

RhoA and RhoC are involved in stromal cell-derived factor-1-induced cell migration by regulating F-actin redistribution and assembly

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Received: 29 December 2016 / Accepted: 16 May 2017 / Published online: 23 May 2017
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Abstract Stromal cell-derived factor-1 (SDF-1) signaling is important to the maintenance and progression of T-cell acute lymphoblastic leukemia by inducing chemotaxis migration. To identify the mechanism of SDF-1 signaling in the migration of T-ALL, Jurkat acute lymphoblastic leukemia cells were used. Results showed that SDF-1 induces Jurkat cell migration by F-actin redistribution and assembly, which is dependent on Rho activity. SDF-1 induced RhoA and RhoC activation, as well as reactive oxygen species (ROS) production, which was inhibited by Rho inhibitor. The Rho-dependent ROS production led to subsequent cytoskeleton redistribution and assembly in the process of migration. Additionally, RhoA and RhoC were involved in SDF-1-induced Jurkat cell migration. Taken together, we found a SDF-1/CXCR4-RhoA and RhoC-ROS-cytoskeleton pathway that regulates Jurkat cell migration in response to SDF-1. This work will contribute to a clearer insight into the migration mechanism of acute lymphoblastic leukemia.

Keywords SDF-1 · Rho · ROS · Cytoskeleton · Migration

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Introduction

Stromal cell-derived factor-1 (SDF-1), which belongs to the CXC chemokine subfamily, is produced in two different forms, including SDF-1 α (CXCL12 α) and SDF-1 β (CXCL12 β) [1]. Endothelial cells and stromal cells of the bone marrow microenvironment express SDF-1, which has multiple functions in retention, migration, and mobilization of hematopoietic stem cells and endothelial progenitor cells [2]. CXC chemokine receptor 4 (CXCR4), the receptor of SDF-1, is commonly expressed on the surface of vascular and hematopoietic progenitor cells. CXCR4 is also found on the surface of a variety of leukemic cells. It was reported that CXCR4 on leukemic cells has an important role in bone marrow invasion [3]. The SDF-1/CXCR4 interaction has also been found to be important in hematological malignancies, such as acute myeloid leukemia, B cell acute lymphoblastic leukemia, and T-cell acute lymphoblastic leukemia (T-ALL) [4–7]. T-ALL is an aggressive cancer that is thought to result from the malignant transformation of T-cell precursors in the thymus [8]. Clinical studies demonstrated that enhanced cell surface expression of CXCR4 in T-ALL cells is correlated with a high incidence of extramedullary infiltration and poor prognosis [9]. Jurkat cell, a typical T-ALL cell line expressing functional CXCR4 at high levels, has been reported to be attracted by SDF-1 [10, 11]. However, the mechanism underlying the migration towards SDF-1 is not well known.

Cytoskeletal remodeling is of key importance in migrating cells [12]. RhoGTPases, including Rho, Rac, and Cdc42, have been proved to have central roles in the regulation of cell migration through remodeling of the cytoskeleton [13–18]. Most RhoGTPases act as molecular switches to control different aspects of actin-dependent

cytoskeletal morphogenesis, including formation of stress fibers, lamellipodia, and filopodia. In migrating lymphocytes, RhoA stimulates myosin-based contractility of the actin cytoskeleton, which drives retraction of the rear of the cell [19]. Cdc42 and Rac1 promote actin polymerization, resulting in the formation of either lamellipodia (Rac1) or filopodia (Cdc42) through activation of the Arp2/3 complex and drives cell protrusion at the leading edge of a migrating cell [20]. It was demonstrated that SDF-1 induces morphological changes in adherent leukocytes and the acquisition of a bipolar shape with front leading edges [21], by stimulating the actin polymerization in a Rac- and Cdc42-dependent way [22, 23]. It was also demonstrated that RhoA activation leads to better cell spreading but lower mechanical properties, while Rac1 activation induces mechanotransduction [24]. The activation of RhoA or Rac1 leads to the assembly of myosin filaments, protrusive lamellipodia, and filopodia [25]. In spite of the great advances in the role of Cdc42 and Rac in SDF-1-induced cell migration, the exact mechanisms of Rho on SDF-1-induced migration remain unclear. In the present study, we will assess a possible regulation of cytoskeleton by Rho in SDF-1-induced Jurkat cell migration.

