Original Article

Role of miRNA-181a-2-3p in cadmium-induced inflammatory responses of human bronchial epithelial cells

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Background: Inflammation is an important priming event in the pathogenesis of pulmonary diseases, including chronic obstructive pulmonary disease (COPD). Increasing evidence indicates that microRNAs (miRNAs) contribute to the pathogenesis of COPD by regulating inflammatory response. Therefore, it is necessary to investigate novel molecular targets in COPD without any validation in COPD samples in airway inflammation. The aim of our study is to reveal novel miRNAs that can influence molecular targets for COPD and to examine the underlying mechanism in airway inflammation.

Methods: We identified the downregulation of miR-181a-2-3p in the serum of COPD patients and further investigated the role of miR-181a-2-3p in cadmium (Cd)-induced inflammation of a human bronchial epithelial cell line (BEAS-2B) and normal human bronchial epithelial (NHBE) cells.

Results: Our results showed that expression of miR-181a-2-3p was significantly decreased in Cd-treated cells and silencing of miR-181a-2-3p enhanced Cd-induced inflammatory responses and inflammasome activation. This negative regulatory effect of miR-181a-2-3p on inflammation is partly mediated by the calcium signaling pathway. Furthermore, global gene expression profiling revealed that Toll-like receptor 4 or sequestosome 1 genes were identified as potential targets of miR-181a-2-3p, which were significantly upregulated by knockdown of miR-181a-2-3p in Cd-treated cells.

Conclusions: Our results strongly suggest that miR-181a-2-3p has a critical role in Cd-induced inflammation of airway by regulating its potential target genes, which could be molecular targets for COPD.

Keywords: Chronic obstructive pulmonary disease (COPD); miR-181a-2-3p; cadmium (Cd); inflammation; inflammasome

Submitted Oct 20, 2018. Accepted for publication Jun 28, 2019.
doi: 10.21037/jtd.2019.07.55
View this article at: http://dx.doi.org/10.21037/jtd.2019.07.55

Introduction

Chronic respiratory diseases are provoked by inflammatory responses to external toxicants, such as smoke, air pollution, and heavy metals (1). Inflammatory signals activate a range of responses in the multiple cell types of airway epithelium (2). As a first-line defense, damaged epithelial cells recruit inflammatory cells to the airway by producing inflammatory cytokines and mediators and increasing mucus secretion and other signals that promote generating reactive oxygen species (ROS). Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation because of airway inflammation and destruction of lung parenchyma. However, the mechanisms underlying COPD

remain unclear. Although its pathogenesis is uncertain, cigarette smoking remains the main environmental risk factor for COPD (3,4), with its involved aberrant inflammatory and dysregulated cellular responses, where airways and lung tissue are affected. However, according to the World Health Organization, only 20% of the smokers suffer from COPD, suggesting that environmental factors and genetic susceptibility may also contribute to the pathogenesis of this disease (5-7).

As an environmental risk factor, cadmium (Cd) is a harmful component of cigarette smoke and particulate matter. Emerging evidence demonstrates that Cd exposure causes its excessive accumulation in the lungs which leads to the airway inflammation and lung dysfunction observed in COPD (8-11). Although the underlying mechanisms associated with Cd exposure have not been addressed in COPD, recent studies have suggested that Cd is closely associated with the severity and progression of COPD (8-10). Furthermore, exposure to Cd leads to epigenetic modulations, including aberrant methylation of DNA repair genes, histone modification, or microRNAs (miRNAs) (11-13).

miRNAs are small noncoding RNAs. These gene regulatory networks are rather complex, because a single miRNA can bind to the 3'-untranslated region (3'-UTR) of multiple target mRNAs, typically resulting in transcriptional or translational regulation of gene expression (14,15). By doing so, miRNAs are involved in various biological processes, such as cellular development, differentiation, proliferation, and apoptosis. In particular, recent studies have reported that several miRNAs have an important role in positive and negative regulation of the inflammatory response and participate in various regulatory network motifs in respiratory disease (16-19). Despite the critical role of miRNAs in inflammatory response, limited studies have focused on its role in inflammation-induced COPD. Numerous studies have revealed the dysregulation of miRNAs and their pathogenic roles in COPD. For instance, miR-26a acts as a regulator of the nuclear factor-κB (NF-κB) pathway (20) by regulating its target gene and activating signal cointegrator 1 complex subunit 3 (ASCC3) (21). In addition, miR-146a is significantly downregulated in lung fibroblasts of COPD patients and correlated positively with cytokine activation (22). Other studies suggested that miR-218-5p is downregulated in recruiting inflammatory cells toward the sites of inflammation through targeting chemokine (C-C motif) ligand 20 and interleukin (IL)-8, thereby assisting in sustaining the inflammation (23). However, knowledge of this state has remained limited to establish their role as biomarkers for early prevention, prognosis, and possible therapy (24). Therefore, it is necessary to develop novel therapeutic methods and targets through understanding the mechanism of COPD pathogenesis mediated by miRNA.

