# The GTPase Rac Regulates the Proliferation and Invasion of Fibroblast-Like Synoviocytes from Rheumatoid Arthritis Patients

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Fibroblast-like synoviocytes (FLS) isolated from joints of rheumatoid arthritis (RA) patients display proliferative and invasive properties reminiscent of those of malignant tumor cells. Rac small GTPases play an important role in tumor cell proliferation and invasion. We therefore investigated the potential role of Rac proteins in the proliferative and invasive behavior of RA-FLS. We showed that inhibiting Rac activity with the Rac-specific small molecule inhibitor NSC23766 causes a strong inhibition of RA-FLS proliferation, without affecting cell survival. Rac inhibition also results in a strong reduction in RA-FLS invasion through reconstituted extracellular matrix and a less marked inhibition of two-dimensional migration as measured by monolayer wound healing. We also showed that small interfering RNA-mediated depletion of Rac1 inhibits RA-FLS proliferation and invasion to a similar extent as NSC23766. These results demonstrate for the first time that Rac proteins play an important role in the aggressive behavior of FLS isolated from RA patients. In addition, we observed that inhibiting Rac proteins prevents JNK activation and that the JNK inhibitor SP600125 strongly inhibits RA-FLS invasion, suggesting that Rac-mediated JNK activation contributes to the role of Rac proteins in the invasive behavior of RA-FLS. In conclusion, Rac-controlled signaling pathways may present a new source of drug targets for therapeutic intervention in RA.

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## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic disorder that causes progressive joint destruction. An important characteristic of the rheumatoid synovium is the marked hyperplasia of the lining layer, which is caused by an increased number of fibroblast-like synoviocytes (FLS) and macrophages (1,2). Accumulating evidence indicates that, in addition to macrophages and T cells, activated FLS deliver distinct contributions to the pathogenesis of RA (3–7). RA-FLS constitute an important source of matrix metalloproteinases (MMPs) and cathepsins, proteases that mediate joint destruction (8–10), and it has been shown that RA-FLS can induce cartilage degradation in the absence of T cells or monocytes in the SCID mouse (11). In addition, FLS may contribute to the initial phases of synovitis via the secretion of chemokines, such as MCP-1, MIP-1α, and IL-16 (12,13), and pro-inflammatory cytokines, such as IL-1 (14,15).

The signaling pathways that are responsible for the hyperplasia and high activation state of RA-FLS to a large extent remain to be defined. RA-FLS share a number of features with transformed cells, including enhanced proliferation and the elaboration of matrix-degrading proteases (14,16,17). Work from our and several other laboratories has shown that the small GTPase Rac1 plays an important role in oncogenic transformation and invasion (18–21). We therefore hypothesized that activated Rac1 contributes to rheumatoid arthritis by stimulating multiple aspects of the activated phenotype of RA-FLS, including enhanced invasion and proliferation.

Rac proteins are members of the Rho family of Ras-like small GTPases. These GTPases essentially function as switches, they are "on" in the GTP-bound and "off" in the GDP-bound state (22). In the active state, they relay signals from growth factors, cytokines, and adhesion

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molecules to a large number of effector proteins. There are three Rac genes in the human genome, which differ in their tissue distribution. Rac1 is ubiquitously expressed, Rac2 is hematopoietically-specific, and Rac3 is predominantly expressed in the brain (23). The three Rac proteins are highly homologous (displaying approximately 92% amino acid identity) and share most of their effector proteins and functions (21,23).

Here, to investigate the role of Rac proteins in the proliferative and invasive properties of RA-FLS, we used both a specific small molecule inhibitor of Rac proteins (24), that is likely to inhibit all three members of the Rac subfamily of Rho GTPases, comprising Rac1, Rac2, and Rac3, and Rac1-specific small interfering RNA (siRNA). We showed that inhibiting Rac proteins causes a significant inhibition in RA-FLS proliferation and invasion in vitro. These results indicate that Rac proteins contribute to the aggressive behavior of RA-FLS.

## MATERIALS AND METHODS

### Inhibitors

The JNK inhibitor SP600125 was purchased from Calbiochem (San Diego, CA, USA). NSC23766 (24) was custom synthesized.

### Cell Culture

Synovial tissues were obtained from RA patients undergoing orthopedic surgery. Tissues were digested with collagenase, hyaluronidase, and DNAse and placed in culture. RA-FLS were used between passages 4–12. Cells were grown at 37° C in DMEM supplemented with 10% FBS and penicillin/streptomycin.

