ROCK1 as a Potential Therapeutic Target in Osteosarcoma

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ABSTRACT: Osteosarcoma is the most common primary malignancy of bone. Patients with localized disease are routinely treated with surgery and chemotherapy. Unfortunately, many of these patients eventually relapse even after high-dose pre- and postoperative chemotherapy. Upon recurrence of the tumor locally or distantly, they have limited treatment options that are usually unsuccessful. Our prior studies screening lentiviral shRNA libraries, searching for kinases involved in osteosarcoma cell growth and proliferation have identified the Rho-associated coiled-coil containing protein kinase 1 (ROCK1) as a possible hit. We show in this study that ROCK1 is highly expressed in various tumor cell lines and tumor tissues from osteosarcoma patients. ROCK1 knockdown by synthetic siRNA decreases cell proliferation, viability and induces apoptosis in osteosarcoma cell lines KHOS and U-2OS. Finally, we established the relationship between expression levels of ROCK1 and clinical prognosis in osteosarcoma patients by using immunohistochemistry. There were significant differences in overall survival between cohorts of patients with ROCK1 levels categorized as high-staining, moderate-staining, and low-staining. High levels of ROCK1 were associated with poor outcomes in clinical osteosarcoma. These findings suggest that knockdown of ROCK1 inhibits proliferation and induces apoptosis in osteosarcoma cell lines. ROCK1 may be a promising therapeutic target for the treatment of osteosarcoma patients. © 2011 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

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Osteosarcoma is a debilitating, high-grade primary bone malignancy affecting rapidly growing bones in mostly children and young adolescents.1 Standard treatment for osteosarcoma includes surgical resection incorporated with chemotherapy.2 Several chemotherapeutic reagents are involved in chemotherapy protocols, such as doxorubicin (DOX), cisplatin (CDP), methotrexate (MTX), and ifosfamide (IFO).3 Patients who receive surgical treatment combined with chemotherapy have a higher long-term disease-free survival rate of >60% compared to 10–20% for those treated with surgery alone.4–7 However, once patients become resistant to these drugs, very few options are available for success. Furthermore, in spite of aggressive chemotherapy, one-third of patients presenting with localized osteosarcoma later experience local recurrence or distant metastasis. If those patients failed to achieve a second surgical remission, the average survival period will be reduced to <1 year.8,9 Therefore, it is imperative to exploit novel therapeutic regimens for the treatment of osteosarcoma to improve outcome in selected patients.

The human kinome contains more than 600 identified protein kinases, phosphorylation by which may regulate almost every property of a protein and is involved in all fundamental cellular processes.10,11 Mutations and deregulations of protein kinases play important roles in various human diseases.12 Kinases, such as polo-like kinase 1, minibrain-related kinase, and insulin-like growth factor 1 receptor, have been found to be overexpressed in sarcomas and correlated with clinical prognosis.10,11,13

In previous studies, we screened human protein kinases by using a lentiviral short hairpin RNA (shRNA) library in osteosarcoma cell lines.10,11 We observed that Rho-associated coiled-coil containing protein kinase 1 (ROCK1) is one of four kinases, when inhibited by shRNA result in decreased cell viability and increased apoptosis in osteosarcoma. Since ROCK1 is a unique kinase with no previously described role in osteosarcoma, it was selected for further study.

The studies of ROCK1 described in this article demonstrate that knockdown of ROCK1 can inhibit osteosarcoma cell proliferation and the expression levels of ROCK1 are much higher in osteosarcoma cell lines and tissues than in normal osteoblast cells. High levels of ROCK1 expression correlate with poor prognosis in osteosarcoma patients.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human osteosarcoma cell line U-2OS, uterine sarcoma cell line MES-SA, and ovarian cancer cell line SKOV-3 were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The osteosarcoma cell line KHOS (originally from ATCC) and the Ewing sarcoma cell line TC-71 were provided by Dr. Efthathios Ganos (Institute of Biological Research & Biotechnology, Athens, Greece) and Dr. Katia. Scotlandi (Institute Orthopedics Rizzoli, Italy). Chondrosarcoma cell line CS-1, synovial sarcoma cell line SS-1, and ovarian cancer cell line 3A were established from...
primary tumor tissues in our laboratory. Human osteoblast cells HOB-c and NHOst were purchased from PromoCell GmbH (Heidelberg, Germany) and Lonza Walkersville, Inc. (Walkersville, MD). Osteoblast cells were cultured in osteoblast growth medium with 50 ml SupplementMix (PromoCell GmbH). All other cell lines were cultured in RPMI 1640 from Invitrogen (Carlsbad, CA) supplemented with 10% FBS, 100-U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were maintained in a humidified atmosphere containing 5% CO₂–95% air at 37°C. Light microscopic images were documented using Zeiss microscope from Carl Zeiss, Inc. (Oberkochen, Germany) with an attached Nikon D 40 digital camera from Nikon Corp. (New York, NY).