Materials and methods

Cell culture and treatment

Jurkat cells obtained from the Chinese Center for Type Cultures Collections were cultured in RPMI-1640 (Gibco) and 10% FBS in a humidified atmosphere with 5% CO₂ at 37 °C. For SDF-1 stimulation, Jurkat cells were collected, washed with PBS, re-suspended with RPMI-1640, and then incubated with indicated concentrations SDF-1 or solvent at 37 °C for indicated time periods. For the inhibition experiments, cells were pretreated with 2 µg/ml Cytochalasin B, 1 µg/ml CT04, 1 mM N-acetyl-L-cysteine (NAC), or equal volume of solvent before SDF-1 stimulation.

RNA extraction and cDNA synthesis

Total RNAs were extracted from Jurkat cells using Trizol Reagent (Takara, Shiga, Japan). The quality and integrity of these total RNA samples were determined by a Biospectrometer (Eppendorf, Germany). The extracted total RNA samples were incubated with gDNA Eraser in PrimeScript RT Reagent kit (Takara, Shiga, Japan) for 30 min at 37 °C to remove genomic DNA. The first strand cDNA was synthesized from 2 µg of total RNA using PrimeScript RT Reagent kit (Takara, Shiga, Japan).

Plasmid construction

The human gene encoding Rho-binding domain (RBD) of Rhotekin was amplified from the synthesized cDNA using a PCR mixture (Takara, Shiga, Japan) with the following parameters: one cycle of 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and one cycle of 72 °C for 10 min. Forward primer: CGGATC-CATCCTGGAGGACCTGAATAT; Reverse primer: GGAATTCCTAGCTTGTCTTCCCCAG. PCR products were separated, extracted, digested by *Bam*H1 and *Xho*1, and then inserted into pGEX-4T-1.

Transwell migration assay

The migration assay was performed using 3.0 µm pore size (Corning Costar) in 24-well culture plates. Before the assay, Jurkat cells were starved overnight in RPMI-1640 with 0.5% FBS. Then cells were re-suspended in serum-free RPMI-1640 at a density of 3×10^6 cells/ml. 100 µl suspension was seeded onto each upper chambers and 500 µl of RPMI-1640 supplemented with different concentrations of SDF-1 or equal volume of RPMI-1640 was added to the lower one. The transwell wells were maintained at 37 °C in a humidified atmosphere with 5% CO₂. After 4 h, the migration rates were analyzed.

Immunofluorescence

To detect the distribution of F-actin, Jurkat cells were treated with SDF-1 for 4 h, and then fixed with 4% paraformaldehyde for 20 min at room temperature. After washing with PBS, Jurkat cells were permeabilized with 0.2% Triton X-100 in PBS (containing 5 mM EDTA and 2% FBS) for 10 min. After washing with PBS, cells were stained with 2×10^{-7} M FITC-conjugated phalloidin at room temperature for 20 min, washed with PBS, and investigated under a confocal microscope.

Flow cytometry

The quantification of F-actin in cells was examined by flow cytometry. After stimulation with SDF-1, Jurkat cells were fixed at room temperature for 20 min, permeabilized for 10 min, stained with 2×10^{-7} M FITC-conjugated phalloidin for 20 min, and then washed with PBS. Cells were examined on flow cytometer, and the results were expressed as relative fluorescence index (RFI) by dividing the fluorescence value of the experimental groups by that of the control group.

Assessment of active Rho levels

Experiment was conducted following the instruction of the active Rho pull-down assay kit (Cytoskeleton). Briefly,

cells were treated with 25 ng/ml SDF-1 at 37 °C for the indicated time and lysed in 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 60 mM MgCl₂, 1% Nonidet P-40, and 5% glycerol, and Rho-GTP in 500 µg extracts was captured by 30 µg GST-Rhotekin RBD immobilized to glutathione resin. After washing with binding buffer, the activated Rho (Rho-GTP) was eluted with Laemmli buffer (0.125 M Tris–HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8). The extracted Rho-GTP was quantified by Western blot analysis.

