Phosphatidylinositol 3-Kinase and Protein Kinase C Signaling Pathways Are Involved in Stromal Cell–derived Factor-1α–mediated Transmigration of Stem Cells from Apical Papilla

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Abstract

Introduction: Previously, we have shown that stem cells from apical papilla (SCAPs) can be chemoattracted by stromal cell–derived factor-1α (SDF-1α). The purpose of this study was to investigate the intracellular signaling pathways involved in SDF-1α–mediated migration of SCAPs. **Methods:** Chemotaxis assays were performed to assess the effect of phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) signaling pathways in the SDF-1α–mediated migration of SCAPs using inhibitors of PI3K (LY294002) or PKC (GF109203X). The Cell Counting Kit-8 assay (Dojindo Laboratories, Kumamoto, Japan) was used to evaluate the effect of the inhibitors on the proliferation of SCAPs. The expression of focal adhesion–related proteins was examined by immunofluorescence staining and Western blot analysis. Phosphorylation of PI3K subunit p85 and PKC after SDF-1α induction was evaluated by Western blot. **Results:** The inhibition of PI3K or PKC signaling pathways significantly reduced SDF-1α–mediated migration of SCAPs. The inhibitors had no effect on the proliferation of SCAPs. Immunofluorescence analysis revealed that SDF-1α stimulated focal adhesion formation and stress fiber assembly in SCAPs, in addition to up-regulation of the expression of focal adhesion molecules, including p-focal adhesion kinase, p-paxillin, and vinculin. Pretreatment with PI3K or PKC inhibitors before SDF-1α induction significantly inhibited focal adhesion molecule expression. Moreover, increased phosphorylation of p85 and PKC were observed after SDF-1α stimulation, whereas these phosphorylations were down-regulated by the inhibition of PI3K or PKC signaling pathways. **Conclusions:** PI3K and PKC signaling pathways appear to be required for SDF-1α–mediated transmigration of SCAPs. These findings provide insights into the signaling mechanisms that underlie SDF-1α–mediated migration of SCAPs. *(J Endod 2016;42:1076–1081)*

Key Words

Migration, phosphatidylinositol 3-kinase, protein kinase C, stem cells from apical papilla, stromal cell–derived factor-1α

Significance

In our previous research, we have demonstrated that SDF-1α could chemoattract SCAP to migrate. In this article, we further demonstrated that PI3K and PKC signaling pathways are involved in SDF-1α-mediated transmigration of SCAP, which lay the foundation for clarifying the mechanism regulating SDF-1α-mediated migration of SCAP.

Regenerative endodontics has been considered as the preferred therapeutic option for the treatment of endodontically involved teeth, particularly immature permanent teeth. When the pulp is lost in an immature tooth resulting from necrosis, dental pulp stem cells (DPSCs) are no longer present. In cases in which apical papilla is still viable, stem cells residing in the apical papilla (SCAPs) may be present and could be attracted into the canal space to regenerate the pulp (1, 2). Chemokines are critical signaling molecules that could instruct stem cells and specific subpopulations of leukocytes in trafficking and the homing process to achieve tissue regeneration (3). Stromal cell–derived factor-1α (SDF-1α) is a widely expressed chemotactic cytokine that belongs to the CXC cytokine subfamily and mediates cell migration through its binding with the CXC chemokine receptor 4 (CXCR4) (4, 5). The SDF-1α/CXCR4 axis has been used as a strategy to regenerate various tissues/organs under multiple physiological and pathological conditions, such as myocardia (6, 7), kidney (8), liver (9), bone (10), and dental pulp (11, 12). In our previous study, we showed for the first time the *in situ* expression of CXCR4 in apical papilla and cultured SCAPs (13). The SDF-1α gradient may induce translocation of cytoplasmic CXCR4 to the membrane and enhance SCAP transmigration (13). However, the molecular mechanism involved in the regulatory process of SCAP migration has not been revealed.