We identified the downregulation of miR-181a-2-3p in serum and lung tissues of COPD patients and further revealed its negative regulatory role in enhancing Cd-induced inflammatory responses of a human bronchial epithelial cell line (BEAS-2B) and normal human primary bronchial epithelial (NHBE) cells. Moreover, silencing of miR-181a-2-3p revealed its potential target genes, which could be implicated in the pathological events of COPD.

Methods

Preparation of serum samples

Serum samples were obtained from 58 COPD patients and 38 subjects with normal lung function registered in the COPD cohort study. The demographic characteristics of donors in each group are shown in *Table 1*. Methods for recruiting the cohort population have been reported previously (25,26). COPD was defined as a post-bronchodilator forced expiratory volume in 1 s/forced vital capacity ratio less than 0.7. The study was approved by the institutional review board of Kangwon National University Hospital (2012-06-007). All participants provided written informed consent.

Cell culture

BEAS-2B was kindly provided by the Biomedical Research Institute at Seoul National University Hospital. BEAS-2B cells were maintained in defined keratinocyte serum-free medium containing epidermal growth factor, 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). NHBE cells (CC-2540; Lonza Group, Allendale, NJ, USA) isolated from the epithelial lining of airways above the bifurcation of a normal human donor lung were cultured in bronchial epithelial growth medium (BulletKit medium, CC-3171, Lonza Group) and used before passage five in all experiments. Cell cultures were incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2.

In vitro transfection experiments of miR-181a-2-3p mimetics or inhibitors

Unless otherwise indicated, all materials for miRNA study

Table 1 Characteristics of COPD and control subjects used for expression profiling of miRNAs

Characteristics	COPD (n=58)	Control (n=38)	P value
Male (%)	40 (69.0)	22 (57.9)	0.27
Age (years)	73.9±5.2	72.0±7.0	0.05
Smoking (pack-years)	20.0±21.7	16.0±22.8	0.78
FEV_1 (% of predicted)	75.3±18.7	97.5±21.2	<0.001
FEV_1/FVC	57.7±6.5	75.4±3.9	<0.001

Unless otherwise stated, data are represented as mean ± standard deviation. FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; COPD, chronic obstructive pulmonary disease.

were purchased from Qiagen (Hilden, Germany). For a transient transfection approach with the aim to inhibit or enhance miR-181a-2-3p function, cells were transfected using the fast-forward HiPerFect Transfection Reagent protocol according to the manufacturer's instructions. A specific miR-181a-2-3p mimic, inhibitors or negative inhibitors were purchased commercially. For the reference to normalize the findings, we used the miScript inhibitor negative control under the same concentrations and conditions used for the mimic/inhibitor (50 nM). Transfected bronchial epithelial cells were incubated under their normal growth conditions, and the effects of miR-181a-2-3p manipulations on changes in gene expression levels were measured by quantitative reverse transcription-polymerase chain reaction (RT-PCR) after 24 h as described above.

Cd treatment

BEAS-2B and NHBE cells were seeded at a density of 2.0×10^5 cells per well in 6-well plates and incubated until they reach 70–80% confluency. Cells were then transfected with miR-181a-2-3p mimics and/or inhibitors, followed by treatment with or without Cd for 24 h (27,28).

ELISA assay

Secreted IL-1β was measured in BEAS-2B cell culture supernatants using the human IL-1β/IL-1F2 Quantikine ELISA Kit (R&D Systems, MN, USA). The ELISA plates were read using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Inflammasome activation

THP-1 cells (1×10^6 cells/well) in 12-well plates were differentiated into macrophage-like cells by incubating with phorbol 12-myristate 13-acetate (100 nM, PMA; InvivoGen, San Diego, CA, USA) for 72 h. THP-1-derived macrophages were primed for 3 h with lipopolysaccharide (LPS) (1 μg/mL), and then cells were stimulated as follows by the NLRP3, NLRC4, or AIM2 activators ATP (2 mM), flagellin (500 μg/mL), or dsDNA (2 μg/mL), respectively, with miR-181a-2-3p mimic/inhibitor (50–100 nM), and then the IL-1β (p17, active form) secretion was assayed.

Pharmacological reagents treatment

Unless otherwise indicated, all pharmacological reagents were obtained from Tocris Bioscience (Bristol, UK) or Cayman Chemical (Ann Arbor, MI, USA) and used at the following working concentrations: Toll-like receptor 4 (TLR4) inhibitor (TAK242, 2.5 μM; Cayman Chemical), phospholipase C (PLC) inhibitor (U73122, 10 μM; Tocris Bioscience), IP3/TRP channel inhibitor [2-aminoethoxydiphenyl borate (2-APB), 10 μM; Tocris], and nicotinamide adenine dinucleotide phosphate oxidase (NOX) inhibitor [diphenyleneiodonium (DPI), 10 μM; Tocris]. Dimethyl sulfoxide at 0.1% concentration was used as the vehicle control. Cells were pretreated with the concerned pharmacological reagents for 1 h followed by transfection with miRNA transfection protocol.

