CMLS, Cell. Mol. Life Sci. 62 (2005) 1167–1175
1420-682X/05/101167-09
DOI 10.1007/s00018-005-5050-2


CMLS Cellular and Molecular Life Sciences

## Research Article

# Emodin inhibits tumor cell migration through suppression of the phosphatidylinositol 3-kinase-Cdc42/Rac1 pathway

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Received 7 February 2005; received after revision 11 March 2005; accepted 18 March 2005

**Abstract.** Enhanced cell migration is one of the underlying mechanisms in cancer invasion and metastasis. Therefore, inhibition of cell migration is considered to be an effective strategy for prevention of cancer metastasis. We found that emodin (3-methyl-1,6,8-trihydroxyanthraquinone), an active component from the rhizome of *Rheum palmatum,* significantly inhibited epidermal growth factor (EGF)-induced migration in various human cancer cell lines. In the search for the underlying molecular mechanisms, we demonstrated that phosphatidylinositol 3-kinase (PI3K) serves as the molecular target for emodin. In addition, emodin markedly suppressed EGF-induced activation of Cdc42 and Rac1 and the corresponding cytoskeleton changes. Moreover, emodin, but not LY294002, was able to block cell migration in cells transfected with constitutively active (CA)-Cdc42 and CA-Rac1 by interference with the formation of Cdc42/Rac1 and the p21-activated kinase complex. Taken together, data from this study suggest that emodin inhibits human cancer cell migration by suppressing the PI3K-Cdc42/Rac1 signaling pathway.

**Key words.** Migration; emodin; PI3K; Cdc42; Rac1; PAK.

Cell migration is an integral cellular response to extracellular stimuli, and a defining feature of tumor invasion and metastasis [1]. Thus, much effort to reduce the spread of malignant tumors has recently focused on cell migration. Many growth factors can produce some of the key features of cell motility. Among them, epidemal growth factor (EGF), has been shown to stimulate the migration of both normal and tumor cells, including normal epithelial cells, fibroblasts, renal and breast carcinoma cells [2–5].

Although a complex molecular interplay has been reported for the mechanism underlying cell migration, several lines of evidence have recently suggested the possible involvement of the phosphatidylinositol 3-kinase (PI3K) signaling pathway in the cell motility process [6–8]. PI3K signaling has been shown to be activated by various growth factors, such as insulin, interleukins, and EGF. Selective activation of PI3K using constitutively active PI3K mutants has been reported to promote cell migration [6, 9] and inhibition of PI3K signaling interfered with cell migration [7, 10].

Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) is an active component contained in the rhizome of *Rheum palmatum L.*, which is traditionally used in Chinese medicine [11]. Emodin has been reported to inhibit cell proliferation in different cancer cells [12]. Furthermore, emodin has been shown to inhibit *HER-2/neu* tyrosine kinase activity [13] and suppress the transformation of *HER-2/neu*-overexpressing breast cancer cells [14]. Recent studies on the anti-cancer effect of emodin have focused on its induction of apoptosis in several cancer cell lines [15–17]. However, its effect against tumor cell invasion and metastasis has not been systematically reported. Our previous work showed that emodin exhibited strong anti-invasion activity in some cancer cell lines [18]. Since cell migration is an important component of cell invasion and metastasis, we examined further the effect of emodin on

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cancer cell migration. Our findings showed that emodin significantly inhibited the migration of EGF-stimulated cancer cells. We further demonstrated that emodin effectively suppressed the PI3K-Cdc42/Rac1 pathway, leading to decreased cell motility. Findings from the present study provide novel insights into the molecular mechanisms of emodin and suggest the potential therapeutic value of emodin in preventing invasion or metastasis of human cancer.

## Materials and methods

### Cell lines

MDA-MB-231 (a human breast cancer cell line) and HepG2 (a human hepatocarcinoma cell line) were purchased from ATCC (Manassas, Va.). HSC5, derived from a human skin squamous carcinoma, was kindly provided by Dr. S. Kondo (Yamagata University, Yamagata, Japan). MDA-MB-231 and HSC5 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). HepG2 cells were grown and passaged in MEM containing 10% FBS.

### Chemicals and reagents

Emodin and FITC-conjugated phalloidin were purchased from Sigma (St. Louis, Mo.). LY294002 (a specific PI3K inhibitor), antibody against phospho-Akt (Ser473), phospho-Akt (Thr308), and Akt were obtained from Cell Signaling (Beverly, Mass.). Antibody against p21-activated kinase1 (PAK1) was purchased from Santa Cruz Technology (Santa Cruz, Calif.). Sepharose 4B was from Amersham Bioscience (Uppsala, Sweden). The Cdc42/Rac1 activation kit was from Upstate (Lake Placid, N.Y.). Recombinant human EGF was from Calbiochem (San Diego, Calif.).

### Plasmids and transfection

Vectors expressing dominant-negative (DN)-Cdc42 and constitutively active (CA)-Cdc42 were gifts from Dr. A. Hall (University College London, London, UK). DN-Rac1 and CA-Rac1 vectors were from Dr. J. T. Parsons (University of Virginia). The DN form of PI3K (DN-p85 and DN-p110) and wild-type (WT)-p110 plasmids were kindly provided by Dr. C. Huang (New York University). An expression vector for red-fluorescent protein (pDsRed) was from Clontech (Palo Alto, Calif.). Transient transfection was performed in MDA-MB-231 cells using lipofectamine 2000 reagent according to the manufacturer's protocol. All transfections were normalized for total DNA using vector plasmid.

