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Mediation of Rac1 Activation by Kindlin-2: An Essential Function in Osteoblast Adhesion, Spreading, and Proliferation

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ABSTRACT

Kindlins are focal adhesion proteins that regulate integrin signaling. Although integrin activation is critical for bone development, little is known about the expression and role of kindlins in osteoblasts. We therefore investigated the function of kindlin-2 in osteoblast adhesion, spreading, and proliferation using small interfering RNA. In MC3T3-E1 cells, only kindlin-2 is highly expressed and localizes to focal adhesion. We found that kindlin-2 was involved in integrin activation in MC3T3-E1 cells and that kindlin-2 knockdown osteoblasts resulted in diminished cell adhesion, spreading, and proliferation. In this process, kindlin-2 knockdown impaired transient Rac1 activation, influencing Akt activation and AP-1 activity. In agreement with these data, pharmacological inhibition of Rac1 reduced MC3T3-E1 cell adhesion, spreading, and proliferation. Overall, these findings demonstrated that kindlin-2 governs Rac1 activation, which controls osteoblast function. Our findings provide the first insights concerning the function of kindlin-2 in osteoblast, and suggest that kindlin-2 is a critical mediator for osteoblast physiology. J. Cell. Biochem. 112: 2541–2548, 2011.

KEY WORDS: KINDLIN-2; OSTEOBLAST; INTEGRIN; Rac1

Osteoblast adhesion to extracellular matrix (ECM) is essential for bone development [Cowles et al., 2000; Siebers et al., 2005]. Integrins, heterodimeric glycoproteins composed of α and β subunits, primarily mediate the interaction between cells and ECM components such as fibronectin, collagen, and vitronectin. Once activated via ligand binding, integrins transduce signals into cells by recruiting adaptor proteins to the cytoplasmic tails, which results in osteoblast adhesion, proliferation, and differentiation [Siebers et al., 2005; Legate and Fässler, 2009; Shattil et al., 2010].

Kindlins are one of the adaptor proteins on the cytoplasmic tails of integrins [Legate and Fässler, 2009; Meves et al., 2009; Malinin et al., 2010; Shattil et al., 2010]. Among three kinds of kindlin family, kindlin-2 is ubiquitously expressed, and is the only kindlin isoform expressed at the protein level in embryonic stem cells [Legate and Fässler, 2009; Shattil et al., 2010]. Kindlin-2 is known as an essential element of the integrin-mediated signaling pathway by binding to integrin-linked kinase (ILK) and migfilin in β1 and β3 integrin [Tu et al., 2003; Montanez et al., 2008; Meves et al., 2009]. The function of kindlin-2 is complex and is dependent on cell type. Previous functional studies have shown that kindlin-2 is required for cell adhesion and migration in endothelial cells, fibroblast, and tumor cells [Montanez et al., 2008; An et al., 2010; He et al., 2011]. Kindlin-2 is involved in vertebrate myocardial formation and function [Dowling et al., 2008a]. Moreover, knockdown of kindlin-2 in myoblasts results in abnormal elongation and myogenesis [Dowling et al., 2008b]. However, although integrin-mediated signaling is important for osteoblast function, the role of kindlin-2 is still unknown in the process of bone formation.

Rho GTPases control a signal transduction pathway that links cell surface receptors to the assembly and disassembly of the actin cytoskeleton and of the associated integrin adhesion complexes [Hall, 2005]. They are involved in many cellular functions, including focal adhesion, cell spreading, and cell migration. Previous studies have shown that integrins can activate Rho GTPases, especially Rac.

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Fibronectin-mediated integrin activation induces Rac1 activation, which promotes cell survival, proliferation, and spreading [Price et al., 1998; Sundberg et al., 2003; Gao et al., 2004]. In addition, the overexpression of β1 integrin increases Rac activity and lamellipodia formation [Miao et al., 2002].

In this study, we investigated the expression and the role of kindlin-2 in osteoblast by examining the changes of MC3T3-E1 osteoblast cell adhesion, spreading, and proliferation after kindlin-2 gene knockdown using small interfering RNA (siRNA) transfection. We also tried to elucidate the downstream signal mediated by kindlin-2 after integrin activation by fibronectin.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

Newborn mouse calvaria-derived MC3T3-E1 (subclone 4) preosteoblastic cells (ATCC) were cultured in Minimum Essential Medium Alpha Medium (Invitrogen, NY) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, and streptomycin (Invitrogen) at 37°C in an atmosphere with 5% CO_2. For experiments, cells were resuspended in antibiotic-free growth medium and seeded at 30–40% confluence. Transfections of mouse kindlin-2 siRNA (Invitrogen, oligo IDs MSS211905/6) or the medium GC-content scrambled control siRNA (Invitrogen) were done with Lipofectamin™ RNAiMAX (Invitrogen) following manufacturer's instructions. Twenty-four hours later, cells were detached with trypsin/EDTA and seeded onto fibronectin-coated plate or glass coverslip. A seeding density of 10,000 cells/cm^2 was used in immunocytochemistry and 25,000 cells/cm^2 was used in all other experiments. Cells were plated with NSC23766 (Tocris, NY) when necessary.