Western blotting analysis

An equal amount of proteins from control and each treatment sample were loaded by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (10% or 15%) and transferred onto polyvinyl difluoride membranes (Millipore, Billerica, MA, USA). Nonspecific binding proteins were blocked with 3% skim milk in 1× phosphate-buffered saline with 0.05% Tween 20 for 60 min. Membranes were incubated with primary antibodies against antihuman

IL-1β antibody (AF-201-NA, R&D Systems, Minneapolis, MN, USA), NF-κB sampler kit antibodies (#9936; Cell Signaling Technology; Danvers, MA, USA), and CEBP Antibody Sampler Kit (#12814, Cell Signaling Technology), PI3 Kinase Antibody Sampler Kit (#9655, Cell Signaling Technology), or anti-β-actin antibody (sc4778, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4 ℃. The membranes were probed further with horseradish peroxidase-conjugated secondary anti-sera (A9917, A6667, or A5420; Sigma-Aldrich Corp., St. Louis, MO, USA) and visualized with PierceFast Western blot kit (Thermo Fisher Scientific) and a cooled CCD camera system (Bio-Rad Laboratories, Hercules, CA, USA).

miRNA and mRNA extraction and real-time PCR

According to the manufacturer's instructions, total miRNA was isolated from the serum using the miRNeasy Serum/Plasma Kit (Qiagen) and reverse transcription was performed using the miScript II RT Kit (Qiagen). Subsequently, complementary DNAs (cDNAs) were amplified from the following 20 miRNAs using the custom miScript miRNA PCR Array (CMIHS02261C; Qiagen): hsa-miR-101-3p, hsa-miR-125b-1-3p, hsa-miR-143-3p, hsa-miR-148a-3p, hsa-miR-151a-3p, hsa-miR-181a-2-3p, hsa-miR-181b-5p, hsa-miR-181c-5p, hsa-miR-181d-5p, hsa-miR-23b-3p, hsa-miR-26a-5p, hsa-miR-28-3p, hsa-miR-30a-5p, hsa-miR-30c-2-3p, hsa-miR-30e-3p, hsa-miR-361-5p, hsa-miR-501-3p, hsa-miR-660-5p, hsa-miR-769-5p, and hsa-mir-191-5p. The following thermal cycling conditions were used: 95 ℃ for 15 min, followed by 40 cycles of 95, 55, and 70 ℃ for 15, 30, and 30 s, respectively. Quantitation was normalized to hsa-miR-RNU6 (reference gene). Data were analyzed using PCR array data analysis tools (Qiagen). For mRNA analysis, total RNA was extracted from cells by TRIzol (Invitrogen) according to the manufacturer's protocol and reverse-transcribed into cDNA using the QuantiTect® reverse transcription kit. cDNA was quantitated using Power SYBR Green PCR (Applied Biosystems by Life Technologies, Warrington, UK) and the QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems). Primer sequences are listed in *Table S1*.

Identification of miRNAs targets and network analysis

Following incubation with/without miR-181a-2-3p inhibitor (50 nM) for 24 h, Cd-treated BEAS-2B cells were collected as described previously and total RNA was extracted using TRIzol. Affymetrix GeneChip Human 2.0 ST Array (Affymetrix, Santa Clara, CA, USA) was hybridized with complementary RNA (cRNA) probes at the Macrogen core facility (Seoul, Korea). The expression value and detection calls were computed from the raw data and gene set enrichment analysis (GSEA) version 4.0 (Broad Institute, Cambridge, MA, USA) to interpret expression profiles from microarrays. GSEA originally was developed to identify cohorts of genes whose functions are integrated into certain biological processes and/or specific signaling pathways. Pathways were ranked according to the significance of the enrichment, and the validation mode measure of significance was used to identify pathways of greatest enrichment. Significance was tested by comparing the observed enrichment with the enrichment seen in data sets in which sample labels were permutated randomly. All data analysis and visualization of differentially expressed genes was conducted using Cytoscape.

Statistical analyses

Statistical analyses were performed with Student's *t*-test and two-way analysis of variance for multiple groups using GraphPad Prism (GraphPad Software, San Diego, CA, USA). P values are indicated in the figures. Moreover, Fisher's exact test was performed to estimate the significance of differential expressions of miRNAs in serum samples using R program (version 3.5). $P<0.05$ was considered as statistically significant, which is indicated in the figures.

Results

miR-181a-2-3p is downregulated in lung tissues and serum of COPD patients compared with normal subjects

Profiling miRNA expression patterns in lung tissues of COPD patients and determining regulatory mechanisms of specific miRNAs enhance our understanding of molecular mechanisms of COPD. On the basis of our previous report of such profiling in COPD patients and normal subjects (29), we selected several miRNAs (10 upregulated and 10 downregulated) to determine their differential expressions in serum samples from COPD patients and normal subjects (*Table 2*). To draw further conclusions about the correlation between selected miRNAs in lung tissues and body fluid, we compared their expression levels in the serum of 58 smokers with COPD and 38 healthy smokers without COPD.

