

# Nischarin inhibits Rac induced migration and invasion of epithelial cells by affecting signaling cascades involving PAK

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Received 2 October 2002, revised version received 13 March 2003

## Abstract

Nischarin, a cytosolic protein that binds the  $\alpha 5 \beta 1$  integrin, plays an important role in fibroblast migration, and in regulation of the actin cytoskeleton. The effect of Nischarin on Rac induced migration and invasion by breast and colon epithelial cell lines has been determined. In these cells, Rac potently induced migration, as well as invasion of matrix; both of these events were strongly inhibited by overexpression of Nischarin. To understand the mechanism of Nischarin's inhibitory role in Rac induced cell migration, several effector domain mutants of Rac1 were employed. Nischarin was able to inhibit migration induced by Rac effector mutants that can activate PAK and JNK, but not migration stimulated by other Rac mutants. Further, Nischarin inhibited PAK induced cell migration, while not affecting migration induced by MEKK1, a Rac effector in the JNK pathway. In addition, Nischarin failed to inhibit migration induced by MEK1, a downstream effector in the Ras-Raf-MEK-Erk signaling cascade. Furthermore, Nischarin does not affect Rac mediated JNK and PI3K activities. However, Rac induced migration and invasion were effectively blocked by pharmacological inhibitors of PI-3 kinase and MEK. These results suggest that several pathways contribute to cell migration, but that Nischarin selectively inhibits Rac driven signaling cascades that affect migration through PAK.

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*Keywords:* Nischarin; Rac; Migration; Invasion; Integrin

## Introduction

Cell migration is an important aspect of many biological processes including wound healing, angiogenesis, embryonic development, and oncogenic progression [1]. Cell invasion is a complicated process that involves partial detachment from intercellular adhesions, and from cell-ECM interactions mediated by integrins, reorganization of the actin cytoskeleton, and movement through the ECM [2]. Rho family GTPases control a wide variety of biological processes, but are particularly involved in the organization of the actin cytoskeleton and in cell migration and invasion [3]. For example, in T47D mammary carcinoma cells [4], activated Rac promotes integrin dependent cell motility, while Tiaml, a guanine nucleotide exchange factor that activates Rac, induces invasion in T-lymphoma cells [5] and

murine breast cancer epithelial cells [6,7]. Thus, there is extensive evidence for a role of Rac in cell motility and invasion.

The biological effects of Rac are exerted through the activation of several downstream effectors [8]. One important family of Rac effectors is comprised of the p21 activated kinases (PAKs) [9] that play a role in cytoskeletal reorganization [10] and cell migration [11–15]. The best understood function of PAK in actin regulation is the phosphorylation and activation of LIM kinase (LIMK); active LIMK inhibits the actin severing protein cofilin, leading to actin depolymerization [16,17]. PAK has also been shown to phosphorylate and inhibit MLCK, leading to inhibition of myosin phosphorylation and reduction of contractility [18]. Rac also activates MEKK1 [19], leading to activation of MKK4 and subsequent activation of JNK and p38 [20–22]. MEKK1 has been shown to associate with active Rac 1 [19], and MEKK1 has been implicated in cell motility [23].

Nischarin is a novel protein that binds selectively to the

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integrin  $\alpha 5$  subunit cytoplasmic tail [24]. Observations of Nischarin's effects on the actin cytoskeleton, cell migration, and Rac driven lamellipodia formation [24], led me to hypothesize that Nischarin may play a role in Rac-regulated cell motility and invasion. However, little is known about the mechanistic bases of Nischarin's actions. Here I demonstrate that Nischarin inhibits Rac induced cell migration and invasion in breast and colon epithelial cells without having a major effect on cell adhesion. I also sought to determine how Nischarin affected migration stimulated by Rac effector domain mutants that differ with respect to their downstream signaling events [25]. My findings indicate that Nischarin impairs Rac-driven migration only for those mutants able to activate PAK and JNK. Further investigation suggested that Nischarin effects on migration are mediated primarily through PAK.

## Materials and methods

### Cell culture

MCF7 cells were maintained in alpha MEM containing sodium pyruvate and insulin, and supplemented with 10% FBS. SW480 cells were maintained in Leibovitz L-15 medium (Sigma, St. Louis, MO, USA) supplemented with 10% FBS in a CO<sub>2</sub>-free incubator. During transfection, SW480 cells were maintained in DMEM supplemented with 10% FBS.

### Antibodies and pharmacological reagents

Mouse monoclonal antibodies to integrin  $\alpha 5$  and integrin  $\beta 1$  subunits were purchased from Chemicon International, Inc (Temecula, CA, USA). Pharmacological inhibitors UO126, LY294002, and Wortmannin were purchased from Calbiochem (San Diego, CA, USA), while PD98059 was from Promega (Madison, WA, USA).

### Immunoblot analysis

Western blotting was performed essentially as described [26]. Briefly, cells were washed twice with cold PBS and lysed in modified RIPA buffer (50 mM Tris (pH 7.5), 1% NP40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM NaF, 1 mM sodium pyrophosphate) with protease inhibitors. The protein lysates were resolved on 10% SDS PAGE gels, transferred onto PVDF membranes and incubated with either antiphospho serine 473 AKT (New England Biolabs, Beverly, MA, USA) or monoclonal antiphospho ERK (Santa Cruz Biotechnology) primary antibodies, followed by their respective secondary antibodies. The signals were detected by enhanced chemiluminescence.

### Immunoprecipitations

MCF7 cells were transfected with HA-JNK1 + Vector, RacQ61L + HA-JNK1 + Vector, RacQ61L + HA-JNK1 + Myc-Nischarin and lysed in modified RIPA buffer. In these experiments, quantity of DNA used was 1  $\mu$ g of HA-JNK1, 2  $\mu$ g of Rac Q61L, and/or 2  $\mu$ g of Myc-Nischarin, and DNA amount was normalized with vector DNA. All lysis buffers contained phosphatase inhibitors (2 mM phenyl methyl sulfonyl fluoride, 1 mM sodium vanadate, 10  $\mu$ g/ml aprotinin, 1 mM nitrophenol phosphate, 5 mM benzamide). HA-JNK was immunoprecipitated from the respective lysates by using anti-HA antibody (Covance) for 2 h at 4°C, followed by the addition of protein-G sepharose (Pharmacia) for 2 h at 4°C.