FLOW CYTOMETRY

Integrin activation was assessed via flow cytometry using antibodies that recognize total (clone MB1.2, Millipore, CA) and activation (clone 9EG7, BD Biosciences, NJ) associated epitopes of β1 integrin. Transfected MC3T3-E1 cells were seeded on fibronectin-coated plates for 15 min and harvested by trypsinization. After washing, cells were incubated with primary antibodies, washed with PBS with 1% BSA and incubated with FITC-conjugated secondary antibody (Invitrogen). Flow cytometry acquisition was performed using the BD FACSCalibur (BD Biosciences).

IMMUNOCYTOCHEMISTRY AND IMAGE QUANTIFICATION

Transfected MC3T3-E1 cells grown on fibronectin-coated coverslips were fixed with 4% paraformaldehyde in PBS. For immunostaining, the following antibodies were used: anti-kindlin-2 (Millipore); rhodamine–phalloidin (Invitrogen); and Alexa flour 488 goat anti-mouse IgG (Invitrogen). After coverslips were mounted on glass microscope slides, digital images were captured by Olympus laser scanning confocal microscope and Fluoview 300 software. Image quantification was determined using the histogram/area functions of the Fluoview software. Cell size was outlined with the free hand tool and pixel area was measured and converted to micrometers based on pixel size values computed for each image by Fluoview software.

RNA EXTRACTION AND REVERSE TRANSCRIPTION PCR (RT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen) as directed by the manufacturer. After quantification, first-strand cDNA was synthesized using 1 μg of total RNA in a 20 μl reverse transcriptase reaction mixture (Takara, Japan) containing RNase inhibitor. The sequences of the forward and reverse primers were: 5′-CTACACCTTCTTTGACTTG-3′ and 5′-AGGGATGTCAGTTATGTC-3′ for kindlin-1; 5′-GCCTTGCTGCTCCGATTCA-3′ and 5′-CTTGGCCTGCTCGTAAAGCTG-3′ for kindlin-2; 5′-AGCTGTCTCTGCTGCGTGCTC-3′ and 5′-ATACCTTGCTGCATGAGGCAC-3′ for kindlin-3. After amplification, PCR products were subjected to a 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

WESTERN BLOT

MC3T3-E1 cells were washed with chilled PBS and lysed with lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF, and protease inhibitor cocktail (Sigma, MO)). Protein quantification was performed with the BCA protein assay reagent (Thermo Fisher Scientific, IL) following the manufacturer's protocol. Protein samples were separated on SDS–PAGE and transferred to nitrocellulose membrane. The membrane was blocked in 5% non-fat skim milk in TBS-T (0.1% Tween-20) for 1 h at room temperature followed by incubation with kindlin-2 (Millipore), phospho-AKT (Cell Signaling Technology, MA), Akt (Cell Signaling Technology), or tubulin (Sigma) antibody at 4°C overnight. After washing, the membranes were incubated with HRP-conjugated secondary antibodies (Jackson Laboratories, PA) for 1 h. Immunoreactive bands were visualized by chemiluminescence using Supersignal blotting kit (Thermo Fisher Scientific).

CELL ADHESION ASSAY

MC3T3-E1 cells transfected with kindlin-2 siRNA or scrambled control siRNA were seed into fibronectin coated 24-well plates and allowed to attach for 1 h at 37°C in an atmosphere with 5% CO_2. After three times washing with PBS to remove non-bound cells, cells were fixed with 4% paraformaldehyde and stained with crystal violet to quantify the number of attached cells. Analysis was done by counting cells on 10 randomly chosen microscopic fields.

METHYL TETRAZOL SULFATE (MTS) ASSAY

Cell proliferation was assessed 3 and 7 days after seeding by using the Cell Titer 96® Aqueous One Solution Reagent (Promega, CA) test. Cells were washed with PBS and placed in a mixture containing complete culture medium and MTS solution in a 5:1 ratio and then incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 3 h incubation, the optical density was read at 490 nm.

RAC1 ACTIVITY ASSAY

The quantity of cellular activated Rac1 was evaluated with the Rac1 G-LISA assay kit (Cytoskeleton, Denver) according to the manufacturer's protocol. Briefly, equal amount of cell lysate was loaded in wells of the Rac1-GTP affinity plate. The well-bound activated Rac1 was detected with an anti-Rac1-specific antibody recognized by a secondary antibody conjugated to HRP. The degree of Rac1 activation was determined by sample reading at 490 nm on a microplate reader (Bio-Rad Laboratories, CA).

