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ARHGAP11A Promotes Lung Adenocarcinoma Proliferation and Invasion through ROCK-LIMK-Cofilin Pathway

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Abstract

Background: We aimed to investigate the clinical significance and biological roles of *ARHGAP11A* in lung adenocarcinoma (LUAD).

Methods: We firstly analyzed the expression level of *ARHGAP11A* in LUAD based on TCGA. Next, quantitative reverse transcription PCR (qRT-PCR) was performed to examine the expression level of *ARHGAP11A* in LUAD cell lines (A549, and Calu-3). After *ARHGAP11A* knockdown in A549 cells, cell counting kit-8 (CCK-8) and trans well assays were used to measure the proliferation and migration ability of A549 cells with or without *ARHGAP11A* silence. Western blotting was utilized to identify the underlying pathway through which *ARHGAP11A* silencing mediates biological roles in LUAD.

Results: *ARHGAP11A* was significantly over-expressed in LUAD tissues. Patients in the high *ARHGAP11A* expression subgroup experienced worse overall survival compared to the low expression subgroup. Univariate and multivariate analysis exhibited that *ARHGAP11A* was an independent prognostic predictor for LUAD. After *ARHGAP11A* silencing in A549 cells, cell proliferation rate, invasive and migratory capacity were observed to be inhibited. Remarkably, western blot results exhibited that reduction of *ARHGAP11A* remarkably inhibited the expression of ROCK, pLIMK2, and pCofilin in A549 cells.

Conclusion: Increased *ARHGAP11A* expression could regulate LUAD cell proliferation and metastasis, and identified it as a poor prognostic biomarker in LUAD.

Keywords: *ARHGAP11A*; Lung adenocarcinoma; Transwell assay; CCK-8; ROCK-LIMK-Cofilin

Introduction

Lung carcinoma is one of the leading cause of cancer-related mortality in the world, and it is classified into small cell and non-small cell lung cancer [1]. Among the non-small cell lung cancers, adenocarcinoma accounts for about 50% [2]. Although the third-generation anti-neoplastic agents as well as targeted treatments are introduced, the overall 5-year survival rate is still not improved [3]. Thus, an in-depth understanding of molecular mechanisms underlying lung adenocarcinoma (LUAD) will facilitate to development novel therapeutic strategy.

The Ras homology (Rho) subfamily of small GTPases is a family of small GTP-binding proteins which are involved in cell migration as well as proliferation. Emerging evidence has demonstrated that these GTP-binding proteins are a new class of biomarkers for cancer prognosis [4-6]. Rho GTPase activating protein 11A (*ARHGAP11A*) is one member of the Rho GTPase activation protein (RhoGAP) family, which promotes the hydrolysis of GTP and inactivates Rho GTPases.

Lawson et al. [7] also have indicated that *ARHGAP11A* serves as oncogene in basal-like breast cancers. Kagawa et al. [8] have demonstrated that the significant up-regulation of *ARHGAP11A* is observed in colon cancers, which is linked to clinical invasion status. However, Xu and the colleagues have indicated that *ARHGAP11A* accumulates in the nuclei of colorectal cancer cells, interacts with p^{53} and then attenuates cell proliferation and induces apoptosis [9]. The function of *ARHGAP11A* in different cancers, however, is inconsistent. Significantly, till now, there is no published study on the role of *ARHGAP11A* in LUAD tissues and its relationship with the clinicopathological factors still remain poor.

Consequently, in the present study, to investigate the *ARHGAP11A* expression levels in human LUAD and further to reveal the potential correlation between the *ARHGAP11A* expression and clinic pathological characteristics, data analysis was implemented based on the data recruited from The Cancer Genome Atlas (TCGA) database. To confirm our results, we used A549 cells to carry out CCK 8 experiment, and transwell assay to explore the effect of *ARHGAP11A* in LUAD *in vitro*. Western blotting analysis was performed to measure the expression level of ROCK-LIMK-Cofilin pathway to illuminate whether the altered expression of *ARHGAP11A* was associated with prognosis. Our findings suggest that the expression of *ARHGAP11A* might act as a potential candidate target for treating LUAD.

Materials and Methods

Tumor gene expression and prognosis based on TCGA database using TCGA database, expression level of *ARHGAP11A* was compared in normal lung tissues (n=59) and LUAD samples (n=535) relying on LIMMA package [10]. The data were downloaded on 5th Dec 2017. In LUAD samples, differences between the gene expression and overall outcomes were compared in the highest vs. lowest *ARHGAP11A* expression.