SiRNA transfection

RhoA siRNA (siRhoA55: sense: GACAUGCUUGCUCUAGUC, antisense: GACUAUGAGCAAGCAUGUC; siRhoA465: sense: GUACAUGGAGUGUUCAGCA, antisense: UGCUAAGACACUCCAUGUAC), RhoC siRNA (siRhoC191: sense: GAAGACUAUGAUCGACUGC, antisense: GCAGUCGAUCAUAGUCUUC; siRhoC391: sense: GCUGGCCAAGAUGAAGCAG; antisense: CUCUUCAGCUUGGCCAGC), and control siRNA (sense: UUCUCCGAACGUGUCACGU, antisense: ACGUGACACGUUCGGAGAA) were synthesized and transfected into Jurkat cells by ESCORTTMIII Transfection Reagent (sigma). Cells were suspended in serum-free growth medium to give a final concentration of 6×10^6 cell/ml. The siRNA/liposome complexes were made in serum-free medium and complexes were formed at room temperature for 15–45 min. After 5–8 h incubation, 4 ml growth medium containing 12.5% serum was added and continued the incubation. After 24 h incubation, the cells were collected and lysate were used for Western blot assay to detect the siRNA efficiency.

Cell viability assay

MTT assay was employed to quantify cell viability. Jurkat cells were starved with 0.5% serum overnight, and then washed once with RPMI-1640. Approximately 3×10^5 cells for each sample were treated with indicated concentrations of CT04 for 2 h. At the end of treatment, MTT reagent (5 mg/ml, 20 µl) was added to each sample and incubated for 3 h. 200 µl DMSO was added to each sample and mixed by gentle shaking. Then mixture of 150 µl was pipetted into a 96-well plate and the optical density value of each well was determined using a plate reader at 570 nm.

Reactive oxygen species assay

To assess the cellular reactive oxygen species (ROS) generation by SDF-1-stimulated cells, Reactive Oxygen Species kit (Beyotime) was used. Jurkat cells were starved with 0.5%

serum overnight, washed once with RPMI-1640 pre-warmed to 37 °C, and then treated with SDF-1 (25 ng/ml) for 1, 2, 3, 4 h, respectively, at 37 °C. After treatment, DCFH-DA were diluted (1:1000) using pre-warmed RPMI-1640, added to each sample, and incubated at 37 °C for another 40 min in dark. Finally, each sample was washed twice with pre-warmed PBS and detected by flow cytometry.

Statistical analysis

Data were expressed as mean \pm SD. Comparisons were analyzed with the Student's *t* test. A *p* value <0.05 was considered statistically significant.

Results

1. SDF-1 induces Jurkat cell migration by F-actin redistribution and assembly.

To investigate whether SDF-1 could regulate cell migration, we performed transwell assay. The results showed that SDF-1 at the concentrations of 25, 50, 100, and 200 ng/ml could induce Jurkat cell migration with relative migration rates of 14.2, 19.7, 33.3, and 51.2%, respectively, compared with the control group (Fig. 1a). When Jurkat cells were pre-incubated with Cytochalasin B, the migration induced by SDF-1 was significantly inhibited, compared with the cells pre-treated with equal volume of solvent DMSO (Fig. 1b), suggesting that Jurkat cell migration requires an intact cytoskeleton.

Next we dedicated to elucidate the relationship of cell migration with cytoskeleton in the context of SDF-1 stimulation. F-actin was observed by confocal microscopy and quantified by flow cytometry after being stimulated by SDF-1 and labeled with FITC-conjugated phalloidin. The result showed that most cells of the SDF-1-stimulated group displayed obvious F-actin rearrangement, while the F-actin distribution of the control group cells remained the same before and after 4 h (Fig. 1c). Additionally, the relative level of F-actin was significantly increased in response to SDF-1 stimulation (Fig. 1d), indicating that SDF-1 could induce F-actin assembly.

2. Rho inhibitor CT04 inhibits SDF-1-induced Jurkat cell cytoskeleton changes and migration.

Since Rho family of the RhoGTPases is critical regulators of cytoskeleton, the direct inhibitor to Rho (CT04) were used to test whether Rho is required for the cytoskeleton changes and subsequent cell migration in response to SDF-1 stimulation. The result showed that CT04 pre-incubation inhibited F-actin redistribution in response to SDF-1, comparing with