### Rac Activity Assay

Activated Rac was detected using an EZ-Detect Rac1 Activation Kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. This assay is based on the fact that Rac effector proteins, such as PAK1 (p21-activated kinase 1), specifically bind to the GTP-bound form of Rac proteins and show negligible binding to the GDP-bound form. One day before the assay, RA-FLS was serum starved with or without 50 μM NSC23766. After 24 h of treatment, cells were stimulated with IL-1β for 5 min and subsequently lysed with lysis buffer. Subsequently, 20 μg of GST-human Pak1-PBD was incubated for 1 h at 4° C with equal amounts of protein from each condition. After incubation, GST-Pak1 beads were centrifuged and washed three times to remove unbound material. Rac-GTP was detached from GST-beads by boiling the samples in 2× SDS sample buffer for 5 min. Rac-GTP and total Rac protein levels were visualized by Western Blotting using an anti-Rac antibody (Upstate Biotechnology, Lake Placid, NY, USA).

### Invasion Assay

Invasion was assayed by measuring cell invasion through Matrigel Invasion Chambers (BD Biosciences Bedford, MA, USA). One day after treatment of RA-FLS with 50 μM NSC23766 or control solution, $4 \times 10^4$ cells were placed in the upper chamber in serum free medium. 500 μL of DMEM containing 10% FBS and 10% human serum was added to the bottom chamber. After 24 h of incubation at 37° C, cells on the upper surface of the filter were wiped off with a Q-tip and the filter was fixed in 4% formaldehyde/PBS. After staining with Crystal Violet, all cells on the bottom of the chamber were counted using an IX70 Olympus inverted microscope.

### Monolayer Wound Healing Assay

$2 \times 10^5$ cells were plated in a 12-well plate and grown to confluency. After overnight incubation with or without 50 μM NSC23766, two parallel wounds of approximately 400 μm were made using a p1000 pipette tip. After rinsing with PBS, cells were allowed to recover for 15 min. Images were collected for each treatment at various times after wounding for the same eight spots, localized on the underside of the dish by a marker. Cell migration distance was determined by measuring the width of the wound divided by two and subtracting this value from the initial half-width of the wound. The rate of cell movement was calculated as the slope of the migration distance over a 21 h time period.

### Fluorescence Microscopy

RA cells were plated on coverslips and treated as described for the monolayer wound healing assay. Cells were washed with PBS 3 h after wounding, fixed in 4% formaldehyde/PBS, permeabilized with 0.1% Triton X-100 dissolved in PBS, and incubated with FITC-conjugated phalloidin (Invitrogen Corporation, Carlsbad, CA, USA) to stain for F-actin. Processed coverslips were mounted in 75% Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were collected using an IX70 Olympus inverted microscope equipped with a 60× (1.4 NA) objective, an Orca II cooled CCD camera (Hamamatsu Photonic Systems, Bridgewater, NJ, USA) and ESee (Inovision, Raleigh, NC, USA) image analysis software.

### Sulphorhodamine B Assay

Cell proliferation was measured using the sulphorhodamine B colorimetric assay (25). Briefly, 1 day after treatment with NSC23766 or control solution, cells were seeded at $2 \times 10^3$ cells/well in a 96-well microtiter plate. At various times, cells were fixed in 10% trichloroacetic acid for 1 h at 4° C, rinsed and subsequently stained for 30 min at room temperature with 0.2% SRB dissolved in 1% acetic acid, followed by air drying. Bound dye was solubilized in 100 μL of 10 mM unbuffered Tris base for 30 min and the OD was read at 490 nm in an ELISA plate reader.

### Apoptosis Assay

The level of DNA fragmentation was quantified using the Cell Death ELISA PLUS kit (Roche, Indianapolis, IN, USA) using the protocol recommended by the manufacturer.

### siRNA Transfections

siRNA duplexes specific for Rac1 were designed according to (Elbashir et al.,

2001). 21-nt RNAs were purchased from Dharmacon (Lafayette, CO, USA) in deprotected and desalted form. The Rac1 siRNA sequence (5′ AAGGAGATTGGTG CTGTAAAA) used corresponds to bp 439–459 after the start codon and GL2 luciferase, corresponds to bp 153–173 after the start codon (5′ AACGTACGCGGAA TACTTCGATT). Transient transfection of siRNA was carried out using the basic fibroblast kit from amaxa biosystems (Gaithersburg, MD, USA). $3 \times 10^5$ cells were transfected with 1 μg of siRNA using nucleoporation program U23. Cells were assayed seven to eight days after transfection. Rac expression was determined by Western blot analysis using a monoclonal anti-Rac antibody (Upstate Biotechnology, Lake Placid, NY, USA).