Cell lines and culture

The human LUAD cell lines A549, and Calu-3, and a normal lung human bronchial epithelial cell line BEAS-2B were obtained from the Shanghai Chinese Academy of Sciences Cell Bank (China). All cells were cultured in RPMI-1640 medium (Life Technologies, Carlsbad,

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CA, US) supplemented with penicillin G (100 U/ml), streptomycin (100 mg/ml) and 10% fetal bovine serum (FBS, Life Technologies) and were grown under a humidified atmosphere of 5% CO_2 at 37°C.

Small interfering RNA (siRNA) transfection

SiRNA against *ARHGAP11A* (si-*ARHGAP11A*) and negative control (si-NC) without definite target were produced and purchased from GenePharma (Shanghai, China). Cells were seeded on six-well plates at a density of 3×10^5/well overnight, and then transfected with siRNA and si-NC at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen, USA). After 24 h of transfection, qRT PCR and western blotting were used to determine the interfering efficiency, and the siRNA with transfection efficacy of more than 80% was selected for subsequent experiments. The details of siRNA sequences were as follows: 5'UAUGGACACAUUCCAAGCU 3'(sense) for siRNA *ARHGAP11A* 1# and 5' GGGCUUUUUCGGAAAUCAG 3' (sense) for siRNA *ARHGAP11A* 2#.

RNA extraction and qRT-PCR

Overall RNA of cell line A549 was extracted using Trizol reagent (Invitrogen, Carlsbad, USA). The primers sequences of *ARHGAP11A* were: F: 5'- GGAGGTAGAGGCAGGCTGAT-3', R: 5' CAACTCTTCCAGTCCCGGTG-3'. The primers sequences of GAPDH were as follows: F: 5' GGAGCGAGATCCCTCCAAAAT-3', R:5'-GGCTGTTGTCATACTTCTCATGG-3'. The following criteria of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 30 min were utilized to determine the expression levels of *ARHGAP11A* and GAPDH. ABI 7500 real-time PCR system was used to collect data. The relative expression of *ARHGAP11A* was computed and normalized relying on the 2−ΔΔCt method relative to GAPDH.

CCK-8 analysis

For CCK-8 analysis, cells (2×10^3) were seeded into each well of 96 well plates, and cell viability was recorded at 24 h, 48 h, 72 h, and 96 h. The details of staining process were implemented according to the previous study [11].

Scratch wound-healing assay and transwell assay

We conducted a scratch wound-ealing assay and transwell assay to further detect the effect of *ARHGAP11A* on the cell migration of A549 cells.Approximately 3×10^5 cells were plated into 6-well dishes and an incision was done in the central area of the confluent culture to create an artificial wound. Photographs of the wound area were recorded by microscope 24 h after injury. To assess the effect of *ARHGAP11A* cell migration and invasion, cells in 100 ul RPMI1640 medium without serum were plated in the upper chamber of the transwells with and without matrigel. The lower chamber was filled with 500 ul of RPMI1640 with 10% FBS as chemo attractants for cells. Then, cells were fixed using 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet solution. After that, migrated and invaded cells were calculated and imaged using microscope.

Western blotting analysis

Cell protein lysates were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to PVDF membrane, following by incubating with specific antibodies (anti-ROCK1, anti-pLIMK2, anti-LIMK2, anti-pCofilin, anti-Cofilin, and anti-β actin). After incubated with horseradish peroxidase-conjugated IgG secondary antibodies, enhanced chemiluminescence was used to visualize the immunoreactive bands. The band density was quantified by densitometry (Quantity One software; Bio-Rad).

Statistical analysis

SPSS 22.0 was used to implement all statistical analyses of our study. All values are expressed as the mean ± standard deviation (SD). A chi-square test was carried out to determine significant differences between two groups. One-way ANOVA was utilized to calculate statistical differences between multiple groups. Survival curve was plotted by means of Kaplan-Meier method and compared using log-rank test. A probability level of 0.05 was regarded significant.