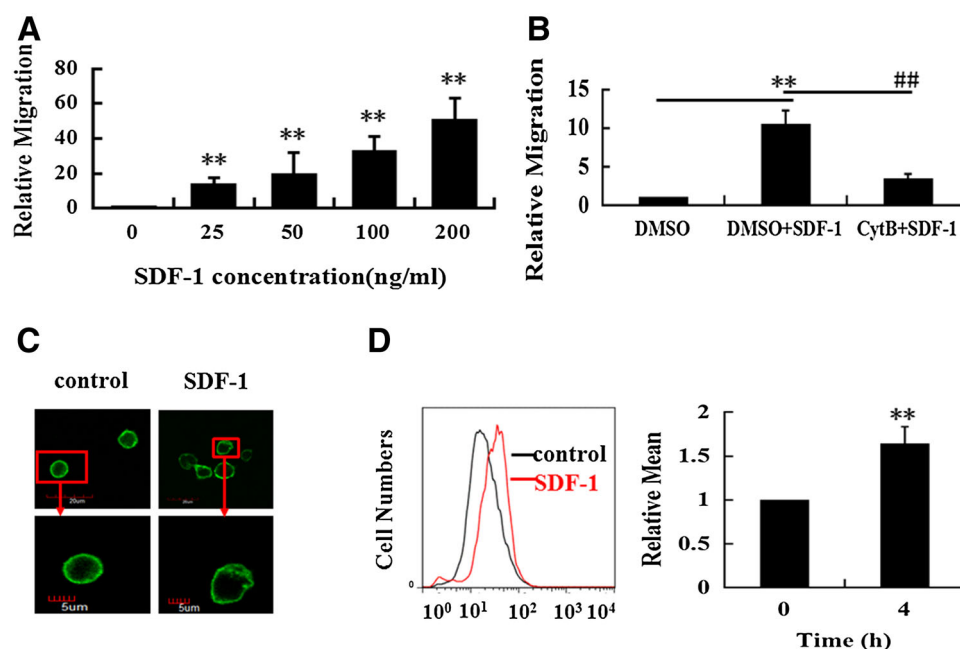


Fig. 1 SDF-1 induces cytoskeleton-dependent cell migration of Jurkat cells. **a** The migration of Jurkat cells in response to SDF-1 was determined at different concentrations by transwell migration assay. **b** Jurkat cells were pretreated with Cytochalasin B (2 μ g/ml) or DMSO in equal volume of solvent for 1 h, followed by incubation with SDF-1 (25 ng/ml) or equal volume of RPMI-1640 medium for 4 h in the transwell assay. **c, d** Cells were stimulated by SDF-1

(25 ng/ml) or equal volume of RPMI-1640 medium for 4 h, and F-actin was labeled with FITC-Phalloidin. The distribution of F-actin was observed under a confocal microscopy (**c**) and the amount of F-actin was quantified by flow cytometry (**d**). Data of three independent experiments are analyzed. ** $p < 0.01$ compared with control, ## $p < 0.01$ compared with SDF-1

the SDF-1-treated positive control cells (Fig. 2a). Meanwhile, CT04 also abolished the F-actin assembly induced by SDF-1 (Fig. 2b). Furthermore, CT04 pretreatment obviously reduced the migration rate towards SDF-1 (Fig. 2c). However, CT04 had no obvious effect on cell viability (Fig. 2d). These data suggested that Rho was required to regulate F-actin changes and the consequent cell migration in response to SDF-1 stimulation.

3. SDF-1 induces RhoA and RhoC activation, rather than RhoB.

As CT04 is inhibitor to Rho A, RhoB, and RhoC, we next verify which isotype of Rho functions in response to SDF-1 stimulation. We found that RhoA and RhoC, rather than RhoB were profoundly expressed in Jurkat cells (Fig. 3a). Therefore, active GTP-bound RhoA and RhoC were analyzed in the following experiments. The result showed that RhoA was activated after 5 min stimulation, and remained active until 15 min (Fig. 3b). RhoC activation was similar to that of RhoA (Fig. 3c, upper). GTP-bound RhoB was not found in the period of SDF-1 stimulation (Fig. 3d). This result demonstrated that RhoA and RhoC are rapidly activated in response to SDF-1 stimulation.

4. ROS functions downstream of Rho in the process of SDF-1-induced cytoskeleton changes and migration. Reactive oxygen species (ROS) play critical roles in signal transduction. To identify whether ROS is involved in the above-mentioned process, we analyzed the ROS levels after SDF-1 treatment. We found that ROS was rapidly increased in response to SDF-1 treatment (Fig. 4a). Furthermore, ROS scavenger, N-acetyl-L-cysteine (NAC), significantly inhibited the F-actin redistribution (Fig. 4b) and assembly (Fig. 4c) induced by SDF-1 stimulation, indirectly indicating the involvement of ROS in cytoskeleton changes. Additional result showed that NAC inhibited cell migration towards SDF-1, suggesting that ROS is required for this process (Fig. 4d).