### Cell migration assay

The migration assay was conducted as described previously [19] with modifications using BD Falcon 8.0-μm cell culture inserts (Becton Dickinson, Franklin Lakes, N. J.). Membranes were coated with purified fibronectin at a concentration of 20 μg/ml. After starvation in serum-free medium for 24 h, cells were collected and resuspended in serum-free medium containing 0.1% bovine serum albumin (BSA) and pretreated with emodin or LY294002 for 1 h. Subsequently, cells ($4\times10^5$/ml) were seeded into transwell chambers in the presence or absence of 20 ng/ml EGF and various concentrations of emodin or LY294002. After 4 h incubation, the lower surfaces of the membrane were fixed with 100% methanol and stained with 0.5% crystal violet solution. Cells that had migrated to the lower surface of the membranes were counted in high-power fields under an inverted microscope.

For the migration assay of transiently transfected cells, pDsRed plasmid was used to mark the transfected cells. As described above, cells were transiently transfected with various plasmids, serum starved, trypsinized, and resuspended in BSA-containing medium. After normalization by counting pDsRed-positive cells (with red fluorescence) under a fluorescence microscope, cells ($2\times10^5$ transfected cells/ml) were treated according to the context and placed in transwell chambers. Only pDsRed-positive cells that migrated through the membrane after 4 h incubation were counted by fluorescence microscopy. The data are presented relative to cells transfected with empty vector and stimulated with EGF, which were normalized to 100%.

### Immunofluorescence

Cells were first seeded on fibronectin-coated coverslips at a density of $2.5\times10^3$ cells /coverslip and then serum starved for 24 h. After various designated treatments, cells were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS, and incubated with 500 ng/ml FITC-conjugated phalloidin for 1 h at room temperature. Cells were visualized under a fluorescence microscope (Nikon, Tokyo, Japan) and photographed. Cytoskeletal features were quantified in fields of view blindly selected at random.

### Western blot

Cells were starved in serum-free medium for 24 h, and then treated as designated. Cell pellets were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mM $Na_3O_4$, 1 mM NaF) with protease inhibitor cocktail (Roche, Basel, Switzerland). Aliquots of the lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with primary antibody followed by secondary antibody and visualized using a SuperSignal West Dura kit (Pierce, Rockford, Ill.), according to the manufacturer's protocol. Densitometric measurements of the bands on Western blots were performed using a program from Kodak (Kodak 1D 3.5).

### Co-immunoprecipitation assay

Cell lysates were prepared with co-immunoprecipitation lysis buffer (50 mM HEPES, pH 7.6, 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 0.5 mM PMSF and the proteinase inhibitor cocktail). Protein concentration was determined by a Bio-Rad DC protein assay (Bio-Rad, Hercules, Calif.). Cell lysates (500 µg) were incubated with 2 µg of PAK1 antibody for 1 h at 4°C. Protein-antibody complexes were collected after incubation with protein A-Sepharose beads for 1 h at 4 °C. The beads were washed with ice-cold co-immunoprecipitation lysis buffer three times, resolved by 10% SDS-PAGE, and immunoblotted with antibody against Cdc42 or Rac1.

### Cdc42 and Rac1 activation assay

Cdc42 and Rac1 activation was analyzed using an activation assay kit (Upstate, Lake Placid, N. Y. ). In brief, cells were lysed with 1×$Mg^{2+}$ lysis/wash buffer and then 10 µl of GST–PAK1 p21-binding domain (PBD) agarose beads was added to 0.5 ml cell lysate and incubated at 4 °C for 60 min. Non-stimulated cell lysate was incubated with recombinant GTP$\gamma$S and GDP and used as positive and negative control, respectively. The agarose beads were collected, washed three times, and resuspended in Laemmli sample buffer and boiled for 5 min. The supernatant was subjected to SDS-PAGE and immunoblotted with antibody against Cdc42 or Rac1.

### Statistics

All numeric data are presented as means ± SD, and analyzed by Student's t-test.

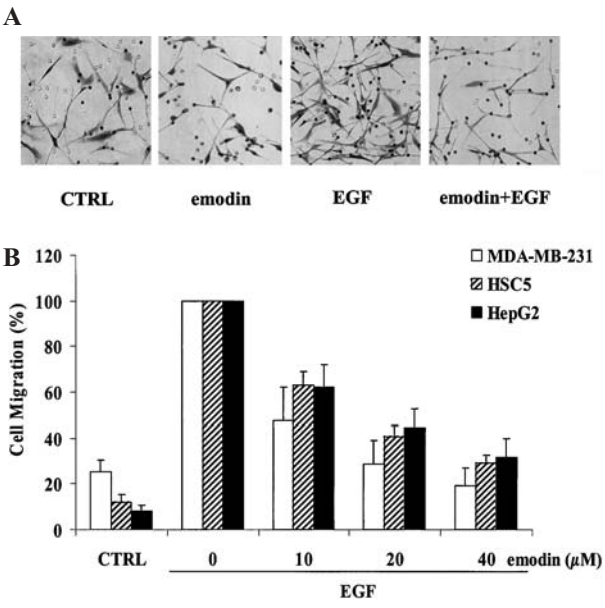


Figure 1. Inhibition of EGF-induced cell migration by emodin. Cells were incubated with 20 ng/ml EGF for 4 h after pretreatment with 0–40 µM emodin for 1 h. Cells that migrated to the lower surface of the membrane were fixed, stained, and counted, as described in Materials and methods. (*A*) Representative images showing the inhibitory effect of emodin on migration of MDA-MB-231 cells. (*B*) Quantification of the inhibitory effect of emodin on migration of MDA-MB-231, HSC5, and HepG2 cells. Migration data are presented as means ± SD of three independent experiments and expressed as percentage.

