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# Rho-Associated Protein Kinase (ROCK) Promotes Proliferation and Migration of PC-3 and DU145 Prostate Cancer Cells by Targeting LIM Kinase 1 (LIMK1) and Matrix Metalloproteinase-2 (MMP-2)

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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
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**Background:** In the pathogenesis and progression of prostate cancer, cell proliferation and cell migration results in tumor invasion and metastasis that is associated with patient morbidity and mortality. Rho-associated protein kinase (ROCK) has previously been shown to be upregulated in prostate cancer, but its biological role remains poorly understood. This study aimed to investigate the role of ROCK in the proliferation and migration of PC-3 and DU145 prostate cancer cells and to identify the possible targets involved by knockdown of ROCK1 and ROCK2 RNA expression.


**Material/Methods:** An RNA interference (RNAi) assay was performed to silence the expression of ROCK1 and ROCK2 in the PC-3 and DU145 human prostate cancer cell lines. Cells were also treated with a specific ROCK inhibitor, Y27632. A cell counting kit-8 (CCK-8) assay was used to determine the proliferation rate of prostate cancer cells, and cell migration and invasion assays were performed. Western blot and polymerase chain reaction were used to measure protein and RNA expression levels.

**Results:** In PC-3 and DU145 prostate cancer cells, knockdown of ROCK1 and ROCK2 reduced cell migration and invasion. ROCK1 and ROCK2 regulated cell proliferation in PC-3 and DU145 prostate cancer cells. Protein levels of phosphorylated LIM kinase 1 (p-LIMK1) and matrix metalloproteinase-2 (MMP-2) were reduced in ROCK1 and ROCK2 siRNA transfected cells.

**Conclusions:** In PC-3 and DU145 human prostate cancer cells, ROCK promoted cell proliferation and migration by targeting LIMK1 and MMP-2.

**MeSH Keywords:** **Matrix Metalloproteinase 2 • Prostatic Neoplasms • rho-Associated Kinases**

**Full-text PDF:** <https://www.medscimonit.com/abstract/index/idArt/912098>

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## Background

Worldwide, prostate cancer is the second most commonly diagnosed cancer in men, resulting in high morbidity and mortality [1,2]. Although there have been recent advances in the diagnosis and management of prostate cancer, in some patients, the prognosis remains poor [3]. Therefore, more effective and targeted approaches for the diagnosis and treatment of prostate cancer are still needed.

Rho-associated protein kinase (ROCK) belongs to the highly conserved serine/threonine kinase family. In humans, a total of two homologs, ROCK1 and ROCK2, have been identified on the 18q11 and 2p24 chromosome regions, respectively. ROCK is involved in several biological processes, including cell proliferation, cell migration, and cell motility [4–6]. The findings from several previously published studies have shown that inhibition of the expression of the ROCK gene inhibits invasion of breast cancer cells *in vitro* [7,8]. However, the role of ROCK in the behavior of prostate cancer cells remains unknown.

LIM kinase 1 (LIMK1) is expressed in the cell cytoplasm and cell nucleus and is upregulated in several human cancers, including prostate and breast cancer [9]. The major functions of LIMK1 in cell migration and cell proliferation are mainly dependent on phosphorylation. Previous have shown that ROCK might be a regulator for the phosphorylation of LIMK1 (p-LIMK1) in some human cancers [4,10,11]. Therefore, studies to evaluate the correlation between ROCK and p-LIMK1 expression and their effects in prostate cancer cells would appear to be an important area of study.

Matrix metalloproteinase-2 (MMP-2) belongs to MMP protein family, which has a key role in the regulation of cell proliferation, migration, and differentiation [12–14]. MMPs have been considered as an attractive therapeutic target for the cancer treatment [14]. MMP-2 is a physiological regulator for vascular remodeling, and the regulation of MMP-2 can affect angiogenesis and the progression, invasion, and metastasis of cancer cells [13,15]. A previously published study has shown that MMP-2 is regulated by ROCK [16]. However, the association between MMP-2 and ROCK in prostate cancer remains to be investigated.

Therefore, this study aimed to investigate the role of ROCK in the proliferation and migration of PC-3 and DU145 prostate cancer cells and to identify the possible targets involved by knockdown of ROCK1 and ROCK2 expression.

## Material and Methods

### Cell culture

Human prostate adenocarcinoma cell lines, DU145 and PC-3 were obtained from the Cell Bank of the Shanghai Biology Institute, Shanghai, China. All culture media were mixed with 10% fetal bovine serum (FBS) (Gibco, Thermofisher Scientific, Waltham, MA, USA), 2 mM L-glutamine and 1% penicillin and streptomycin (Solarbio, Beijing, China). DU145 and PC-3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis MO, USA). Cell lines were maintained at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

### Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from prostate cancer cell samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using a cDNA synthesis kit (Fermentas, Burlington, ON, Canada), according to the manufacturer's instructions. A quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed with SYBR<sup>®</sup> Green real-time PCR Master Mix (Thermofisher Scientific, Waltham, MA, USA) on an ABI 7300 ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following cycling parameters, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 45s, and normalized to GAPDH. The relative gene relative expression was calculated by the 2<sup>-ΔΔCt</sup> method. All data represented the average of three replicates. The primer sequences used were as follows:

The homo sapiens rho-associated coiled-coil containing protein kinase 1 (ROCK1), mRNA NM\_005406.2:

Forward: 5' CCCAAGGAGATGTGTATAG 3';

Reverse: 5' GGAAAGTGGTAGAGTGTAG 3';

Positive: 4480–4657 C;

Amplified product size: 178 bps;

Product GC: 35%.