Studies of lymphocytes have shown that SDF-1α, after binding to CXCR4, causes mobilization of calcium and the activation of multiple signaling pathways, including phosphatidylinositol 3-kinase (PI3K) and phospholipase C gamma (PLC-γ)/protein kinase C (PKC) (14–16). SDF-1α stimulation enhances the tyrosine phosphorylation of multiple focal adhesion components, including focal adhesion kinase (FAK), paxillin, p130Cas, CrkII, and CrkL, thus modulating the formation and function of focal

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adhesions in hematopoietic progenitor cells (16, 17). These adhesions are directly associated with chemotaxis and cell migration (16). PI3K as well as PKC signaling pathways appear to be required for the SDF-1α–induced phosphorylation of these focal adhesion components as well as for cell migration in hematopoietic progenitor cells (18).

In the present study, we hypothesized that PI3K and PKC signaling pathways are involved in SDF-1α/CXCR4 axis–mediated SCAP transmigration. We investigated *in vitro* transmigration with inhibitors of PI3K and PKC signaling pathways. We also evaluated the expression of key focal adhesion components (FAK, paxillin, and vinculin) and the phosphorylated downstream proteins of PI3K and PKC after SDF-1α stimulation to determine the potential role of PI3K and PKC signaling pathways in SCAP transmigration.

Materials and Methods

Cell Isolation and Culture

SCAPs were isolated as described previously (13, 19). Human impacted third molars (N = 8) with an open apex were collected from healthy patients (8 donors aged 16–22 years) in the Oral and Maxillofacial Surgery Department at Peking University School of Stomatology, Beijing, P.R. China, with a protocol approved by the Ethical Committee of Peking University Health Science Center. Briefly, immediately upon extraction, the apical papilla was carefully cut off from the root apex. The tissues were minced and digested in a solution of 3 mg/mL collagenase type I (Worthington, Lakewood, NJ) and 4 mg/mL dispase (Sigma-Aldrich, St Louis, MO) for 30 to 60 minutes at 37°C. The isolated cells were seeded and cultured with alpha modification of Eagle's medium (Gibco, Grand Island, NY) supplemented with 15% fetal bovine serum (Gibco), 2 mmol/L L-glutamine (Gibco), 100 U/mL penicillin G, and 100 mg/mL streptomycin (Gibco) and maintained in 5% CO_2 at 37°C. Colony formation units of fibroblastic cells were normally observed within 1 to 2 weeks. These heterogeneous populations of adherent, clonogenic dental stem/progenitor cells were tested for their cell surface marker expression by flow cytometry analysis (positive for STRO-1, CD146, CD90, and CD105 and negative for CD45). Cells at passage (p) 2 or p3 were used for experiments. Cells isolated from each tooth/donor were grown, maintained, and used separately for each independent experiment.

Cell Transmigration Assay

SCAP (p3, 5×10^3) were loaded into the upper chamber of an 8-μm-pore Transwell insert (Corning, New York, NY). There were 4 experimental groups:

1. *The negative control group:* The lower chamber contained only culture medium.
2. *The SDF-1α group:* The lower chamber medium was supplemented with 100 ng/mL SDF-1α (R&D Systems, Abington, UK) (13).
3. *LY/SDF-1α group:* SCAPs were preincubated with 10 μmol/L LY294002 (PI3K Inhibitor, Sigma-Aldrich) for 1 hour before loading into the upper chamber. The lower chamber medium was supplemented with 100 ng/mL SDF-1α.
4. *GF/SDF-1α group:* SCAPs were preincubated with 10 μmol/L GF109203X (PKC Inhibitor, Sigma-Aldrich) for 1 hour before loading into the upper chamber. The lower chamber medium was supplemented with 100 ng/mL SDF-1α.

The chambers were incubated in 5% CO_2 at 37°C for 24 hours. The nonmigrated cells on the upper side of the membranes were wiped off, and cells migrated to the lower surface of the membranes were fixed with 95% ethanol and stained with 0.1% crystal violet for evaluation under a microscope (DP72; Olympus, Tokyo, Japan). Nine fields under a 200-fold magnification were randomly selected for cell counting.

Cell Counting Kit-8 Assay for Cell Proliferation

SCAPs (p2) were seeded into 96-well plates at 4×10^3 cells/well. Cells were divided into 3 groups: the negative control group and 2 experimental groups each treated with 10 μmol/L LY294002 or GF109203X. Cells in the LY294002 and GF109203X groups were pretreated with inhibitors for 1 hour before being seeded into the 96-well plates. Cell viability was determined at 24, 36, and 72 hours using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The optical density was measured with an Elx808 enzyme-linked immunosorbent assay reader (BioTek, Winooski, VT) at 450 nm.