**Lentiviral Human Kinase shRNA Library Screen**

Screening of lentiviral human kinase shRNA library was performed as previously described.10

**Synthetic ROCK1 siRNA and Transfection**

ROCK1 knockdown phenotype changes in osteosarcoma were further confirmed by transfection of synthetic human ROCK1 siRNA (ID: s12098), which was purchased from Ambion at Applied Biosystems (Foster City, CA). Nonspecific siRNA oligonucleotides were purchased from Dharmacon (Chicago, IL) and used as nonspecifics. 3 × 10⁵ cells per well were seeded in 96-well plates with complete growth medium without antibiotics and transfected with nonspecific siRNA or ROCK1 siRNA. Transfections were performed with Lipofectamine™ RNAiMAX (Invitrogen) following the manufacturer’s instructions. Each 96-well plate received 0.1 μg siRNA per well in a volume of 100 μl in triplicate. Medium was replaced with RPMI 1640 supplemented with 10% fetal bovine serum, 100-U/ml penicillin, and 100 μg/ml streptomycin 24 h after transfection.

**Proliferation Assay**

Briefly, KHOS and U-2OS cells were seeded in 96-well plates with complete growth medium without antibiotics and transfected with nonspecific siRNA or ROCK1 siRNA. Twenty microliters of CellTiter 96®AQueous One Solution Reagent was added to each well containing the samples in 100 μl of culture medium every 24 h. The absorbance was measured at a wavelength of 490 nm on a SPECTRAmax® Microplate Spectrophotometer from Molecular Devices (Sunnyvale, CA). Experiments were performed in triplicate. All data were processed with the use of GraphPad Prism 4 software from GraphPad Software, Inc. (San Diego, CA).

**Apoptosis Assay**

Quantification of apoptosis was evaluated by using the ApoONE Homogeneous Caspase-3/7 Assay kit from Promega according to manufacturer’s instructions (Madison, WI). U-2OS and KHOS cells were seeded per well in a white 96-well plate and transfected with ROCK1 siRNA. Seventy-two hours after transfection, the fluorescence of each well was measured at an emission wavelength of 521 nm and an excitation wavelength of 490 nm on a SPECTRAmax GeminiXS Microplate Spectrophotometer from Molecular Devices.

**Western Blotting**

Protein lysates were harvested from osteosarcoma cells with 1× RIPA Lysis Buffer from Upstate Biotechnology (Charlottesville, VA) and the protein concentrations were determined by Protein Assay Reagents (Bio-Rad, Hercules, CA) and spectrophotometer quantification from Beckman DU-640, Beckman Instruments, Inc. (Columbia, MD). Western blotting and densitometric analysis of Western blotting results were carried out as previously described.14

**Immunofluorescence**

For the purposes of immunofluorescence, KHOS and U-2OS were transfected with nonspecific siRNA or ROCK1 siRNA and continued to incubate for 72 h. Detection was performed by using secondary biotinylated antibodies. Osteosarcoma cells were then visualized on a Nikon Eclipse Ti-U fluorescence microscope (Nikon Corp.) equipped with a SPOT RT digital camera from Diagnostic Instruments, Inc. (Sterling Heights, MI).