The homo sapiens rho-associated coiled-coil containing protein kinase 2 (ROCK2), transcript variant 2, mRNA NM\_001321643.1:

Forward: 5' TGATTGGTGGTCTGTAGG 3';

Reverse; 5' GCTGCCGTTTCTTATAG 3';

Positive: 818–1099 C;

Amplified product size: 282 bps;

Product GC: 40%.

The homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 2, mRNA NM\_001256799.2:

Forward: 5' AATCCCATCACCATCTTC 3';

Reverse: 5' AGGCTGTTGCATACTTC 3';

Positive: 436–653 C;

Amplified product size: 218 bps;

Product GC: 56%.

**Table 1.** Homo sapiens ROCK1 and ROCK2 RNAi targeting locus information.

RNAi targeting locus			Sequence
Name	Locus position (bp)		
ROCK1	RNAi1-1	1410–1428	CCTGGTGGAGATCTTGTA
	RNAi1-2	1998–2016	GCACCAGTTGTACCCGATT
	RNAi1-3	3476–3494	GCTTGAAGCTGAGCAATAT
ROCK2	RNAi2-1	2273–2291	GCTGGAGACTGCCAAGTTA
	RNAi2-2	3224–3242	GCAACTGGCTCGTTCAATT
	RNAi2-3	3452–3470	GCAACTGTCAAGATTGAAA

### RNA interference (RNAi)

Two short interfering RNA (siRNA) targeting positions of human ROCK1 (NM\_005406.2) and ROCK2 (NM\_001321643.1) were synthesized. A non-specific scramble siRNA sequence was used as a negative control (NC). All of the siRNAs were transiently transfected into DU145 or PC-3 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Assays were performed 48 h after transfection. Sequence information for the ROCK siRNAs is shown in Table 1.

### Western blot

Whole protein lysates were extracted from indicated cells using RIPA lysis buffer (JRDUN, Shanghai, China) with an EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration was estimated using an enhanced BCA protein assay kit (ThermoFisher Scientific, Waltham, MA, USA). Equal amounts of total protein (25 µg) were fractionated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane overnight (Millipore, Burlington, MA, USA). After blocking with 5% dried skimmed milk powder for 1 h at room temperature, the membranes were incubated at 4°C overnight with the primary antibodies, followed by secondary anti-mouse IgG (1: 1000) (Beyotime, Shanghai, China) for 1 h at 37°C. The enhanced chemiluminescence (ECL) system (Tanon, Shanghai, China) was used to detect the protein expression levels. The names, source, and dilutions of the primary antibodies used are shown in Table 2.

### Cell proliferation assay

Cell counting kit-8 (CCK-8) assay kits (Sigma-Aldrich, St. Louis MO, USA) were used to examine the cell proliferation rate, according to manufacturer's protocol. Briefly, cells transfected with siROCK were seeded in 96-well plates and cultured for 0, 24, 48, and 72h. CCK-8 solution (10 µl in 100 µl DMEM medium) was added to each well and incubated for 1 h.

**Table 2.** The primary antibodies used in Western blot.

Antibody name	Source	Dilution
ROCK1	Abcam, Cambridge, UK	1: 2000
ROCK2	Abcam, Cambridge, UK	1: 1000
LIMK1	Abcam, Cambridge, UK	1: 1000
p-LIMK1	Abcam, Cambridge, UK	1: 500
MMP-2	Abcam, Cambridge, UK	1: 1000
GAPDH	CST, Danvers, MA, USA	1: 2000

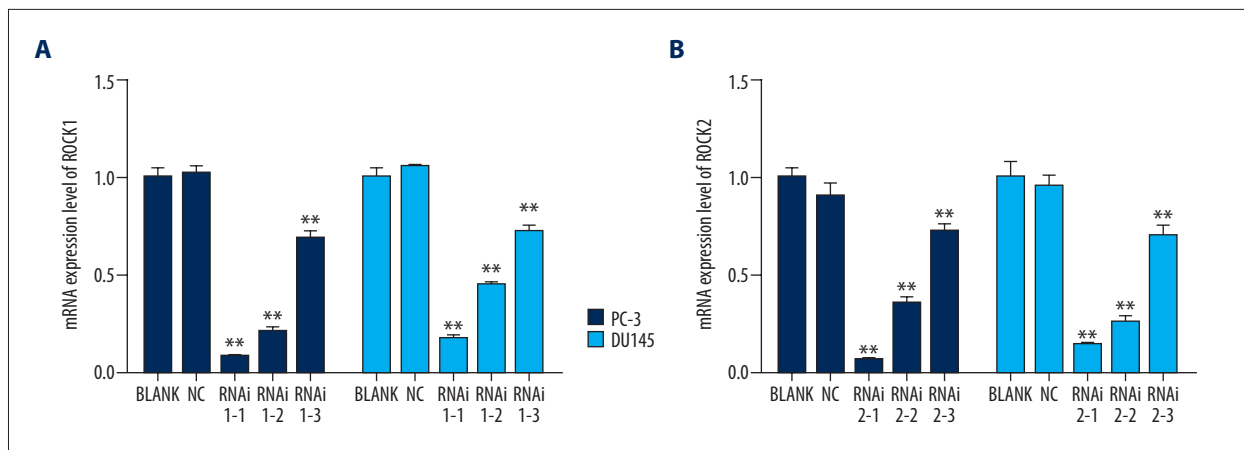
Optical density (OD) values at a wavelength of 450 nm were measured using a microplate reader (Pulangxin, Beijing, China). All experiments were performed in triplicate at each time point.