Cell Double-labeling Immunofluorescence Assay

SCAPs (p2) were cultured on coverslips at 60% confluence. The treated cells were fixed in 4% paraformaldehyde (Solarbio, Beijing, China) and washed with 0.01 mol/L phosphate-buffered saline. After permeabilization with 0.5% Triton X-100 (Sigma-Aldrich) for 15 minutes, cells were blocked with 10% goat serum albumin (ZSGB-Bio, Beijing, China) and incubated with primary rabbit antihuman antibodies specific to phosphorylated (p)-paxillin (phospho-paxillin [Tyr 118]; Cell Signaling Technology, Inc, Danvers, MA), p-FAK (phospho-FAK [Tyr 397]; Cell Signaling Technology, Inc), or mouse antihuman vinculin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) followed by secondary antibodies Rhodamine Red-X–conjugated goat antirabbit immunoglobulin G or Rhodamine Red-X–conjugated goat antimouse immunoglobulin G (CW-BIO, Beijing, China). Cells were then incubated with fluorescein isothiocyanate (FITC)-phalloidin (binds F-action; Sigma-Aldrich) for 30 minutes. The coverslips were counterstained with 4′,6-diamidino-2-phenylindole (ZSGB-Bio, Beijing, China) and mounted for images analysis under a fluorescence microscope (LSM5; Carl Zeiss, Jena, Germany).

Western Blot

The treated SCAPs (p2) were harvested in radio immunoprecipitation assay (RIPA) lysis buffer (Huangxing Bio, Beijing, China) with protease inhibitor (Huangxing Bio) cocktail on ice for 15 minutes. The supernatant was collected, and protein concentrations were determined using a bicinchoninic acid protein assay (Huangxing Bio). Whole cell lysates were fractionated with 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane (Huangxing Bio), blocked with 5% nonfat dry milk (Huangxing Bio), and incubated with primary antibodies to selected protein antigens. The following primary antibodies were used: anti–p-FAK, anti–p-paxillin, antivinculin, anti–p-p85 (Cell Signaling Technology, Inc), anti–p-PKC (Cell Signaling Technology, Inc), and anti–β-actin (Huangxing Bio). Immunoreactive bands were visualized by using horseradish peroxidase–conjugated secondary antibody and Super Enhanced Chemiluminescence Plus (Huangxing Bio) using the LAS-3000 (Fujifilm, Tokyo, Japan) luminescent image analyzer. Immunoblots were semiquantified using Image J 2X software (National Institutes of Health, Bethesda, MD). The levels of p-FAK/FAK, p-paxillin/paxillin, vinculin/β-actin, p-p85/p85, and p-PKC/PKC were determined.

Data Analysis

All experiments were repeated at least 3 times using cells cultured from at least 3 different donors. All values are presented as mean ± standard deviation. Statistical analysis was performed with the

SPSS software (version 16.0; IBM SPSS Statistics, Armonk, NY). One-way analysis of variance was used followed by the least significant difference test. *P* values less than .05 were considered statistically significant.

Results

Blocking PI3K or PKC Signaling Pathway Inhibited SDF-1α–induced Migration of SCAPs

We observed that significantly more cells transmigrated in SDF-1α groups compared with the other 3 groups ($F = 61.286$, $P < .001$). When SCAPs were pretreated with the inhibitor of PI3K (LY294002) or PKC (GF109203X), the number of migrated cells in the LY/SDF-1α and GF/SDF-1α groups significantly decreased compared with that in the SDF-1α group ($P < .05$) and the negative control group (LY/SDF-1α group vs negative control group: $P < .05$; GF/SDF-1α group vs negative control group: $P < .05$), indicating that the chemotactic migration induced by SDF-1α appeared to be regulated via the PI3K or PKC pathways (Fig. 1*A* and *B*).

To verify that such reduced cell migration by the treatment of the 2 inhibitors was not because of their effect on reduced cell proliferation, we tested their effects on cell proliferation. As shown in Figure 1*C*, LY294002 and GF109203X had no effect on the proliferation of SCAPs. There was no significant difference between the negative control and the inhibitor-treated groups ($P > .05$).