ELECTROPHORESIS MOBILITY SHIFT ASSAY (EMSA)

MC3T3-E1 cells were lysed in ice-cold hypotonic lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 1 μg/ml aprotinin, 10 μg/ml leupeptin). After 15 min, 0.5% NP-40 was added; nuclei were collected at 10,000*g* for 1 min, resuspended in hypertonic extraction buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM PMSF), and centrifuged at 10,000*g* for 10 min. Supernatants were nuclear extracts which were used for EMSA. The oligonucleotide (0.3 pmol) for AP-1 containing consensus binding sequence (Santa Cruz Biotechnology, CA) was radiolabeled with [γ-^{32}P]-dATP by T4 polynucleotide kinase (New England Biolabs, MA) to produce double-stranded DNA probes. Ten micrograms of nuclear proteins were added to 20 μl of binding buffer (1 mM $MgCl_2$, 4% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 1 μg of poly (dI-dC), and 10 mM Tris–HCl, pH7.5) containing ^{32}P-labeled double-stranded DNA (100,000 cpm) and incubated at room temperature for 20 min. After incubation, bound and free probes were separated by electrophoresis on 6% polyacrylamide gel in non-denaturing condition and visualized by autoradiography.

STATISTICAL ANALYSIS

Results were expressed as mean ± standard deviation (SD). Data were evaluated for statistical significance using Student's *t*-test. Experiments were repeated at least three times. In all analyses, a *P*-value of <0.05 was considered statistically significant.

RESULTS

It has been known that kindlins are expressed in a tissue-specific manner. Kindlin-1 is mainly expressed in epithelial cells; kindlin-2 has ubiquitous expression in all tissues; and kindlin-3 is expressed primarily in hematopoietic cells [Malinin et al., 2010]. To examine the expression of kindlins in osteoblast, we performed RT-PCT using the mRNA of MC3T3-E1 osteoblast. mRNA from 2-week-old C57BL/6 mouse kidney and thymus was used for positive control [Ussar et al., 2006]. Results demonstrated that only kindlin-2 was detected in MC3T3-E1, whereas little or no kindlin-1 and kindlin-3 was expressed at the RNA level (Fig. 1A). Using immunocytochemistry, we found that kindlin-2 is well distributed in focal adhesion areas in MC3T3-E1 cells (Fig. 1B). As only kindlin-2 was expressed in MC3T3-E1 cells, we adopted the siRNA of murine kindlin-2 gene to scrutinize the role of kindlin-2 in osteoblasts. Based on the Western blot data, we found that the level of kindlin-2 knockdown was nearly 90% 24 h after siRNA transfection (Fig. 1C).

Kindlins have been known to affect integrin activation by binding to integrin β cytoplasmic tails and relaying integrin-mediated signals that influence cell physiology [Meves et al., 2009; Malinin et al., 2010]. To elucidate the possible function of kindlin-2 in osteoblasts, MC3T3-E1 cells were seeded on fibronectin-coated plates after kindlin-2 siRNA transfection. Cells with scrambled siRNA transfection were used as a control. First, we assessed the affinity states of integrin by determining the binding of the 9EG7 antibody, which recognizes only activated β1 integrin. Fluorescence-activated cell sorter analysis with the 9EG7 antibody demonstrated reduced signals in kindlin-2 siRNA treatment cells, whereas the total amount of β1 integrin was not changed (Fig. 2). Cells with scrambled siRNA treatment showed similar expression patterns of activated β1 integrin with parental cells. Next, we tried to elucidate the role of kindlin-2 in osteoblast function after integrin activation. Adherent cells had been significantly reduced by kindlin-2 siRNA transfection by the time a cell-adhesion assay was performed, 1 h after seeding to the fibronectin-coated coverslip (Fig. 3A). We then examined the effect of kindlin-2 in cell proliferation. MTS assay result showed that kindlin-2 knockdown