**Osteosarcoma Tissue Microarray and Immunohistochemistry**

The osteosarcoma tissue microarray was purchased from Imgenex Corp. (San Diego, CA), which contained 58 samples from 58 osteosarcoma patients. There were three samples without tumor cells and information of another four samples was lost. Thirty-seven of 51 samples were from male patients and the other 14 were from female, with the mean age of 20.5 years old (range 5–61 years old) and the average follow-up of 63.0 months (range 3–180 months). Twenty-seven of 51 patients were dead during the period of follow-up.

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**Figure 1.** Representational 96-well plate demonstrates the screening results of the MISSION® LentiExpress™ Kinase shRNA library and lentiviral shRNA targets ROCK1 in KHOS cells. KHOS cells were seeded into the LentiExpress™ 96-well plate and then subjected to cell viability assay to check protein kinases required for proliferation. (A) Simple visual inspection was sufficient to determine C3–C5 as potential "hits." B11-C5: ROCK1 shRNA lentiviral transduction particles. (B) Results of lentiviral shRNA targeted ROCK1 in KHOS cells from the initial screening and the data were quantified by CellTiter 96®AQueous One Solution as described in the Materials and Methods Section. C, Empty vector control; M, media control; N, nontarget shRNA control.
Twenty-three patients died of cancer and two died of heart disease. The causes of death for another two patients were unknown. Immunohistochemical staining was performed with cell and tissue staining kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

Evaluation of Tissue Microarray Slide
Tissue microarray slides were evaluated under the microscope by two independent operators. The percentage of positive cytoplasm staining for ROCK1 was calculated by reviewing the entire slide. ROCK1 positive samples were defined as those showing cytoplasm staining pattern of tumor tissues. The staining intensity was graded into three groups: 0–1, absent or weak cell cytoplasm staining; 2+, moderate cell cytoplasm staining; 3+, strong cell cytoplasm staining.

Statistical Analysis
GraphPad PRISM® 4 software from GraphPad Software, Inc. was used to statistically analyze the data. Expression levels of ROCK1 and prognosis was analyzed by Kaplan–Meier survival analysis. A two-sided Student’s t-test was applied to compare the differences of ROCK1 intensity scores between survival and nonsurvival group. The effects of ROCK1 knockdown in osteosarcoma cells were also evaluated using the two-sided Student’s t-test. Errors were SD of averaged results and p values <0.05 were accepted as a significant difference between means.

RESULTS
ROCK1 Is a New Biomarker of Regulating Osteosarcoma Cell Survival
To investigate the potential functions of kinases in the proliferation and survival of osteosarcoma cells, a screen that targeted 673 human kinase genes was performed by using a lentiviral shRNA library in KHOS and U-2OS.10,11 We observed that ROCK1 knockdown decreases cell viability and induces cell death in osteosarcoma cell lines. Once KHOS cells were infected with lentiviral shRNA directing against five sites of ROCK1 mRNA, the cell growth in three (C3–C5) out of five wells (B11–C5) were impaired (Fig. 1A). As compared with control wells, the survival percentages of cells in C3–C5 were significantly decreased (Fig. 1B). The reason to choose ROCK1 for further studies was the lack of existing research on the biological characteristics of ROCK1 in osteosarcoma.

ROCK1 Is Highly Expressed in Various Tumor Cell Lines and Osteosarcoma Tissues
ROCK1 protein was detected by Western blotting in osteosarcoma cell lines (U-2OS and KHOS), uterine sarcoma cell line (MES-SA), chondrosarcoma cell line (CS-1), synovial sarcoma cell line (SS-1), Ewing sarcoma cell line (TC-71), and ovarian cancer (SKOV-3, 3A, and 2008). (B) High levels of ROCK1 expression in osteosarcoma tissues. OST 1–6 stand for different tumor samples from six patients with osteosarcoma. (C) Western blotting results of A and B were analyzed by densitometry, which was performed in Adobe Photoshop and normalized to β-actin expression. Quantitative results of ROCK1 protein for each cell line were presented as relative expression, which was obtained by assigning the expression value of SS-1 to 1.