### JNK kinase assays

Protein G Sepharose bound material was washed two times in modified RIPA, and two times in wash buffer (0.25 M Tris (pH 7.5), 0.1 N NaCl), and 40  $\mu$ l of kinase assay buffer (10 mM Tris, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 10  $\mu$ M ATP, 5  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP and 2  $\mu$ g of GST-c-Jun (1-133)) was added, and incubated at 30°C for 30 min with gentle shaking. Reactions were stopped by the addition of 10  $\mu$ l 6XSDS Laemmli sample buffer and boiling for 5 min. Proteins were fractionated on 15% SDS-PAGE, and the gels were dried and exposed to X-ray films for overnight.

### AKT phosphorylation assays

MCF7 cells were transfected with HA-AKT1 + vector, RacQ61L + HA-AKT1 + vector, or RacQ61L + HA-AKT1 + Myc-Nischarin and lysed in modified RIPA as described above. HA-AKT was immunoprecipitated from the lysates by using anti-HA antibody and the samples were subjected to SDS-PAGE, transferred to PVDF membrane, and immunoblotted with antiphospho specific (P473) AKT antibody, or anti-AKT antibody.

### Transwell cell migration assay

MCF7 or SW480 cells were transiently transfected with a vector control (pAX 164), constitutively active Rac (pAX Rac Q61L), or active Rac along with Nischarin. In all transfections, 3  $\mu$ g of pAX164 Rac and 2  $\mu$ g of pcDNA myc Nischarin were used. A GFP expression plasmid (1  $\mu$ g) was used to mark the transfected cells. Rac effector domain mutants (see Table 1), pCMV PAK 165 (gift from Melanie Cobb) [27], pcDNA V5 PAK T423E (originally gift from Jonathan Chernoff [28], and subcloned into pcDNA V5 vector (Invitrogen)) or pRCMEKK1 (Stratagene), with or without Nischarin vector, were transfected in the same way. In some cases 1  $\mu$ g of pRC  $\beta$ -gal plasmid (Stratagene, La Jolla, CA) was used as marker. All transfections were normalized for total DNA using vector plasmid. Assays were

Table 1  
Migratory properties of effector domain mutants and Nischarin's effects

Protein	Lamellipodia	JNK	PAK	Migration
pAX Rac Q61L	Strong	Strong	Strong	Strong/inhibited by Nisch
pCGN 61L43D	Strong	Strong	Strong	Strong/inhibited by Nisch
pCGN 61L40C	Strong	No	No	Strong/not Inhibited by Nisch
pcDNA 12V40H	Strong	No	No	Strong/not Inhibited by Nisch
pCGN 61L 31V	Weak	Strong	Strong	No effect
pCGT 12V37L	No	Weak	Weak	No effect

*Note.* Induction of lamellipodia and activation of JNK and PAK were previously determined [25]. Migration was indicated as strong or weak, and Nischarin's effects were indicated as inhibited or not inhibited. No, no activity was seen.

performed essentially as described earlier [24]. Briefly, after overnight transfection, cells were washed with PBS, maintained in 0.5% serum overnight, harvested using a trypsin EDTA solution, and resuspended in BSA medium. Transfection efficiency of these cells is about 10%. The underside of the transwell was coated with fibronectin and 200,000 cells (approximately 20,000 transfected cells) were added on the upper surface. The GFP positive cells that migrated through the membrane during an overnight incubation were counted by fluorescence microscopy. Approximately 10% of Rac transfected cells migrated to the other side of the membrane, while only 2% of vector transfected cells migrated. In the case of cells transfected with a  $\beta$ -gal plasmid, cells were fixed and stained for  $\beta$ -gal as discussed previously [24]. The ratio of migrant transfected cells to total transfected cells was plotted. The data are presented relative to cells transfected only with the pAX vector, which is normalized to 1.0. Treatment with pharmacological inhibitors, or antibodies, was performed as described [4].

#### Cell adhesion assay

MCF7 cells were cotransfected with 2  $\mu$ g of Nischarin and 250 ng of pGL3 luciferase plasmid (as an indicator). After 48 h of transfection, cells were replated on fibronectin coated dishes for various times. Cells not adhered to fibronectin were removed by washing with PBS. The adhered cells were lysed in luciferase lysis buffer (Promega, Madison, WI, USA). After a brief spin to remove nonsolubilized material, the cell lysates were tested for luciferase activity on a luminometer (Analytical Luminiscence Laboratory). In all transfections, DNA quantities were normalized with the pcDNA vector. Luciferase activity was measured by normalizing for total protein content.

#### Cell invasion assay

Invasion assays using transwell membrane filters were essentially the same as described [4], with minor modifications. The under surfaces of transwell membranes were coated with 20  $\mu$ g/ml fibronectin for several hours at 37°C, and the upper surfaces were coated with 1 mg/ml Matrigel

matrix (Becton Dickinson Labware) at 37°C for 30 min. As described above, cells were transiently transfected with various plasmids, serum starved, trypsinized, and resuspended in BSA containing medium.  $2 \times 10^6$  cells (about  $2 \times 10^5$  transfected cells) were added to transwell chambers and allowed to invade through the membrane for 48 h toward the lower chamber, which contained medium with 10% serum. About 0.5% of Rac transfected cells invaded, while only 0.1% vector transfected cells invaded. All transfections were normalized for total DNA using vector plasmid. Non-invading cells were removed with sterile cotton swabs, and invading cells were fixed, and GFP positive cells were counted by fluorescence microscopy. The ratio of invading transfected cells to total transfected cells was plotted. The data are presented relative to cells transfected with pAX vector only and normalized to 1. For experiments with pharmacological inhibitors or antibodies, cells were harvested as described above, the cells were treated with the inhibitors at 37°C for 30 min, and invasion assays were performed in the presence of inhibitors or antibodies.