PI3K and PKC Signaling Pathways Were Involved in SDF-1α–stimulated Cytoskeletal Reorganization and Focal Adhesion Formation in SCAPs

To examine the downstream effects of PI3K and PKC signaling on SCAP migration stimulated by SDF-1α, we visualized the cytoskeletal arrangement and focal adhesion formation of treated SCAPs by double labeling of F-actin (by phalloidin) and 1 of the cell adhesion–associated proteins (p-FAK, p-paxillin, or vinculin). Our immunofluorescence data shown in Figure 2 indicate stress fiber assembly and a higher expression of focal adhesion components, including p-FAK and vinculin, in the SDF-1α group, whereas disordered fuzzy stress fiber and a lower expression of focal adhesion were observed in the inhibitor groups (Fig. 2). The expression levels of p-paxillin were similar between the SDF-1α group and the negative group, whereas they were lower in the inhibitor groups.

We further confirmed these findings using Western blot analysis (Fig. 3*A*). The levels of p-FAK/FAK and vinculin/β-actin in the SDF-1α group were significantly increased compared with those in the other 3 groups (p-FAK/FAK: $F = 4.142$, $P < .05$; vinculin/β-actin: $F = 8.161$, $P < .05$) (Fig. 3*B*). However, there was no significant difference in the levels of p-paxillin/paxillin between the SDF-1α group and the negative control group ($P > .05$). The p-paxillin levels in the LY/SDF-1α group and the GF/SDF-1α group significantly decreased compared with those in the SDF-1α group ($P < .05$). These results indicate that SDF-1α stimulated cytoskeletal reorganization and focal adhesion formation in SCAPs, whereas specific PI3K and PKC signaling pathways inhibitors could block these effects.

Phosphorylation of p85 and PKC Was Involved in the Activated SDF-1α/CXCR4 Axis in SCAPs

To further dissect the specific reactions in the involved PI3K or PKC signaling pathways after SDF-1α stimulation, we examined the levels of the p-p85 (PI3K subunit) and p-PKC. As shown in Figure 4*A* and *B*, SDF-1α treatment induced phosphorylation of