### Statistical Analysis

Data are expressed as the mean ± SEM. Statistical significance was performed using the two-tailed Student's *t* test. *P* values of < 0.05 were considered statistically significant.

## RESULTS

### Rac Regulates RA-FLS Cell Proliferation

To modulate Rac signaling in RA-FLS cells, we used NSC23766, a Rac-specific small molecule inhibitor that specifically binds to Rac proteins and prevents their activation (24). To verify that NSC23766 inhibits Rac1 activity in RA-FLS cells, we quantified the level of activated (GTP-bound) Rac by selectively extracting GTP-bound Rac from cell lysates using a GST-PAK1 fusion protein (26). NSC23766 at 50 μM significantly inhibits Rac1-GTP levels in RA-FLS cells stimulated with IL-1β (Figure 1A).

To examine the role of Rac proteins in RA-FLS proliferation, we quantified cell proliferation in optimal growth conditions over a nine-day period using the sulphorhodamine B (SRB) colorimetric assay (25). NSC23766 inhibits RA-FLS proliferation in a dose-dependent manner (Figure 1B). We observed the inhibitory effect of NSC23766 on cell pro-

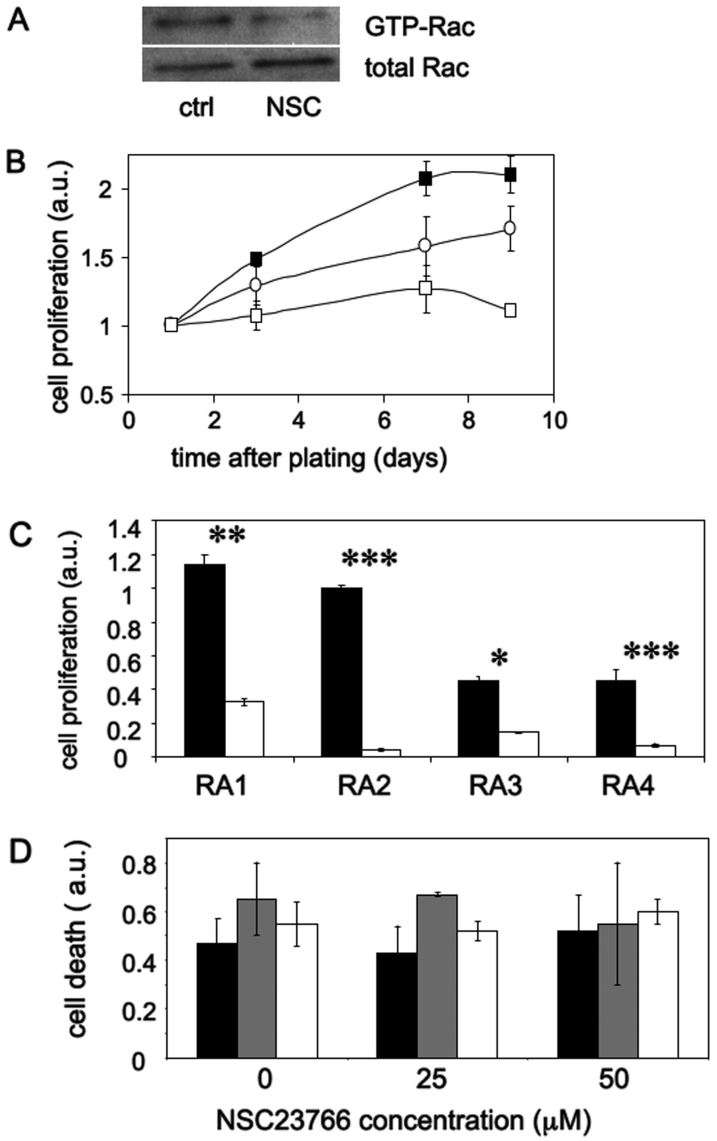


**Figure 1.** NSC23766 blocks Rac activity and RA-FLS proliferation. (A) RA-FLS were serum starved (control) in the presence or absence of 50 μM NSC23766 (NSC) for 24 h and subsequently stimulated with 10 ng/mL IL-1β for 5 min in the presence or absence of 50 μM NSC23766. Activated Rac (GTP-Rac) was extracted from cell lysates using GST-PAK and visualized by Western blotting using anti-Rac antibody. Total Rac from cell lysates before extraction was determined as loading control. Data shown are representative of two independent experiments. (B) RA-FLS (RA2) were grown overnight in 10 % serum in the absence (closed squares) or presence of 25 μM NSC23766 (open circles) or 50 μM NSC23766 (open squares). Subsequently, cells were plated on 96 well plates and cell growth was quantified using SRB staining. (C) Effect of NSC23766 on the proliferative activity of different RA-FLS cultures. Conditions as in (B). Solid bars: controls, empty bars: 50 μM NSC23766. Shown