## Results

#### *Nischarin blocks Rac induced cell migration*

Earlier work indicated that Nischarin inhibits fibroblast migration and actin organization. Here I investigated whether Nischarin affects Rac induced carcinoma cell migration. MCF7 or SW480 epithelial cells transiently expressing constitutively active Rac with or without Nischarin were subjected to cell migration assays. As shown in Fig. 1A, Rac induced MCF7 cell migration was threefold greater than for vector controls, and was strongly inhibited by Nischarin. Rac induces cell migration in SW480 cells as well, and Nischarin had a moderate but significant inhibitory effect on Rac dependent migration in these cells (Fig. 1A). These inhibitory effects are statistically significant. Western blot analysis revealed a strong expression of constitutively active Rac as compared with endogenous Rac; Nischarin expression had no effect on the expression of Rac (not shown). As described previously [24], transfection with

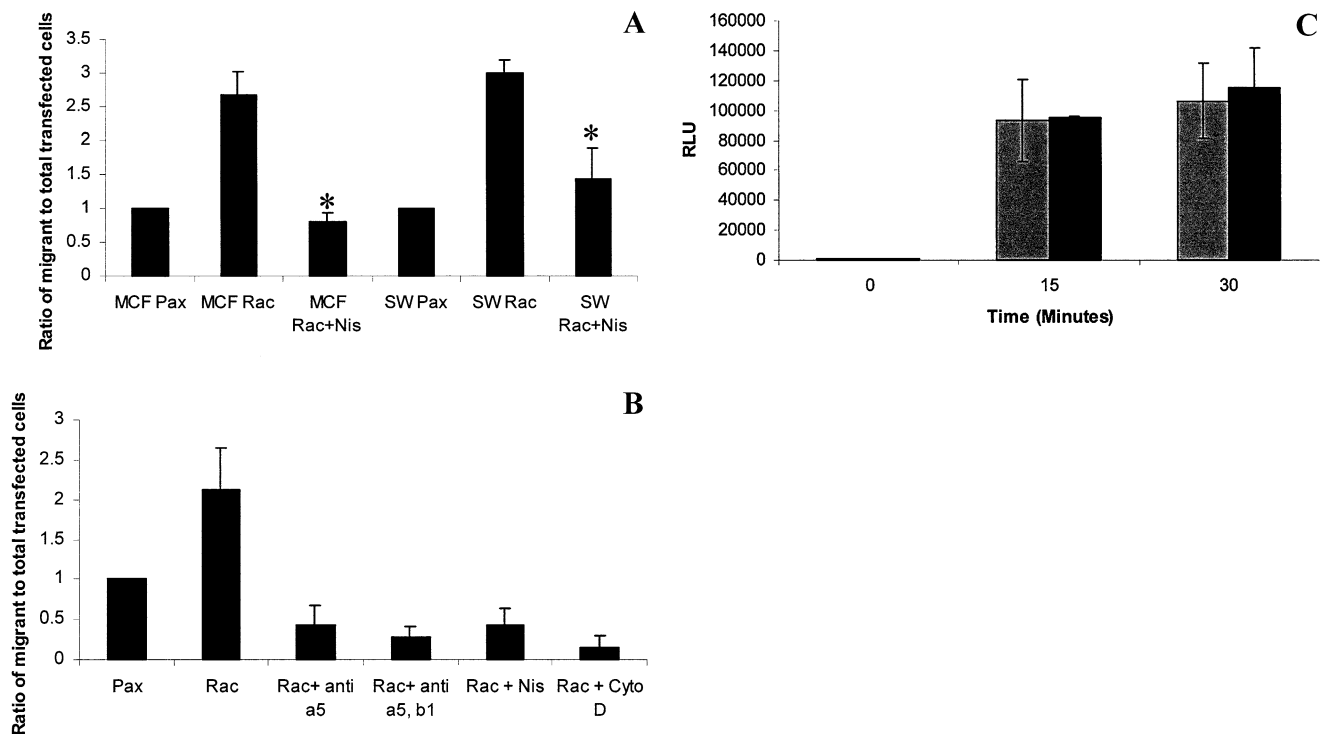


Fig. 1. Effect of Nischarin on Rac induced cell migration. (A) Nischarin inhibits Rac induced migration in MCF7 and SW480 cells. Cell migration assays were performed as described in the methodology section. Cells were transiently transfected with pAX vector + pcDNA3 vector, pAXRac Q61L + pcDNA vector, or pAX Rac Q61L + pcDNA3 Nischarin, and plated onto transwells, and migrating cells were counted. Inhibitory effects are statistically significant. MCF7 cells  $*P < 0.01$  ( $P = 0.009$ , migration between Nischarin (Rac + Nischarin) expressing cells and vector (Rac + vector) transfected cells was compared); SW480 cells  $*P < 0.01$  ( $P = 0.008$ , migration between Nischarin (Rac + Nischarin) expressing cells and vector (Rac + vector) transfected cells was compared). (B) Cell migration on fibronectin is mediated via  $\alpha 5\beta 1$ . This assay was performed exactly as in 1A and the transfected MCF7 cells were incubated with 10  $\mu\text{g}$  of each of anti- $\alpha 5$ , anti  $\alpha 5$  + anti- $\beta 1$  or anti-IgG for 30 min and migration was performed in the presence of antibodies. The results in A and B represent the means and standard errors of three determinations. (C) Effect of Nischarin overexpression on cell adhesion. MCF7 cells were transfected with a plasmid expressing Nischarin (Myc-Nischarin), and cotransfected with a luciferase plasmid. The transfected cell populations were analyzed for cell adhesion behavior, and the figure illustrates the kinetics of adhesion to a fibronectin-coated surface for Nischarin transfectants vs vector transfected MCF7 cells. At 0 min time point, hardly any cells adhered and thus luciferase values are in background levels. Results are the means and standard errors of three determinations. The gray bars represent the control luciferase transfectants; the black bars represent cells transfected with 2  $\mu\text{g}$  of Nischarin plasmid.

Nischarin did not impair cell viability; thus, the reduced migration due to the overexpression of Nischarin was not caused by cell loss.