### Cell migration assay

Each ROCK siRNA cell group was serum starved for 24 h followed by seeding in the upper chamber, while the medium was supplemented with 30% FBS (Gibco, ThermoFisher Scientific, Waltham, MA, USA), which was placed in the lower chamber. After 24 h of incubation, cells in the upper side of the filters were removed and the remaining cells were fixed in 4% formaldehyde and stained with 0.01% crystal violet (Solarbio, Beijing, China). Cells in the lower chamber were stained with crystal violet and counted under the light microscope, at a magnification of ×200. All the procedures were performed using Boyden chambers (Costar Technologies, Inc., China), according to the method previously described [17]. Cell migration was evaluated using Boyden chambers that contained a polycarbonate filter coated with Matrigel on the upper surface. Cell migration was viewed by light microscopy following cell staining with crystal violet.

### Murine *in vivo* model of pulmonary metastasis

The animal study was performed following the Guidelines for the Animal Care and Use, Shanghai Eastern Hospital, China.



**Figure 1.** The mRNA expression of ROCK1 and ROCK2 were inhibited by ROCK short-interfering RNAs (siRNAs) in prostate cancer cells, PC-3 and DU145. **(A)** ROCK1 siRNA mRNA expression levels in prostate cancer cells. **(B)** ROCK2 siRNA mRNA expression levels in prostate cancer cells. \*\* P<0.01.

PC-3 cells transfected with the ROCK siRNAs, or treated with the ROCK inhibitor, Y27632 ( $2 \times 10^6$ ) were injected into the lateral tail vein of nude mice, at 4-weeks-of-age (n=16). Mouse survival was monitored daily. After four weeks, four mice in each group were sacrificed and portions of the lungs were processed for histology and light microscopy using hematoxylin and eosin (H&E) staining. The lung tissue sections were reviewed at a magnification of  $\times 200$ .

### Statistical analysis

GraphPad Prism software, version 6.0 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. Data were shown as the mean  $\pm$  standard deviation (SD). One-way analysis of variance (ANOVA) was used for multiple comparisons. A p-value <0.05 indicated statistical significance.

## Results

### Knockdown of the rho-associated protein kinase (ROCK) gene in PC-3 and DU145 prostate cancer cells

Three siRNAs targeting human ROCK1 or ROCK2 (siROCK1: RNAi1-1, RNAi1-2, RNAi1-3; siROCK2: RNAi2-1, RNAi2-2, RNAi2-3) and a non-specific scramble siRNA (siNC) were synthesized and transfected into PC-3 and DU145 cell lines. Untreated cells served as a control.

As shown in Figures 1 and 2, both the mRNA and protein of ROCK were efficiently suppressed by ROCK siRNAs. Also, cells transfected with RNAi1-1 and RNAi2-1 had a greater effect than that of other siROCKs in two cell lines. Therefore, RNAi1-1 and RNAi2-1 transfected cells were chosen for further studies.

All transfected ROCK siRNAs showed highly specific characteristic for silencing ROCK (Figure 3).

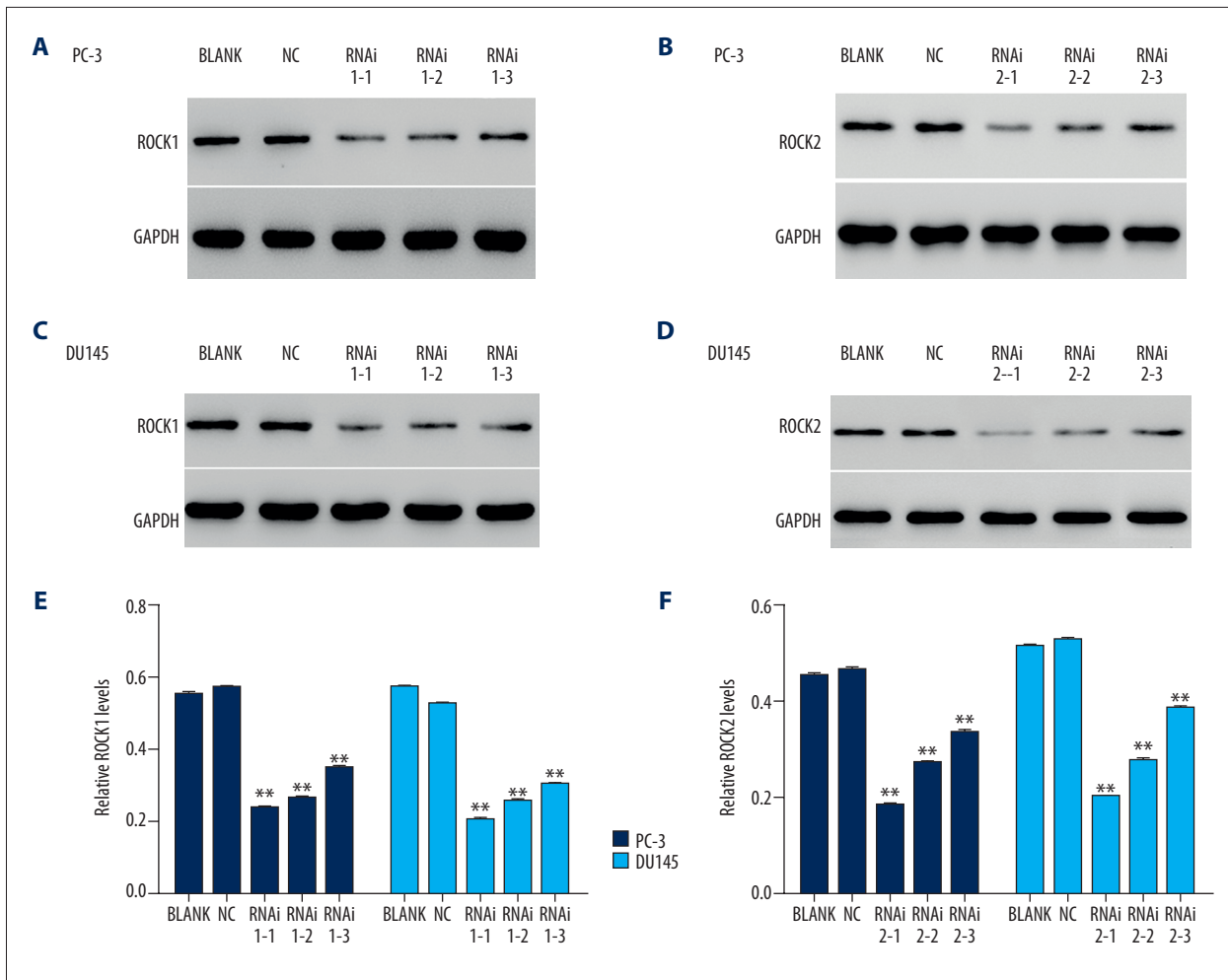
### Knockdown of ROCK inhibited the proliferation of PC-3 and DU145 prostate cancer cells