is the mean (± SEM) of five wells. For some of the data points, the error is smaller than the symbol size. * = $P < 0.02$, ** = $P < 0.01$ and *** = $P < 0.001$, two-tailed *t* test. Data for RA1 and RA2 are representative of respectively of three and two experiments. (D) Effect of NSC23766 on RA-FLS survival. RA-FLS (RA2) cells were treated with the indicated concentration of NSC23766 and apoptosis was measured using an ELISA assay that quantifies histone-associated DNA fragments. Shown are the mean values (± SEM) of three independent experiments from three RA-FLS cell lines.

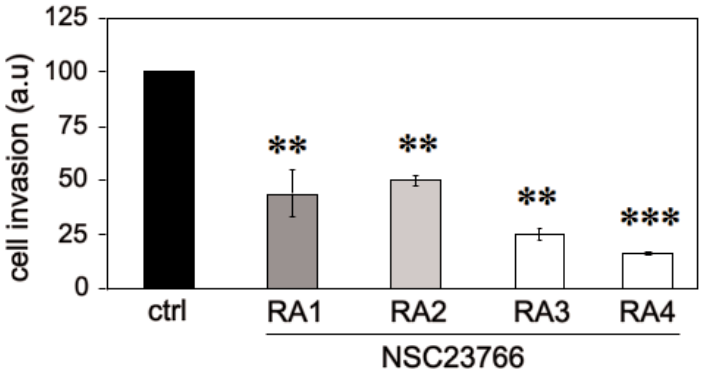


**Figure 2.** Rac activity is necessary for RA-FLS invasion. Four different RA-FLS cultures were treated with 50 μM NSC23766 or control solution for 24 h. Cell invasion through Matrigel was quantified as described in Materials and Methods. Cell invasion numbers obtained with the different cultures were normalized to the respective controls. Shown is the mean (± SEM) of at least two independent experiments. ** = $P < 0.01$, two-tailed *t* test.

liferation in all RA-FLS cultures examined, although the extent of inhibition shows patient-dependent variability (Figure 1C). To determine the effect of NSC23766 on RA-FLS survival, we used a histone-associated DNA fragmentation ELISA that quantifies histone-associated DNA fragmentation (Roche), a very sensitive apoptosis assay. No significant effect of NSC23766 on RA-FLS survival could be detected however (Figure 1D). Taken together, these results indicate that Rac proteins regulate RA-FLS proliferation mainly by stimulating mitogenesis.

### Rac Regulates RA-FLS Invasion, Migration, and Actin Organization

The in vitro invasion potential of RA-FLS is a patient-specific characteristic that correlates well with the rate of joint destruction in RA patients (27). To examine the role of Rac proteins in the invasive behavior of RA-FLS in vitro, we determined the effect of NSC23766 on RA-FLS invasion through a thin layer of reconstituted extracellular matrix (Matrigel), an assay that is well-established for the evaluation of FLS in