## Results

### Emodin inhibited EGF-induced migration in human cancer cells

Previous studies in our laboratory have demonstrated the strong anti-invasion activity of emodin in some cancer cell lines [18]. Since cell migration is one of the critical steps leading to cell invasion, we investigated further the effect of emodin on cancer cell migration. Here, we first measured the effect of emodin on EGF-stimulated cell migration in human breast cancer MDA-MB-231 cells. As shown in figure 1A, emodin treatment alone did not significantly affect cell migration, while it completely suppressed EGF-stimulated cell motility in MDA-MB-231 cells. To confirm that such inhibition is not cell type specific, we also tested the effect of emodin on other cancer cells, including HSC5 and HepG2. As shown in figure 1B, emodin showed a similar inhibitory effect in both HSC5 and HepG2 cells.

To rule out the possibility that such inhibition is due to its cytotoxicity, the cell viability of emodin-treated cells was determined using an MTT assay. The result showed that emodin had no significant effect on cell viability at concentrations ranging from 10 to 40 µM up to 12 h of treatment (data not shown), suggesting that emodin inhibits cell migration without obvious cellular cytotoxicity.

### Emodin blocked EGF-induced PI3K activation

The PI3K signaling pathway promotes cell migration leading to cancer cell invasion and metastasis [8, 20]. PI3K is a heterodimer composed of two subunits, a p85 regulatory subunit and a 110-kDa catalytic subunit [8]. Here, we first confirmed that PI3K was required for the EGF-induced migratory response in MDA-MB-231 cells. Our data showed that cells overexpressing DN mutants of PI3K, DN-p85 and DN-p110, failed to respond to EGF treatment in the cell migration assay (fig. 2A). Furthermore, DN-p110 overexpression suppressed the EGF-induced formation of filopodia and lamellipodia, two main cytoskeleton changes that are known to play an important role in the onset and maintenance of cell migration [21] (fig. 2B). These data suggest a pivotal PI3K involvement in EGF-induced cell migration.

Then we examined whether emodin inhibits EGF-stimulated cancer cell migration via the PI3K pathway. One well-defined downstream effector of PI3K is Akt [8]. As activation of PI3K correlated well with phosphorylation at $Thr^{308}$ and $Ser^{473}$ residues of Akt [22], we investigated

