCF-7. Inhibition of Rac1 or VASP by siRNA, respectively, decreased the migration and invasion ability of breast cancer cells and the transfection of Rac1 siRNA-mediated reduction of VASP expression at mRNA and protein levels. Collectively, our data showed that the higher expression level of VASP contributed to a higher invasive migration capacity of human breast cancer cells which was controlled by the Rac1 pathway.

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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 makes this disease refractory and incurable. The phenotypes associated with metastatic breast cancer cells include up-regulated cell growth and survival, decreased cell-cell adhesion, increased ability to degrade the extracellular matrix and increased cell motility and the ability of invasion. Although the physiology of the breast has become better understood, the precise molecular and genetic mechanisms of metastasis leading to these malignant phenotypes remain unclear.

The GTPase proteins Rac, Cdc42 and Rho are members of the Rho family, a subset of the Ras superfamily. They are involved in many signal transduction pathways that control a broad array of essential cellular functions such as reorganization of the actin cytoskeleton, gene expression, cell cycle

progression and cell-cell adhesion (1). Rearranging the actin cytoskeletal proteins in response to Rho GTPase is important for the ability of tumor cells to metastasize. Overexpression of Rho GTPase has been linked to the pathogenesis and metastasis of human breast cancer. To characterize the contribution of Rho GTPases to cancer initiation and progression requires an understanding of how Rho GTPase is regulated in non-invasive and invasive tumor cells. As a significant member of the Rho family, Rac1 has been implicated in tumorigenesis (2), tumor angiogenesis (3), invasion and metastasis (4), cell-cycle control and apoptosis (5). During cell migration, activated Rac and Cdc42 induce reorganization of the actin cytoskeleton at the leading edge (6). Localized actin polymerization at the leading edge pushes the membrane forward in finger-like structures known as filopodia and in sheet-like structures known as lamellipodia. These structures generate the locomotive force in migrating cells. Our attention was drawn to the fact that the relative contribution of each Rho GTPase to cytoskeletal rearrangements depends on the specific cell condition and cell type. In some cell types Rac1 up-regulation was shown to result in the inhibition of migration such as Rac1 overexpression in Madin-Darby canine kidney (MDCK) epithelial cells which resulted in the inhibition of migration and invasion (7). Migration was shown to be unchanged in Rac1-deficient colon carcinoma cells (8), rat fibroblasts (9) and macrophages (10). However, experimental data exist supporting the view that the increased Rac1 expression was related to higher TNM stages of gastric carcinoma (11). The expression of a dominant-negative form of Rac in elongated cells, such as B16F10 melanoma cells, inhibits the formation of such protrusive membrane structures, causing decreased cell migration (12). Thus, we aimed to investigate the role Rac1 plays in breast cancer cells.

Ena/VASP proteins are actin-binding proteins that localize to actin stress fibers, the tips of filopodia and the lamellipodial leading edge. A number of apparently conflicting studies have confused the Ena/VASP field, pointing to roles for these proteins in the promotion and inhibition of actin-dependent processes (13). Deletion of the Ena/VASP-binding sites within the bacterial protein ActA led to a decrease in the actin-dependent intracellular motility of Listeria monocytogenes (14). However, fibroblasts devoid of Ena/VASP proteins exhibit increased rates of cell motility (15). Therefore, there is controversy concerning the function of Ena/VASP proteins in differential cell motility and migration. VASP regulation of the cytoskeleton is mediated through interaction with or regulation of Rho GTPases. Fryer and Field (16) indicated that there was overlap in the phenotypic regulation by PKG, Rac and Pak especially in endothelial permeability, focal adhesion remodeling, and angiogenesis. PKG and its substrate VASP appear to regulate Rac/Pak activity. An experiment was performed showing that cardiac fibroblasts from VASP knockout mice display increased spreading, prolonged Rac and Pak activation in response to PDGF, and an inability to properly orient in a monolayer wound-healing assay (17). The data provide evidence that VASP is a negative regulator of Rac and Pak, and VASP is a common convergence point for cGMP and Rho GTPase signaling. However, in the presence of N17 rac1 which suppressed progression, dominant negative GTPase were transfected into malignantly transformed cancer cells, VASP was down-regulated, and vinculin and F-actin colocalization restored (18). Therefore, there is no final conclusion regarding the relationship between Rac1 and VASP in tumor cells.

Double-stranded (ds) RNA has been known to mediate sequence-specific gene silencing in a range of organisms, including plants and *C. elegans*. It has been found that 21 short nucleotide (nt) ds RNA molecules, known as short interfering RNAs (siRNAs), can mediate RNAi in mammalian cell lines (19). Pan has successfully suppressed the Rac1 expression by using Rac1-specific siRNA and demonstrated that the conditioned medium from these Rac1 siRNA-targeted cancer cells can effectively inhibit endothelial cell proliferation (12). We used siRNA oligos which is a process whereby the introduction of double-stranded RNA (dsRNA) into cells results in the degradation of homologous mRNA strategies on MCF-7 and MDA-MB-231 cells to knock down Rac1 level. In our study, in order to gain insight into whether a higher activity of Rac1 and an increased expression of VASP were related to a higher invasive migration capacity of breast cancer cells, we investigated the mRNA and protein levels of MCF-7 and MDA-MB-231 cells. We have characterized for the first time the effects of direct inhibition of Rac1 GTPase and VASP on the intrinsic migratory behaviors of the two breast cancer cell lines that possessed a differential capacity of migration and invasion. Our study demonstrated that by increasing its activity Rac1 promoted the migration and invasion capacity of breast tumor cells by controlling VASP expression, located on the downstream of Rac1, positively. Therefore, Rac1 and its effectors may serve as important targets for cancer therapeutics.

Materials and methods

Materials and chemicals. Human breast cancer MCF-7 and MDA-MB-231 cells were preserved by the Department of Pathophysiology, Medical College of Wuhan University. RPMI-1640 medium was from Hyclone Company. The monoclonal mouse anti-VASP antibody (IE273) was obtained from ImmunoGlobe Company. Anti-Rac1 (23A8 clone) and Rac1 activation assay kit were purchased from Upstate Biotechnology. The DAB kit was purchased from Beijing Zhongshan Biotechnology Co., Ltd. The 24-well transwell system was from Costar Corporation. fMLP was purchased from Sigma Company.

Cell culture and siRNA transfection. Human breast cancer MCF-7 and MDA-MB-231 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, MCF-7 or MDA-MB-231 cells were seeded in 6-well plates and grown overnight in RPMI-1640 medium supplemented without calf serum or antibiotics to reach ~60-70% confluence. The RNA oligos containing 21 nucleotides were synthesized in sense and anti-sense directions corresponding to human Rac1 (Genbank accession number AF498964) siRNA1 at nucleotides 439-459 bp (sense: 5'-GGAGATTG GTGCTGTAAAA-3' and anti-sense: 5'-UUUUACAGCACC AAUCUCC-3'), siRNA2 at nucleotides 199-217 bp (sense: 5'-CUACUGUCUUUGACAAUUA-3' and anti-sense: 5'-UAA

UUGUCAAAGACAGUAG-3'), and siRNA3 at nucleotides 328-346 bp (sense: 5'-CAUCCUAGUGGGAACUAAA-3' and anti-sense: 5'-UUUAGUUCCCACUAGGAUG3') with dTdT overhangs at each 3' terminus (Shanghai GenePharma Co., Ltd.). The RNA oligos containing the 21 nucleotides were synthesized in sense and anti-sense directions corresponding to human VASP (Genbank accession number BC038224) siRNA1 at nucleotides 942-960 bp (sense: 5'-CAACCUUGC CAAGGAUGAATT-3' and anti-sense: 5'-UUCAUCCUUGG CAAGGUUGTG-3'), siRNA2 at nucleotides 1002-1020 bp (sense 5'-CGCCCAGCUCCAGUGAUUATT-3' and anti-sense: 5'-UAAUCACUGGAGCUGGGCGTG-3') and siRNA3 at nucleotides 1024-1042 bp (sense 5'-GGACCUACAGAGG GUGAAATT-3' and anti-sense: 5'-UUUCACCCUCUGUAG GUCCGA-3'). The selected sequences were submitted to a BLAST search against the human genome to ensure that only the selected genes were targeted. One negative siRNA control (sense 5'-UUCUCCGAACGUGUCACGU-3' and anti-sense 5'-ACGUGACACGUUCGGAGAA-3') was supplied. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen Co., Ltd.). The day prior to transfection, cells were trypsinized, diluted with fresh medium and then transferred to 6-well plates. Transfection of siRNAs was carried out using Lipofectamine 2000 reagent. Lipids and siRNAs were diluted into the RPMI-1640 medium, respectively. Diluted lipids were mixed with diluted siRNAs and the mixture was incubated for 20 min at room temperature for complex formation. After the addition of RPMI-1640 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, the cells were fixed for RT-PCR analysis or scraped for Western blotting.