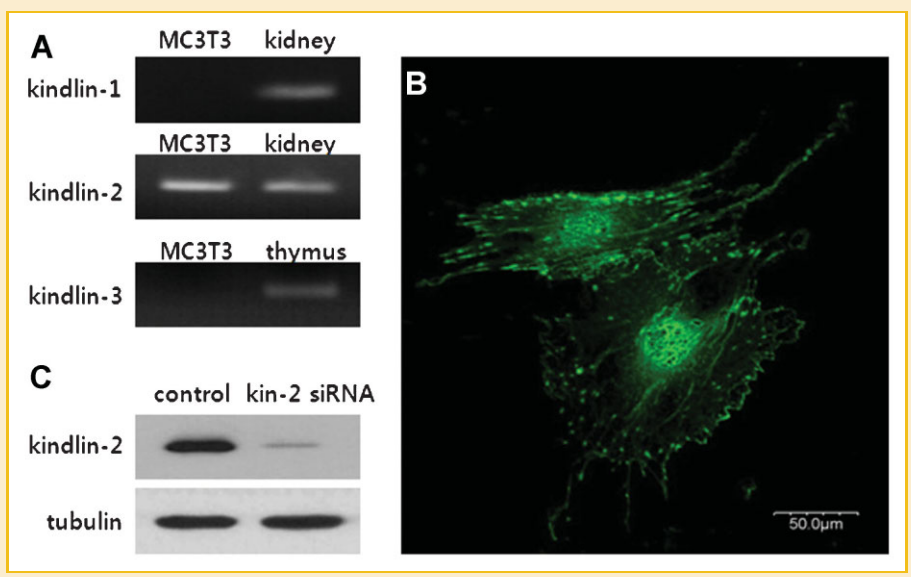

Fig. 1. Kindlin-2 is highly expressed in MC3T3-E1 cell. A: mRNA expression of kindlin-1, 2, and 3 in MC3T3-E1 cells determined in RT-PCR. mRNAs from mouse kidney and thymus were used in positive control. B: Representative picture of immunocytochemistry using anti-kindlin-2 antibodies on MC3T3-E1 cell. C: Western blot of MC3T3-E1 cells treated with scrambled siRNA (control) or kindlin-2 siRNA (50 nM, kin-2 siRNA) for 24 h. Tubulin was used as loading control.

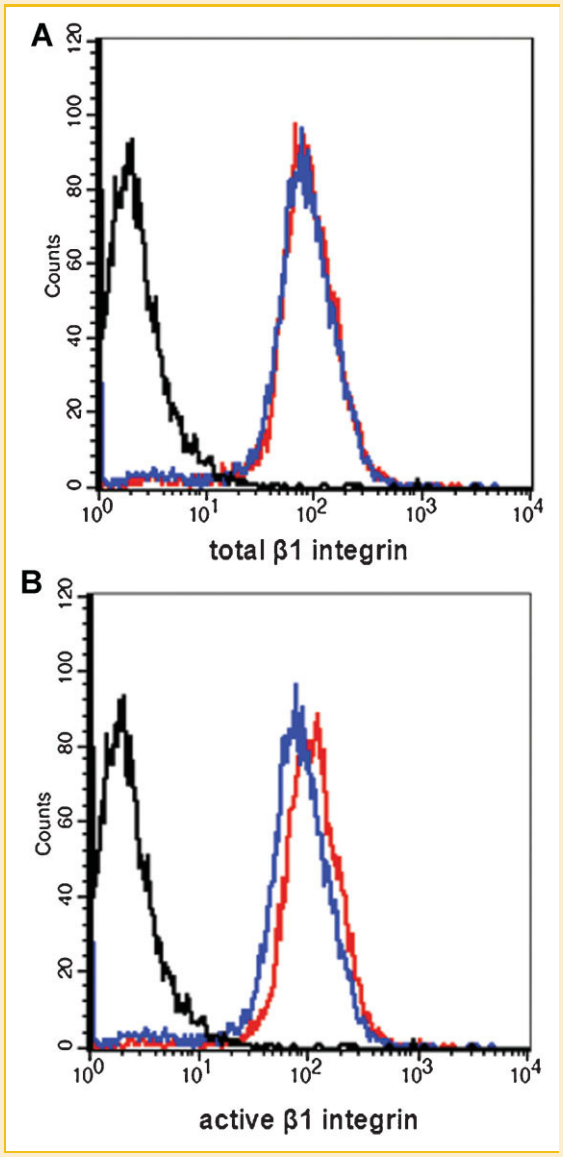

Fig. 2. Impaired β1 integrin activation in kindlin-2 knockout osteoblast. Surface expression of flow cytometry of total (A) and active (B) β1 integrin at the surface of the scrambled siRNA treated (red) and kindlin-2 siRNA treated (blue) MC3T3-E1 cells. Isotype background staining is shown in black curves.