Table 2 Expression levels of miRNAs in the COPD and control group

miRNAs	COPD *vs.* control		
	Fold regulation	95% CI	P value
hsa-miR-769-5p	2.0108	0.77, 3.26	1.000
hsa-miR-181d-5p	1.6559	0.81, 2.50	1.000
hsa-miR-501-3p	1.4094	0.61, 2.21	0.502
hsa-miR-125b-1-3p	1.2192	0.60, 1.83	<0.0001
hsa-miR-191-5p	1.0708	0.95, 1.19	0.835
hsa-miR-143-3p	1.0675	0.68, 1.46	0.536
hsa-miR-26a-5p	1.0577	0.94, 1.18	0.835
hsa-miR-23b-3p	1.0504	0.90, 1.20	0.522
hsa-miR-30a-5p	1.0456	0.97, 1.12	1.000
hsa-miR-151a-3p	1.0011	(0.89, 1.12)	0.537
hsa-miR-361-3p	–1.0044	(0.65, 1.34)	0.835
hsa-miR-28-3p	–1.011	(0.86, 1.12)	1.000
hsa-miR-148a-3p	–1.0189	(0.83, 1.14)	0.679
hsa-miR-30e-3p	–1.0218	(0.74, 1.21)	<0.0001
hsa-miR-361-5p	–1.0354	(0.58, 1.35)	0.302
hsa-miR-181b-5p	–1.0709	(0.83, 1.04)	0.659
hsa-miR-101-3p	–1.1287	(0.74, 1.03)	0.679
hsa-miR-660-5p	–1.1339	(0.62, 1.15)	1.000
hsa-miR-181c-5p	–1.1594	(0.61, 1.11)	0.831
hsa-miR-181a-2-3p	–1.5998	(0.28, 0.97)	0.008

Fold change ($2^{-\Delta\Delta Ct}$) is normalized gene expression. ($2^{-\Delta Ct}$) in test sample divided the normalized gene expression ($2^{-\Delta Ct}$) in the control sample. Quantitation was normalized to reference control. COPD, chronic obstructive pulmonary disease; CI, confidence interval.

Among these miRNAs, miR-181a-2-3p was significantly downregulated in the serum samples of COPD patients, whereas miR-181d-5p, miR-501-3p, miR-769-5p, and miR-191-5p were upregulated (*Figure 1A,B*). Also, miR-181a-2-3p and miR-501-3p were similarly dysregulated between lung tissues and serum of the COPD patients (*Figure 1C*).

Cd, a major component of cigarette smoke and particulate matter, induces inflammatory responses and endoplasmic reticulum stress in human bronchial epithelial cells via the C/EBP-DDIT3 signaling pathway (28). Thus, we investigated whether expression of miRNAs is influenced by Cd treatment in BEAS-2B and NHBE cells. We found that expression of miR-28-3p, miR-101-3p, miR-148-3p, miR-151-3p, miR-181a-2-3p, and miR-501-3p was influenced by Cd treatment in BEAS-2B and/or NHBE cells (*Figure 2*). Interestingly, miR-181a-2-3p was downregulated in Cd-treated bronchial epithelial cells as well as lung tissue and serum of COPD patients, suggesting that miR-181a-2-3p may have an important role in promoting inflammatory response associated with the pathogenesis of pulmonary diseases.

Knockdown of miR-181a-2-3p enhances inflammatory responses and inflammasome activation in human bronchial epithelial cells

To determine if downregulation of miR-181a-2-3p is involved in regulating inflammatory responses, we measured transcriptional levels of pro-inflammatory cytokines in Cd-treated BEAS-2B cells in the presence/