The cell counting kit-8 (CCK-8) assay was performed to examine the proliferation rate of siNC, siROCK1, siROCK2, and siROCK1 and siROCK2 transfected cells. Cells were cultured with a specific ROCK inhibitor, Y27632 ( $10 \mu\text{M}$ ), and served as positive control (PC).

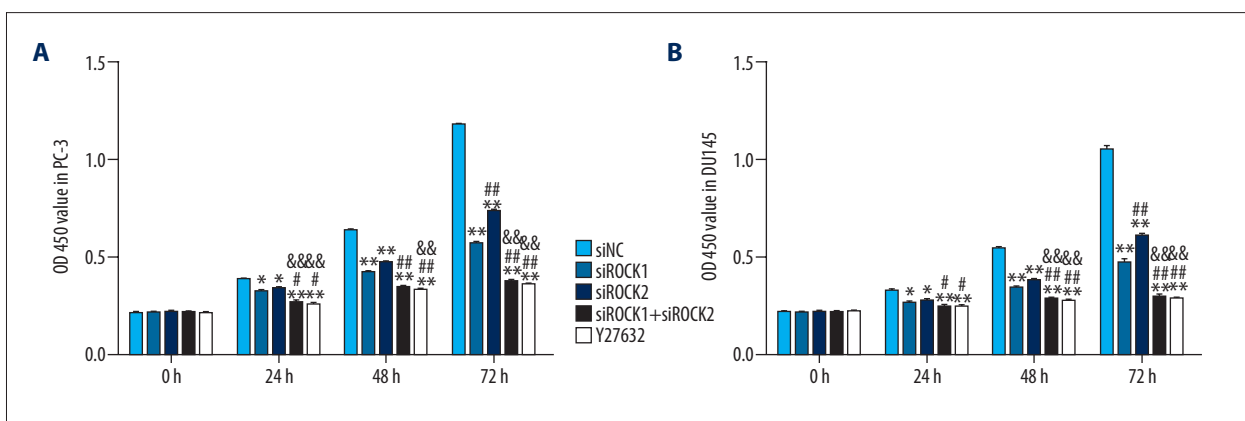
As shown in Figure 4, the cell proliferation rate of the siROCK transfected cells was significantly decreased when compared with siNC transfected cells. The combined siROCK1 and siROCK2 transfected cells had a lower proliferation rates when compared with the siROCK1 or siROCK2 transfected cells alone, which showed no significant difference with the control cells. These results supported an anti-proliferation effect of ROCK siRNAs on prostate cancer cells *in vitro*.

### Silencing of ROCK suppressed cell migration and invasion of PC-3 and DU145 prostate cancer cells

Figure 5 shows that the rate of cell migration and invasion was significantly reduced in siROCK transfected cells compared with the siNC transfected cells. Also, the migration and invasion rate of the combined siROCK1 and siROCK2 transfected cells was reduced when compared with the siROCK1 or siROCK2 transfected cells alone. These results supported the role of ROCK in promoting the invasive properties of prostate cancer cells *in vitro*.