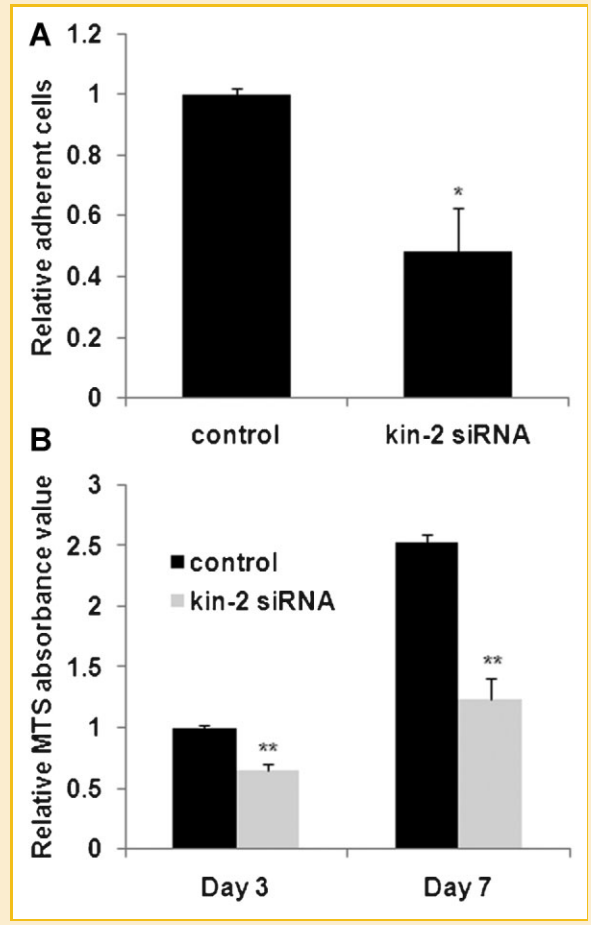

Fig. 3. Kindlin-2 knockdown prevents MC3T3-E1 cell adhesion and proliferation. Kindlin-2 siRNA or scrambled siRNA (control) transfected MC3T3-E1 cells were plated onto fibronectin-coated culture plates. A: One hour after seeding, adherent cells were stained with crystal violet and counted. The number of kindlin-2 siRNA treated cells on 10 microscopic fields are averaged and expressed as a fold increase relative to the number of control cells. B: At defined time points, MTS activity was measured in cells on culture plate. The values of optical density were expressed as a fold increase relative to the value of control cells (day 3). All data are means ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

diminished the MC3T3-E1 cell proliferation rate by 35% and 51% 3 and 7 days after seeding, respectively (Fig. 3B). In addition, kindlin-2 knockdown osteoblasts displayed reduced spreading (Fig. 4A–C). Six hours after seeding, the mean area for the osteoblasts was $2846.1 \pm 412.3\ \mu m^2$ in control cells, versus $1669.5 \pm 499.5\ \mu m^2$ in kindlin-2 knockdown cells. These findings demonstrated that kindlin-2 is required for cell spreading as well as for osteoblast proliferation and adhesion.

Next we investigated the Rac, a member of Rho GTPase, because it is activated by cell adhesion to fibronectin and is known to be involved in cytoskeletal organization and proliferation [Price et al., 1998; Sundberg et al., 2003]. When measuring Rac1 activation using ELISA, GTP-bound Rac1 was transiently increased and peaked at 1 h. The level of active Rac1 was diminished in kindlin-2 siRNA transfected cells (Fig. 5). Next, we tried to confirm the Rac1 to influence cellular function in MC3T3-E1 cells. Results showed that the Rac1 inhibitor, NSC23766, significantly suppressed cell spreading, adhesion, and proliferation (Fig. 6A–C).

We then investigated the Rac1-mediated downstream signals. Western blot results showed that fibronectin-induced integrin activation led to Akt phosphorylation, which was inhibited by kindlin-2 knockdown and NSC23766 (Fig. 7A). These results supported the suggestion that kindlin-2 mediate Rac1 activation, which leads to Akt phosphorylation. Integrin-mediated osteoblast adhesion to fibronectin is known to regulate gene expression via transcription factors such as AP-1 [Cowles et al., 2000]. Moreover, Rac1 activation influences the gene expression by affecting AP-1, which controls cell proliferation and adhesion [Genot et al., 1998; Troussard et al., 1999]. To confirm whether