Western blotting. The 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 zNaF, 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 x g for 15 min at 4˚C. The protein concentration of the supernatant lysate was determined by Coomassie brilliant blue assay. For direct immunoblotting, lysate aliquots were mixed with 5X sample buffer containing 2-mercaptoethanol and boiled for 5 min before loading on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel with a 5% (v/v) stacking 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, and 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 a different dilution of different antibodies according to their descriptions. After extensive washing, membrane strips were incubated with a different dilution 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) or ECL immune enzymic reaction. The former was stopped by rinsing in distilled water.

Reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR was used to determine the target sequences of Rac1 and VASP in human breast cancer cells including MCF-7 and MDA-MB-231. Total RNA was purified using total RNA isolation system (Promega) and RT-PCR was performed using the Acess RT-PCR system (Promega). The specific primers and thermocycler for Rac1 and VASP were as follows: the upstream and downstream primers of Rac1 were 5'-ATGTGCTCTCCCGAGTAACC-3' and 5'-GGAGGAAA CTGAGGCAACAC-3', respectively. Rac1 reaction parameters were 94˚C, 40 sec; 54˚C, 30 sec and 72˚C, 30 sec for 30 cycles. The upstream and downstream primers of VASP were 5'-CCACTGAACTTCTGATTCGC-3' and 5'-GCGTGCT AGCTGGATGTCTT-3'. VASP reaction parameters were 94˚C, 40 sec; 60˚C, 30 sec and 72˚C, 30 sec for 30 cycles. GAPDH was employed as an internal control for the mRNA and protein analyses.

Rac1 activation assay. The GTPase activity of Rac1 was measured as described by the instructions. Cells were rinsed with cold PBS and lysed in MLB buffer. 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 the reaction mixture was gently rocked at 4˚C for 60 min, and the agarose beads were collected by pulsing for 5 sec in the microcentrifuge at 14,000 x g to remove and discard the supernatant. The beads were washed 3 times with MLB, resuspended in 40 μl of 2X 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 anti-Rac1 antibody as described above. The experiments were carried out in triplicate.

Two-dimensional (2D) cell migration assays. A two-dimensional cell migration assay using a wound-healing model was performed as previously described (20). After being serum-starved for 12 h in serum-free RPMI-1640 medium in 6-well plates, the former monolayer was scraped with a sterile 200 μl pipette tip into a 6-well-plate and washed with PBS. We then transfected a series of different segments of Rac1- or VASP-siRNA to the monolayer with the control cells treated with nothing. Time-lapse images were captured using an inverted phase-contrast microscope at x100 magnification for 48 h. The cell migration was evaluated by calculating the average cell migration velocity using the formula: average cell migration velocity = migration distance (μm)/migration time (h), and migration index = migration velocity of experimental group/migration velocity of nc group.

Three-dimensional (3D) cell migration and invasion assays. A three-dimensional cell migration assay was performed with the transwell system, which allows cells to migrate through an 8-μm pore size polycarbonate membrane with or without a layer of 25 μg/cm^2 Matrigel® (Becton-Dickinson, Heidelberg, Germany) on it. 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

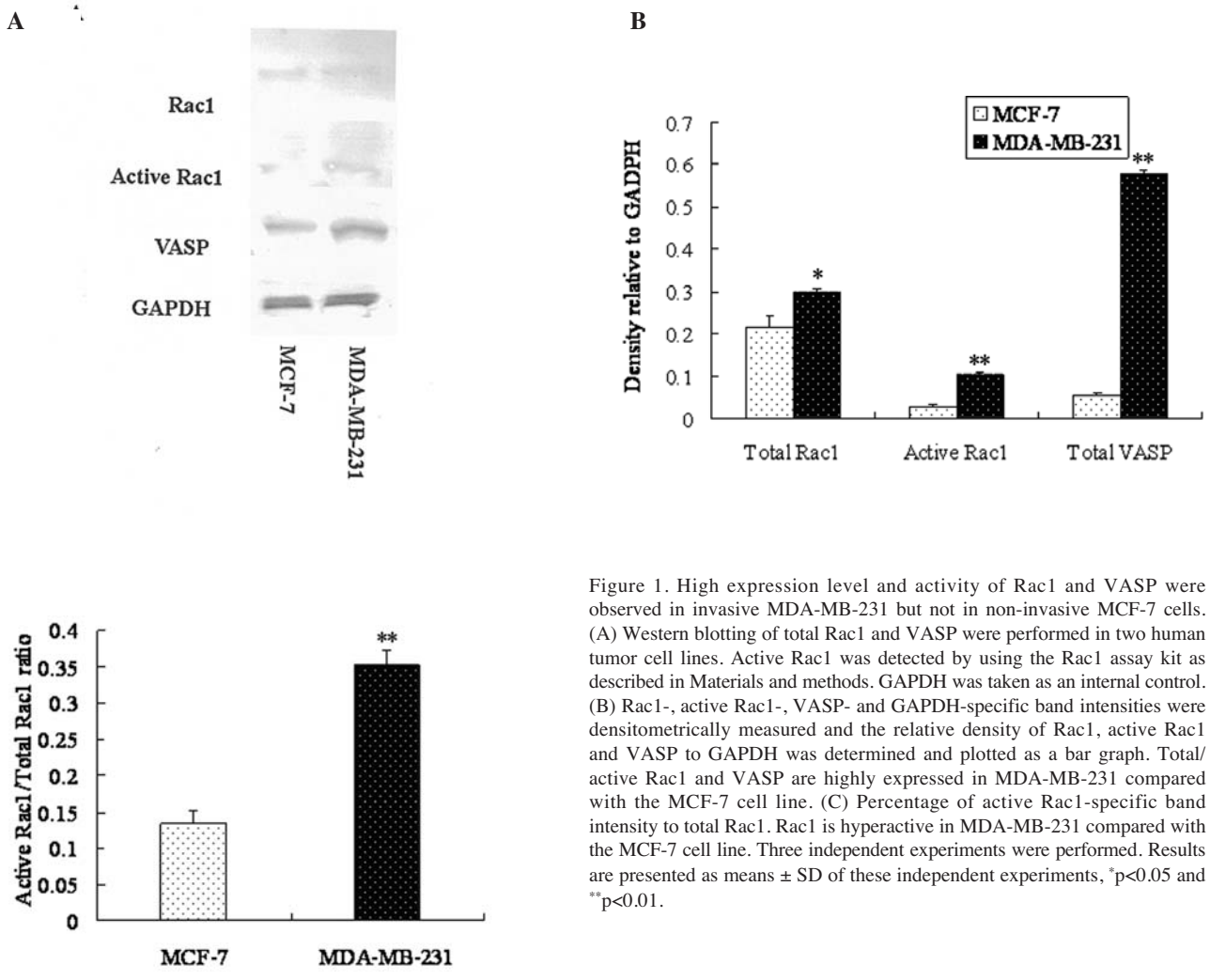

Figure 1. High expression level and activity of Rac1 and VASP were observed in invasive MDA-MB-231 but not in non-invasive MCF-7 cells. (A) Western blotting of total Rac1 and VASP were performed in two human tumor cell lines. Active Rac1 was detected by using the Rac1 assay kit as described in Materials and methods. GAPDH was taken as an internal control. (B) Rac1-, active Rac1-, VASP- and GAPDH-specific band intensities were densitometrically measured and the relative density of Rac1, active Rac1 and VASP to GAPDH was determined and plotted as a bar graph. Total/active Rac1 and VASP are highly expressed in MDA-MB-231 compared with the MCF-7 cell line. (C) Percentage of active Rac1-specific band intensity to total Rac1. Rac1 is hyperactive in MDA-MB-231 compared with the MCF-7 cell line. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments, $^{*}p<0.05$ and $^{**}p<0.01$.