Figure 2. ROCK1 is highly expressed in various tumor cell lines and osteosarcoma tissues. (A) Compared with normal osteoblasts (HOB-c and NHOST), ROCK1 were found abundantly expressed in various tumor cell lines, including osteosarcoma (U-2OS and KHOS), uterine sarcoma (MES-SA), chondrosarcoma (CS-1) synovial sarcoma (SS-1), Ewing sarcoma (TC-71), and ovarian cancer (SKOV-3, 3A, and 2008). (B) High levels of ROCK1 expression in osteosarcoma tissues. OST 1–6 stand for different tumor samples from six patients with osteosarcoma. (C) Western blotting results of A and B were analyzed by densitometry, which was performed in Adobe Photoshop and normalized to β-actin expression. Quantitative results of ROCK1 protein for each cell line were presented as relative expression, which was obtained by assigning the expression value of SS-1 to 1.
sarcoma cell line (TC-71), ovarian cancer cell lines (SKOV-3, 3A, and 2008), normal osteoblasts (HOB-c and NHOST), and osteosarcoma tissues. The amounts of protein for ROCK1 were significantly greater in various tumor cell lines than in normal osteoblasts (Fig. 2A). To preclude the possibility that ROCK1 expression is an artifact induced by in vitro culture propagation, we also examined six freshly isolated primary osteosarcoma tissues. In five out of six osteosarcoma patient samples, ROCK1 was observed to be highly expressed (Fig. 2B). Quantification of Western blot results showed that the expression levels of ROCK1 in various tumor cell lines and osteosarcoma tissues were at least 10 times more than in osteoblasts (Fig. 2C).

ROCK1 Knockdown by Synthetic siRNA Decreases Cell Proliferation, Viability, and Induces Apoptosis in KHOS and U-2OS Cells

This siRNA oligonucleotide has been validated in other cell lines to inhibit ROCK1 expression. Cell proliferation, apoptosis, and growth characteristics were measured to test the impacts of knocking down the expression of ROCK1 by synthetic siRNA on inhibition of cell proliferation and induction of apoptosis. We observed that incubation with ROCK1 siRNA extensively inhibited cell proliferation, decreased cell viability, and induced cell apoptosis in both osteosarcoma cell lines KHOS and U-2OS (Fig. 3A,B,D).

Western blotting showed that transfection with ROCK1 siRNA efficiently depleted ROCK1 protein expression in both osteosarcoma cell lines (Fig. 3C). Immunofluorescence analysis further confirmed that, compared with cells in control groups that were transfected with nonspecific siRNA, KHOS, and U-2OS cells expressed much lower levels of ROCK1 after being transfected with ROCK1 siRNA (Fig. 4A,B). We used a cell growth assay to determine that ROCK1 knockdown by siRNA had no effects on the cell growth of normal human osteoblasts but significantly decreased KHOS and U-2OS cell survival rate (Fig. 5A,B).

ROCK1 Overexpression Is Correlated With Poor Clinical Outcomes in Osteosarcoma Patients

The relationship between ROCK1 expression levels and clinical outcomes of osteosarcoma patients were investigated by immunohistochemistry using osteosarcoma tissue microarray. According to the grading system of staining intensity described in the Materials and Methods Section, all the tissue blots from 51 patients were evaluated under the microscope. 25 (49.0%), 21 (41.2%), and 5 (9.80%) patients were classified as low, moderate, and high staining, respectively (Fig. 6C and S-Table 1). Average follow-up periods for those three groups were 81.2, 41.7, and 11.5 months (S-Table 1). Comparison of Kaplan–Meier survival
proportion in patients with different expression levels of ROCK1 protein linked high ROCK1 expression with shortened overall survival ($p = 0.0104$, Fig. 6A). Patients who died after 60-month follow-up expressed higher levels of ROCK1 than those who survived; mean staining intensity 1.807 versus 1.233 ($p = 0.0069$, Fig. 6B). There were no statistical correlations between ROCK1 expression and other clinical characteristics, such as age, gender, tumor site, and tumor type ($p > 0.05$, S-Table 1).