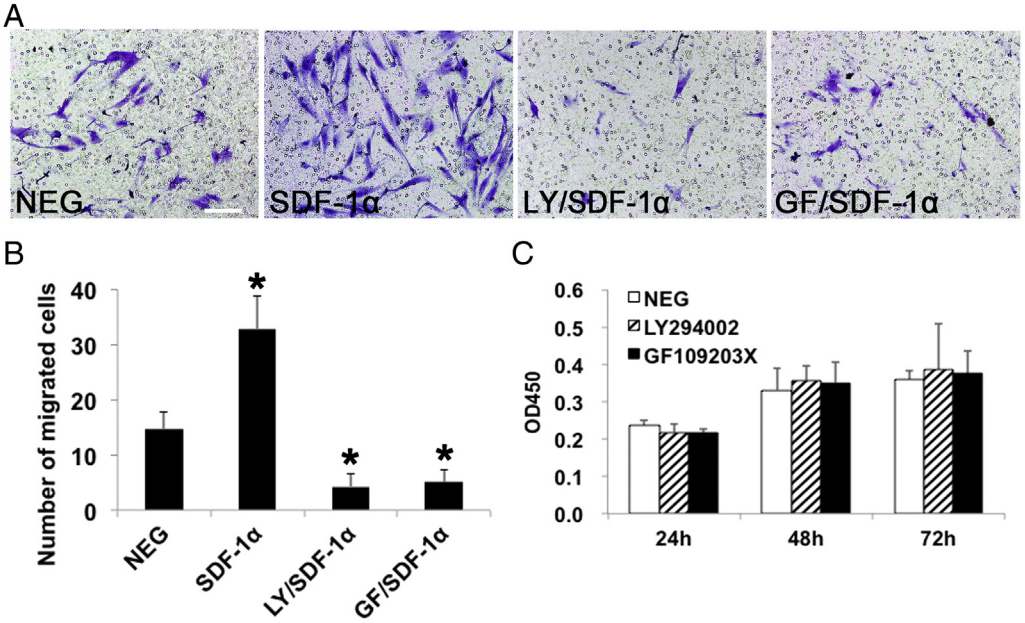

Figure 1. Inhibition of PI3K or PKC signaling pathways blocked SDF-1α–induced migration of SCAPs. (*A*) Transmembrane migration of SCAPs stained with 0.1% crystal violet. Images are representative data of independent experiments with consistent results. SCAPs were from sample #1 (22-year-old woman, 3rd molar at p3). Scale bar = 100 μm. NEG, negative control. (*B*) The number of migrated cells in the LY/SDF-1α group and the GF/SDF-1α group, which were pretreated with inhibitors for 1 hour before loading into the upper chamber, significantly decreased compared with those in the negative control group (*$P < .05$). SCAPs were from sample #1, sample #2 (21-year-old woman), and sample #3 (19-year-old man) (all third molars, p3). (*C*) The effect of the inhibitors on the proliferation of SCAPs determined by Cell Counting Kit-8 assays. The optical density at 450 nm values represented relative numbers of cells (mean $\pm$ standard deviation). Cells in the LY294002 group and GF109203X group were pretreated with inhibitors for 1 hour before seeded into the 96-well plates. There was no significant difference between the negative control (nontreated) and the inhibitor groups ($P > .05$). SCAPs were from sample #1, sample #2, and sample #3 (all third molars, p2).

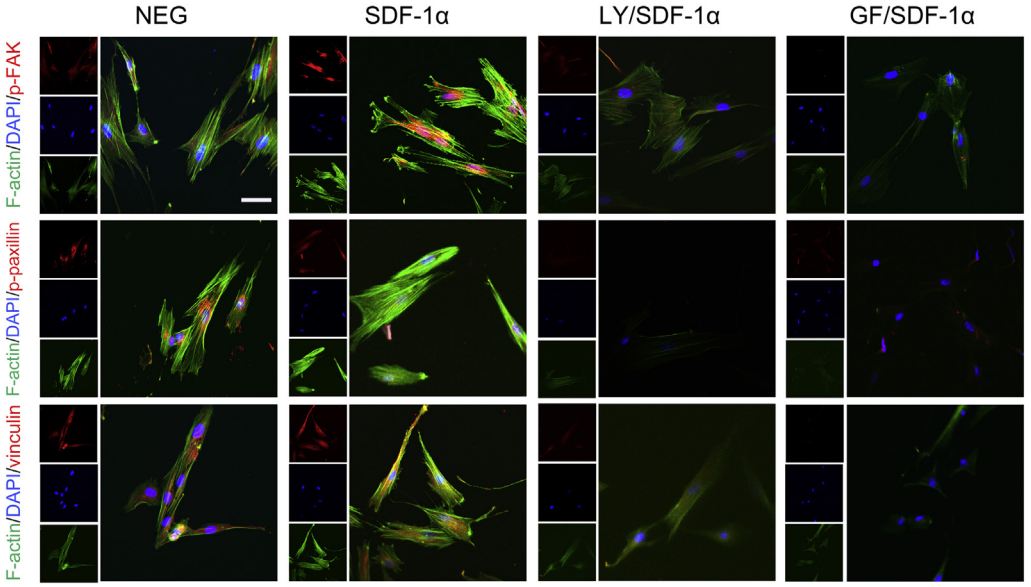

Figure 2. Immunofluorescence analysis of SDF-1α–stimulated cytoskeletal reorganization and focal adhesion formation in SCAPs. SCAPs were pretreated with LY294002 (PI3K inhibitor) or GF109203X (PKC inhibitor) for 1 hour and then incubated with SDF-1α for 24 hours. Immunofluorescence labeling of F-actin stress fiber assembly (*green*) and a higher expression of focal adhesion components (p-FAK/vinculin, *red*) were noted in the SDF-1α group, whereas disordered fuzzy stress fiber and a lower expression of focal adhesion were observed in the inhibitor groups. The expression of p-paxillin is similar between the SDF-1α and negative control groups, whereas it was lower in the inhibitor groups. Nuclear counterstaining was done with 4′,6-diamidino-2-phenylindole DAPI (*blue*). Images are representative data of independent experiments with consistent results. SCAPs were from sample #1 at p2. Scale bar = 100 μm (for the large merged image).