To identify the relationship of Rho and ROS, CT04 and NAC were used. The result showed that CT04 significantly inhibited ROS production (Fig. 5a), suggesting that the SDF-1-induced ROS production is Rho dependent. Furthermore, NAC reduced RhoA and RhoC activation induced by SDF-1 (Fig. 5b, c). Therefore, ROS and Rho function reciprocally.

5. RhoA and RhoC are involved in SDF-1-induced migration.

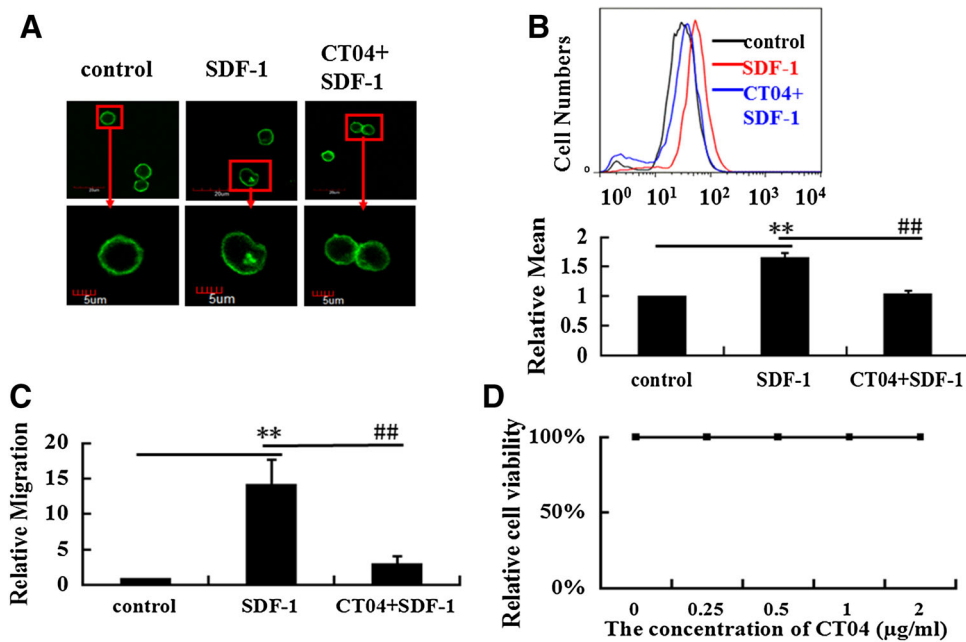
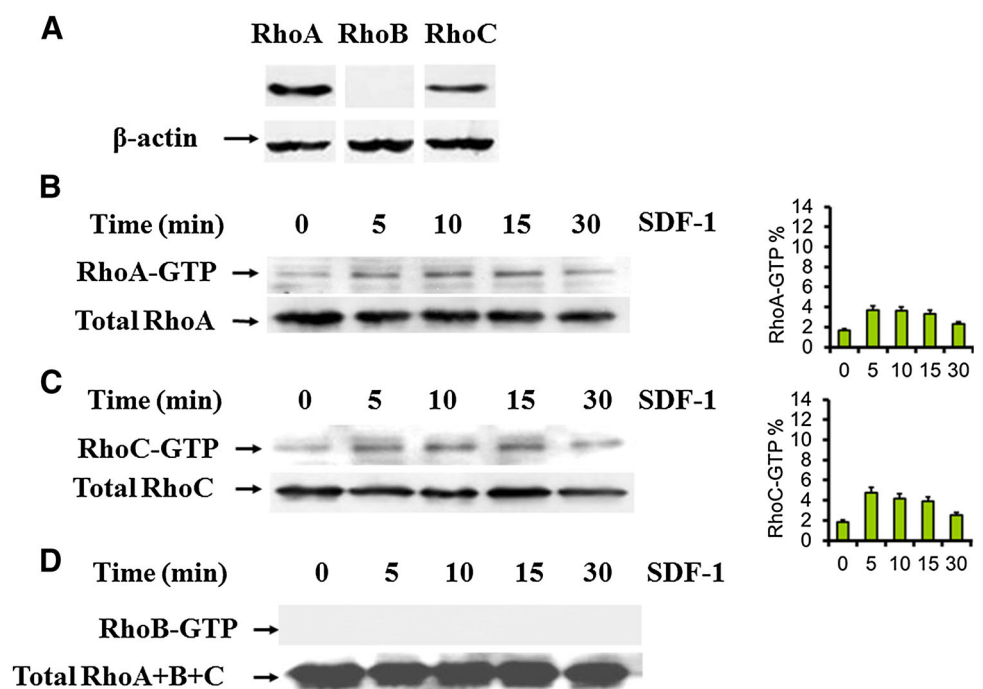