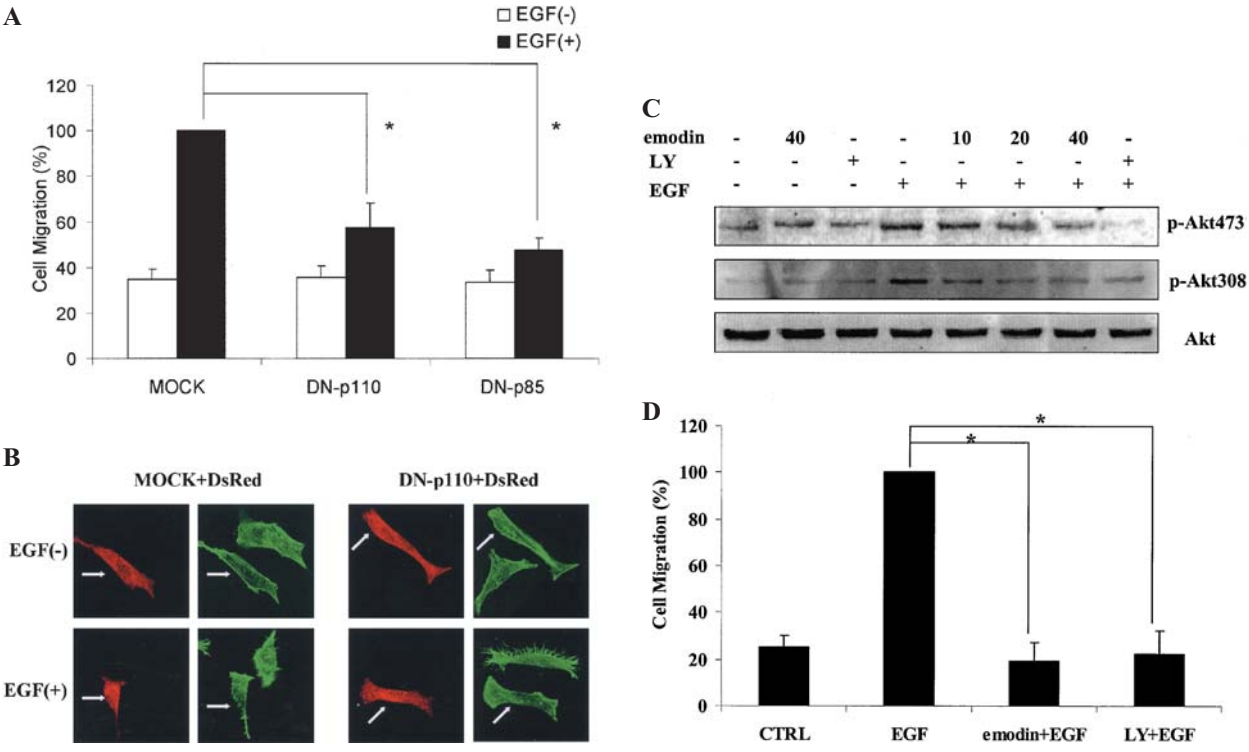


Figure 2. Emodin blocked EGF-induced PI3K activation. (*A*) Overexpression of DN-p110 and DN-p85 abolished EGF-induced cell migration. MDA-MB-231 cells were transfected with empty vector, DN-p110, or DN-p85, together with a pDsRed (a red fluorescence protein) expression vector used to mark the transfected cells. Cells were subjected to a cell migration assay as described in Materials and method, in the absence (open columns) or presence (solid columns) of 20 ng/ml EGF. Transfected cells (with red fluorescence) that had migrated to the lower surface of the membranes were counted under a fluorescence microscope. (*B*) Overexpression of DN-p110 abolished EGF-induced actin cytoskeleton changes. After transfection with empty vector or DN-p110, together with pDsRed, cells were incubated with or without EGF for 30 min. Then cells were fixed and stained with FITC-conjugated phalloidin to detect cytoskeleton changes. Arrows indicate transfected cells (with red fluorescence). (*C*) Inhibitory effect of emodin and LY294002 on EGF-induced phosphorylation of Akt. MDA-MB-231 cells were pretreated with 0–40 μM emodin, or 10 μM LY294002 for 1 h, followed by 15 min EGF incubation. Western blot of whole-cell lysates was performed as described in Materials and methods. (*D*) Inhibitory effect of emodin and LY294002 on EGF-induced cell migration. Cells were stimulated for 4 h with EGF after 1 h pretreatment with 40 μM emodin or 10 μM LY294002. A migration assay was performed as described in Materials and methods. Data are presented as means ± SD of three independent experiments and expressed as percentage (*$p<0.05$ compared to the EGF group and analyzed by Student's t test).

the effect of emodin on the phosphorylation of Akt. As shown in figure 2C, emodin markedly decreased the EGF-induced phosphorylation of Akt, which was completely abolished by LY294002, in a concentration-dependent manner. Emodin or LY294002 alone did not affect the phosphorylation of Akt. Consistently, both emodin and LY294002 suppressed EGF-induced cell migration (fig. 2D). Emodin and LY294002 also showed similar inhibitory effects on EGF-induced migration in two other cell lines, HSC5 and HepG2 (data not shown). These data suggested that emodin might suppress EGF-induced cell migration by interfering with the PI3K pathway.

**Involvement of the PI3K signaling pathway in the effect of emodin on cell migration**

To further confirm the involvement of PI3K in the inhibitory effect of emodin on EGF-induced cell migration, we measured cell migration after transfection with wild-type p110. As shown in figure 3, overexpression of p110 alone significantly enhanced cell migration and EGF was unable to further increase cell migration in those cells. Emodin, as well as LY294002, clearly inhibited the migration of p110-overexpressing cells with or without EGF. Together with the results in figure 2, these findings clearly suggested that emodin suppressed cell migration by interfering with the PI3K signaling pathway.

**Inhibitory effect of emodin on EGF-induced actin cytoskeleton changes and Cdc42/Rac1 activation**

Certain cytoskeleton changes, such as filopodia and lamellipodia, are closely related to cell migration [23, 24]. To further confirm that emodin suppresses cell migration through a PI3K-dependent pathway, we measured the cytoskeleton changes using an immunofluorescence method. As shown in figure 4A, EGF induced a significant increase in filopodia and lamellipodia formation; 49% and 63% of cells formed filopodia and lamellipodia,

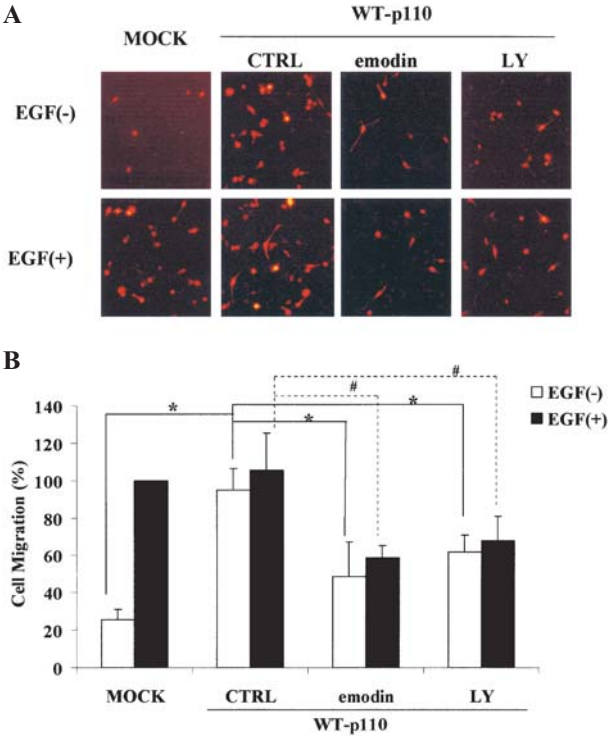


Figure 3. Involvement of PI3K in the inhibitory effect of emodin on cell migration. (*A*) Representative images showing the inhibitory effect of emodin and LY294002 on migration of WT-p110 transfected cells. (*B*) Quantification of the inhibitory effect of emodin on migration of WT-p110-transfected cells. Cells were transfected with empty vector or WT-p110, together with pDsRed. After 1 h pretreatment with 40 μM emodin or 10 μM LY294002, cells were subjected to a migration assay in the absence (open columns) or presence (solid columns) of 20 ng/ml EGF. Data are presented as means ± SD of three independent experiments and expressed as percentage (*p<0.05 compared to the control group without EGF; #p<0.05 compared to the control group with EGF).