**DISCUSSION**

ROCK1-mediated oncogenic characteristics in various tumors have been demonstrated by a dominant active mutant of ROCK1, which enhanced the invasion of cancer cells and direct phosphorylation of myosin light chain, which leads to increased cell migration and invasion, activation of AKT/mTOR/eIF4E signaling, and upregulated M-CSF cytokine production to facilitate osteolytic metastasis. ROCK1 is one of four kinase hits in our previous kinase lentiviral shRNA

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**Figure 4.** Immunofluorescence analysis shows that transfection with ROCK1 siRNA decreases ROCK1 expression in KHOS and U-2OS cells. (A,B) KHOS and U-2OS were transfected with nonspecific siRNA or ROCK1 siRNA. Cells were then visualized on a Nikon Eclipse Ti-U fluorescence microscope equipped with a SPOT RT digital camera. The expression levels of ROCK1 were significantly decreased in cells transfected with ROCK1 siRNA.
screening in osteosarcoma. In this study, we observed that knockdown of ROCK1 decreased cell proliferation and induced cell death in both osteosarcoma cell lines. In addition, ROCK1 overexpression is associated with poor prognosis in osteosarcoma patients, indicating that ROCK1 might be a prognosis marker and therapeutic target for the treatment of osteosarcoma.

Several recent studies report the cell survival effects mediated by ROCK1 in epithelial, endothelial, and cancer cells. We also found that transfection with either ROCK1 shRNA or siRNA significantly inhibited ROCK1 expression and decreased cell viability, proliferation, and eventually induced cell death. These phenomena were not only found in sensitive but also in multidrug resistant osteosarcoma cell lines (S-Fig. 1). However, normal osteoblasts are much less sensitive to ROCK1 knockdown via RNA interference targeting, implying that ROCK1 is required for maintaining cell proliferation of malignant osteoblasts. The effects of ROCK1 knockdown on cell growth, proliferation, and apoptosis in osteosarcoma may be mediated through the regulation of cytoskeletal actin–myosin contraction. Recently, it has been reported that ROCK1 is a target of miRNA146a (mir-146a), one of eight miRNAs significantly down-regulated in prostate cancer. Transfection of mir-146a results in decreased expression of ROCK1 in prostate cancer cells, significantly reduced cell proliferation, invasion, and metastasis to human bone marrow. This finding demonstrates that ROCK1 plays an important role in cancers.

Figure 5. Knockdown ROCK1 decreases cell viability and induces cell death in multiple osteosarcoma cell lines. (A) Proliferation of each cell line was determined by MTT. KHOS, U-2OS, and human osteoblast cell HOB-c were transfected with nonspecific siRNA or ROCK1 siRNA. The absorbance values were normalized by assigning the survival percentage of control cells (nontransfected) to 100% and the relative survival percentages of nonspecific siRNA and ROCK1 siRNA transfected cells were then quantified. Knockdown of ROCK1 by siRNA decreased proliferation of osteosarcoma cells but not normal osteoblast cells. Transfection of nonspecific siRNA had no effects on proliferation of osteosarcoma cell lines. (B) Phase-contrast photomicrographs of cells were taken after transfection of nonspecific siRNA and ROCK1 siRNA. ROCK1 knockdown induces significantly cell death in osteosarcoma cell lines KHOS, U-2OS but not in human osteoblast cell HOB-c.
As upregulation of ROCK1 has been observed in various types of cancer cells,²⁰,²¹ our study showed that, as compared with normal osteoblasts, expression of ROCK1 at the protein level was much higher in various tumor cell lines and in osteosarcoma tissues. Furthermore, the results are also supported by several reports regarding the correlation between the expression of ROCK1 and clinical outcome in human bladder and breast cancers.²²,²³ By using immunohistochemistry and tissue microarray analysis, we established the relationship between ROCK1 expression level and prognosis in osteosarcoma patients. In our study, for those patients who survived after 60-month follow-up, the levels of ROCK1 staining were significantly lower than those who died within 60-month follow-up, suggesting that high expression levels of ROCK1 were correlated with shorter overall survival of patients.

In conclusion, this study demonstrated that knockdown ROCK1 inhibited proliferation and induced apoptosis in osteosarcoma cells. These results, together with the correlation between ROCK1 and survival of osteosarcoma patients, point towards the importance of targeting ROCK 1 as a novel approach for the treatment of osteosarcoma.

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