Fig. 2 RhoA + B+C were required for the SDF-1-induced cytoskeleton changes and migration of Jurkat cells. **a, b** Cells were treated with CT04 (1 μg/ml) or equal volume of RPMI-1640 medium for 2 h, treated with SDF-1(25 ng/ml) for another 4 h, labeled with FITC-Phalloidin, and analyzed by confocal microscopy or flow cytometry. **c** Jurkat cells were pretreated with CT04 (1 μg/ml) or

equal volume of RPMI-1640 medium for 2 h, then incubated with SDF-1(25 ng/ml) or equal volume of RPMI-1640 medium for 4 h in the transwell assay. **d** Effect of different concentrations of CT04 on cell variability was detected by MTT assay. Data of three independent experiments are analyzed. ***p* < 0.01 compared with control, ##*p* < 0.01 compared with SDF-1

Fig. 3 SDF-1 activates RhoA and RhoC. **a** 20 μg of total Jurkat lysate was loaded and the expressions of RhoA, RhoB, and RhoC were detected by Western blot. β-actin is used as a loading control. **b–d** Jurkat cells stimulated with SDF-1 or equal volume of PBS for indicated time and then cell lysates were prepared. Active Rho was captured by GST-Rhotekin RBD, and RhoA, RhoB, RhoC or RhoA + B+C were detected by Western blots. Total RhoA, RhoC, or RhoA + B+C was used as loading controls



Since SDF-1 induced RhoA and RhoC activation, we investigated the role of RhoA and RhoC in SDF-1-induced migration. Our results showed that the expression levels of RhoA and RhoC were reduced

by siRhoA55, siRhoA465, and siRhoC191 correspondingly, as detected by Western blot assay (Fig. 6a). SiRhoA55 and siRhoC191 successfully inhibited the SDF-1-induced migration (Fig. 6b).

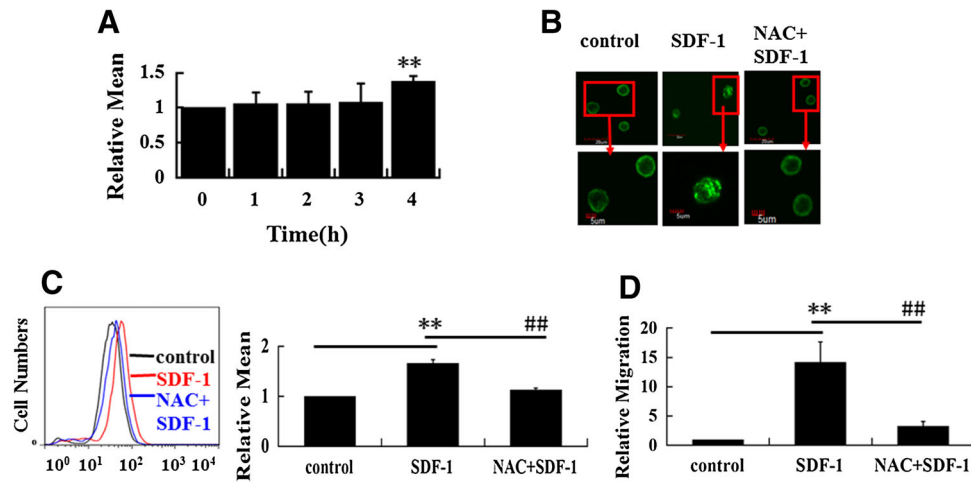


Fig. 4 ROS is involved in the SDF-1-induced cytoskeleton changes and cell migration of Jurkat cells. **a** ROS levels of Jurkat cells were examined by flow cytometry after SDF-1 treatment. **b, c** Jurkat cells were pretreated with NAC (1 mM) or equal volume of RPMI-1640 medium for 2 h, stimulated with SDF-1 (25 ng/ml) for 4 h, labeled with FITC-phalloidin, and analyzed by confocal microscopy or flow