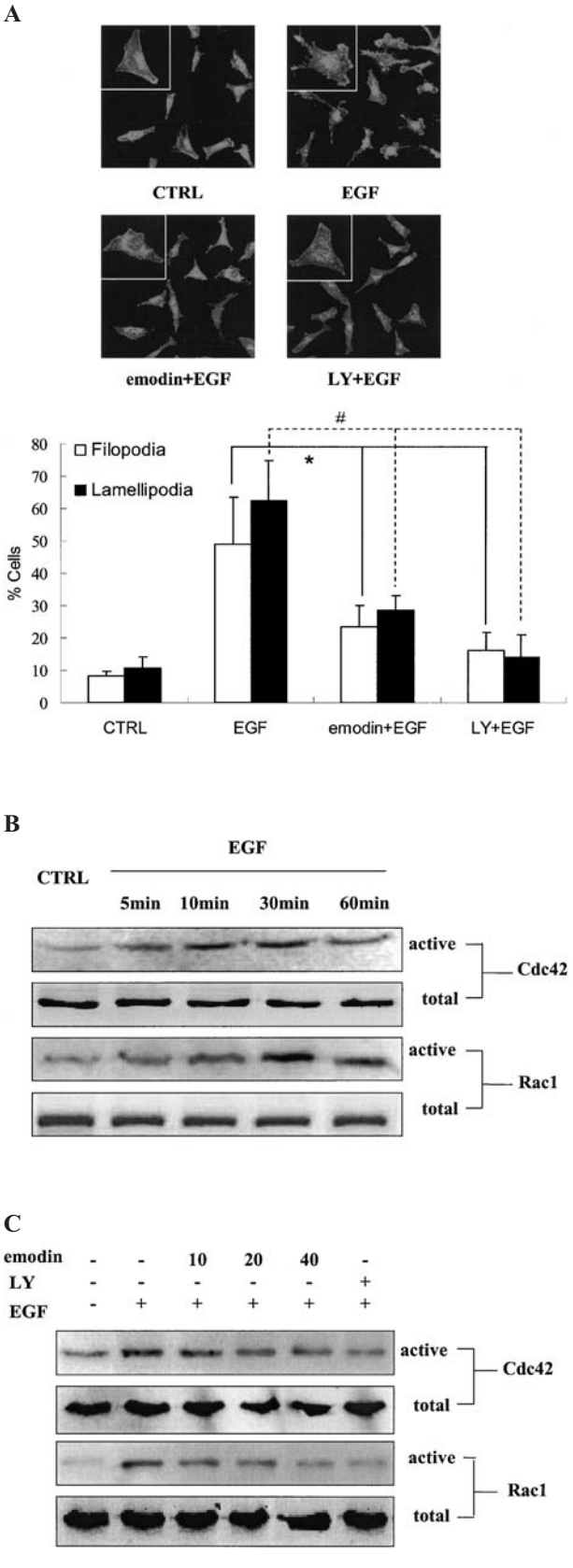


Figure 4. Emodin inhibited EGF-induced cytoskeleton changes and Cdc42 and Rac1 activation. (*A*) Inhibitory effect of emodin on EGF-induced filopodia and lamellipodia formation. Cells were stimulated with 20 ng/ml EGF for 30 min after 1 h pretreatment with 40 μM emodin. Cells were fixed and stained with FITC-conjugated phalloidin as indicated in Materials and methods. Cells at higher magnification are presented as inserts. (*B*) Cdc42 and Rac1 activation induced by EGF. (*C*) Inhibitory effect of emodin and LY294002 on EGF-induced activation of Cdc42 and Rac1. Cells were pretreated with 0–40 μM emodin or 10 μM LY294002 for 1 h followed by 30-min stimulation with 20 ng/ml EGF. Cells were assayed for Cdc42/Rac1 activation using a PAK/GST-PBD pull-down assay as described in Materials and methods. The levels of total cellular Cdc42/Rac1 were determined by Western blotting.

respectively, after 30 min of EGF stimulation. With emodin treatment, the number of cells with filopodia extensions was decreased to 23%, and that with lamellipodia extensions was decreased to 29%. A similar inhibitory effect was also observed with LY294002. Taken together, these results further support the notion that emodin inhibits an EGF-induced PI3K-dependent pathway, leading to the suppression of cytoskeleton changes and cell migration.

Cdc42 and Rac1, two main members of Rho GTPases, are crucial in the formation of filopodia and lamellipodia [25]. Moreover, both Cdc42 and Rac1 were reported to act as downstream effectors of PI3K in several growth-factor-stimulated pathways [5, 8, 26]. To investigate whether the inhibitory effect of emodin on cell migration pertains to the activation of Cdc42 and Rac1, we first examined EGF-mediated Cdc42 and Rac1 activation. We used a Cdc42/Rac1 activation assay kit (from Upstate) to measure the activation level of Cdc42 or Rac1. This is a direct pull-down experiment performed by measuring the binding capability of GTP-bound Cdc42 or Rac1 to GST-PAK1 PBD fusion protein immobilized onto glutathione-agarose beads. As shown in figure 4B, both Cdc42 and Rac1 were readily activated after 20 ng/ml EGF stimulation. EGF induced a substantial but transient increase in active Cdc42. Maximum Cdc42 activation occurred between 10–30 min after EGF stimulation. In contrast, Rac1 is activated in a modest but sustained manner, reaching the maximum level after 30 min. Next we measured the effect of emodin and LY294002 on the activation of Cdc42 and Rac1. As shown in figure 4C, emodin showed an evident inhibitory effect on EGF-induced activation of both Cdc42 and Rac1 in a concentration-dependent manner. LY294002 showed a similar suppressive effect. These results suggest that Cdc42 and Rac1, as downstream effector molecules of PI3K, are likely to be involved in the inhibitory effect of emodin on EGF-induced cell migration.