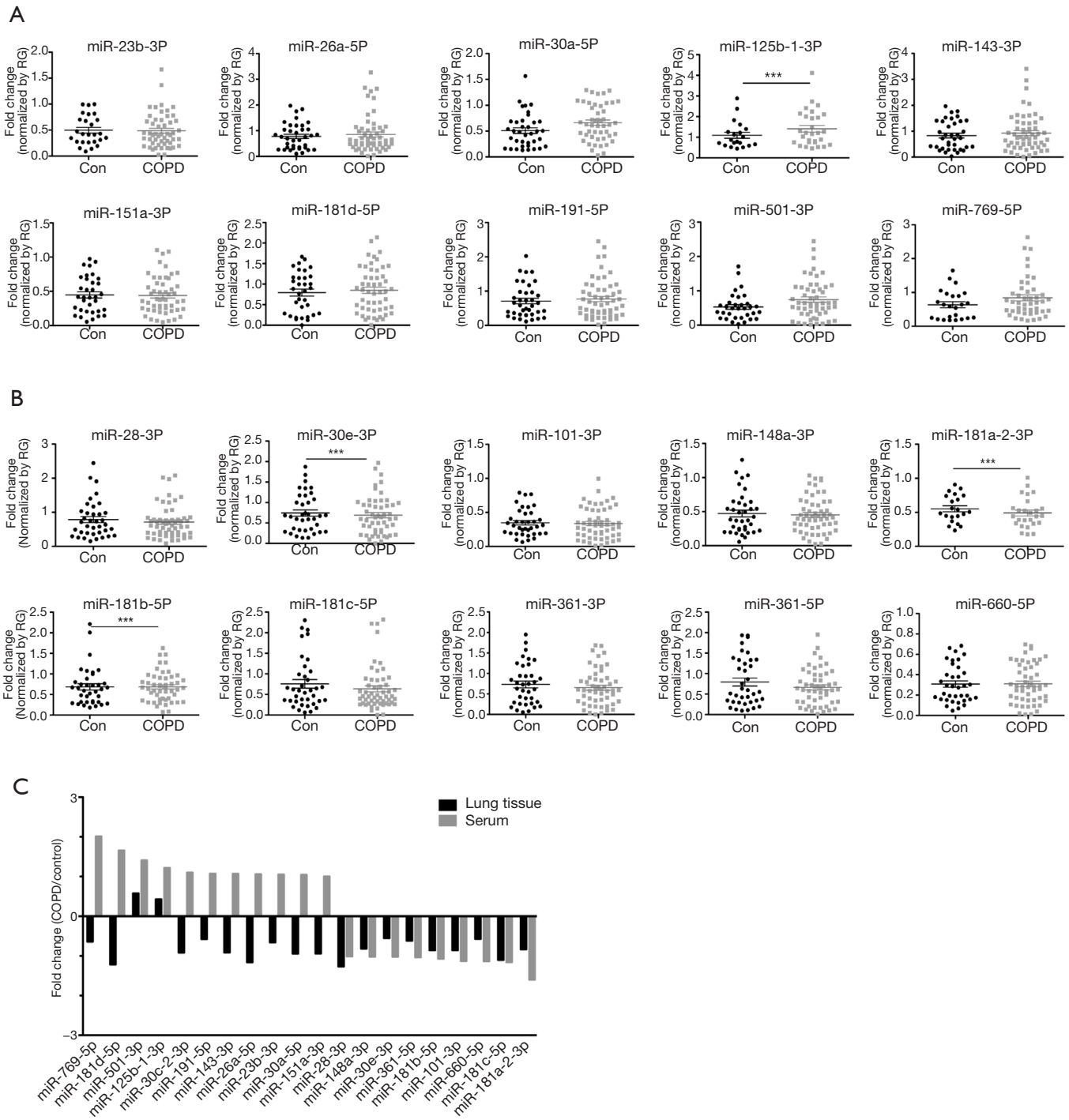

Figure 1 Selection of candidate miRNAs. Differentially expressed miRNAs in the serum from subjects with COPD compared with healthy smoker. (A) Ten miRNAs (miR-23b-3p, miR-26a-5p, miR-30a-5p, miR-125b-1-3p, miR-143-3p, miR-151a-3p, miR-181d-5p, miR-191-5p, miR-501-3p, and miR-769-5p) were substantially upregulated; (B) 10 miRNAs (miR-28-3p, miR-30e-3p, miR-101-3p, miR-148a-3p, miR-181a-2-3p, miR-181b-5p, miR-181c-5p, miR-361-3p, miR-361-5p, and miR-660-5p) were downregulated in COPD patients; (C) analysis of differential miRNA expression profiles from serum and lung tissues (published miRNA profile) of COPD patients, represented by fold-change. ***, P<0.001 were considered significant. COPD, chronic obstructive pulmonary disease.