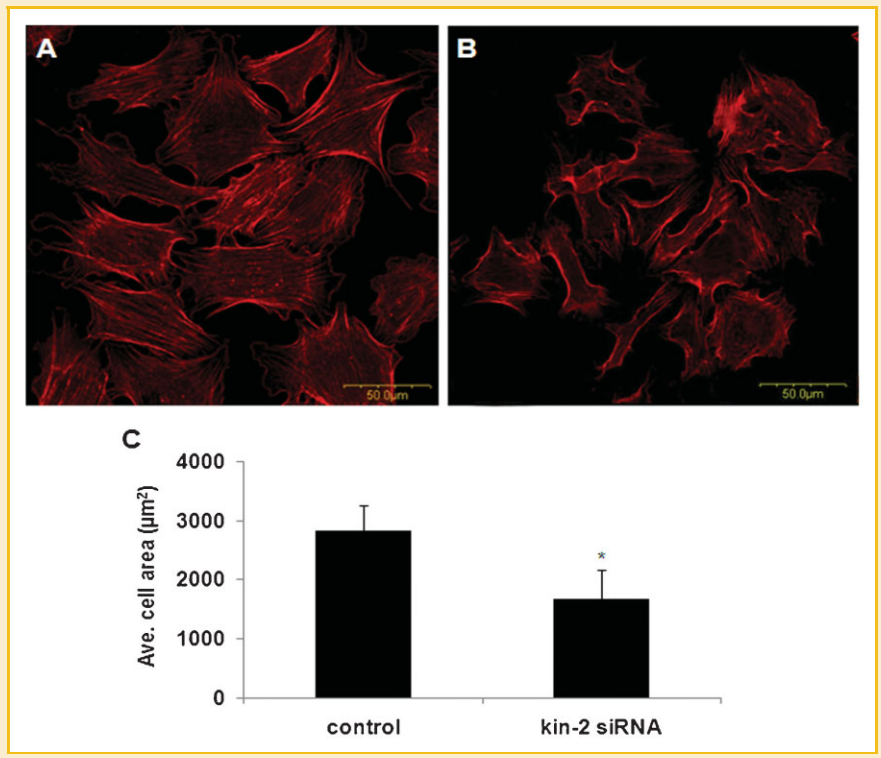

Fig. 4. Kindlin-2 depletion impairs osteoblast spreading. Scrambled siRNA (A) or kindlin-2 siRNA (B) transfected MC3T3-E1 cells were plated onto fibronectin-coated glass coverslips. Six hours after seeding, cells were fixed and stained with rhodamine–phalloidin. On each coverslip, 30 cells were selected and cell area was determined using Fluoview software. Values are mean ± SD of three different experiments. $^{*}P < 0.05$.

kindlin-2 influences AP-1 activity, we conducted EMSA. Result demonstrated that AP-1 DNA binding ability was decreased by kindlin-2 knockdown and Rac1 inhibition (Fig. 7B). Taken together, these results support a pivotal role of kindlin-2 in integrin-mediated osteoblast cell functions.

DISCUSSION

It is known that integrin activation by binding to ECM is critical for osteoblast function. However, the recruitment of adaptor proteins to the cytoplasmic tails of integrins, and their role in osteoblast physiology, is not well known. In this study, we demonstrate the role of kindlin-2 as an integrin adaptor protein in osteoblast adhesion, spreading, and proliferation. First of all, we confirmed that only kindlin-2 is highly expressed in osteoblast. Previous studies have shown that kindlin-2 is expressed ubiquitously and cooperates integrin activation with talins via binding the distal NxxY motif on integrin β cytoplasmic tails [Harburger et al., 2009; Malinin et al., 2010]. Kindlin-2 facilitates talin function although it is not sufficient to shift integrins to a high-affinity state [Montanez et al., 2008]. In this experiment, we tried to elucidate the effect of kindlin-2 on integrin activation in osteoblast using conformation sensitive antibodies. Similar with a previous report [Montanez et al., 2008], our result demonstrated that the antibody 9EG7 binding to MC3T3-E1 cells was reduced by kindlin-2 depletion. This means that osteoblasts require kindlin-2 to mediate β1 integrin activation by binding to fibronectin. Therefore, kindlin-2 is essential for β1 integrin-mediated osteoblast function.

Previous studies showed cell function is promoted by integrin activation via interaction between the integrin of an osteoblast with fibronectin. The anti-integrin α5β1 antibodies or RGD peptides

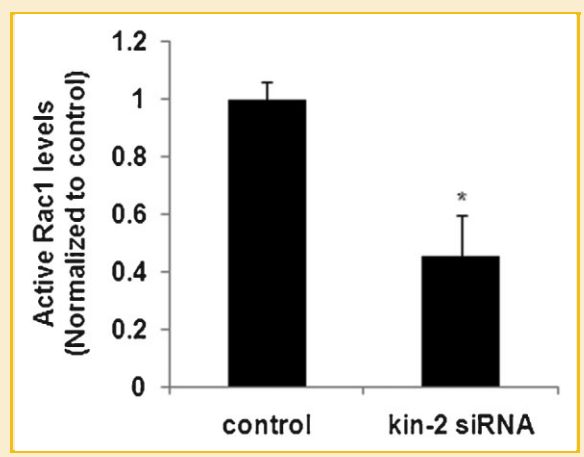

Fig. 5. Kindlin-2 is involved in Rac1 activation in MC3T3-E1 cells. MC3T3-E1 cells were transfected with scrambled siRNA or kindlin-2 siRNA and then reseeded on fibronectin-coated culture plates. One hour after seeding, active Rac1 levels were measured by ELISA. Data represent mean ± SD relative to control cell level ($n = 3$, $^{*}P < 0.01$).