(the lower chamber of transwell), and then to the transwell insert (the upper chamber of transwell), which was equilibrated overnight to allow cell attachment at 37˚C in an incubator supplemented with 5% CO_2. Cells were trypsinized, washed, and resuspended in RPMI-1640 medium containing 10% calf serum ($1x10^6$ 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. Cells in the two situations referred to previously adhered to the transwell filters during a 2-h incubation at 37˚C in the presence of 5% CO_2. The RPMI-1640 medium containing 10% calf serum in the upper chamber was replaced with serum-free RPMI-1640 medium or Rac1-siRNA segment. 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. The filters were 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 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 x200 magnification.

Statistical analysis. Values are expressed as mean ± standard error of the mean (SEM). The mean difference among the multiple groups was assessed using analysis of variance (ANOVA). Statistical significance was established between the two groups using the t-test. A probability value of $p<0.05$ was considered statistically significant.

Results

High expression level and activity of Rac1 and VASP were observed in invasive MDA-MB-231 but not in non-invasive MCF-7 cells. To study the relationship between breast cancer cell invasive ability and the expression and activity of Rac1 and VASP, we conducted Western blotting (Fig. 1). The result showed that the expression levels of Rac1 and VASP in the invasive cell line were markedly higher than those in the non-invasive one (Fig. 1A). The significant differences were quantified by the densitometry analysis of Western blotting (Fig. 1B, $p<0.05$ or $p<0.01$). The total Rac1 protein level in the invasive cell line was 1.4-fold of the expression level in the non-invasive one. Almost 2-fold of the VASP protein level was observed in the invasive cell line compared to the level in the non-invasive one. We further analyzed the activity of Rac1 between the two cell lines by a pull-down experiment

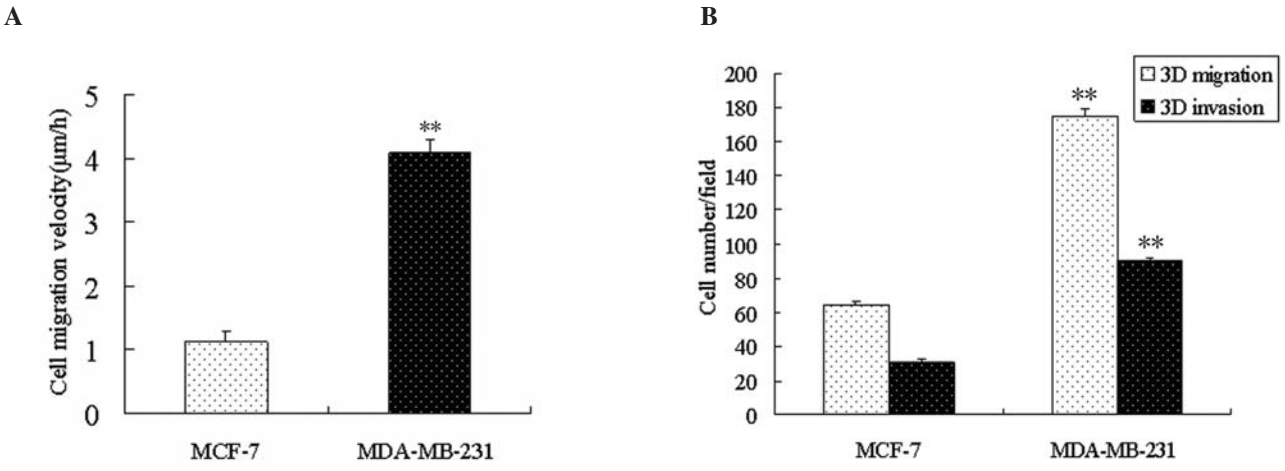

Figure 2. More aggressive migration and invasive ability were found in invasive MDA-MB-231 than in the non-invasive MCF-7 cells. (A) MDA-MB-231 cells exhibited a higher cell migration velocity than MCF-7 in 2D migration cell assays. Three independent experiments were performed. Results are presented as mean cell migration velocity ± SD of the independent experiments, $^{**}p<0.01$. (B) MDA-MB-231 cells revealed a stronger 3D migration and invasion ability, respectively, in 3D migration and invasion assays (upper chambers of 24-well transwell inserts were overlaid with or without diluted matrigel gel). Three independent experiments were performed. Results are presented as means ± SD of these independent experiments, $^{**}p<0.01$.

followed by the Western blot. Consistent with the total Rac1 expression result, the activity of Rac1 (measured by the ratio of active Rac1 over total Rac1) in the invasive cell line was 0.352 compared to 0.135 in the non-invasive cell line, a 2.6-fold difference between the two cell lines (Fig. 1A and C), suggesting a potential correlation between the invasive ability of breast cancer and the expression level and activity of Rac1 and VASP.

More aggressive migration and invasive ability were found in invasive MDA-MB-231 than in non-invasive MCF-7 cells. To characterize the invasive migration potential of the two cell lines, the cell migration and invasion ability were assessed by 2D, 3D migration and 3D invasion assay, respectively (Matrigel 25 $\mu g/cm^2$). The invasive cell line showed a 2.7- and 3.6-fold migration ability compared to the non-invasive cell line in the 2D and 3D migration assay, respectively ($p<0.01$) (Fig. 2A and B). Concomitant with the high migration ability, the invasive MDA-MB-231 cells showed the more aggressive invasion (90.0 cells/field in matrigel) versus the non-invasive MCF-7 cells (31.0 cells/field in matrigel) (Fig. 2B, $p<0.01$). The result indicated that the invasive breast cancer cells tend to be more aggressive than the non-invasive ones as regards cell migration and invasion.

Specific siRNA for Rac1 was sufficient to knock down Rac1 and depress VASP gene transcription as well as translation. To test whether the gene transcription and translation of VASP were affected by the expression level of Rac1 within breast cancer cells, loss-of-function approach was applied. Invasive and non-invasive breast cancer cells were transfected, respectively, with three different segments of siRNA specific for Rac1 (Rac1-siRNA1, 2 and 3) with Lipofactamine 2000. At 48 h after trasfection, the mRNA expression of Rac1 and VASP was detected with RT-PCR, and the protein expression was measured by Western blotting (Fig. 3A). The Rac1-siRNA1 insert effectively inhibited Rac1 gene transcription and translation in MCF-7 cells, which resulted in the mRNA expression level of Rac1 being reduced to 48% (Fig. 3B, $p<0.05$) and the protein expression level being decreased to 47% (Fig. 3D, $p<0.01$), respectively, in contrast to the negative control group (nc) after trasfection. Furthermore, we found that in MCF-7 cells, the Rac1-siRNA1 construct transduction diminished VASP expression at the mRNA and protein levels to 50% ($p<0.05$) (Fig. 3C) and 53% ($p<0.05$) (Fig. 3E) compared with the nc group. However, Rac1-siRNA2 and Rac1-siRNA3 segments revealed no inhibitory effect compared with the nc group ($p>0.05$) (Fig. 3B-E). Consistent with the result in MCF-7 cells, specific siRNA1 for Rac1 was sufficient to knock down Rac1 and depress VASP gene transcription as well as the translation in MDA-MB-231 cells (data not shown). These data showed that in invasive and non-invasive breast cancer cell lines decrease of the Rac1 expression level resulted in the depression of VASP gene transcription and translation, which suggested that VASP expression was positively regulated by Rac1.