Because  $\alpha 5\beta 1$  is a major receptor for fibronectin, I anticipated that antibodies to this integrin would block cell migration on fibronectin. Thus, Rac transfected cells were treated with anti- $\alpha 5$  and anti- $\beta 1$  antibodies. In the presence of an anti- $\alpha 5$  blocking antibody, cell migration was dramatically inhibited in both cell lines (Fig. 1B, and data not shown for SW480 cells). When the anti- $\alpha 5$  antibody was used in combination with an anti- $\beta 1$  antibody, the extent of inhibition was further enhanced. Migration was almost completely abolished when Rac expressing cells were treated with the actin disrupting drug cytochalasin D. These data indicate that the  $\alpha 5\beta 1$  integrin and actin filament organization are important for Rac induced cell migration on fibronectin in MCF7 and SW480 cells, as anticipated. However, it should be noted that Rac expressing MCF7 cells can migrate on other substrata (such as collagen) and Nischarin expression can block this (data not shown).

#### *Nischarin does not affect cell adhesion*

In order to examine whether the effects on motility are due to changes in cell adhesion, MCF7 cells were cotransfected with Myc-Nischarin and with pGL3 luciferase plasmid (as an indicator). As shown in Fig. 1C, Nischarin transfected cells adhered to fibronectin as strongly as vector control transfected cells, indicating that Nischarin expression does not lead to reduce adhesion. Thus, overexpression of full length Nischarin does not inhibit integrin-dependent adhesion to fibronectin in MCF7 cells as well as in SW480 cells (data not shown).

#### *PI-3-kinase and Erk are involved in Rac induced carcinoma cell migration*

It is known that PI3K often plays an important role in growth factor and Rac induced cell migration. A majority of studies implicate PI3K upstream of Rac, however, some reports placed PI3K downstream of Rac [29,30]. Here I

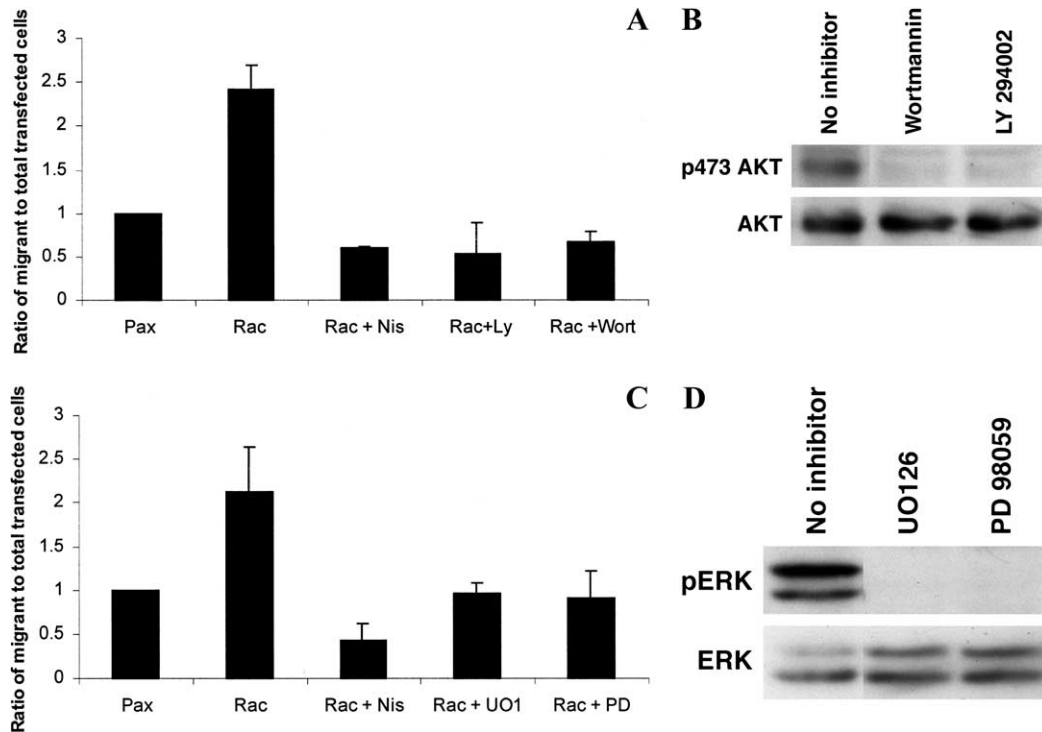


Fig. 2. Rac induced migration requires PI3K and ERK. (A) PI3K is required for Rac induced migration. MCF7 cells or SW480 cells (data not shown) were transfected with various plasmids as described, and the cells were incubated with 50  $\mu$ M of LY294002 or 50  $\mu$ M Wortmannin and the migration assay was performed in the presence of the inhibitors. (B) PI3K inhibitors modulate AKT phosphorylation in MCF7 and SW480 cells. MCF7 or SW480 cells (data not shown) were treated with 50  $\mu$ M of LY294002 or 50  $\mu$ M Wortmannin overnight and Western blotting was performed on the lysates made from these cells using phospho-specific p473 anti-AKT (New England Biolabs) (top panel) and the same blot was stripped and blotted with anti-AKT antibody (New England Biolabs) (bottom panel). (C) ERK is required for Rac induced cell migration. Cell migration assays were performed in the presence of 20  $\mu$ M UO126 or 20  $\mu$ M PD98059 compounds. (D) MEK inhibitors modulate phosphorylation of ERK. These experiments were performed exactly as described in 2B except UO126 or PD 98059 were used instead of PI3K inhibitors as well as the appropriate anti ERK and anti-phospho ERK antibodies (Santa Cruz Biotechnology).

examined the role of PI3K in Rac driven cell migration in the epithelial cell lines using the pharmacological inhibitors LY294002 and Wortmannin. In the presence of these inhibitors, Rac promoted cell migration was almost completely blocked in both MCF7 and SW480 cells, while vector control cells showed only a minimal effect (Fig. 2A and data not shown). The concentrations of inhibitor used did not affect the expression levels of Rac1 Q61L (data not shown), however, as expected, phosphorylation of AKT, a downstream target of PI3K, was inhibited [26] (Fig. 2B). These data suggest that Rac induced migration in MCF7 and SW480 cells involve a major contribution from the PI3K pathway.