cytometry. **d** Jurkat cells were pretreated with NAC (1 mM) or equal volume of RPMI-1640 medium for 2 h. The cell migration was detected by transwell assay in the presence of SDF-1 (25 ng/ml) or equal volume of RPMI-1640 medium. Data of three independent experiments are analyzed. ** $p < 0.01$ compared with control, ## $p < 0.01$ compared with SDF-1

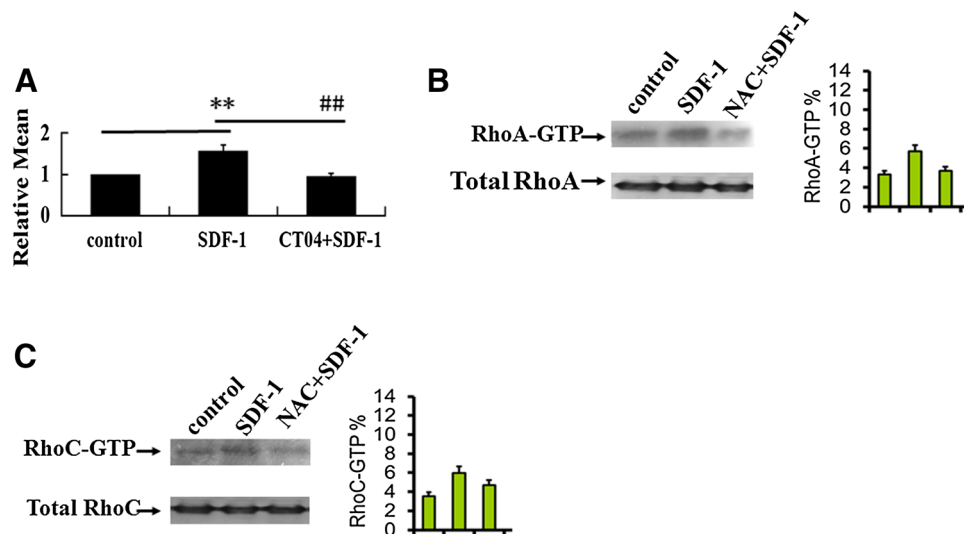


Fig. 5 ROS and Rho function reciprocally. **a** Cells were pretreated with CT04 (1 μ g/ml) or equal volume of RPMI-1640 medium, stimulated with SDF-1 (25 ng/ml) or equal volume of RPMI-1640 medium for 4 h in the measurement of ROS levels. **b, c** Jurkat cells were pretreated with NAC (1 mM) or equal volume of RPMI-1640

medium for 2 h, washed, and stimulated with SDF-1 or equal volume of PBS for 5 min, and then cell lysates were prepared. Active Rho was captured by GST-Rhotekin RBD. RhoA and RhoC were detected by Western blots. Total RhoA and RhoC were used as loading controls

Discussion

SDF-1, in association with its cognate receptor CXCR4, plays a central role in the morphogenesis of leukocytes, and migration of hematopoietic progenitors, mature T cells, and monocytes [26–30]. Meanwhile, lots of studies demonstrated the essential role for CXCR4 signaling in the progression of T-ALL [6, 7], suggesting distinct requirements for SDF-1/CXCR4 signaling between physiology

and disease. However, the underlying mechanism is not well studied. Now, our work extends these findings by showing the importance of Rho-regulated F-actin changes in the CXCL12/CXCR4 signaling of Jurkat acute lymphoblastic leukemia cells (Figs. 1, 2).