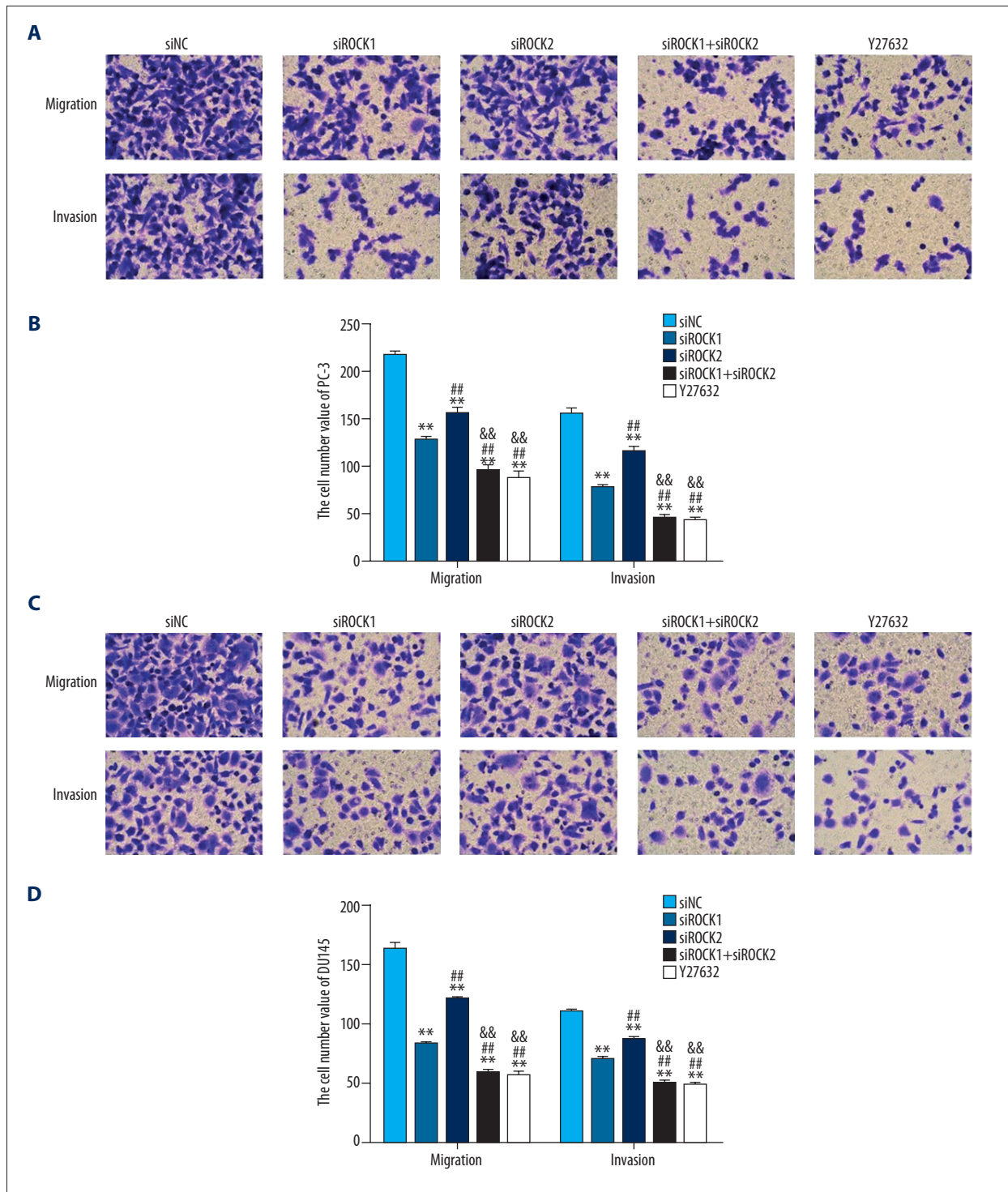


**Figure 2.** The protein levels of ROCK1 and ROCK2 were inhibited by ROCK short-interfering RNAs (siRNAs) in prostate cancer cells, PC-3 and DU145. (A, C, E) The protein levels of ROCK1 were inhibited in siROCK1 (RNAi1-1, RNAi1-2, and RNAi1-3) transfected cells. (B, D, F) The protein levels of ROCK2 were inhibited in siROCK2 (RNAi2-1, RNAi2-2, and RNAi2-3) transfected cells. \*\* P<0.01 vs. normal control (NC).



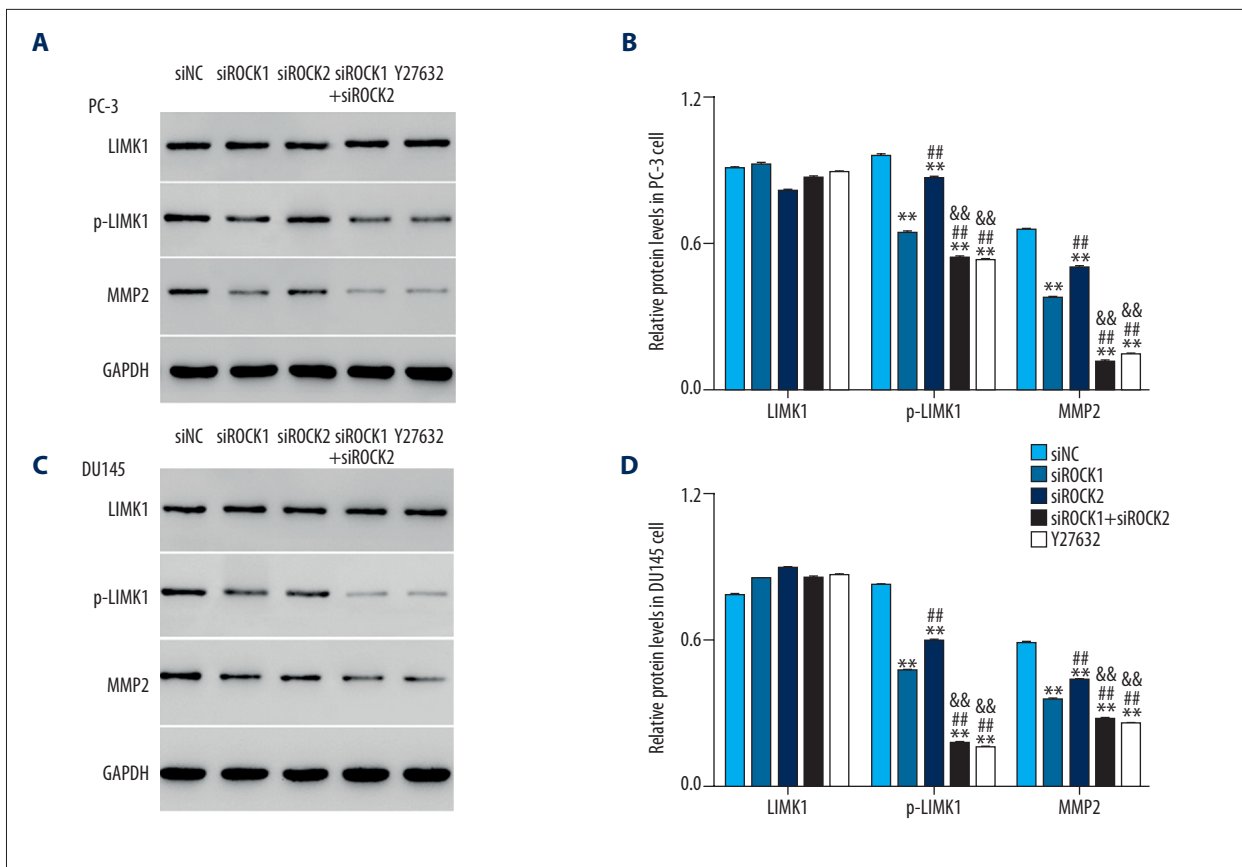
**Figure 3.** ROCK siRNAs showed highly specific characteristic for silencing ROCKs in prostate cancer cells, PC-3 and DU145. (A, B) The mRNA and protein level of ROCK1 in different siROCK2 transfected cells. (C, D) The mRNA and protein level of ROCK2 in different siROCK1 transfected cells.