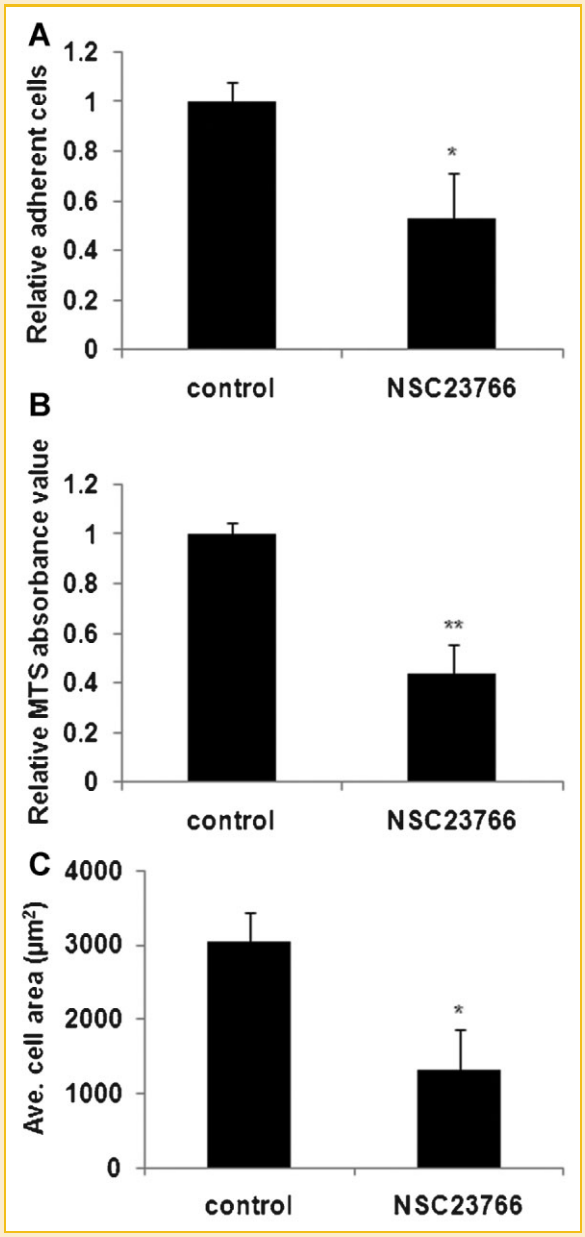

Fig. 6. Pharmacological inhibition of Rac1 activation reduces osteoblast cell adhesion, spreading, and proliferation. MC3T3-E1 cells were plated onto fibronectin-coated culture plate with or without NSC23766 (50 μM). A: One hour after seeding, adherent cells were stained and counted. The number of cells were averaged and represented relative to control. B: MTS assay were performed three days after seeding. Values are expressed relative to control absorbance level. C: Six hours after seeding, cells were fixed and stained with rhodamine–phalloidin. Cell area was measured with Fluoview software ($n = 60$). $^{*}P < 0.05$; $^{**}P < 0.01$.

inhibit c-fos expression, which affects osteoblast proliferation [Cowles et al., 2000]. Treatment of osteoblast with α5- or β1-integrin subunit antibody or αVβ3 integrin antibody prevents osteoblast mineralization [Schneider et al., 2001]. Our findings showed that deficiency of β1 integrin activation by kindlin-2 knockdown resulted in impaired MC3T3-E1 cell adhesion, spreading, and proliferation on fibronectin-coated plates. Scrambled siRNA treatment to MC3T3-E1 cells did not affect osteoblast adhesion, spreading, and proliferation significantly when compared with parental cells. This is in agreement with the result of the surface expression of activated β1 integrin.

Several reports have demonstrated that kindlin-2 knockdown impaired cell spreading, adhesion, and migration [Dowling et al., 2008b; Montanez et al., 2008; An et al., 2010; He et al., 2011]. These processes require the integrity and reorganization of the actin cytoskeleton, which is mainly governed by Rho GTPases. Integrins modulate the activity and localization of Rho GTPases and their effector proteins [Schwartz and Shattil, 2000]. By transforming from GDP bound state to GTP bound state, Rho GTPases are able to bind and activate a variety of effector proteins that modulate the organization of the actin cytoskeleton [Hall and Nobes, 2000]. Rac1 and Cdc42 stimulate the formation of small cell matrix adhesions while RhoA is implicated in the formation of actin stress fibers and the maturation of cell matrix adhesions [Danen et al., 2002]. Our findings showed that Rac1 transiently increases within 1 h after cells bind to fibronectin. In the absence of kindlin-2, the integrin failed to activate Rac1, which resulted into abnormal cell adhesion, spreading, and proliferation in osteoblast. This result is similar to that in a previous report showing that knockdown of kindlin-1 prevented Rac1 activation in keratinocytes [Has et al., 2009]. Kindlin-2 depletion also inhibited Rac1 activation, which affected cell adhesion, spreading, and migration in fibroblasts [He et al., 2011]. Moreover, kindlin-1 is associated with RhoA activation in keratinocytes [Has et al., 2009]. Fibroblasts on fibronectin-coated plates exhibit early activation of Cdc42 and Rac and delayed activation of Rho [Price et al., 1998; Huveneers and Danen, 2009]. In our unpublished observation, actin stress fiber density was significantly decreased in kindlin-2 knockdown MC3T3-E1 cells 24 h after seeding. Thus, although further studies are needed, it might be suggested that RhoA is activated after downregulation of Rac1, and RhoA activity is also modulated by kindlin-2.