p85 and PKC. Pretreatment with PI3K or PKC inhibitors blocked SDF-1α–induced p-p85 and p-PKC levels. The ratios of p-p85/p85 and p-PKC/PKC in the SDF-1α group were significantly increased compared with the other 3 groups (p-p85/p85: $F = 12.867$, $P < .05$; p-PKC/PKC: $F = 10.360$, $P < .05$). The phosphoprotein levels in the LY/SDF-1α and the GF/SDF-1α groups significantly decreased compared with those in the SDF-1α and negative control groups ($P < .05$). The results indicate that SDF-1α stimulation led to a significant increase

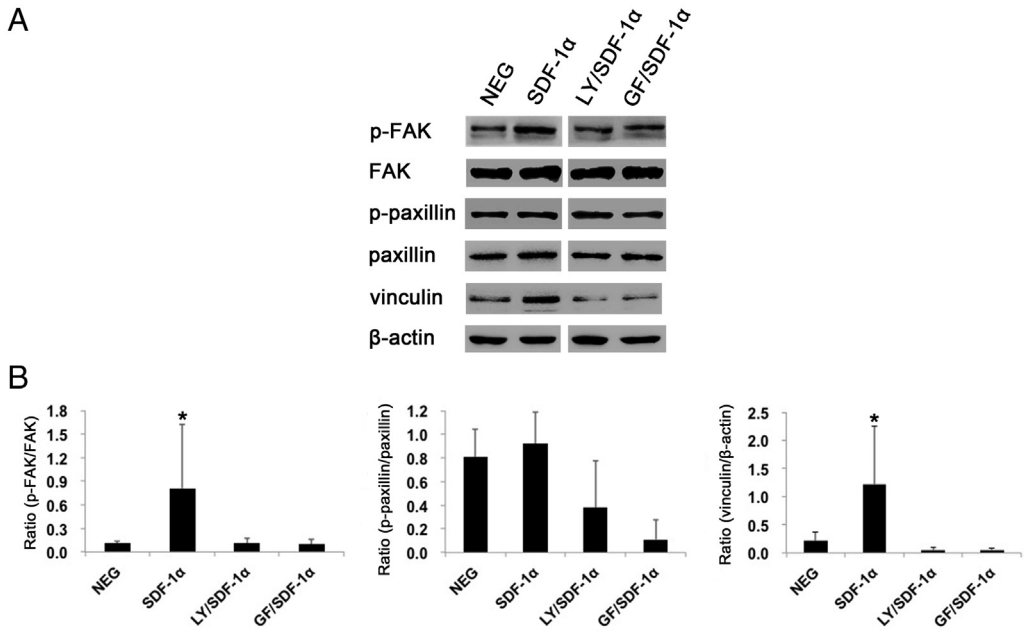

Figure 3. Western blot analysis of adhesion protein expression in SCAPs stimulated by SDF-1α. (*A*) Cells were pretreated with LY294002 or GF109203X for 1 hour and then incubated with SDF-1α for 1 hour. Cells were then harvested for Western blotting and antibody detection of the expression levels of p-FAK, FAK, p-paxillin, paxillin, vinculin, and β-actin. Images are representative data of independent experiments with consistent results. SCAPs were from sample #4 (19-year-old woman, third molar at p2). (*B*) The ratios of p-FAK/FAK, p-paxillin/paxillin, and vinculin/β-actin were determined after semiquantitative analysis of the band intensities using Image J 2X software. Data from 3 different donors are shown as means ± standard deviation. *$P < .05$ compared with the negative group. SCAPs were from sample #4, sample #5 (21-year-old woman), and sample #6 (16-year-old female) (all third molars, p2.). NEG, negative control.