Results

ARHGAP11A is significantly up-regulated in LUAD tissues and cell lines

To determine the role of *ARHGAP11A* in LUAD progression, we analyzed the *ARHGAP11A* expression in LUAD dataset from TCGA and the results showed that *ARHGAP11A* expression was over-expressed in LUAD tissues (n=535) compared that in the normal (n=59) (Figure 1A, p=1.79E 33). Next, we measured the *ARHGAP11A* expression levels in two human LUAD cell lines (A549, and Calu-3) and a human bronchial epithelial cell line (BEAS-2B). Up-regulation of *ARHGAP11A* was observed in both LUAD cell lines tested compared with the human bronchial epithelial cell line (Figure 2). All data demonstrated that *ARHGAP11A* was frequently over-expressed in LUAD. From the Figure 2, we found that the level of *ARHGAP11A* in

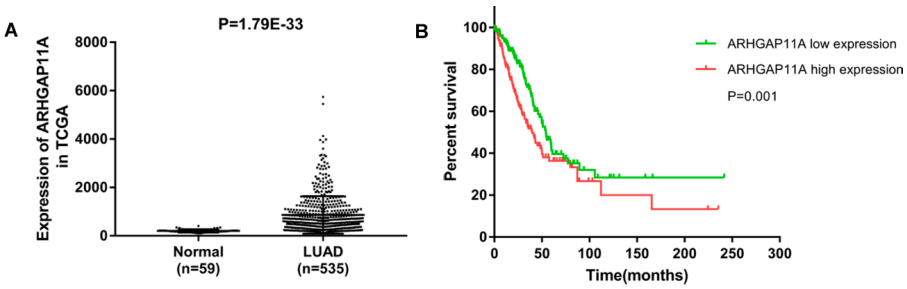

Figure 1: Expression level of ARHGAP11A in lung adenocarcinoma (LUAD) was significantly over-expressed and associated with the prognosis of patients. **(A)** ARHGAP11A expression level in the patients with LUAD (n=535) and normal samples (n=59) recruited from TCGA database (p=1.79E-33). **(B)** The Kaplan–Meier plot suggested that ARHGAP11A high expression (red line) had a worse overall survival, relative to the low expression group (green line) (p=0.001).

A549 was higher than that in Calu-3 cells, thus, in subsequent study, A549 cell line was used.

ARHGAP11A is an independent predictor for survival in LUAD patients

The correlation between the *ARHGAP11A* expression status and the clinic pathologic characteristics of 535 LUAD tissues was further evaluated, and the results are listed in Table 1. A positive correlation was found between the *ARHGAP11A* over-expression and gender (p=0.000) and pathologic-node (p=0.040) (Table 1). Moreover, Kaplan-Meier survival analysis suggested that the survival rate was significantly lower in patients with *ARHGAP11A* high-expression than in the patients with lower *ARHGAP11A* expression (p=0.001, Figure 1B). Further, univariate and multivariate analysis exhibited that the up-regulation of *ARHGAP11A* (p=0.004), pathologic tumor (p=0.008), and pathologic node (p=0.001) were independent prognostic predictors for LUAD patients (Table 2).

Silence of *ARHGAP11A* abrogates cell growth, migration and invasion of A549 cells *in vitro*

The effects of *ARHGAP11A* on cell proliferation, invasion and migration were monitored by loss of function studies in LUAD cells. We developed A549 cell line with silenced *ARHGAP11A* using two siRNA sequence (Figure 3). From Figure 3, we found that both of siRNA *ARHGAP11A* 1# and siRNA *ARHGAP11A* 2# decreased mRNA and protein level of *ARHGAP11A* (p<0.001 both in RT-PCR and western blotting). Significantly, siRNA *ARHGAP11A* 1# the owned the higher transfection efficiency (more than 80%). Thus, we used the siRNA 1# for the subsequent transfection.

To gain an insight into the role of *ARHGAP11A* in cell proliferation, CCK-8 assay was implemented. We found that silence of *ARHGAP11A* decreased viability in A549 cells (Figure 4, p<0.001 for 72 h and 96 h).

Moreover, the wound healing assay and transwell system were used to measuer the migration ability of the A549 cells. After 24 h of seeding, the monitored wound and transwell system exhibited that the silence of *ARHGAP11A* remarkably restrained cell migration (Figure 5A, 5B, and 5C, p<0.001). Taken together, *ARHGAP11A* served as an oncogene in human LUAD, and *ARHGAP11A* up regulation significantly increased the tumorigenicity.