### Emodin interfered with the formation of the Cdc42/Rac1 and PAK1 complex

To further confirm the involvement of Cdc42 and Rac1 in EGF-induced cell migration, we transfected the cells with DN-Cdc42 or DN-Rac1 vector and found that DN-Cdc42 and DN-Rac1 plasmids significantly reduced EGF-induced cell migration (fig. 5A, 6A), suggesting that activation of Cdc42 and Rac1 is necessary for EGF stimulation of cell migration.

To clarify whether the inhibitory effect of emodin on cell migration and activation of Cdc42/Rac1 is not just a consequence of inhibition of PI3K activity, cells were first transfected with CA-Cdc42 or CA-Rac1 plasmid, then treated with emodin or LY294002 for 1 h, followed by measuring the level of Cdc42 or Rac1 activation and a cell migration assay. As shown in figures 5B and 6B,

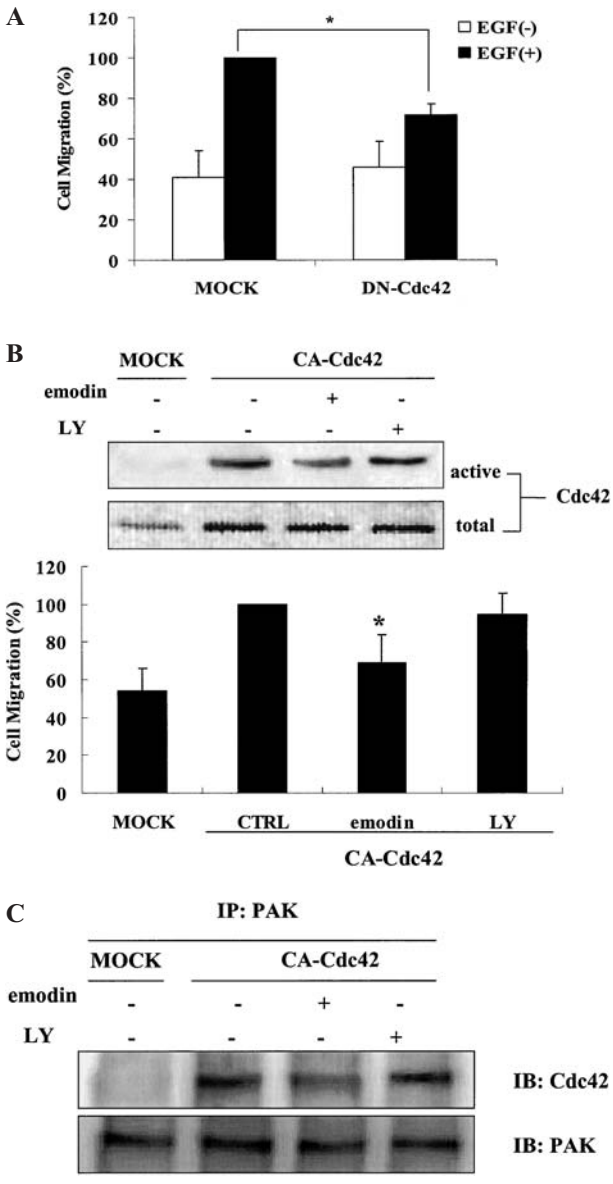


Figure 5. Emodin interfered with Cdc42-PAK1 complex formation. (*A*) Overexpression of DN-Cdc42 inhibited EGF-induced cell migration. MDA-MB-231 cells were transfected with empty vector or DN-Cdc42, together with pDsRed. A cell migration assay was conducted as described in Materials and methods. (*B*) Emodin inhibited migration of CA-Cdc42-transfected cells. Twenty-four hours after transfection with empty vector or CA-Cdc42, together with pDsRed, cells were starved in FBS-free medium for another 24 h. Then they were incubated with or without 40 μM emodin or 10 μM LY294002 for 1 h. Cells were then assayed for Cdc42 activation and motility, as described in Materials and methods. Migration data are presented as means ± SD of three independent experiments and expressed as percentage (*$p<0.05$ compared to the control group). (*C*) Emodin interfered with the formation of Cdc42 and PAK1 complex. Cells were transfected with empty vector or CA-Cdc42, together with pDsRed, starved and treated with 40 μM emodin or 10 μM LY294002 for 1 h. Cell lysates were immunopricipitated (IP) with anti-PAK1 antibody following by anti-Cdc42 immunoblotting (IB). Data shown are representative of three experiments with similar results.