The Ras-Raf-MEK-ERK cascade has also been implicated in cell migration [31,32]. To date there are no reports suggesting a direct role of Rac in activating the ERK pathway, however Rac plays a supporting role in Raf mediated activation of ERK [33]. In order to study the role of ERK in Rac driven cell migration, I used the MEK1/2 specific inhibitors UO126 and PD98059. Inhibition of ERK activation by these compounds significantly affected Rac1 induced cell migration (Fig. 2C), indicating that ERK activation is also important for Rac1 induced cell migration in these epithelial cells. To confirm that UO126 and PD 98059

inhibit MAPK in these cells, a Western blot analysis was performed using antibodies that recognize phospho-ERK1 and 2. Consistent with earlier studies [26,33], the treatment of cells with the MEK inhibitors prevented the activation of ERK (Fig. 2D).

#### *Suppression of Rac induced cell invasion by Nischarin*

To investigate the effect of Nischarin on Rac induced cell invasion, MCF7 or SW480 cells were transfected with active Rac along with Nischarin, and transfected cells were identified using a GFP plasmid as described in methods. Consistent with effects on Rac induced motility, the in vitro invasiveness of Rac expressing cells was increased more than twofold relative to that of vector transfected cells. As shown in Fig. 3A, cell invasion was decreased by ~50% in cells expressing Rac plus Nischarin compared to Rac plus empty vector controls. In contrast to the migration data, Nischarin had a somewhat greater effect on invasion of Matrigel by SW480 cells compared to invasion by MCF7 cells. These inhibitory effects are statistically significant. No significant differences in transfection efficiency in the different cell populations were observed, as determined by counting total transfected cells (data not shown).

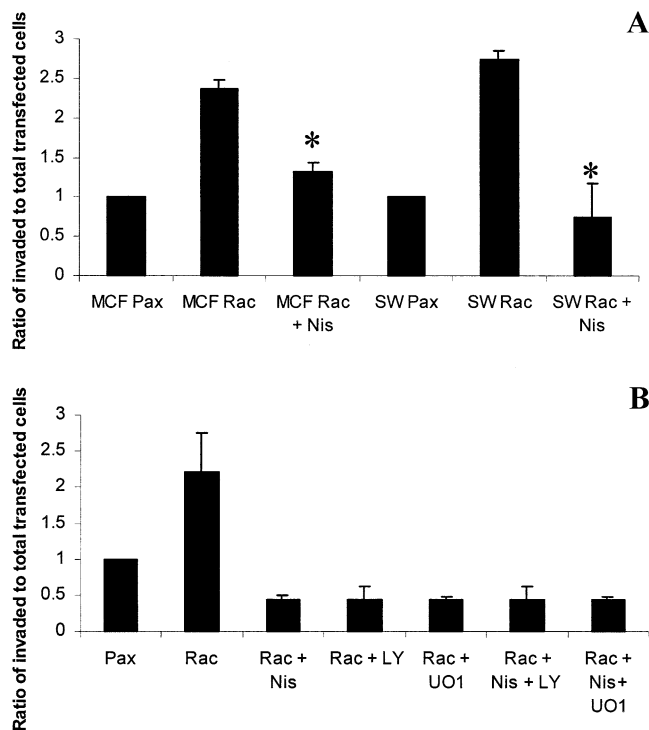


Fig. 3. Inhibition of Rac induced invasion by Nischarin. (A) Constitutively active Rac driven cell invasion into Matrigel. Cells expressing active Rac with or without Nischarin were plated in transwells coated with Matrigel. Transfection with the pAX142 vector (pax) served as a negative control. Invasion was carried out using MCF7 (MCF) and SW480 (SW) cells. (B) PI3K and ERK required for Rac induced invasion. SW480 cells were transfected, and preincubated with the pharmacological inhibitors UO126, or LY294002 for 30 min and the invasion assay was performed for 48 h as described in the Materials and methods section. Results in A and B represent the means and standard errors of three determinations. Inhibitory effects are statistically significant. MCF7 cells,  $*P < 0.01$  ( $P = 0.01$ , invasion between Nischarin (Rac + Nischarin) expressing cells and vector (Rac + vector) transfected cells was compared); SW480 cells,  $P^* < 0.02$ , ( $P = 0.02$ , invasion between Nischarin (Rac + Nischarin) expressing cells and vector (Rac + vector) transfected cells was compared).

I next examined the effects of MEK inhibitor UO126 on invasion of Matrigel by SW480 cells. As with the cell migration studies, treatment of cells with UO126 inhibited Rac induced cell invasion, indicating a role for MAPK in this process (Fig. 3B). Rac mediated invasion was also blocked by the presence of the PI3K inhibitor LY294002 (Fig. 3B). I also attempted to determine whether the action of Nischarin is affected by the presence of PI3K or MEK inhibitors. However, the inhibitory effect of Nischarin on Rac driven invasion was not further enhanced by the presence of LY294002 or UO126, and vice versa (Fig. 3B). This suggests that, rather than making parallel independent contributions to cell motility and invasion, the pathways blocked by PI-3-K inhibitors, MEK inhibitors, and Nischarin are all essential for migration.

### Nischarin selectively influences migration induced by effector domain mutants of Rac1 that act through PAK

Rac effector domain mutants have been useful in dissecting the downstream signaling events of Rac [25]. I employed such mutants in order to understand the migration events regulated by Nischarin. As shown in Fig. 4A, and in Table 1, mutants that cannot induce lamellipodia formation (61L31V, 12V37L) were unable to induce cell migration in MCF7 cells. Like Rac Q61L, Rac 61L43D is a strong inducer of lamellipodia, as well as an activator of PAK and JNK. Nischarin effectively inhibited migration induced by Rac Q61L and Rac61L43D mutants. By contrast, migration induced by mutants (61L40C, 12V40H) that cannot activate PAK and JNK, was not inhibited by Nischarin (Fig. 4A and Table 1).