Specific siRNA for VASP was sufficient to knock down VASP gene transcription and translation but not to affect Rac1 expression. To further confirm the relationship between Rac1 and VASP, we designed three different pairs of siRNA specific for VASP to silence VASP expression in invasive and non-invasive breast cancer cell lines. The cells were transfected with the three different VASP-siRNA segments 1, 2 and 3, respectively, for 48 h. Then, the mRNA expression level of VASP was detected by RT-PCR, and the protein expression was measured by Western blotting. The most effective siRNA segment to reduce VASP expression was found to be VASP-siRNA1 compared with the nc group, in MCF-7 (Fig. 5A) and MDA-MB-231 cells (Fig. 5B). The VASP expression was decreased by VASP-siRNA1 to ~46% ($p<0.01$) (Fig. 5C) and 32% ($p<0.01$) (Fig. 5D) at a level of mRNA and protein compared with the nc group in MCF-7 cells. The change in MDA-MB-231 cells was similar (Fig. 5E). In order to demonstrate the interaction between VASP and Rac1 more rigorously, we detected the mRNA expression of

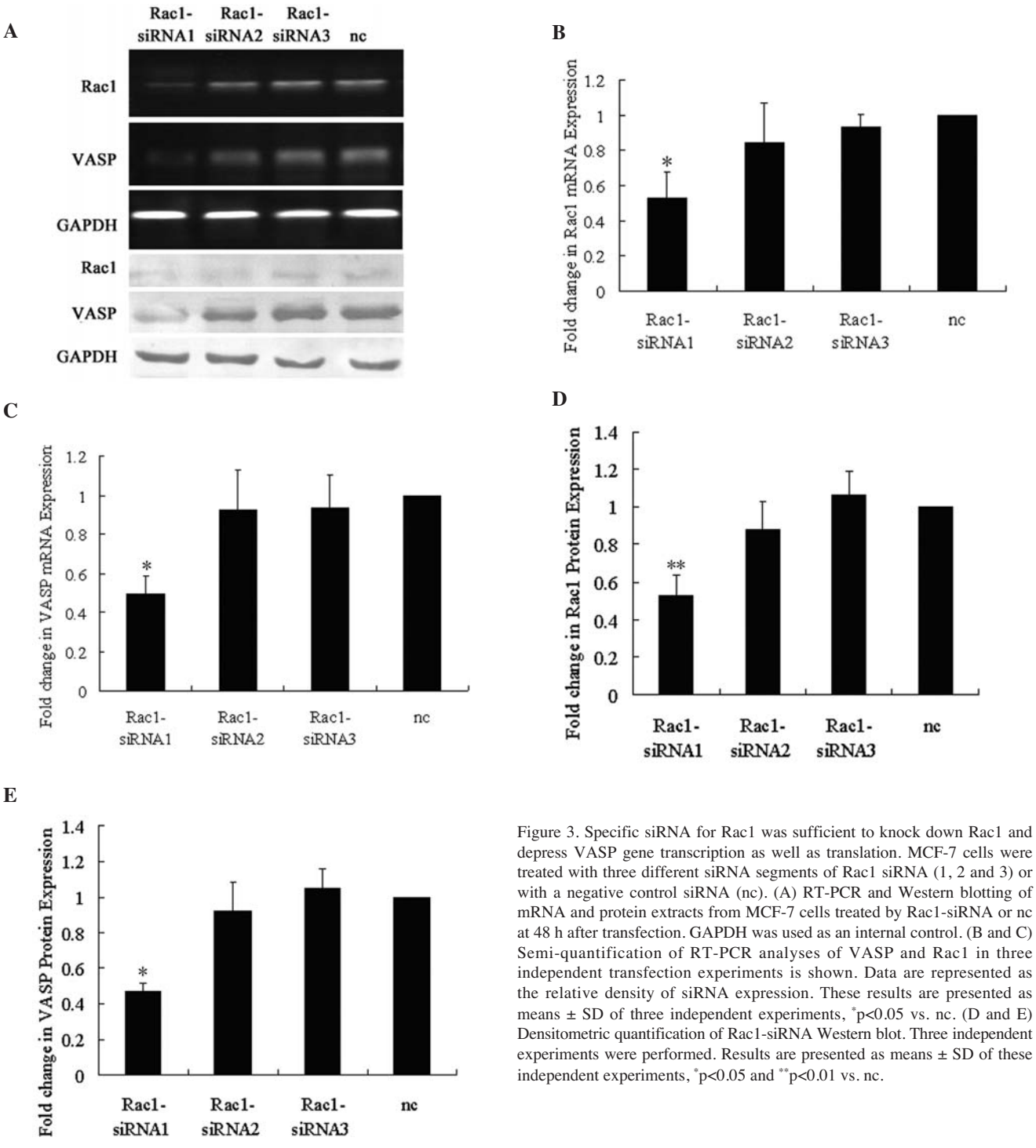

Figure 3. Specific siRNA for Rac1 was sufficient to knock down Rac1 and depress VASP gene transcription as well as translation. MCF-7 cells were treated with three different siRNA segments of Rac1 siRNA (1, 2 and 3) or with a negative control siRNA (nc). (A) RT-PCR and Western blotting of mRNA and protein extracts from MCF-7 cells treated by Rac1-siRNA or nc at 48 h after transfection. GAPDH was used as an internal control. (B and C) Semi-quantification of RT-PCR analyses of VASP and Rac1 in three independent transfection experiments is shown. Data are represented as the relative density of siRNA expression. These results are presented as means ± SD of three independent experiments, $^{*}p<0.05$ vs. nc. (D and E) Densitometric quantification of Rac1-siRNA Western blot. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments, $^{*}p<0.05$ and $^{**}p<0.01$ vs. nc.

Rac1 in VASP-siRNA-trasfected MDA-MB-231 cells. The RT-PCR result showed that the mRNA expression level of Rac1 was not reduced in the three VASP-siRNA segment-treated group compared with the nc group ($p>0.05$) (Fig. 5B and F). It revealed that the change of VASP expression level does not affect the gene transcription of Rac1.

Specific siRNAs for Rac1 and VASP weakened the migration and invasion capacity of breast cancer cells. To observe whether the expression level of Rac1 and VASP was associated with cancer cell migration and invasion, specific siRNAs for Rac1 or VASP knocked down Rac1 or VASP in breast cancer cells. Cell migration and invasion capacity were then assessed in 2D migration as well as 3D migration and invasion assays. The experimental data showed that Rac1-siRNA1- and VASP-siRNA1-transfected breast cancer cells presented a low migration and invasion ability. The migration index of Rac1-siRNA1-treated MCF-7 cells was reduced to 42% ($p<0.01$) of the nc group at 48 h after transfection, while the Rac1-siRNA segments 2 and 3 did not produce an inhibitory effect ($p>0.05$) (Fig. 4A). A similar phenomenon was observed in MDA-MB-231 cells (Fig. 4B). As for the 3D migration and invasion assays, cells in each field of the Rac1-siRNA1-treated group were much less than that of the nc group in MCF-7 and MDA-MB-231 cells (48% of nc group in 3D migration assay for MCF-7 cells, $p<0.01$; 19% of nc group in 3D invasion