**Figure 4. (A–D)** Knockdown of ROCK gene expression reduced the proliferation rate of prostate cancer cells, PC-3 and DU145.

(A) The proliferation rate was detected at 12, 24, 48, and 72 hours after the PC-3 cells were transfected with siNC, siROCK1, siROCK2, siROCK1 and iROCK2, and treated with the ROCK inhibitor, Y27632, respectively. (B) The proliferation rate was detected 12, 24, 48, and 72 hours after the DU145 cells were transfected with siNC, siROCK1, siROCK2, and siROCK2, and treated with the ROCK inhibitor, Y27632, respectively. \*  $P < 0.05$  vs. siNC, \*\*  $P < 0.01$  vs. siNC, #  $P < 0.05$  vs. siROCK1, ##  $P < 0.01$  vs. the siROCK1 group, &&  $P < 0.01$  vs. the siROCK2 group.



**Figure 5.** Silencing the ROCK gene inhibited the motility and migration of prostate cancer cells, PC-3 and DU145. **(A, B)** Cell migration and invasion rate was analyzed in PC-3 cells that were transfected with siNC, siROCK1, siROCK2, siROCK1 and siROCK2 and treated with the ROCK inhibitor, Y27632, respectively. **(C, D)** Cell migration and invasion rate was analyzed in DU145 cells that were transfected with siNC, siROCK1, siROCK2, siROCK1 and siROCK2, and treated with the ROCK inhibitor, Y27632, respectively. Magnification  $\times 200$ . \*  $P < 0.05$  vs. siNC, \*\*  $P < 0.01$  vs. siNC, #  $P < 0.05$  vs. siROCK1, ##  $P < 0.01$  vs. siROCK1, &&  $P < 0.01$  vs. siROCK2.

### Knockdown of ROCK reduced the expression of p-LIMK1 and MMP-2

Western blot was used to examine the protein level of LIMK1, p-LIMK1, and MMP-2 in siROCK transfected cells. As shown in Figure 6, the protein level of LIMK1 showed no significant difference among all transfected cells. Protein expression levels of p-LIMK1 and MMP-2 were significantly reduced in ROCK siRNAs transfected cells, particularly in the combined siROCK1 and siROCK2 transfected cells. These results supported the role of ROCK in targeting p-LIMK1 and MMP-2 in prostate cancer cells *in vitro*.

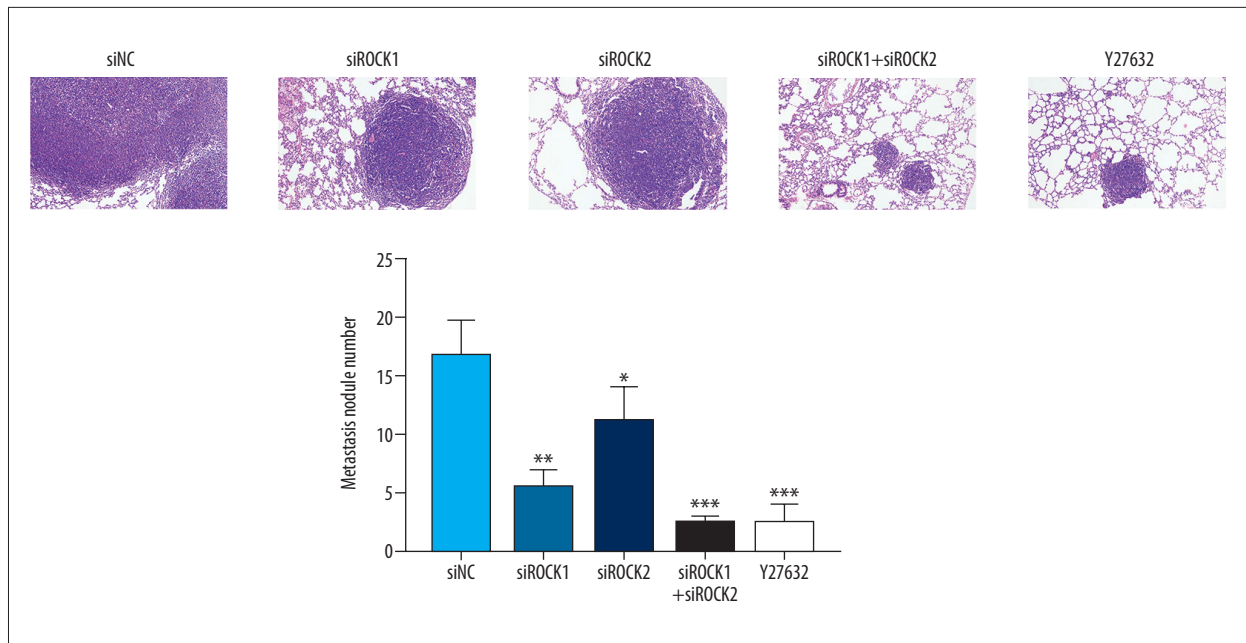
### Knockdown of ROCK reduced pulmonary metastasis *in vivo* in nude mice