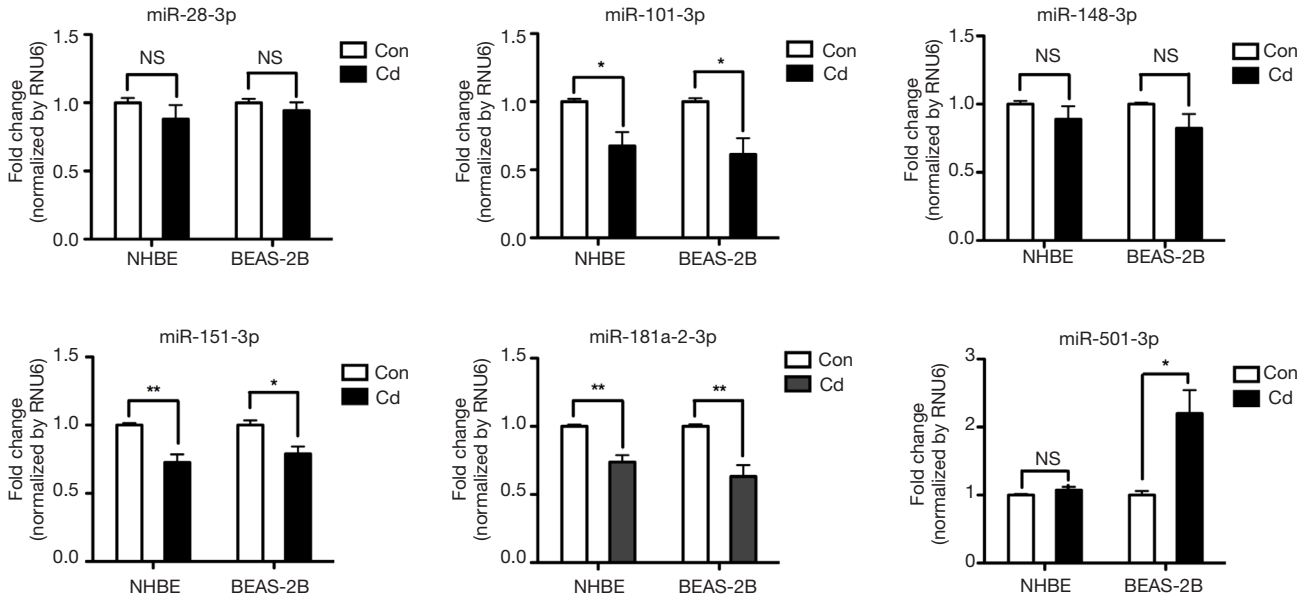

Figure 2 Analysis of dysregulated miRNAs expression of Cd-treated cells. Bronchial epithelial cells (BEAS-2B and primary NHBE cells) were stimulated with 10 μM Cd for 24 h. The miRNA levels were analyzed using SYBR green-based quantitative real-time PCR assays. All data are representative of at least three independent experiments. *, P<0.05; **, P<0.01 were considered significant. Cd, cadmium; NS, not significant; NHBE, normal human bronchial epithelial.

absence of miR-181a-2-3p inhibitor. As shown in *Figure 3A*, transfection of cells with miR-181a-2-3p inhibitor or negative inhibitor (control miRNA inhibitor) alone did not affect the expression of genes associated with inflammatory responses in BEAS-2B cells. However, knockdown of miR-181a-2-3p followed by Cd-exposure significantly elevated the expression levels of inflammatory cytokines and mediators, such as IL-1α, IL-1β, IL-6, IL-8, TNFα, and COX2 (*Figure 3A*). To reinforce the biological relevance of this finding in Cd-treated BEAS-2B cells, we investigated whether knockdown of miR-181a-2-3p promotes inflammatory responses in translational level. As seen in mRNA levels, silencing of miR-181a-2-3p resulted in an increase in the production of IL-1β (*Figure 3B*) in Cd-exposed NHBE cells. In contrast, miR-181a-2-3p mimics ameliorated inflammatory responses (*Figure 3B*). Inflammasome not only regulates the inflammatory response but also has a central role in the development of COPD (30). To further investigate the mechanism involved in the inflammatory response of miR-181a-2-3p, we evaluated whether overexpression or knockdown of miR-181a-2-3p is able to activate the inflammasome. Although knockdown of miR-181a-2-3p significantly induced NLRP3 and caspase 1 (Casp1) expressions and promoted IL-1β secretion in ATP-treated LPS-primed macrophage-like cells, its overexpression attenuates the secretion of IL-1β through three inflammatory response mechanisms, namely activation of NLRP3, NLRC4, or AIM2 inflammasomes (*Figure 4*). Our findings indicated that miR-181a-2-3p increased sensitivity to inflammatory responses induced by Cd exposure in human bronchial epithelial cells.

Blockade of intracellular calcium signaling partly attenuates Cd-induced IL-1β expression in human bronchial epithelial cells

Elevation of intracellular calcium, superoxide generation by the NOXs, and activation of TLR4 signaling are involved in inducing inflammatory responses and inflammation activation in the lung tissues (31,32). Thus, we asked if the above signaling pathways are involved in reducing the negative regulatory effect of miR-181a-2-3p on inflammation in human bronchial epithelial cells treated with Cd. We found that blockage of PLC by U73122 attenuated Cd-induced IL-1β expression in human bronchial epithelial cells, whereas 2-APB (inhibitor of inositol triphosphate receptor and transient receptor