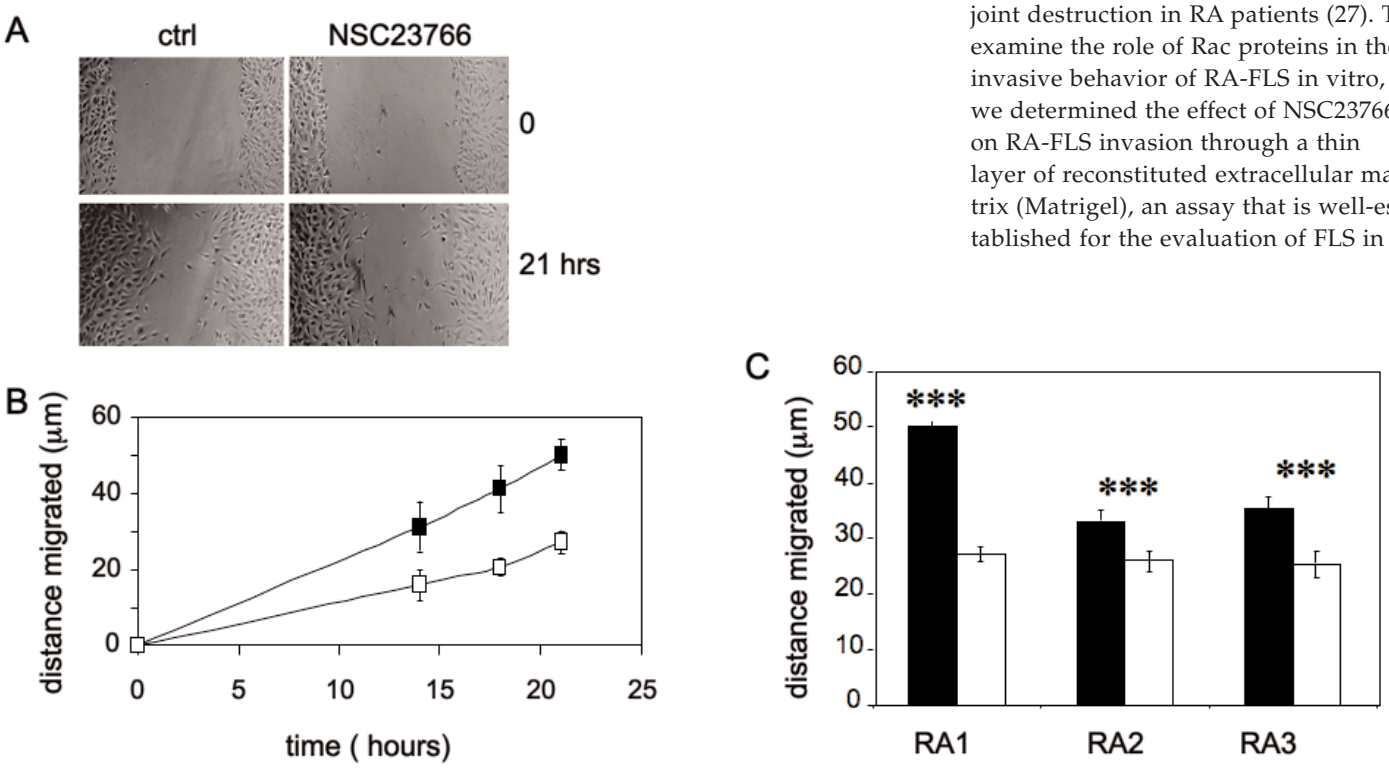


**Figure 3.** Decreased Rac activity inhibits cell migration. (A) Phase contrast micrographs of RA-FLS immediately and 21 h after wounding. RA-FLS (RA1) were pre-treated with 50 μM NSC23766 or control solution for 24 h and maintained in the presence or absence of 50 μM NSC23766 after wounding. Images of RA-FLS were taken at time 0 and 21 h after wounding. (B) Quantification of wound healing. RA-FLS (RA1) were treated with 50 μM NSC23766 (open squares) or control solution (closed squares) as described in (A). The migration distance was quantified as described in Materials and Methods. Shown are the mean values (± SEM) of eight measurements for each time point. Data are representative of two independent experiments. (C) Quantification of migration of control cells (solid bars) or cells treated with 50 μM NSC23766 (empty bars) after 21 h of various RA-FLS lines. *** = $P < 0.001$, two-tailed *t* test. Data shown are representative of two independent experiments.

vitro invasion (27–29). NSC23766 significantly inhibits Matrigel invasion by all RA-FLS cell lines examined (Figure 2), indicating that Rac proteins play an important role in the invasive behavior of RA-FLS.

In principle, the inhibitory effect of NSC23766 on RA-FLS proliferation could contribute to the inhibition of invasion by NSC23766. However, because the RA-FLS in the top chamber are cultured in the absence of serum and because the invasion assay time (24 h) is much shorter than the RA-FLS doubling time (approximately one week), the contribution of diminished cell proliferation appears to be insignificant.

Rac proteins have been implicated in the regulation of cell migration in two-dimensional conditions (30,31), a function that is likely to contribute to invasion through a three-dimensional matrix. We therefore also determined the effect of NSC23766 on cell migration using a monolayer wound healing assay (21,31). Wound closing was significantly slowed down in NSC23766-treated RA-FLS versus controls (Figure 3). This inhibitory effect was observed in all RA-FLS cultures examined, albeit it was less pronounced than the inhibitory effect of NSC23766 on invasion (Figure 3C).