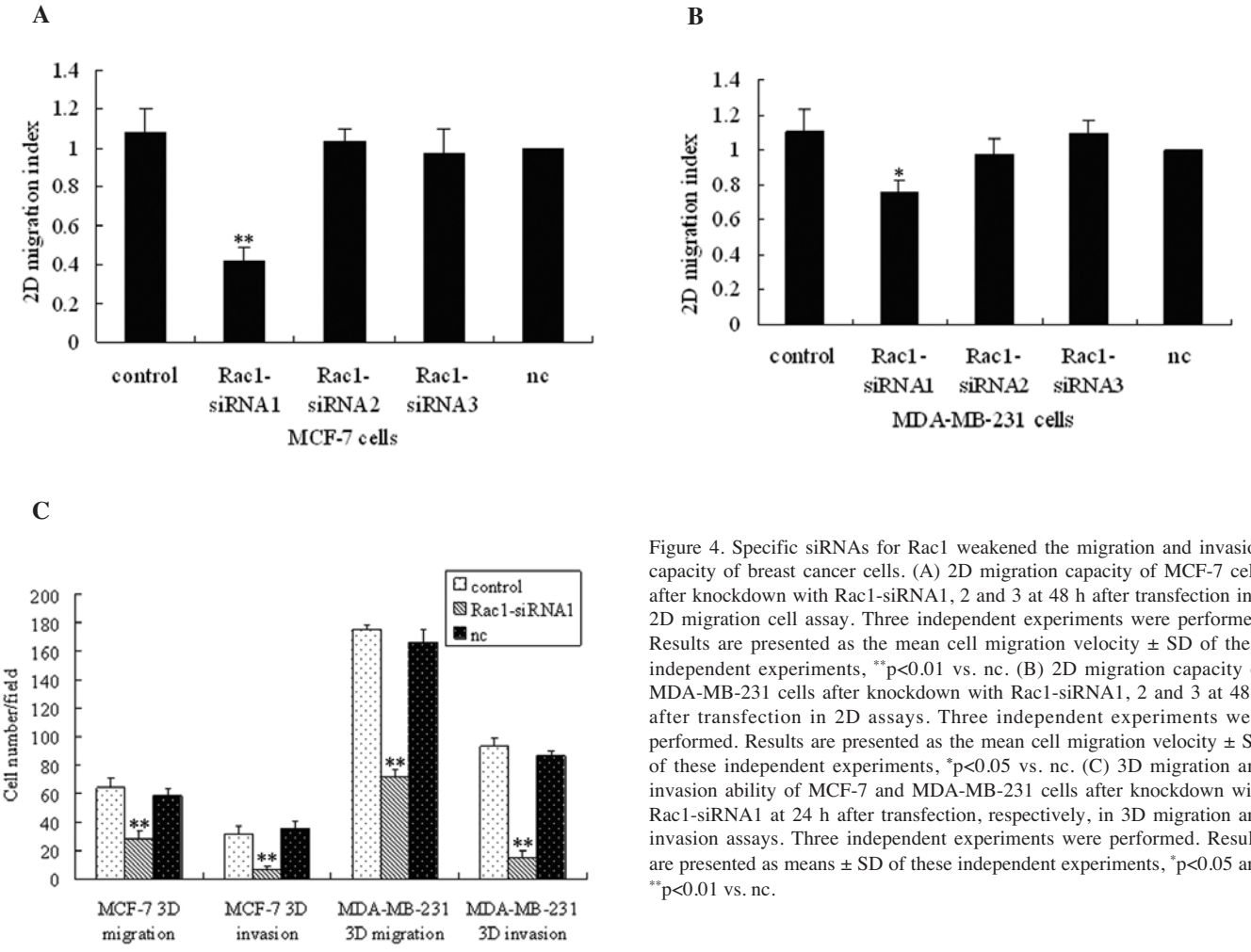

Figure 4. Specific siRNAs for Rac1 weakened the migration and invasion capacity of breast cancer cells. (A) 2D migration capacity of MCF-7 cells after knockdown with Rac1-siRNA1, 2 and 3 at 48 h after transfection in a 2D migration cell assay. Three independent experiments were performed. Results are presented as the mean cell migration velocity ± SD of these independent experiments, $^{**}p<0.01$ vs. nc. (B) 2D migration capacity of MDA-MB-231 cells after knockdown with Rac1-siRNA1, 2 and 3 at 48 h after transfection in 2D assays. Three independent experiments were performed. Results are presented as the mean cell migration velocity ± SD of these independent experiments, $^{*}p<0.05$ vs. nc. (C) 3D migration and invasion ability of MCF-7 and MDA-MB-231 cells after knockdown with Rac1-siRNA1 at 24 h after transfection, respectively, in 3D migration and invasion assays. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments, $^{*}p<0.05$ and $^{**}p<0.01$ vs. nc.

assay for MCF-7 cells, p<0.01; 44% of nc group in 3D migration assay for MDA-MB-231 cells, p<0.01 and 17% of nc group in 3D invasion assay for MDA-MB-231 cells, p<0.01) (Fig. 4C). VASP silencing also blocked breast cancer cells migration. The migration index of the VASP-siRNA1 group was 61% compared with the nc group (p<0.01) (Fig. 6). The result revealed that loss-of-function of Rac1 or VASP in breast cancer cells inhibited their migration and invasion.

fMLP activated Rac1 and up-regulated the expression of VASP in MDA-MB-231 cells. We measured Rac1 activity by using GST-PBD to pull down GTP-Rac. fMLP was known as the Rac1 activator which induces rapid Rac1 activation (21,22). Xu *et al* showed a rapid and transient increase in Rac1 activity ~1 min after exposure to fMLP in HL-60 cells (23). In this study, we found that there was a peak of Rac1 activity stimulated by fMLP at 3 min which then declined in MDA-MB-231 cells (Fig. 7B). The maximum Rac1 activity was 2.0-fold of the control group (p<0.05). At the same time, according to the expression of VASP, we have found that there was a delayed peak of VASP expression which was 3.5-fold of the control group (p<0.01) at 5 min after fMLP stimulation in MDA-MB-231 cells which then decreased (Fig. 7C). Taken together, we suspected that VASP located on the downstream of Rac1.

Discussion

Numerous studies have shown that the migration and invasion ability of tumor is correlated with Rac1 or VASP. However, a mechanism showing the signal pathway that interacts in breast cancer cells has yet to be established. In this study, we used the siRNA technique to silence the expression of Rac1 or VASP, respectively, in MCF-7 and MDA-MB-231 cells and assessed their migration and invasion ability changes by examining 2D and 3D migration/invasion assays accordingly. We have shown that VASP expression and Rac1 activity were concomitant with the migratory and invasive ability of human breast cancer cells. Furthermore, VASP regulated the migration and invasion of breast cancer cells positively *via* Rac1 which located upstream of VASP.

The Rho GTPases are proteins of a ubiquitous family of proteins that are widely appreciated as having the two signaling functions often associated with gene expression, and important roles in the regulation of the actin cytoskeleton (24). There is increasing evidence that Rho proteins influence a variety of important processes in cancer, including cell transformation, survival, proliferation, invasion, metastasis and angiogenesis (25). Among the Rho protein family members, Rac1 plays an important regulatory role in tumor cellular processes, such as gene expression, superoxide production,