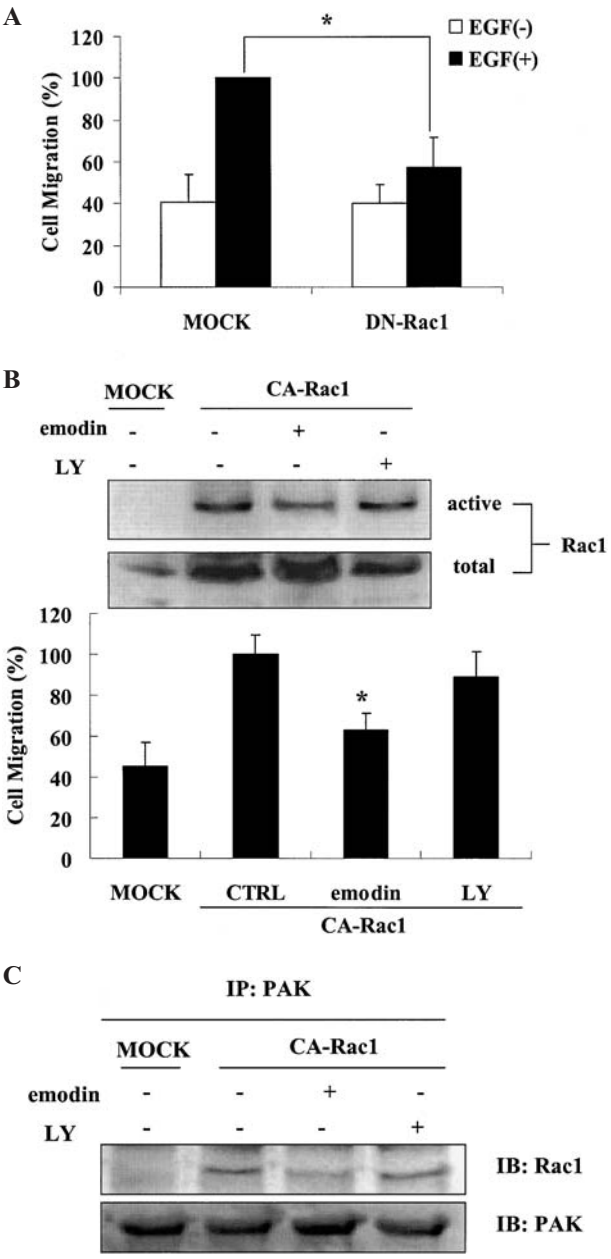


Figure 6. Emodin interfered with the formation of Rac1 and the PAK1 complex. (*A*) Overexpression of DN-Rac1 inhibited EGF-induced cell migration. MDA-MB-231 cells were transfected with empty vector or DN-Rac1, together with pDsRed. A cell migration assay was conducted as described in Materials and methods. (*B*) Emodin inhibited migration of CA-Rac1-transfected cells. Twenty-four hours after transfection with empty vector or CA-Cdc42, together with pDsRed, cells were starved for another 24 h. Then cells were treated with or without 40 μM emodin or 10 μM LY294002 for 1 h. Cells were assayed for Rac1 activation and motility, as described in Materials and methods. Data are presented as means ± SD of three independent experiments and expressed as percentage (*$p<0.05$ compared to the control group). (*C*) Interference by emodin with the formation of Rac1 and PAK1 complex. Cells were transfected with empty vector or CA-Rac1, together with pDsRed, starved and treated with 40 μM emodin or 10 μM LY294002 for 1 h. Cell lysates were immunopricipitated (IP) with anti-PAK1 antibody following by anti-Rac1 immunoblotting (IB). Data shown are representative of three experiments with similar results.

emodin significantly suppressed cell migration in CA-Cdc42- and CA-Rac1-overexpressing cells, while LY294002 had no obvious effect. This data clearly suggest that the inhibitory effect of emodin on cell migration is not only a consequence of suppressing PI3K activation.

One interesting finding here was that the Cdc42- and Rac1 activation level was deceased after emodin treatment in CA-Cdc42- and CA-Rac1-overexpressing cells, respectively (figs 5B, 6B). How could emodin 'inhibit' the activity of CA-Cdc42 and CA-Rac1 in the cell? In our experimental system, the Cdc42/Rac1 activation assay is basically a direct pull-down experiment performed by measuring the binding of the active form of Cdc42 or Rac1 to GST-PAK1 PBD. Therefore, the 'reduced activity' of Cdc42/Rac1 after emodin treatment in CA-Cdc42- or CA-Rac1-overexpressing cells might be due to interference by emodin with the formation of an active Cdc42/Rac1 and PAK1 complex. To test this possibility, a co-immunoprecipitation assay was performed in CA-Cdc42- or CA-Rac1-overexpressing cells to examine the effect of emodin on the protein-protein interaction between Cdc42/Rac1 and PAK1. As shown in the figures 5C and 6C, emodin, but not LY294002, significantly suppressed the association of active Cdc42/Rac1 with PAK1. These data therefore confirm our hypothesis that emodin may disrupt the formation of the Cdc42-PAK1 and Rac1-PAK1 complex.

## Discussion

Emodin is an active component from the rhizome of *R. palmatum* and has been reported to possess anti-tumor effects in various cancer cells [27]. However, little is known about its effect against tumor invasion and metastasis. We previously reported that emodin inhibited cancer cell invasion in both HSC5 and MDA-MB-231 cells through suppression of activator protein-1 and nuclear factor kappaB [18]. In this study, we further investigated the effect of emodin against cancer cell migration, and the molecular mechanisms involved. We found that non-toxic levels of emodin could efficiently suppress the migration of EGF-treated cancer cells, and this suppression is achieved through inhibiting PI3K activity and interfering with Cdc42/Rac1-PAK complex formation.