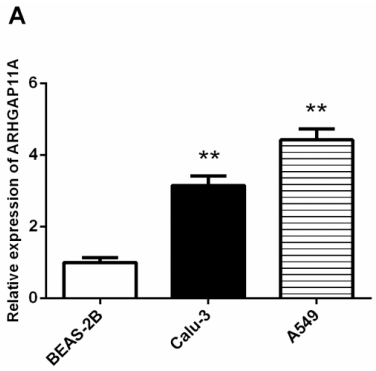

Figure 2: qRT-PCR indicating the relative expression levels of ARHGAP11A in LUAD cell lines (A549, and Calu-3) compared to normal lung human bronchial epithelial cell line BEAS-2B. ARHGAP11A expression level in LUAD cell lines was normalized to GAPDH. Data are exhibited as the means ± S.D. ¨ p<0.001.

Characteristics	Expression of ARHGAP11A		p-value
	Low	High	
Age			
<60	62	74	0.237
≥60	183	172	
Gender			
female	156	111	**0.000***
male	89	135	
Pathologic-Stage			
I+II	195	184	0.099
III+IV	44	60	
Pathologic-T			
T1+T2	213	212	0.711
T3+T4	30	33	
Pathologic-N			
N0	167	151	**0.040***
N1	69	93	
Pathologic-M			
M0	162	161	0.112
M1	8	16	
Note: T: Tumor; N: Node; M: Metastasis.			

Table 1: Correlation between ARHGAP11A expression and clinicopathological parameters of lung adenocarcinoma (LUAD).

Variables	Univariate analysis			Multivariate analysis		
	p-value	HR	95%CI	p-value	HR	95% CI
ARHGAP11A expression (high/low)	**0.001***	1.664	1.235-2.243	**0.004***	1.673	1.175-2.383
Clinical-tage (I+II/III+IV)	**0.000***	2.470	1.808-3.375	0.177	1.407	0.857-2.310
Pathologic-T (T1+T2/T3+T4)	**0.000***	2.278	1.561-3.324	**0.008***	1.900	1.186-3.045
Pathologic-M (M0/M1)	**0.044***	1.769	1.016-3.082	0.943	1.025	0.527-1.992
Pathologic-N (N0/N1+N2+N3)	**0.000***	2.537	1.883-3.420	**0.001***	1.956	1.310-2.921
Age (<60/≥ 60)	0.734	1.059	0.762-1.471	--	--	--
Gender (Female/Male)	0.422	1.128	0.841-1.513	--	--	--

Table 2: Univariate and multivariate analysis of clinical prognostic factors of LUAD.

Down-regulation of *ARHGAP11A* inhibits the expression of ROCK-LIMK-Cofilin pathway

ROCK/LIMK/Cofilin pathway plays important roles in actin fiber assembly and cell–cell adhesion [12]. So, we measured the effects of *ARHGAP11A* on the expression level of ROCK/LIMK/Cofilin pathway (ROCK1, pLIMK2, LIMK2, pCofilin, and Cofilin) using western blotting. Figure 6 showed that silence of *ARHGAP11A* significantly inhibited the expression of ROCK, pLIMK2, and pCofilin in A549 cells (p<0.001).

Discussion

As the most common type of non-small cell lung cancers, LUAD is attracting considerable attention especially since its drug resistance makes treatment difficult. A critical problem in the LUAD progression is the metastasis property of cancer cells, which influences the prognosis of the patients. Consequently, in-depth understanding the mechanisms of LUAD progression and metastasis is crucially important for improving the therapy and overall prognosis of LUAD.

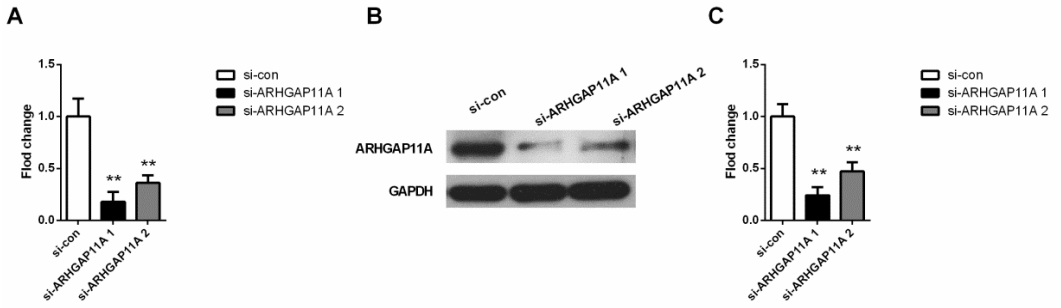

Figure 3: ARHGAP11A expression, measured by qRT-PCR and western blotting, following the treatment of A549 cells with siRNA ARHGAP11A 1# and siRNA ARHGAP11A 2#.