Rac1 is known as a survival factor so pharmacological inhibition of Rac1 has been shown to block cell growth [Gao et al., 2004; Nassar et al., 2006; Liu et al., 2007]. In addition, Rac1 is involved in AP-1 transcriptional activation [Malliri et al., 1998; Sundberg et al., 2003; Engers et al., 2006]. AP-1 activation influences cytoskeletal rearrangement and cell motility as well as cell proliferation [Malliri et al., 1998; Shaulian and Karin, 2001]. Fibronectin-mediated integrin activation leads to AP-1 transcription activation, which regulates proliferation in osteoblasts [Cowles et al., 2000]. In this study, we demonstrated that knockdown of kindlin-2 or inhibition of Rac1 activity diminished phosphorylation of Akt and activity of AP-1. These results suggest that kindlin-2 influences Akt and AP-1 activity by regulation of Rac1.

In summary, we report that only kindlin-2 is highly expressed in MC3T3-E1 osteoblast and that kindlin-2 is required for integrin activation and integrin-mediated cell adhesion, spreading, and proliferation in these cells. In this process, kindlin-2 is involved in transient Rac1 activation, which influences Akt activation and AP-1 DNA binding activity. Our findings provide the first insights concerning the function of kindlin-2 in osteoblast and suggest that kindlin-2 could be critical mediator for bone formation.

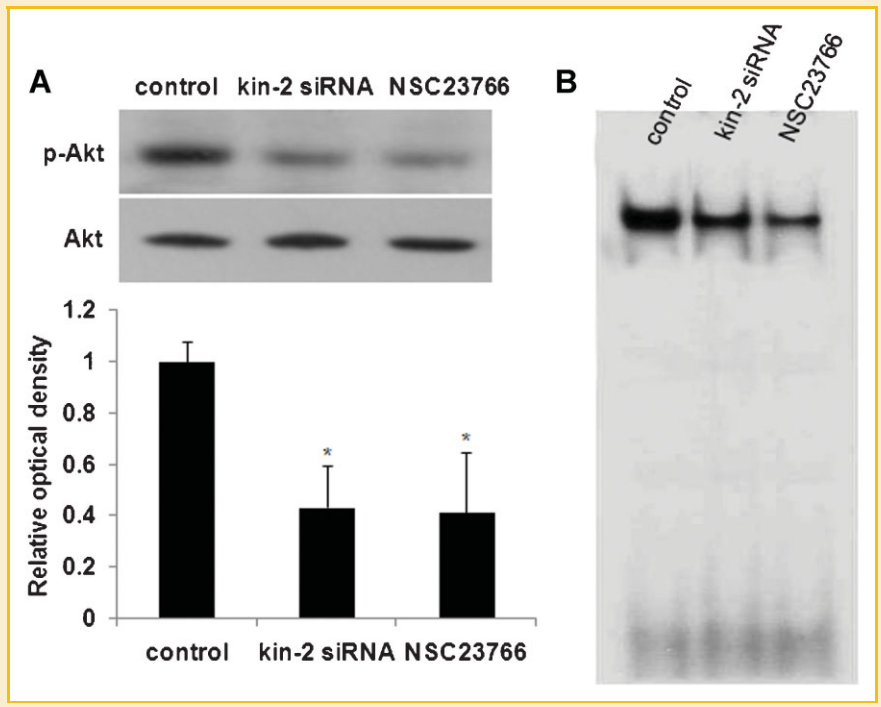

Fig. 7. Kindlin-2 knockdown inhibits Rac1-mediated Akt activation and AP-1 activity. A: One hour after seeding, cells were lysed and used for performing Western blot of phospho-Akt. Western blots were performed three times independently and optical densities were measured. Data are expressed relative to the level of Akt and are the means ± SD $^{*}P < 0.05$. B: siRNA or NSC23766 (50 μM) treated MC3T3-E1 cells were harvested 4 h after seeding. Nuclear extracts were mixed AP-1 consensus sequence containing ^{32}P-labeled oligonucleotide and then EMSA was performed. The autoradiograph is the representative of three separate experiments.

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