Rac proteins play an important regulatory role in the organization of the actin cytoskeleton (32). In particular, in a number of different cell types, the activity of Rac has been shown to be critical for the formation of lamellipodia (21,33), thin actin-rich extensions of the cytoplasm that are thought to play a role in directed cell migration (34). To examine the role of Rac proteins in the organization of the actin cytoskeleton in RA-FLS, we used fluorescent phalloidin staining to visualize polymerized actin in migrating cells shortly after wound healing in the absence or presence of NSC23766. Whereas control cells display flat or ruffling lamellipodia at their leading edge, cells treated with NSC23766 are strongly inhibited in lamellipodia formation (Figure 4). Thus, the functions of Rac proteins in actin dynamics and cell migration may contribute to their role in RA-FLS invasion.

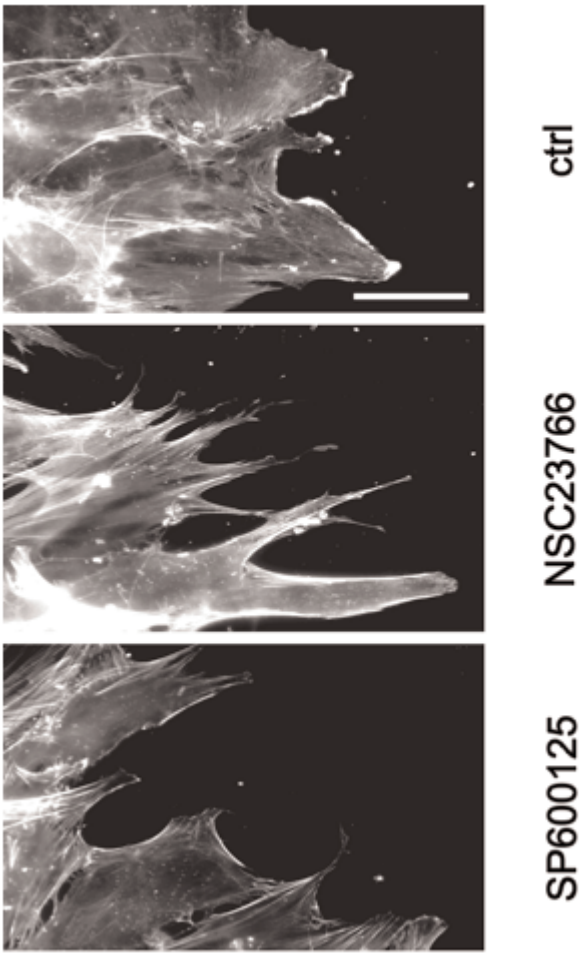


**Figure 4.** Rac is necessary for lamellipodia formation in RA-FLS. RA-FLS (RA1) were plated overnight on coverslips and pre-treated for 1 h before wounding in the presence of 50 μM NSC23766, 20 μM SP600125 or DMSO. Cells were fixed and stained with fluorescent phalloidin 3 h after wounding to visualize polymerized actin in migrating cells. Data shown are representative of two independent experiments. Size bar represents 10 μm.

To confirm the role of Rac proteins in RA-FLS proliferation and invasion, we employed small interfering RNA (siRNA)-mediated depletion of Rac1. Transfection of Rac1-directed siRNA using nucleofection of an siRNA duplex that we extensively characterized previously (21) causes strong inhibition of Rac1 expression, that is optimal at around day eight post-transfection and was sustained up to day eleven post-transfection (Figure 5A). siRNA-mediated depletion of Rac1 inhibits RA-FLS proliferation and invasion to a similar extent as NSC23766 (compare Figure 5B,C with Figures 1 and 2). Together, these data suggest that inhibition of Rac1 is largely responsible for the effects of NSC23766.

### Rac Regulates JNK Activation in RA-FLS

C-Jun N-terminal kinase (JNK) is a critical regulator of joint destruction in RA (9,35). In addition, JNK plays an important role in cell migration in a number of different cell systems (36). Because Rac1 has been implicated in the activation of the JNK cascade by TNFα or growth factors in other cell systems (37,38), we also examined the role of Rac