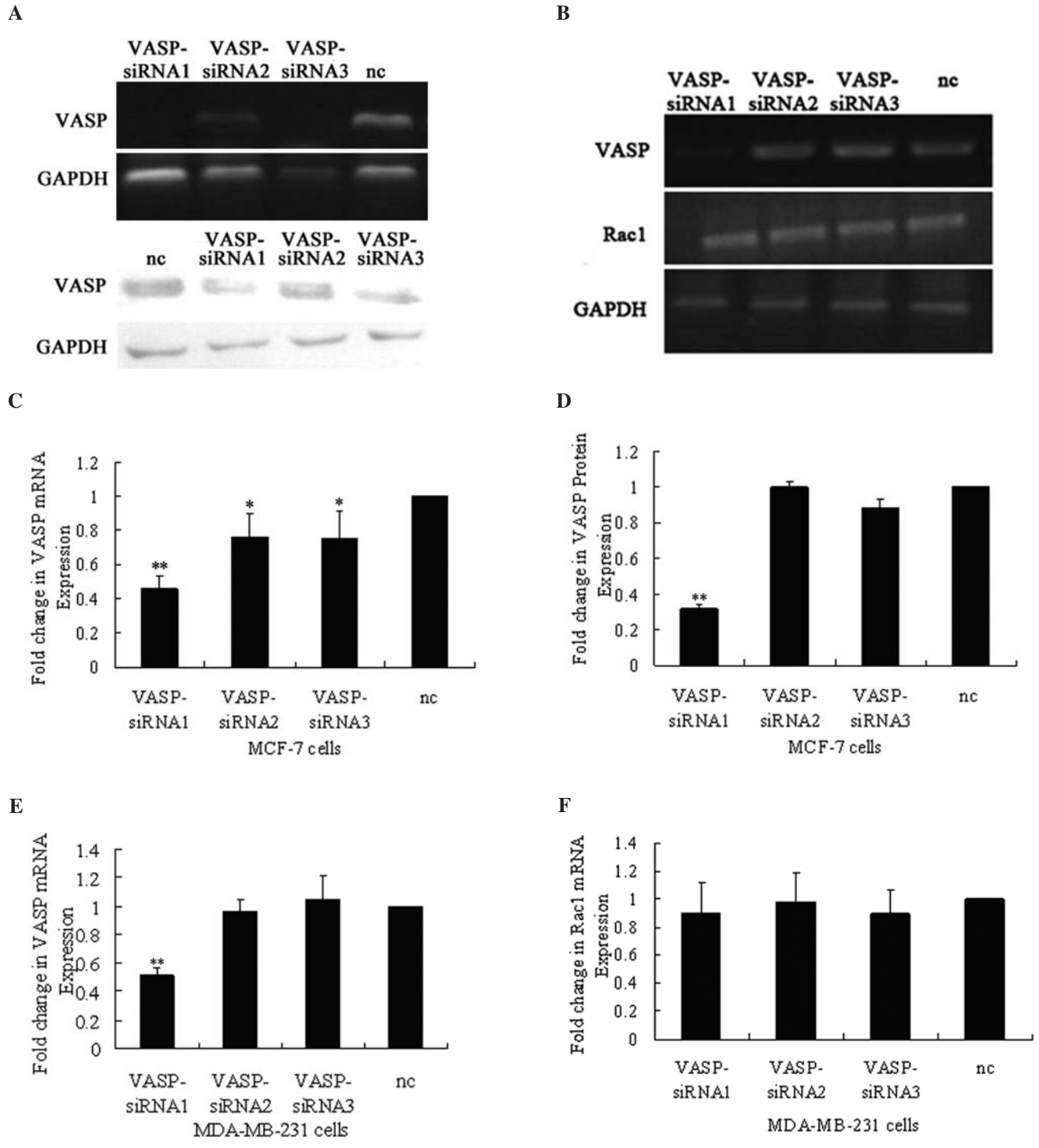

Figure 5. Specific siRNA for VASP was sufficient to knock down VASP gene transcription and translation but not to affect Rac1 expression. MCF-7 cells were treated with three different siRNA oligos of VASP or with nc siRNA. (A) VASP Western blotting and RT-PCR analysis of MCF-7 cells treated by VASP-siRNA or nc at 48 h after transfection. (B) RT-PCR analyses of VASP and Rac1 in the VASP-siRNA expressing MDA-MB-231 cells compared with nc at 48 h after transfection. (C) Relative VASP mRNA quantification, related to GAPDH mRNA by RT-PCR in MCF-7, which were treated by VASP-siRNA or nc at 48 h after transfection. (D) Densitometric quantification of VASP protein expression. (E) Relative VASP mRNA quantification, related to GAPDH mRNA by RT-PCR in MDA-MB-231 cells, which were treated by VASP-siRNA or nc for 48 h. (F) The histogram represented the Rac1 mRNA fold change relative to GAPDH after MDA-MB-231 cells were treated with VASP-siRNA or nc for 48 h. GAPDH was taken as an internal control. Three independent experiments were performed. Results are presented as means ± SD of these independent experiments, $^{*}p<0.05$ and $^{**}p<0.01$ vs. nc.

cell migration and invasion, proliferation and apoptosis. The present study showed that mRNA levels of Rac1 were significantly higher in gastric carcinoma tissues than those in unaffected portions. Furthermore, the expression levels of Rac1 in tumor tissues were correlated with the clinical stage of the malignancy. Activated Rac1 localizes to the plasma membrane where it is thought to function in actin remodeling which contributes to membrane ruffling (26,27). The VASP regulation of the cytoskeleton is mediated through interaction with or regulation of Rho GTPases. VASP binds to WASP, a

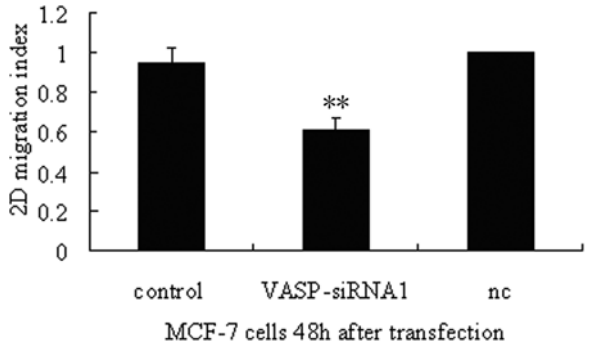

Figure 6. Specific siRNAs for VASP weakened the migration and invasion capacity of MCF-7 cells. The 2D migration capacity of MCF-7 cells after knockdown with VASP-siRNA1 at 48 h after transfection in 2D migration cell assays is shown. Three independent experiments were performed. Results are presented as mean cell migration velocity ± SD of the three independent experiments, $^{**}p<0.01$ vs. nc.

Cdc42-binding effector, to regulate actin assembly and membrane protrusion at the cellular leading edge (28).

Upon examination of the two human breast cancer cell lines with different invasive abilities, we found that Rac1 activity and VASP expression levels correlated with metastatic potential in the breast cancer cells. The high activity of Rac1 accompanied with the high expression level of VASP was correlated with the enhanced ability of 3D migration and invasion of breast cancer cell lines. Thus, we designed three different siRNA oligos to identify the most efficient oligos in silencing Rac1 expression in MCF-7 and MDA-MB-231 cells. We showed that transfection of the breast cancer cells with Rac1 siRNA down-regulated VASP expression and suppressed their migration and invasion ability. To reinforce these data, a diminution of VASP expression following Rac1 siRNA transfection was also shown in invasive MDA-MB-231 breast cancer cells. Herein, we were able to hypothesize Rac1 regulated VASP expression positively in invasive and non-invasive breast cancer cells. In agreement with previous studies, the study by Fryer *et al* on endothelial cells proved that PKG controls cell morphology through Pak by regulation of the Pak/VASP association while Pak is a substrate of Rac (29). However, cardiac fibroblasts from VASP knockout mice display increased spreading, prolonged Rac and Pak activation in response to PDGF (platelet-derived growth factor), and an inability to properly orient in a monolayer wound-healing assay (17). This experiment provided circumstantial evidence that VASP is a negative regulator of Rac. To confirm the hypothesis that Rac1 played a significant role as an upstream regulator of VASP, we transfected VASP-siRNA in MCF-7 (data not shown) and MDA-MB-231 cells. We observed that the mRNA expression of Rac1 showed no difference compared