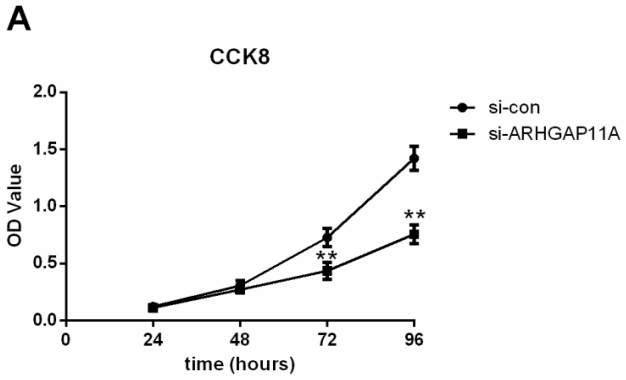

Figure 4: CCK-8 assay was conducted to analyze the proliferation rate of si- ARHGAP11A-transfected A549 cells. Asterisk stands for statistically significant differences relative to the negative control (**p<0.001).

Significantly, *ARHGAP11A* activation has been implicated to play key roles in cancer development. Nevertheless, the potential roles and the related molecular mechanisms of *ARHGAP11A* in LUAD are yet to be clarified.

Our study is the first direct investigation of the relationship between *ARHGAP11A* and LUAD. In the current report, we observed that the average expression level of *ARHGAP11A* in LUAD tissues was significantly higher than those in adjacent normal lung tissues. Furthermore, the TCGA dataset exhibited that *ARHGAP11A* could act as a prognostic factor for LUAD. Additionally, *ARHGAP11A* knockdown significantly inhibited LUAD cell viability, migration, and invasion *in vitro*. In a nutshell, the observations of our study demonstrate that *ARHGAP11A* may serve as an oncogene and may exert important functions in LUAD development and progression.

Cancer progression is a dynamic process involving adhesion, migration, invasion, as well as morphogenesis, which is controlled by the dynamics of actin cytoskeleton [13]. Cofilin is a kind of actin-binding proteins that play a crucial role in mediating actin filament dynamics and reorganization via catalyzing polymerization or depolymerization of actin filaments in migrating cells [14,15]. Cofilin phosphorylation is needed for actin polymerization [15]. Cofilin is inactivated by phosphorylation by LIMK1, and the p-cofilin can in turn reflect the activity of LIMK1. LIMKs is phosphorylated and activated by Rho-associated kinase (ROCK), which facilitates actin polymerization through inactivating cofilin by phosphorylation at Ser3 to prevent cleavage of actin fibers [16]. In cancer cells, phosphorylation of cofilin is controlled by RhoC/ROCK/LIMK pathway [17]. Abundant published evidence has demonstrated that Rho-related pathways, including that mediated by ROCK, participated in polarity control, invasion, infiltration, migration and metastasis [18-20]. Accordingly, our study highlighted the importance of the ROCK/LIMK/cofilin pathway in controlling actin dynamics, which participates in invasion and metastasis of cancer cells. Significantly, silence of *ARHGAP11A* remarkably inhibited the expression of ROCK, pLIMK2, and pCofilin in A549 cells in the current study. Accordingly, our results suggest that the *ARHGAP11A* low-expression might be closely related with progression and prognosis of LUAD, which is mediated through the inactivation of ROCK/LIMK/cofilin signaling pathway.

Conclusion

Taken together, our study identifies *ARHGAP11A* as a novel potential oncogene in LUAD that acts by influencing the changes in action cutoskeleton. *ARHGAP11A* may serve as a prognostic factor in LUAD. The silencing of *ARHGAP11A* in LUAD cell line A549 dramatically inhibited tumor growth and migration *in vitro*, raising the possibility that *ARHGAP11A* could be a promising new therapeutic target for LUAD. However, our findings were not verified using animal mode or patient tissues. Thus, in the late work, further studies are required to advance our understanding of the involvement of *ARHGAP11A* in LUAD progression using animal model or patient samples, because this gene is a potential candidate for LUAD diagnosis and treatment.