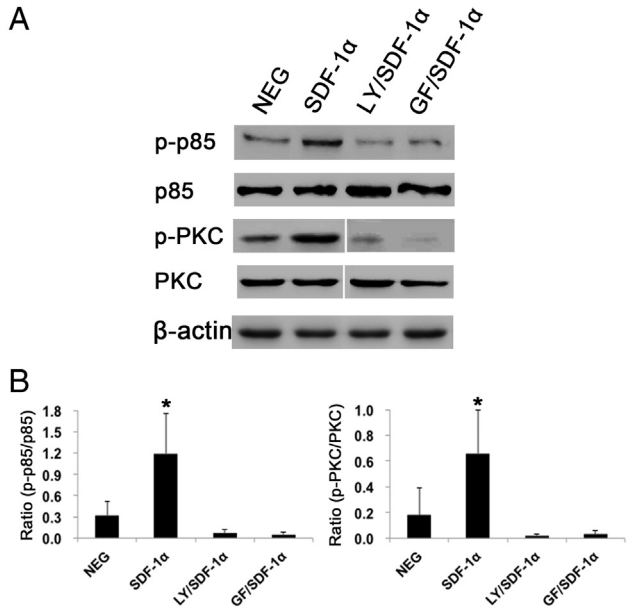

Figure 4. Western blot analysis of phosphorylation of p85 and PKC after SDF-1α stimulation of SCAPs. (*A*) SCAPs were pretreated with LY294002 or GF109203X for 1 hour and then incubated with SDF-1α for 1 hour. Cells were then harvested for Western blotting and antibody detection of the expression levels of p-p85, p85, p-PKC, PKC, and β-actin. Images are representative data of independent experiments with consistent results. SCAPs were from sample #4. (*B*) The ratios of p-p85/p85 and p-PKC/PKC were determined by the same method used for data in Figure 3*B*. Data from 3 different donors are shown as means ± standard deviation. *$P < .05$ compared with the negative group. SCAPs were from sample #4, 5, and 6 at p2. NEG, negative control.

of phosphorylation of p85 and PKC, thereby activating the PI3K and PKC signaling pathways.

Discussion

The present study shows the molecular mechanisms involved in the SDF-1α–induced transmigration of SCAPs. We found that SDF-1α–mediated transmigration of SCAPs involved PI3K or PKC signaling pathways that regulate cytoskeletal reorganization and focal adhesion formation in SCAPs. SDF-1α treatment can significantly promote the phosphorylation of p85 and PKC, indicating that PI3K and PKC signaling pathways are activated after SDF-1α stimulation in SCAPs. These findings provide insights into the signaling mechanisms that underlie SDF-1α–mediated migration of SCAPs.

In our previous study, we observed that SDF-1α could induce transmigration of SCAPs (13); here we extended our study to characterize the signaling transduction pathways associated with SDF-1α–induced migration of SCAPs. Accumulating data have implied that multiple signaling pathways exist to regulate cell migration, such as PI3K (20, 21) and PKC signaling pathways (16). Our results revealed that these 2 signaling pathways are also involved in SDF-1α–induced SCAP transmigration. We verified that the inhibitors did not affect the proliferation of SCAPs (Fig. 1*C*), which corresponds to our previous finding that SDF-1α has no effect on the proliferation of SCAPs (13). This suggests that although SDF-1α activates PI3K and PKC pathways, it does not lead to the pathways that induce cell proliferation. Our present study verified that SCAPs, similar to the migration of hematopoietic cells in response to SDF-1α/CXCR4 axis activation (17), also use PI3K and PKC pathways for cell transmigration effects. We showed that SDF-1α–treated SCAPs presented a highly organized stress fiber assembly. Such formation of focal adhesion complexes is important to provide anchoring sites for cell migration (22). Phosphorylation of FAK increases the catalytic activity of FAK and is important for the tyrosine phosphorylation of paxillin (23). P-paxillin could bind to vinculin, which promotes its localization (24). We observed that focal adhesion components, including p-FAK and vinculin, were up-regulated on SDF-1α stimulation. However, we did not observe a p-paxillin increase in this study. This may be because focal adhesion dynamics during cell migration is a continuous process involving assembly and disassembly during which the level of p-paxillin might fluctuate. A kinetic study of different time points may be needed to detect the change of p-paxillin.

In conclusion, our present study indicates that both PI3K and PKC signaling pathways are required to enhance the phosphorylation of focal adhesion proteins, the stress fiber assembly, and SCAP migration by SDF-1α stimulation. These findings provide a better understanding of the molecular mechanisms that regulate the SDF-1α–mediated migration of SCAPs, which may help establish strategies for cell migration–mediated dental pulp regeneration.

Acknowledgments

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The authors deny any conflicts of interest related to this study.

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