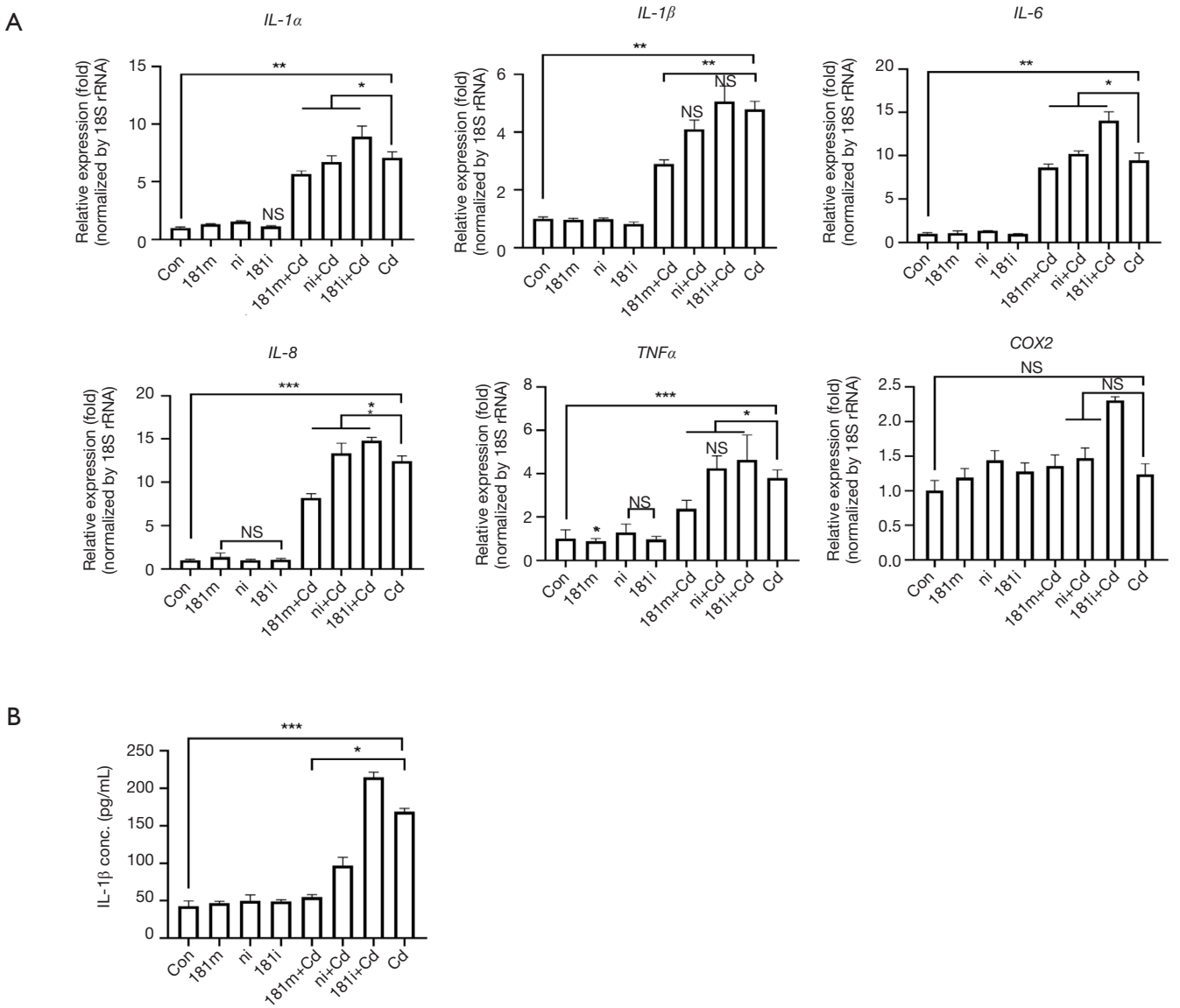

Figure 3 Effect of miRNA 181a-2-3p on pro-inflammatory cytokines expression. Airway epithelial cells (BEAS-2B and primary NHBE cells) were transfected with miRNA 181a-2-3p mimics or inhibitors (50 nM) when the cells were stimulated with 10 μM Cd for 24 h. mRNA levels of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, TNFα, and COX2) were analyzed using SYBR green-based quantitative real-time PCR. (A) BEAS-2B cells; (B) secreted IL-1β levels were measured in NHBE cells culture supernatants by enzyme-linked immunosorbent assay (ELISA). All data shown are representative of at least three independent experiments. Statistically significant differences were analyzed with the t-test. *, P<0.05; **, P<0.01, and ***, P<0.001 were considered significant. NS, not significant; NHBE, normal human bronchial epithelial; Cd, cadmium; 181m, 181 mimics; 181i, 181 inhibitor; ni, miRNA inhibitor negative control.

potential channels), DPI (NOX inhibitor), and TAK242 (Resatorvid, TRL4 inhibitor) did not inhibit IL-1β production enhanced by silencing of miR-181a-2-3p (*Figure 5*). We further investigated miR-181a-2-3p target genes by seven well known miRNA target prediction programs, namely TargetScan, miRWalk, MicroCosm, PITA, miRanda, DIANA LAB, or PicTar. After selecting overlapping genes, a total of 52 genes were predicted as targets of miR-181a-2-3p (*Tables S2*,*S3*). Once predicted genes were analyzed, we further verified that calcium signaling is associated