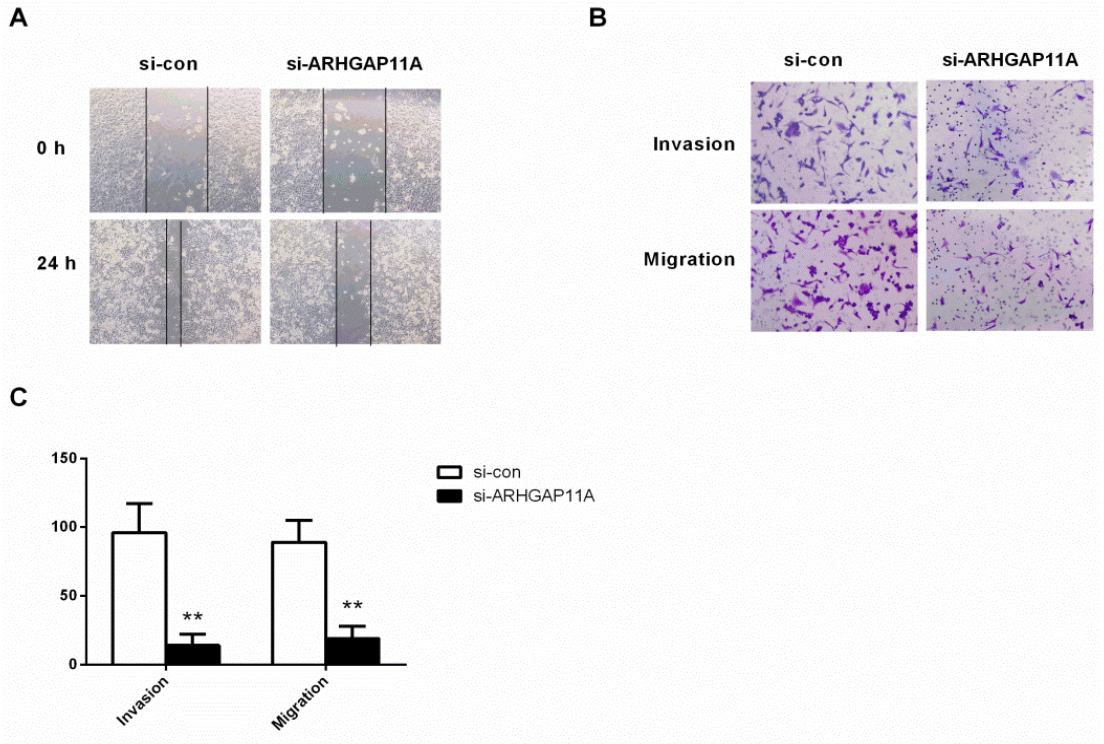

Figure 5: Effects of ARHGAP11A silence on tumor migration in vitro (A, B, C). A. Wound healing assay was used to measure the migration ability of A549 cells with or without ARHGAP11A silence. B and C. Transwell assays were conducted to determine the migratory and invasive abilities of si-ARHGAP11A-transfected A549 cells.

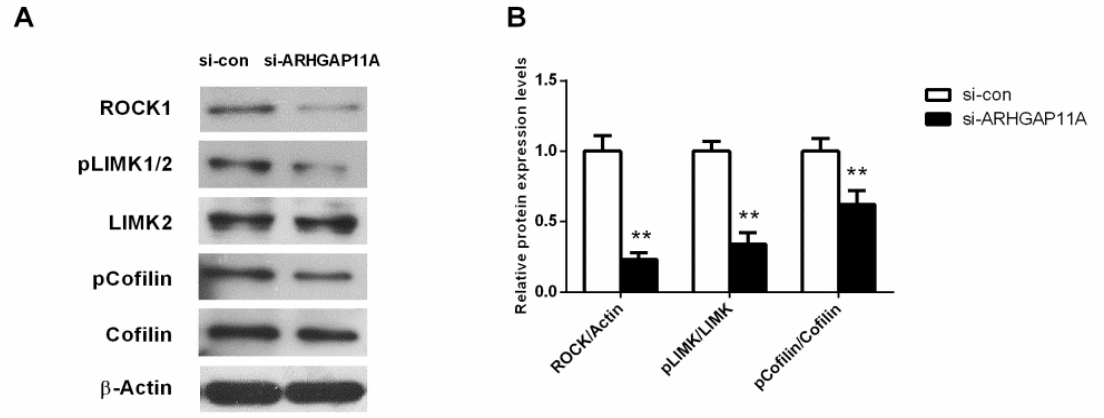

Figure 6: Analysis of ROCK1, pLIMK2, LIMK2, pCofilin, and Cofilin expression in A549 cells treated with siRNA ARHGAP11A by western blotting. The mean content of protein was normalized with the expression of β-actin. ¨p<0.001 compared with the respective negative control group.

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