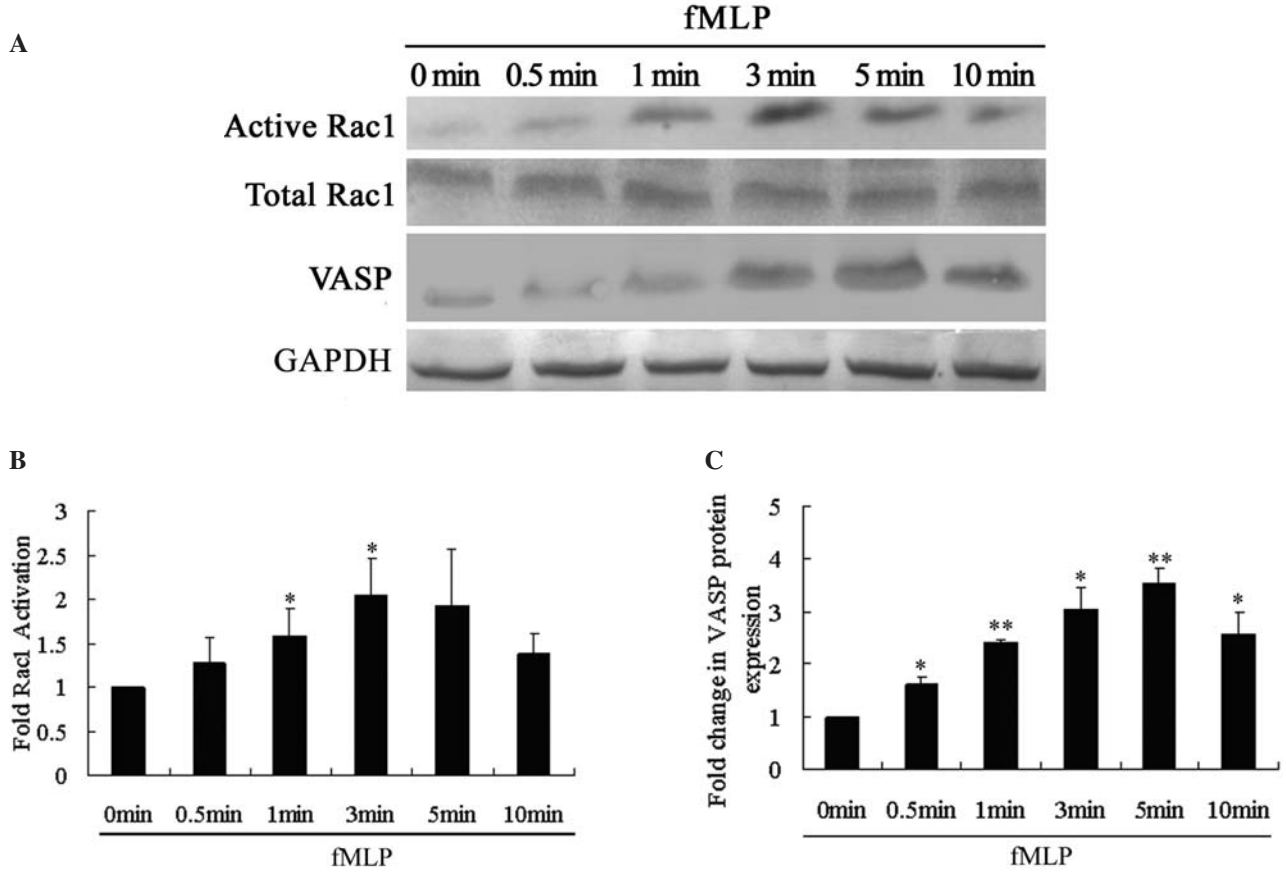

Figure 7. fMLP activated Rac1 and up-regulated the expression of VASP in MDA-MB-231 cells. (A) MDA-MB-231 cells were serum-starved for 8 h, treated with 1 μmol/l fMLP for 0-10 min and subsequently assayed for Rac1 activity as described in Materials and methods. The upper panel shows active Rac1 level after fMLP stimulation. The lower left panel shows total Rac1 level. The third panel shows the protein level of VASP. (B) The histogram shows quantification of the increase in Rac1 activity after fMLP stimulation in three independent experiments. (C) The histogram shows quantification of the VASP level after fMLP stimulation in three independent experiments, $^{*}p<0.05$ and $^{**}p<0.01$ vs. control group.

with the nc group. These results indicated that Rac1 located on the upstream of VASP in breast cancer cell lines. We suspected that the relative contribution of Rac1 GTPases to cytoskeletal rearrangements and its relationship with VASP depended on cell types.

To investigate the relationship between VASP and Rac1, we observed that fMLP, an activator of Rac, time depended stimulation Rac1 activity, would promote VASP expression at protein level in MDA-MB-231 and MCF-7 cells (data not shown). The peak of Rac1 activity occurred 2 min earlier than that of VASP expression, hinting that Rac1 located on the upstream of VASP. Together with our observations that the expression of VASP and Rac1 associated with migratory and invasive abilities, these results suggested that Rac1 contributes to the up-regulation of VASP transcription such as that which occurs during breast cancer cell migration and invasion.

The mechanism by which Rac1 regulates VASP remains unclear. PKG may facilitate the complex formation between Rac1 and an exchange factor by modification of a scaffolding protein such as members of the ENA/VASP family of proteins. This is particularly attractive since PKG colocalizes with VASP in dynamic membrane regions, and the latter is a well-characterized substrate for PKG (30). We have demonstrated that fMLP activated Rac1 activity and VASP expression, while Pak1 was a substrate of activated Rac1 (31). We suspect that the VASP-Rac interaction is mediated by VASP binding a Pak polyproline motif because Pak contains five poly-proline domains, and VASP often associates with proline-rich domains through its EVH1 domain (32).

The vasodilator-stimulated phosphoprotein (VASP), which was first described in human platelets, is one member of the Ena/VASP family of cytoskeletal regulatory proteins (32). Ena/VASP proteins are actin-binding proteins that localize to actin stress fibers, the tips of filopodia and the lamellipodial leading edge (33). That VASP can affect the cell movements of bacteria, mouse melanoma cells, human platelet, fibroblasts and neurons by regulating cytoskeleton has been detected (14,15,34,35). However, the conclusions of these investigations concerning the function of VASP affecting cell migration are contradictory. The deletion of the Ena/VASP-binding sites within the bacterial protein ActA led to a decrease in the actin-dependent intracellular motility of Listeria monocytogenes (14). Furthermore, the addition of VASP resulted in an increase in bacterial speed in the *in vitro* reconstitution assays of Listeria motility (34). In contrast, platelets isolated from VASP knockout mice exhibit increased rates of collagen-induced platelet aggregation (an actin-dependent process) compared with wild-type platelets. Fibroblasts devoid of Ena/VASP proteins exhibit increased rates of cell motility (15). The aberrant superficial placement of Ena/VASP-inhibited neurons was cell autonomous, and the phenotype is consistent with increased rates of neuronal migration (35).

We designed this experiment to investigate the effect of VASP on breast cancer cell migration and invasive ability. We initially observed VASP expression of the protein level as well as the 2D and 3D migration rate and 3D invasive capacity of MCF-7 and MDA-MB-231 cells without any treatment. We found that MDA-MB-231 cells revealed an inherently higher VASP level together with a stronger migration and invasive ability compared with the MCF-7 cells. We then designed VASP-specific siRNA to treat with MCF-7 cells in order to inhibit VASP expression and observe its function on cell movement. The result showed that when VASP was knocked down by VASP-siRNA, the expression of VASP in MCF-7 cells was decreased on the mRNA and protein level, while the 2D migration ability of MCF-7 cells was weakened. The experiment in MDA-MB-231 had similar results, but data are not shown here. These data revealed that the decrease of cell migration and invasion was parallel to the decrease of the expression of VASP, which means that VASP has a positive function in breast cancer cell migration.

Our results were consistent with the findings of Liu *et al* (36) that VASP had a previously unsuspected role in tumorigenesis and/or cancer progression. Besides, many studies had indicated that VASP is overexpressed in lung cancer, gastric cancer and colon carcinoma (37-39). As for breast cancer, it has been reported that hMena protein (another member of Ena/VASP family) was overexpressed in the majority of breast cancer cell lines and in 75% of primary breast tumor lesions evaluated, which was analyzed by Western blotting and immunohistochemistry (35). Based on our investigations we assumed that VASP may accelerate the breast cancer cell migration rate and enhance its invasive ability to facilitate breast cancer invasion and progression.

In conclusion, our results demonstrated that the activity of Rac1 and VASP expression correlated with a migratory and invasive ability and that Rac1 regulated VASP expression positively in breast cancer cells. Due to the identified functional role of VASP in cell migration, the regulation of VASP by Rac1 may be implicated for processes requiring tumor cell migration including tumor cell invasion. This study provided a basis for further characterization of the molecular mechanism of Rac1 and VASP increased cell migration and invasion by siRNA, yielding potential insights in the diagnostic or therapeutic applications of breast cancer.