To investigate whether ROCK siRNAs influenced metastasis of prostate cancer cells *in vivo*, PC-3 and DU145 prostate cancer cells transfected with siROCK were injected into the lateral tail

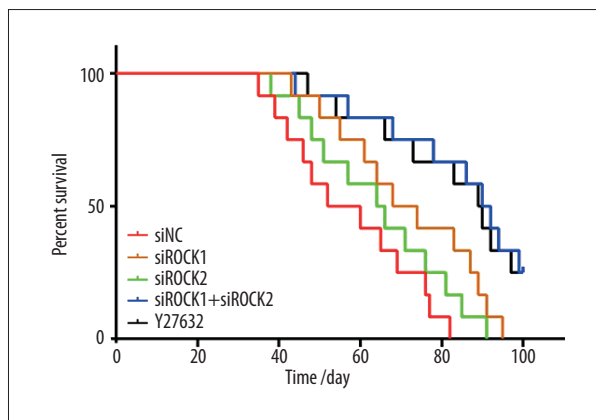
vein of nude mice. As shown in Figure 7, knockdown of ROCK reduced the number of metastatic lung tumor nodules in mice, particularly in the combined siROCK1 and siROCK2 group. ROCK siRNA transfected cells were associated with a reduced number of lung metastases in mice, and inoculation with the combined siROCK1 and siROCK2 transfected cells was associated with increased survival (Figure 8).

### Discussion

The rho-associated protein kinase (ROCK) signaling pathway is required for the regulation of cell morphology, adhesion, and motility [7]. The findings from a previously published study showed that ROCK had a key role in the regulation of the actin cytoskeleton, and the expression level of ROCK was associated with cancer progression [18]. In the present study, the role of ROCK in human PC-3 and DU145 prostate cancer cells was studied *in vitro*. The RNA interference (RNAi) assay was



**Figure 6.** The protein levels of phospho-LIM kinase 1 (p-LIMK1) and matrix metalloproteinase-2 (MMP-2) were decreased in ROCK siRNA PC-3 and DU145 transfected cells. (A, B) The protein levels of LIMK1, p-LIMK1, and MMP-2 detected in PC-3 cells that were transfected with siNC, siROCK1, siROCK2, siROCK1 and iROCK2, and cells treated with the ROCK inhibitor, Y27632, respectively. (C, D) The protein levels of LIMK1, p-LIMK1, and MMP-2 detected in DU145 cells transfected with siNC, siROCK1, siROCK2, siROCK1 and siROCK2, and cells treated with the ROCK inhibitor, Y27632, respectively. \* P<0.05 vs. siNC, \*\* P<0.01 vs. siNC, # P<0.05 vs. siROCK1, ## P<0.01 vs. siROCK1, && P<0.01 vs. siROCK2.



**Figure 7.** Knockdown of ROCK gene expression inhibited pulmonary metastasis in an *in vivo* mouse model. PC-3 cells ( $2 \times 10^6$ ) transfected with the ROCK siRNA were injected into the lateral tail vein of 4-week-old nude mice. At day 35, the mice were sacrificed and portions of the lungs were processed for histology. Hematoxylin and eosin (H&E) staining. Magnification,  $\times 200$ .