PI3K is one of the most important regulatory proteins, involved in controlling several key functions of the cell, such as cell growth, aging, and transformation. It consists of two subunits: p85 (85-kDa regulatory subunit) and p110 (110-kDa catalytic subunit). The activation of PI3K occurs through interaction with receptor (growth factor receptors) or non-receptor tyrosine kinases [8]. Among the main downstream effectors of PI3K, Akt is mainly

involved in the control of apoptosis; and the Ras/Raf/Erk pathway regulates cell growth and division. A role for PI3K in the regulation of cell migration has also been well documented [6, 7]. Activation of the PI3K p110 subunit in breast cancer cells has been reported to be necessary for EGF-induced actin nucleation [28], an important cytoskeleton change for cell migration. In our study, we first confirmed the critical role of PI3K in EGF-induced cell migration in MDA-MB-231 cells, based on the following observations. First, EGF enhanced Akt phosphorylation which is blocked by a specific PI3K inhibitor (LY294002) (fig. 2C); second, overexpression of DN-p110 and DN-p85, as well as LY294002 abolished EGF-induced cell migration (fig. 2A, 2D), and third, LY294002 and DN-p110 inhibited EGF-induced Cdc42 and Rac1 activation and the formation of filopodia and lamellipodia (figs. 2B, 4B, and data not shown). These data indicate that PI3K activation plays a critical role in mediating EGF-induced cell migration in MDA-MB-231 cells.

In this study, we found that emodin suppressed EGF-induced migration in a number of human cancer cell lines (fig. 1). A previous report demonstrated that emodin is an inhibitor of PI3K in a cell-free kinase assay system [29]. We thus hypothesized that inhibition of the PI3K pathway was the underlying mechanism responsible for the effect of emodin against cell migration. This hypothesis is supported by the observation that emodin suppressed the phosphorylation level of Akt, a well-defined downstream effector of PI3K (fig. 2C). In addition, emodin inhibited the migration of cells transfected with CA-p110 vector (fig. 3). The above finding is basically consistent with previous reports on inhibition of the PI3K pathway and suppression of cell migration by other chemopreventive agents such as red wine polyphenols [30], sulindac sulfide, and caffeic acid phenethyl ester [31].

The organized polymerization of actin filaments is believed to be an important mechanism required for cell migration [21]. Among several cell structures related to cell migration, lamellipodia are broad flat membrane extensions generally formed through the polymerization of filamentous (F)-actin, while filopodia are often found at the edges of the lamellipodia and are also the result of actin polymerization [25]. The Rho GTPases are reported to be key regulators of actin dynamics that lead to organized actin-based structures associated with cell migration. Activated Cdc42 and Rac1 stimulate filopodia and lamellipodia formation, respectively [23]. In the present study, EGF induced significant actin cytoskeleton changes and emodin markedly inhibited the EGF-induced formation of filopodia and lamellipodia (fig. 4A), consistent with the finding that emodin inhibited EGF-induced activation of Cdc42 and Rac1 (fig. 4C).

Being downstream of PI3K, Cdc42 and Rac1 are the two important GTPases that have been implicated in many cellular processes, such as cytoskeleton rearrangement, cell adhesion, and transcriptional activation, and are believed to be involved in cancer cell migration, invasion, and metastasis [23, 24, 26]. One important Cdc42/Rac1 effector are PAKs, which also play a critical role in cytoskeleton reorganization and cell migration [32]. By direct interaction of their PBD with GTP-bound Cdc42 or Rac1, PAKs undergo a conformational change that allows autophosphorylation and activation [33]. Downstream targets for PAKs include myosin light-chain kinase (MLCK) and LIM kinase (LIMK) that are known to be involved in migration. MLCK phosphorylates the myosin light chain, which is important in regulating actin cytoskeletonl dynamics [34]. In addition PAK phosphorylates and activiates LIMK, which in turn phosphorylates and inactivates the actin-severing protein cofilin, thus promoting filament assembly [35]. In the present study, we found that emodin, but not LY294002, inhibited the migration of CA-Cdc42- or CA-Rac1-overexpressing cells (figs 5B, 6B). Furthermore, emodin significantly interfered with the formation of the Cdc42/Rac1 and PAK1 complex (figs 5C, 6C), while LY294002 had no such effect. This interference might lead to PAK1 inactivation and, in turn, affect the downstream cytoskeletal targets of PAK. These results suggest that, beside its inhibitory effect on PI3K activity, emodin might also inhibit cell migration through another mechanism – disrupting the formation of an active Cdc42/Rac1 and PAK complex.

Active migration of tumor cells is a prerequisite for tumor metastatsis and cancer development and progression. Inhibition of tumor cell motility has been considered an effective approach for the suppression of cancer invasion and metastasis. In this study, we provide convincing evidence that emodin was able to inhibit EGF-induced cell migration through two independent mechanisms: (i) by suppressing PI3K activity and (ii) by disrupting the formation of the Cdc42/Rac1 and PAK complex. Such findings suggest that this anthroquinone interferes with tumor progression at several pivotal points and it could be used as a chemotherapeutic agent in preventing invasion or metastasis of human cancer.

*Acknowledgements.* We would like to thank Dr. P. Rose for valuable discussion. We also wish to thank Dr. Y. Qian (National Institute for Occupational Safety and Health) and Dr. L. H. Wang (Mount Sinai University of New York) for their assistance in providing DN-Cdc42, CA-Cdc42, DN-Rac1, and CA-Rac1 vectors.

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