RhoA, RhoB, and RhoC are highly homologous. RhoA regulates actomyosin contractility, cytokinesis, cell polarity, and focal adhesion assembly [31]. RhoB regulates cell shapes and CXCR2-mediated chemotaxis [32]. RhoC is

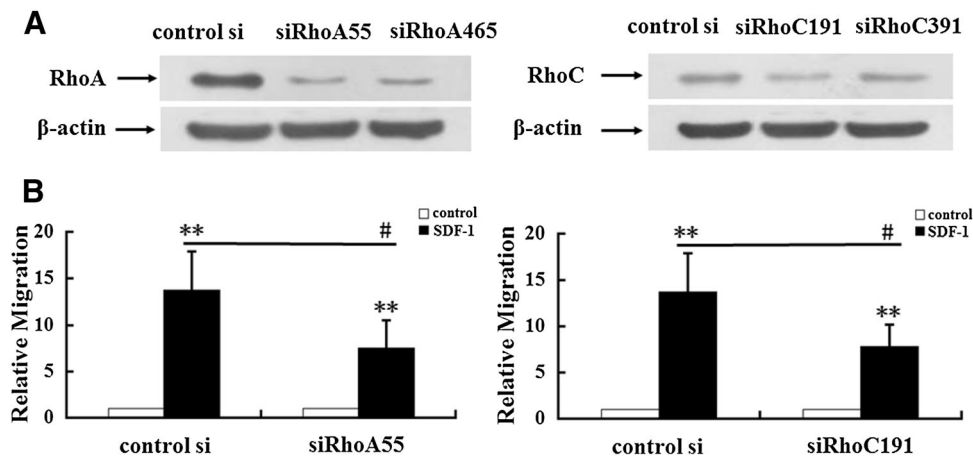


Fig. 6 RhoA and RhoC are required for SDF-1-induced Jurkat cell migration. **a** Jurkat cells were transfected with control siRNA and siRNA targeting RhoA or RhoC. Transfection efficiency was detected by antibody to RhoA or RhoC. Actin antibody was used as a loading control. **b** Transfected cells were stimulated with SDF-1 (25 ng/ml) or

equal volume of RPMI-1640 medium for 4 h by transwell assay. Data of three independent experiments are analyzed. ** $p < 0.01$ compared with control, # $p < 0.05$ compared with SDF-1 treatment in the control si groups

important in tumor cell invasion [33, 34]. We found that RhoA and RhoC were required for Jurkat cell chemotaxis in response to SDF-1. However, we did not find the expression of RhoB in Jurkat cells (Fig. 3). We speculate that RhoB is not involved in the SDF-1-induced Jurkat chemotaxis, or the expression level of RhoB is out of the sensitivity of Western blot assay. Besides, our results showed that RhoA and RhoC were both essential to SDF-1-induced cell migration (Fig. 6b). So we confirm that RhoA and RhoC are involved in the SDF-1-induced Jurkat cell migration. The divergent biological functions of these three proteins may be explained by the different modifications in the carboxy-terminus [35, 36].

Actin filament reorganization is a dynamic process that requires both actin polymerizing and depolymerizing factors, including mDia [37] and cofilins [38, 39]. Rho-mediated activation of mDia1 has been linked to the formation of stress fibers [35], membrane ruffles [36], and lamellipodia [31, 40]. Therefore, we speculate that Rho may activate mDia1 and involve in the migration of Jurkat cells towards SDF-1; however, this hypothesis needs further experiments. Meanwhile, redox activation of cofilin through redox activation of slingshot homolog 1 (SSH-1L) has been demonstrated [32, 41–43]. Interestingly, we found that ROS was induced by SDF-1 (Fig. 4a), which was dependent on Rho (Fig. 5a). Similarly, Rac1-mediated ROS production was shown to play a role in migration and invasion of B16 mouse melanoma cell line [44]. It is probable that the linkers between Rho, SSH-1L, and cofilins may be ROS. To identify how this redox activation by ROS occurred, substantial evidences for the redox-mediated activation of cofilins and SSH-1L are needed in the future work. In addition, Rho and ROS interplay with each

other complicatedly in the process of cell migration. As described in the review by Stanley et al. [45], ROS also functions as regulators of RhoGTPase and affects actin cytoskeleton reorganization. Recent studies have shown that NOX4-derived ROS caused the activation of RhoA in the initiation of lung fibroblast migration [46]. NOX-derived ROS generation led to vascular smooth muscle cell (VSMC) migration by upregulating the RhoA/Rho kinase (Rock) pathway [47, 48]. However, it remains unclear about the effect of oxidative stress on SDF-1-Rho pathway in lymphocyte chemotaxis migration. We found that ROS and Rho function reciprocally (Fig. 5a–c).

Acknowledgements This work was supported by the National Natural Science Foundation of China (31401216, 31471332).

Compliance with ethical standards

Conflict of interest All authors have no financial conflict of interest.

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