Acknowledgements

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References

1. Takai Y, Sasaki T and Matozaki T: Small GTP-binding proteins. Physiol Rev 81: 153-208, 2001.
2. Qiu RG, Chen J, Kirn D, McCormick F and Symons M: An essential role for Rac in Ras transformation. Nature 374: 457-459, 1995.
3. Soga N, Namba N, McAllister S, Cornelius L, Teitelbaum SL, Dowdy SF, Kawamura J and Hruska KA: Rho family GTPases regulate VEGF-stimulated endothelial cell motility. Exp Cell Res 269: 73-87, 2001.
4. Zhuge Y and Xu J: Rac1 mediates type I collagen-dependent MMP-2 activation. role in cell invasion across collagen barrier. J Biol Chem 276: 16248-16256, 2001.
5. Sherr CJ and Roberts JM: CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13: 1501-1512, 1999.
6. Small JV, Stradal T, Vignal E and Rottner K: The lamellipodium: where motility begins. Trends Cell Biol 12: 112-120, 2002.
7. Hordijk PL, ten Klooster JP, van der Kammen RA, Michiels F, Oomen LC and Collard JG: Inhibition of invasion of epithelial cells by Tiam1-Rac signaling. Science 278: 1464-1466, 1997.
8. Ahram M, Sameni M, Qiu RG, Linebaugh B, Kirn D and Sloane BF: Rac1-induced endocytosis is associated with intracellular proteolysis during migration through a three-dimensional matrix. Exp Cell Res 260: 292-303, 2000.

9. O'Connor KL, Nguyen BK and Mercurio AM: RhoA function in lamellae formation and migration is regulated by the alpha6-beta4 integrin and cAMP metabolism. J Cell Biol 148: 253-258, 2000.
10. Wells CM, Walmsley M, Ooi S, Tybulewicz V and Ridley AJ: Rac1-deficient macrophages exhibit defects in cell spreading and membrane ruffling but not migration. J Cell Sci 117: 1259-1268, 2004.
11. Pan Y, Bi F, Liu N, Xue Y, Yao X, Zheng Y and Fan D: Expression of seven main Rho family members in gastric carcinoma. Biochem Biophys Res Commun 315: 686-691, 2004.
12. Kurisu S, Suetsugu S, Yamazaki D, Yamaguchi H and Takenawa T: Rac-WAVE2 signaling is involved in the invasive and metastatic phenotypes of murine melanoma cells. Oncogene 24: 1309-1319, 2005.
13. Krause M, Bear JE, Loureiro JJ and Gertler FB: The Ena/VASP enigma. J Cell Sci 115: 4721-4726, 2002.
14. Niebuhr K, Ebel F, Frank R, Reinhard M, Domann E, Carl UD, Walter U, Gertler FB, Wehland J and Chakraborty T: A novel proline-rich motif present in ActA of Listeria monocytogenes and cytoskeletal proteins is the ligand for the EVH1 domain, a protein module present in the Ena/VASP family. EMBO J 16: 5433-5444, 1997.
15. Bear JE, Loureiro JJ, Libova I, Fassler R, Wehland J and Gertler FB: Negative regulation of fibroblast motility by Ena/VASP proteins. Cell 101: 717-728, 2000.
16. Fryer BH and Field J: Rho, Rac, Pak and angiogenesis: old roles and newly identified responsibilities in endothelial cells. Cancer Lett 229: 13-23, 2005.
17. Garcia Arguinzonis MI, Galler AB, Walter U, Reinhard M and Simm A: Increased spreading, Rac/p21-activated kinase (PAK) activity, and compromised cell motility in cells deficient in vasodilator-stimulated phosphoprotein (VASP). J Biol Chem 277: 45604-45610, 2002.
18. Quinlan MP: Suppression of epithelial cell transformation and induction of actin dependent differentiation by dominant negative Rac1, but not Ras, Rho or Cdc42. Cancer Biol Ther 3: 65-70, 2004.
19. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K and Tuschl T: Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411: 494-498, 2001.
20. Zhang L, Wang T, Wen X, Wei Y, Peng X, Li H and Wei L: Effect of matrine on HeLa cell adhesion and migration. Eur J Pharmacol 563: 69-76, 2007.
21. Nijhara R, van Hennik PB, Gignac ML, Kruhlak MJ, Hordijk PL, Delon J and Shaw S: Rac1 mediates collapse of microvilli on chemokine-activated T lymphocytes. J Immunol 173: 4985-4993, 2004.
22. Geijsen N, van Delft S, Raaijmakers JA, Lammers JW, Collard JG, Koenderman L and Coffer PJ: Regulation of p21rac activation in human neutrophils. Blood 94: 1121-1130, 1999.
23. Xu J, Wang F, Van Keymeulen A, Herzmark P, Straight A, Kelly K, Takuwa Y, Sugimoto N, Mitchison T and Bourne HR: Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. Cell 114: 201-214, 2003.
24. Bokoch GM: Regulation of cell function by Rho family GTPases. Immunol Res 21: 139-148, 2000.
25. Ellenbroek SI and Collard JG: Rho GTPases: functions and association with cancer. Clin Exp Metastasis 24: 657-672, 2007.
26. Kraynov VS, Chamberlain C, Bokoch GM, Schwartz MA, Slabaugh S and Hahn KM: Localized Rac activation dynamics visualized in living cells. Science 290: 333-337, 2000.
27. Dharmawardhane S, Brownson D, Lennartz M and Bokoch GM: Localization of p21-activated kinase 1 (PAK1) to pseudopodia, membrane ruffles, and phagocytic cups in activated human neutrophils. J Leukoc Biol 66: 521-527, 1999.
28. Castellano F, Le Clainche C, Patin D, Carlier MF and Chavrier P: A WASp-VASP complex regulates actin polymerization at the plasma membrane. EMBO J 20: 5603-5614, 2001.
29. Fryer BH, Wang C, Vedantam S, Zhou GL, Jin S, Fletcher L, Simon MC and Field J: cGMP-dependent protein kinase phosphorylates p21-activated kinase (Pak) 1, inhibiting Pak/Nck binding and stimulating Pak/vasodilator-stimulated phosphoprotein association. J Biol Chem 281: 11487-11495, 2006.
30. Butt E, Abel K, Krieger M, Palm D, Hoppe V, Hoppe J and Walter U: cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. J Biol Chem 269: 14509-14517, 1994.
31. Weed SA, Du Y and Parsons JT: Translocation of cortactin to the cell periphery is mediated by the small GTPase Rac1. J Cell Sci 111: 2433-2443, 1998.
32. Krause M, Dent EW, Bear JE, Loureiro JJ and Gertler FB: Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. Annu Rev Cell Dev Biol 19: 541-564, 2003.
33. Reinhard M, Jarchau T and Walter U: Actin-based motility: stop and go with Ena/VASP proteins. Trends Biochem Sci 26: 243-249, 2001.
34. Loisel TP, Boujemaa R, Pantaloni D and Carlier MF: Reconstitution of actin-based motility of Listeria and Shigella using pure proteins. Nature 401: 613-616, 1999.
35. Goh KL, Cai L, Cepko CL and Gertler FB: Ena/VASP proteins regulate cortical neuronal positioning. Curr Biol 12: 565-569, 2002.
36. Liu K, Li L, Nisson PE, Gruber C, Jessee J and Cohen SN: Reversible tumorigenesis induced by deficiency of vasodilator-stimulated phosphoprotein. Mol Cell Biol 19: 3696-3703, 1999.
37. Dertsiz L, Ozbilim G, Kayisli Y, Gokhan GA, Demircan A and Kayisli UA: Differential expression of VASP in normal lung tissue and lung adenocarcinomas. Thorax 60: 576-581, 2005.
38. Tao Y, Chen YC, Wang Y, Zhang ZJ and Xu WR: Phosphorylated vasodilator-stimulated phosphoprotein is localized on mitotic spindles of the gastric cancer cell line SGC-7901. World J Gastroenterol 12: 7478-7481, 2006.
39. Deguchi A, Soh JW, Li H, Pamukcu R, Thompson WJ and Weinstein IB: Vasodilator-stimulated phosphoprotein (VASP) phosphorylation provides a biomarker for the action of exisulind and related agents that activate protein kinase G. Mol Cancer Ther 1: 803-809, 2002.