PAK has been clearly implicated in cell motility; for example, PAK was shown to increase migration in NIH3T3 cells [12]. In order to understand the role of downstream effectors of Rac in carcinoma cell migration, I investigated the direct effects of active PAK. As shown in Fig. 4B, constitutively active PAK [27] that lacks the auto inhibitory region (PAK 165), as well as a constitutively active PAK mutant (T423E) [28], strongly induced cell migration in MCF7 cells, and this was significantly inhibited by Nischarin.

MEKK1 is another downstream effector of Rac that has been shown to activate JNK [19], and that has been implicated in cell migration [23,34]. To determine the effect of Nischarin on migration induced by MEKK1, constitutively active MEKK1 was used in migration assays. Overexpression of MEKK1 in MCF7 cells produced robust enhancement of cell migration compared to vector controls. Interestingly, Nischarin did not have any effect on MEKK1 induced cell migration (Fig. 4B), suggesting that Nischarin does not have a major role in the arm of the Rac cascade downstream of MEKK1. The above findings demonstrate that Nischarin affects Rac driven cell migration by preferentially affecting processes downstream of PAK.

The ERK/MAPK cascade has also been shown to induce cell migration, as discussed above. I examined the effect of Nischarin in MAPK induced cell migration. As shown in Fig. 4C, migration induced by constitutively active MEK was not inhibited by Nischarin, suggesting that Nischarin seems to selectively affect Rac driven signaling pathways. This result is consistent with previous work in which Nischarin affected only Rac driven c-fos activation, but not ERK/MAPK driven c-fos activation [24].

### Activities of JNK and phosphorylation of AKT are not inhibited by Nischarin

Previous studies have demonstrated that Rac induces JNK [25] and AKT through the activation of PI3K [35]. I further investigated if Nischarin has any effect on Rac mediated JNK and AKT functions in MCF 7 cells. In order

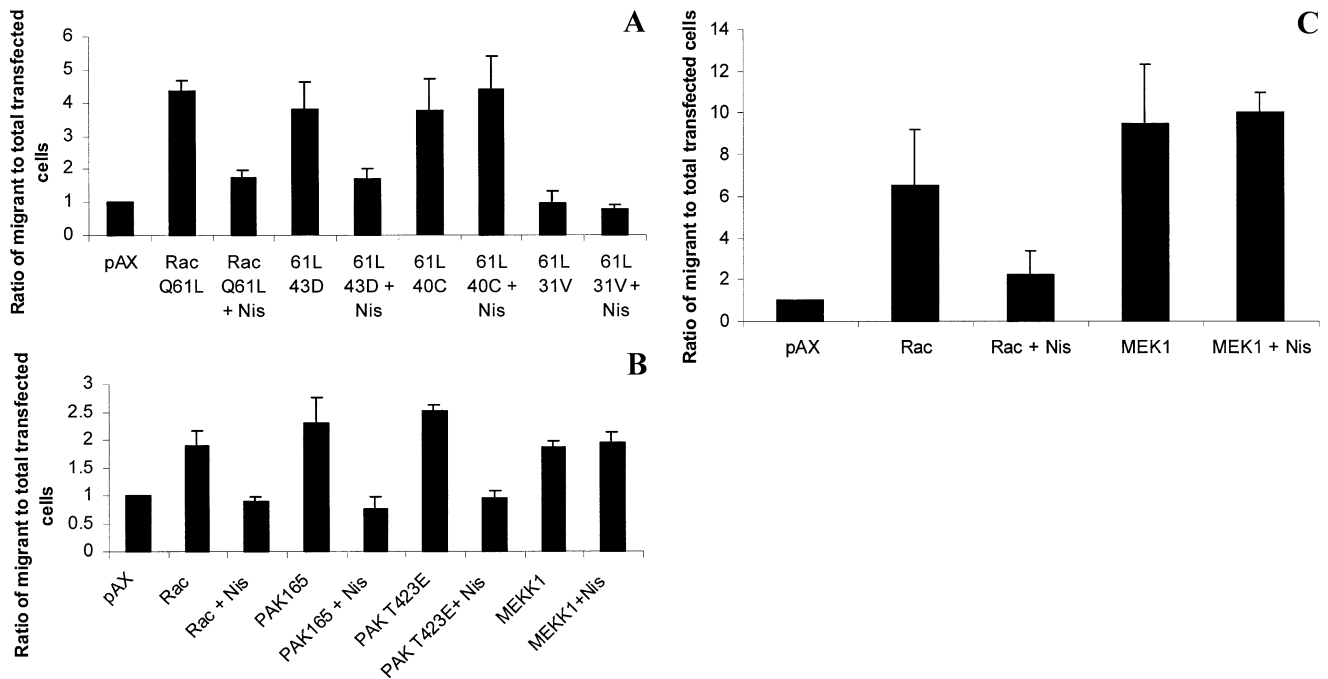


Fig. 4. Nischarin effects on pathways downstream of Rac. (A) Effect of Nischarin on cell migration induced by various effector domain mutants. MCF7 cells were transiently transfected with different Rac effector domain mutant plasmids (Table 1), or with plasmids expressing activated MEKK, or activated MEK, and with full length myc tagged Nischarin plasmid, and with a  $\beta$ -galactosidase plasmid. All transfections were normalized for total DNA using vector DNA plasmid. pcDNA myc vector + pAX 164 vector (pAX), Rac Q61L + pcDNA vector, Rac Q61L + pcDNA Nischarin, 61L43D + pcDNA vector, 61L43D + pcDNA Nischarin, 61L40C + pcDNA vector, 61L40C + pcDNA Nischarin, 61L31V + pcDNA vector, or 61L31V + pcDNA Nischarin. Migrating cells were counted as described in Methods. (B) Nischarin inhibits migration driven by constitutively active PAK but not constitutively activated MEKK1. pAX Rac Q61L (Rac), pFC MEKK1 (MEKK1), pCMV PAK165 (PAK165), or pcDNA V5 PAK (T423E) were transfected with pcDNA myc-Nischarin (Nis), or pcDNA vector, and migrating cells were counted.  $\beta$ -galactosidase plasmid was used as a marker. Transfection with pAX164 served as a negative control. (C) MEK1 induced migration is unaffected by Nischarin. pAX 164 (pAX), Rac Q61L (Rac), or pFC MEK1 (MEK1) were transfected with pcDNA vector DNA or pcDNA myc-Nischarin, and migrating cells were counted as described above. Results in A–C represent the means and standard errors of three determinations.