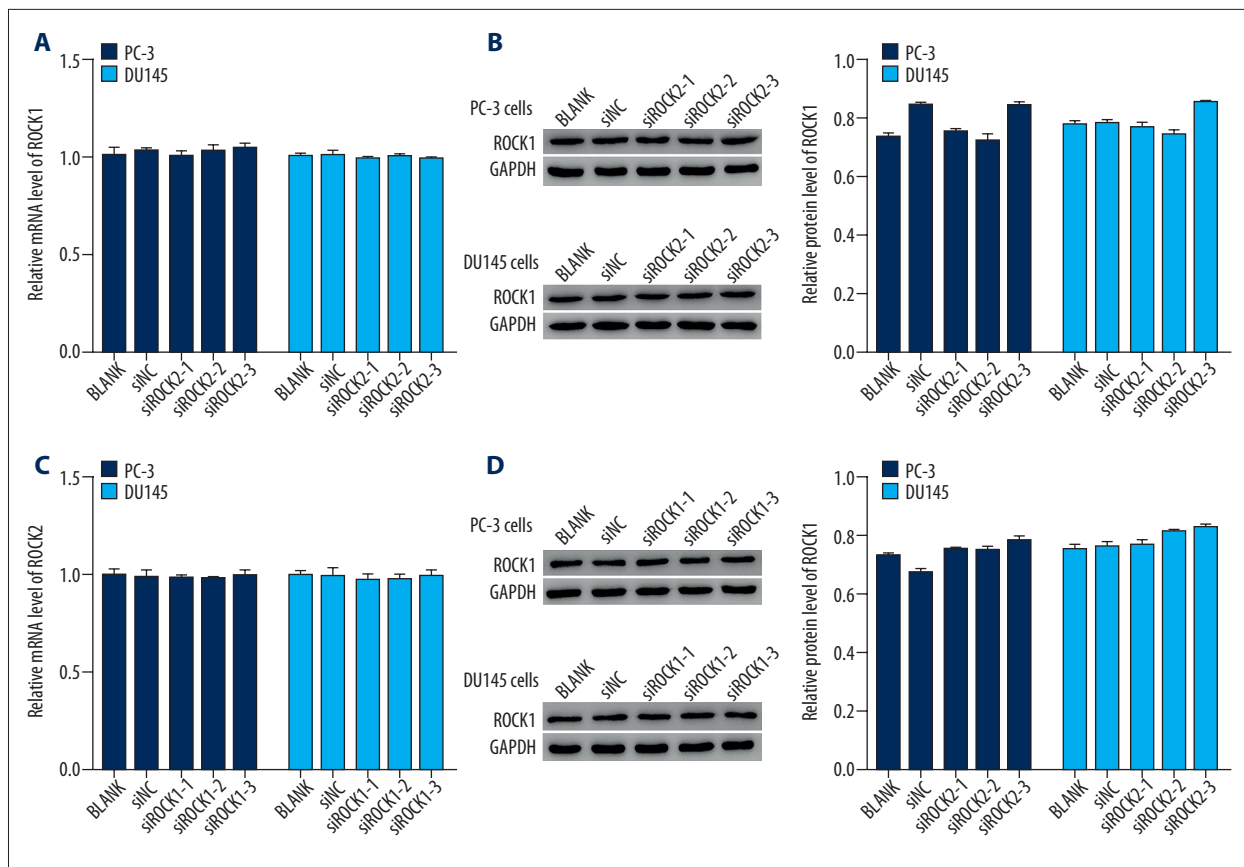
performed to silence the expression of ROCK1 and ROCK2 and a specific ROCK inhibitor, Y27632, was used to inhibit the expression of ROCK. The concordant findings from these two approaches that inhibited ROCK expression supported the validity of the results.

Previous studies have shown that ROCK expression was associated with cell proliferation [19,20]. The findings of the present study supported the findings of these previous studies and also showed that the combined effects of the short-interfering RNAs, siROCK1 and siROCK2 had a greater effect on prostate cancer cells when compared with the findings from siROCK1 or siROCK2 treatment alone. Therefore, the results of the present study supported the effect of ROCK in the regulation of cell proliferation on prostate cancer cells.

Worldwide, cancer is a major public health concern with increased morbidity and mortality from all cancers, including prostate cancer [18,21]. The properties of tumor cell invasion and metastasis are important factors that determine cancer progression [22,23]. Therefore, the inhibition of tumor cell invasion and metastasis might be a promising approach for cancer treatment. Previous reports have shown that ROCK was associated with the progression of cancer and its expression was increased in several types of cancer [7,24]. In the present study, knockdown of expression of ROCK reduced the migration and invasion of human prostate cancer cells, PC-3 and DU145 *in vitro*, and promoted metastasis in a mouse model *in vivo*.

It has previously been reported that the suppression of the expression of LIM kinase 1 (LIMK1) inhibited the invasion and proliferation of gastric cancer cells [25]. Phosphorylate LIMK1





**Figure 8.** ROCK siRNAs showed highly specific characteristic for silencing ROCKs in Pca cells. **A** and **B** stands for the mRNA and protein level of ROCK1 in different siROCK2 transfected cells. **C** and **D** stands for the mRNA and protein level of ROCK2 in different siROCK1 transfected cells.

(p-LIMK1) is the biologically active form of LIMK1 [25,26]. ROCK is reported to be a mediator in the phosphorylation of LIMK1 [27]. The findings of the present study showed that the protein level of p-LIMK1 was significantly inhibited in siROCK transfected PC-3 and DU145 human prostate cancer cells. Therefore, ROCK might regulate the metastasis of prostate cancer cells through the regulation of p-LIMK1.

Matrix metalloproteinase-2 (MMP-2) is a member of the MMPs gelatinase family with increased expression associated with tumor cell invasion due to its effects on degrading the extracellular matrix (ECM), accelerating tumor growth, and promoting tumor angiogenesis [28]. In the present study, the expression levels of MMP-2 were significantly decreased in prostate cancer cells transfected with ROCK siRNAs, especially for combined siROCK1 and siROCK2 transfected cells. These findings were supported by the findings from previous

studies [29,30]. Therefore, MMP-2 might be the novel component in the ROCK/LIMK1 signaling pathway in prostate cancer cells and requires further study to investigate the roles of MMP-2 and LIMK1 in prostate cancer *in vivo*.

## Conclusions

The findings of this preliminary study showed that in PC-3 and DU145 human prostate cancer cells, the expression of rho-associated protein kinase (ROCK) promoted cell proliferation and migration by targeting phosphorylated LIM kinase 1 (p-LIMK1) and matrix metalloproteinase-2 (MMP-2). Further *in vitro* and *in vivo* studies are warranted to determine the roles of ROCK, LIMK1, and MMP-2 in human prostate cancer to identify potential diagnostic and therapeutic targets.

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