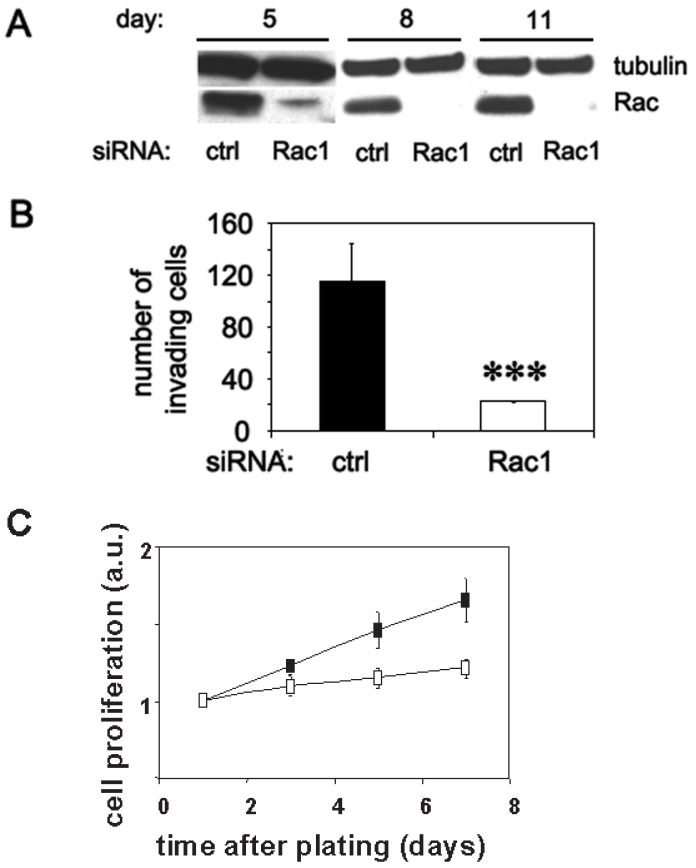


**Figure 5.** Inhibition of RA-FLS proliferation and invasion by siRNA-mediated depletion of Rac1. (A) Rac1 siRNA inhibits Rac expression. RA-FLS were transfected with Rac1 siRNA by nucleoporation. Rac protein levels were determined by Western blotting of cell lysates at the indicated days. Tubulin levels are shown as loading control. (B) Rac1-directed siRNA inhibits RA-FLS invasion. Matrigel invasion assays were performed on day seven post-transfection. Shown is the mean (± range) of two independent experiments. (C) Rac1-directed siRNA inhibits RA-FLS proliferation. SRB cell proliferation assays were initiated four days post-transfection. Shown is the mean (± range) of two independent experiments. *** = $P < 0.001$, two-tailed *t* test

proteins in JNK activation in RA-FLS. NSC23766 strongly inhibits JNK activity in serum starved RA-FLS, as measured by Western blotting with an antibody specific for phospho-JNK (Figure 6A). p54 JNK is the major JNK isoform activated in RA-FLS. We also showed that the specific JNK inhibitor SP600125 (39) strongly inhibits RA-FLS invasion (Figure 6B). Together, these data suggest that Rac-mediated JNK activation contributes to the role of Rac proteins in RA-FLS invasion. Interestingly, treatment with SP600125 also inhibited the formation of lamellipodia in RA-FLS located at the wound edge (Figure 4), suggesting that Rac-mediated JNK activation contributes to the role of Rac proteins in leading edge actin dynamics in RA-FLS.

## DISCUSSION

This study indicates that Rac proteins play an important role in the maintenance of the activated phenotype of FLS isolated from RA patients. We have used both a Rac-specific small molecule and Rac1-directed siRNA and have shown that inhibition of Rac proteins strongly inhibits both the proliferative and invasive behavior of RA-FLS.

The high degree of similarity between the respective Rac sub-family members implies that the NSC23766 compound likely interferes with the activation of all three Rac GTPases. Expression of Rac2 however is restricted to hematopoietic lineages, whereas quantitative PCR showed that in RA-FLS, Rac3 mRNA levels are about 50 fold lower than those of Rac1 (data not shown). Moreover, our observations that siRNA-mediated depletion of Rac1 inhibits RA-FLS proliferation and invasion to a similar extent as NSC23766, indicate that Rac1 is the critical target of NSC23766 in RA-FLS.

We also have examined the effect of NSC23766 on FLS obtained from osteoarthritis (OA) patients. OA-FLS display a lower degree of proliferation and elaboration of matrix degrading proteases and cytokines than RA-FLS (14,40) and therefore are often used as controls for RA-FLS. We found that NSC23766 also significantly reduced the proliferative and invasive behavior of OA-FLS (data not shown). However, because of the lower baseline proliferation and invasion of normal FLS, it is not clear whether inhibiting Rac would affect non-inflamed synovium in vivo in a biologically significant manner.