to examine the effect of Nischarin on Rac mediated JNK activity, *in vitro* kinase assays were performed using c-Jun as a substrate. As shown in the Fig. 5a, Rac Q61L significantly enhanced JNK activity compared to vector control and Nischarin had no effect on Rac induced JNK activity. These data indicate that Nischarin has no effect on the Rac-JNK cascade, which is consistent with the observation that Nischarin does not inhibit MEKK induced cell migration. In several cell types, Rac has been shown to activate PI3K, which also has been shown to induce cell migration [4,30]. Furthermore, AKT has been shown to be essential for Rac driven cell motility [30]. Thus, I examined if Nischarin affects Rac mediated phosphorylation of AKT, a downstream effector of PI3K. As shown in Fig. 5b, RacQ61L indeed induced AKT phosphorylation while vector alone was unable to increase AKT phosphorylation. Interestingly, Nischarin does not have any effect on AKT phosphorylation. PI3K specific inhibitors (LY and Wortmannin) blocked AKT phosphorylation induced by Rac (data not shown), indicating that AKT phosphorylation is mediated through a Rac-PI3K pathway. These data suggest that Nischarin does not affect this arm of Rac signaling as well.

## Discussion

Integrins are a major family of ECM receptors that are required for migration and invasion. They are involved in interactions with the ECM, and in regulating actin dynamics that in turn regulate cell movement [2]. Metastasis and invasion are important and complex characteristics of tumor cells, and cell motility is believed to contribute to these events. Continuous turnover of cytoskeletal elements to form lamellipodia and filopodia, regulated by Rac and CDC42, respectively, results in cell movement [36]; thus these GTPases are thought to be involved in tumor cell motility and invasiveness [4,37,38]. Although active Rac induces cell invasion in most cell types, it can lead to suppression of invasion [39] in some cells, suggesting cell-type differences may exist. My data indicate that constitutively active Rac stimulates cell migration and invasion in MCF7 and SW480 carcinoma cells and corroborates results of Keely et al. [4] concerning the role of Rac in carcinoma cell motility.

Current results demonstrate that Nischarin can strongly inhibit Rac-driven cell motility and invasion. In order to further understand this phenomenon, I examined the role of

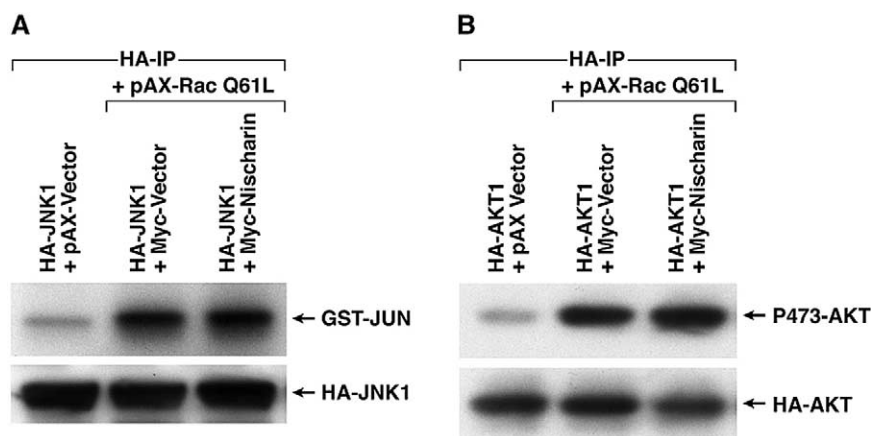


Fig. 5. Nischarin does not affect Rac induced activation of JNK, or phosphorylation of AKT. (A) JNK activation. MCF7 cells were transiently transfected with pAX vector + HA-JNK1, pAX-Rac Q61L + HA-JNK1 + Myc vector, or pAX-Rac Q61L + HA JNK1 + Myc-Nischarin. JNK activity was measured using c-Jun as a substrate following immunoprecipitation of HA-JNK as described in Materials and methods. Top panel, autoradiograph of a JNK assay; bottom panel, Western blot of immunoprecipitates of HA-JNK to demonstrate equivalent amounts of HA-JNK loaded on the gel. (B) AKT-phosphorylation. MCF7 cells were transiently transfected with pAX-vector + HA-AKT1, pAX-Rac Q61L + HA AKT1 + Myc vector, or pAX-RacQ61L + HA-AKT1 + Myc-Nischarin. AKT phosphorylation was measured using the HA immunoprecipitates of lysates made from these cells as described in Materials and Methods. Top panel, Western blotting was performed on the HA immunoprecipitates of lysates transfected with different plasmids as described above using phospho specific anti-AKT antibody (P473) (New England Biolabs); The same samples were blotted with total AKT antibody (New England Biolabs) (middle panel) to demonstrate equivalent loading of AKT. All transfections were normalized for total DNA using vector DNA.

signaling components that have been previously implicated in cell migration. Several signaling pathways converge on the actinomyosin cytoskeletal rearrangements that are essential for cell motility [2,40]. Thus, the Erk-MAP kinase, and several pathways downstream of Rac, including those involving PAK, PI3K, and MEKK1, have been linked to motility. My studies with pharmacological inhibitors clearly show that PI-3-kinase and Erk both play a role in Rac-driven migration in the cells examined here; in addition, activation of Erk can directly drive migration, as indicated by the effect of constitutively active MEK.

Because Nischarin blocks the promigratory effects of a GTPase deficient Rac mutant (Rac Q61L), I suspected Nischarin might function downstream of Rac rather than by regulating Rac activation. This led to the use of effector domain mutants as a powerful approach to dissect the contributions of Rac to downstream events. As expected, I found that only mutants that can induce lamellipodia formation were able to stimulate cell migration. Interestingly, however, Nischarin inhibited motility only for those mutants that could activate PAK and JNK, as well as lamellipodia. This suggested that either the PAK or JNK pathways, or both, were the locus of Nischarin inhibition of cell motility. Further studies with constitutively active PAK or constitutively active MEKK1 (which activates the JNK pathway) indicated that Nischarin blocked motility driven by PAK but not that driven by MEKK1. This, coupled with the observation that Nischarin also failed to block motility driven by activation of the Erk-MAP kinase pathway, indicated that Nischarin selectively affected signaling to the cytoskeleton via PAK.

As discussed above, Rac can regulate several different

pathways through different effectors. Rac activates PI3K and AKT independent of JNK [35], and activates the MEKK-JNK cascade independent of PAK [19]. In addition to the Rac-MEKK-JNK and the ERK cascades, PI3K-AKT has also been shown to promote cell migration [4,30]. Thus, to further confirm if Nischarin effects are mediated exclusively through PAK signaling cascade or if other Rac signaling cascades are affected by Nischarin, I investigated the effects of Nischarin on Rac driven JNK, and PI3K activities. Nischarin had no inhibitory effect on Rac induced JNK activation. These data corroborate the migration data showing that MEKK driven migration is unaffected by Nischarin. In addition, AKT phosphorylation induced by Rac is unaffected. It is known that Rac alone cannot strongly activate ERK cascade [41]. Consistent with this, I was unable to detect any significant Rac mediated ERK activation (data not shown), suggesting that ERK is not a downstream effector of Rac. However my data reveal that MEK inhibitors are able to block migration, which suggests that a basal level of ERK activity may be required for Rac driven cell migration. Overall, these observations further point to the direction that Nischarin primarily affects PAK driven signaling events, while not affecting Rac-JNK, and Rac-AKT signaling cascades.

The PAK family of kinases has been implicated in control of actin filaments and in cell motility; for example, PAK is required for directional migration in fibroblasts [12]. Targets for PAK likely to be involved in migration include myosin light chain kinase (MLCK) and LIM kinase. MLCK phosphorylates MLC, and this phosphorylation has been shown to be important in regulating actin cytoskeletal dynamics [18]. In addition, PAK phosphorylates and activates

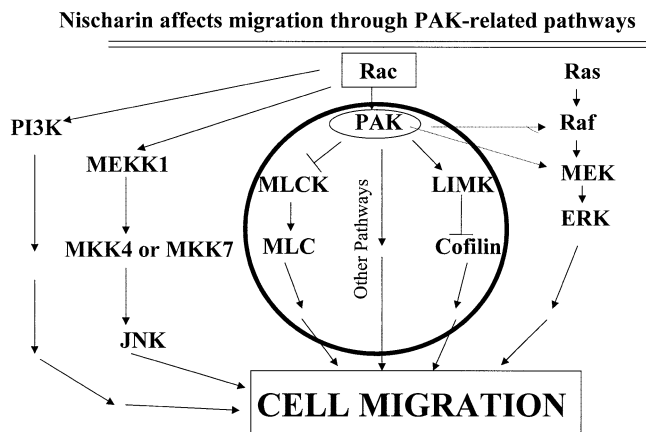


Fig. 6. Proposed model for the role of Nischarin in Rac stimulated cell migration. Rac 1 can be activated by various external stimuli and activates several downstream pathways. MEKK1 and PAK are activated by GTP loaded Rac. PI(3)K activity has been shown to be required for Rac induced migration. MEKK1 activates JNK through the activation of MKK4 or MKK7. Rac also activates PAK, which in turn phosphorylates LIMK leading to phosphorylation of Cofilin, and thus lead to inactivation of Cofilin. PAK inhibits MLCK phosphorylation, which in turn regulates MLC phosphorylation. Ras activates ERK through the activation of Raf and MEK. PAK also can phosphorylate Raf and MEK, enhancing the activation of ERK. Previous studies indicate that JNK, MLC, Cofilin, and ERK regulate cell migration. Nischarin does not affect cell migration mediated through the JNK or ERK pathways. Nischarin retards migration through PAK mediated pathways (shown in a circle).

LIMK, which in turn phosphorylates and inhibits the actin severing protein cofilin, thus promoting filament assembly. The PAK interacting guanine nucleotide exchange factor PIX, the ARF GTPase activating protein PKL, and the adaptor protein Nck, are also mediators of Rho GTPase signaling; they form a complex with PAK to regulate the actin cytoskeleton and stimulate focal complex formation through paxillin interactions [42,43]. G protein coupled receptor kinase interacting protein (GIT1) links PAK to FAK and results in focal contact turnover [44], while another protein, p95 APP 1, of the GIT family, has been implicated in membrane recycling during locomotion [45], suggesting a role of GIT family members in cell motility [46]. Thus, PAK plays an important role in Rac-driven cell motility through several different signaling cascades. It is likely that Nischarin affects one of these pathways, or other novel PAK mediated pathways that are yet to be explored, in regulating cell migration. In future studies I intend to investigate the mechanisms by which Nischarin blocks PAK driven cell motility.

In summary, my data suggest that several different signaling pathways converge, and are each essential, for effective migration of carcinoma cells (see Fig. 6). Contributions from PI-3-kinase and from the Erk MAP kinase pathway are clearly important. Active Rac also stimulates several downstream pathways that contribute to cell motility, including those involving PAK, PI3K-AKT, and the MEKK1-JNK cascade. The results of this study indicate that Nischarin

rather selectively influences the contribution of PAK to Rac-driven cell migration, rather than contributions from other downstream effectors of Rac. At this point the precise locus of Nischarin regulation of motility is unknown. Furthermore, it is not known whether Nischarin regulates cell invasion through PAK. Future studies will address the possible involvement of MLCK, of LIM kinase, or of other effectors influenced by PAK. However, current findings implicate Nischarin in regulating Rac mediated cell migration via PAK.

### Acknowledgments

I am grateful to Dr. R.L. Juliano for his support, guidance, and advice. I thank Dr. C.J. Der (UNC-CH) for providing Rac effector domain mutants. I also thank Hani Nasrallah for excellent technical assistance. This work was supported by grants from the NIH to RLJ.

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