Whereas the signaling pathways that mediate the role of Rac proteins in the control of RA-FLS proliferation remain to be identified, in this work we focused more on the mechanisms that are responsible for Rac-regulated invasion of RA-FLS. The JNK cascade plays an important role in the malignant behavior of RA-FLS. JNK is activated in synovial tissue from RA patients and in RA-FLS in vitro (41,42) and the small molecule JNK inhibitor SP600125 has been shown to strongly inhibit bone destruction in rat adjuvant-induced arthritis (9). We showed that NSC23766 inhibits JNK activity in RA-FLS and that chemical inhibition of JNK inhibits the in vitro invasive behavior of these cells. This strongly suggests that Rac-mediated JNK activation plays an important role in Rac-regulated RA-FLS invasion.

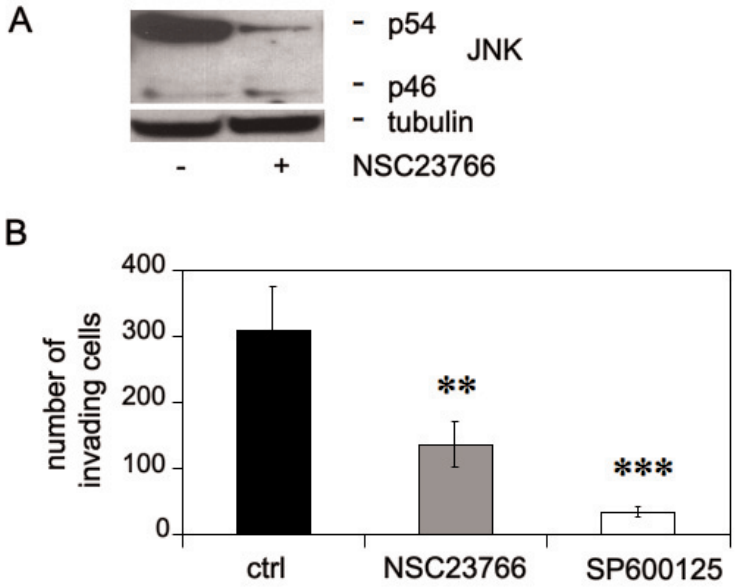


**Figure 6.** Rac is necessary for JNK activation in RA-FLS. (A) RA-FLS (RA3) were serum starved (control) or treated with 50 μM NSC23766 for 24 h. JNK activity was determined by western blotting using phospho-JNK antibody, tubulin expression was determined as loading control. Similar results were obtained with the RA1 and RA2 cultures. (B) The JNK-specific inhibitor SP600125 inhibits RA-FLS invasion. RA-FLS were pre-treated with 50 μM NSC23766 or 20 μM SP600125 or DMSO for 24 h. Cell invasion through Matrigel was quantified as described in Materials and Methods. Shown is the mean (± SEM) of three independent experiments performed in duplicate. ** = $P < 0.01$ and *** = $P < 0.001$, two-tailed *t* test.

How Rac1 activates JNK in FLS is not known. In other cell types, however, a number of Rac1 effectors have been implicated as potential mediators of JNK activation. These include the serine/threonine kinases p21-activated kinase (PAK) (43), mitogen-activated protein kinase/ERK kinase kinase-1 and -4 (MEKK1 and MEKK4) (44), the mixed lineage kinase MLK3 (45) and the scaffold protein plenty of SH3 domains (POSH) (46). Which of these effectors mediate(s) JNK activation by Rac1 in FLS remains to be determined.

Rac GTPases stimulate lamellipodia formation and cell migration in part by regulating actin polymerization (34,47). It is likely that Rac proteins control actin polymerization by activating a number of different signaling pathways in concert (32,48). Thus, Rac1 is thought to stimulate Arp2/3-dependent actin nucleation by regulating a multiprotein complex that includes WAVE (49). In addition, Rac1 may stimulate actin nucleation by activating cofilin-dependent filament severing (50). Our finding that the specific JNK inhibitor SP600125 interferes with lamellipodia formation (Figure 4) suggests that Rac-mediated JNK activation also may contribute to leading edge actin dynamics.

In conclusion, these results demonstrate for the first time that Rac proteins play an important role in the proliferative and invasive behavior of RA-FLS. This work also raises the possibility that Rac proteins contribute to synovial hyperplasia and cartilage invasion and destruction, suggesting that signaling elements in Rac-regulated pathways may include novel drug targets for therapeutic intervention in RA.

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