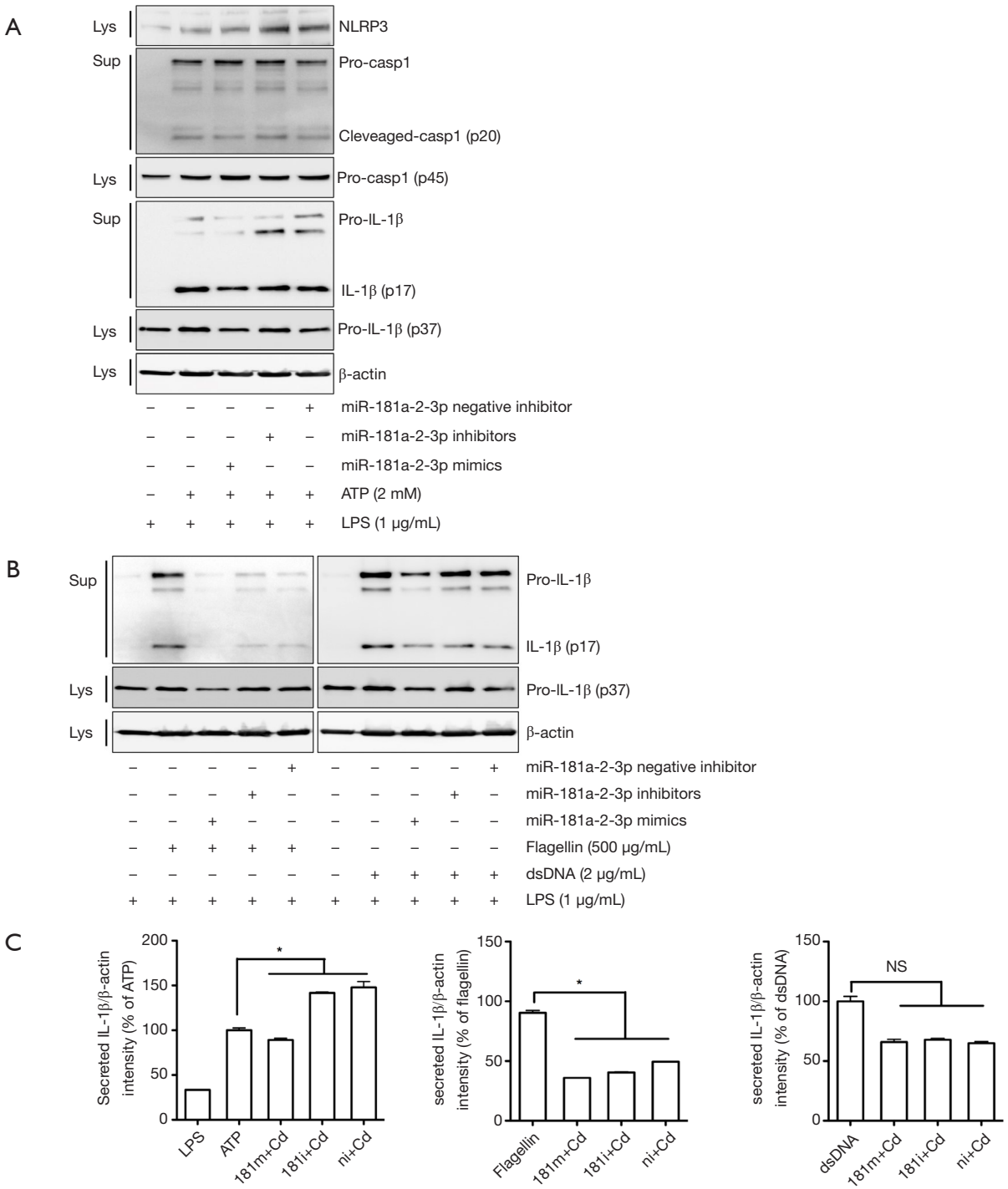

Figure 4 Effect of miR-181a-2-3p on inflammasome activation. miR-181a-2-3p promotes inflammasome activation in human THP-1-derived macrophages. Human THP-1-derived macrophages were pretreated with miR-181a-2-3p mimic/inhibitor (50 nM) and then stimulated with 2 mM ATP, 500 μg/mL flagellin, or 2 μg/mL dsDNA to activate NLRP3, NLRC4 and AIM2 inflammasomes, respectively. (A,B) Western blot analysis of proteins associated with the activation of NLRP3, NLRC4 and AIM2 inflammasomes, such as IL-1β and caspase 1. (C) Quantitation of secreted IL-1β band intensities and their normalization with internal control β-actin by Image J software. All data shown are representative of at least three independent experiments. *, P<0.05 was considered significant. NS, not significant; Lys, lysate; Sup, supernatant; LPS, lipopolysaccharide; 181m, 181 mimics; 181i, 181 inhibitor; ni, miRNA inhibitor negative control; Cd, cadmium.

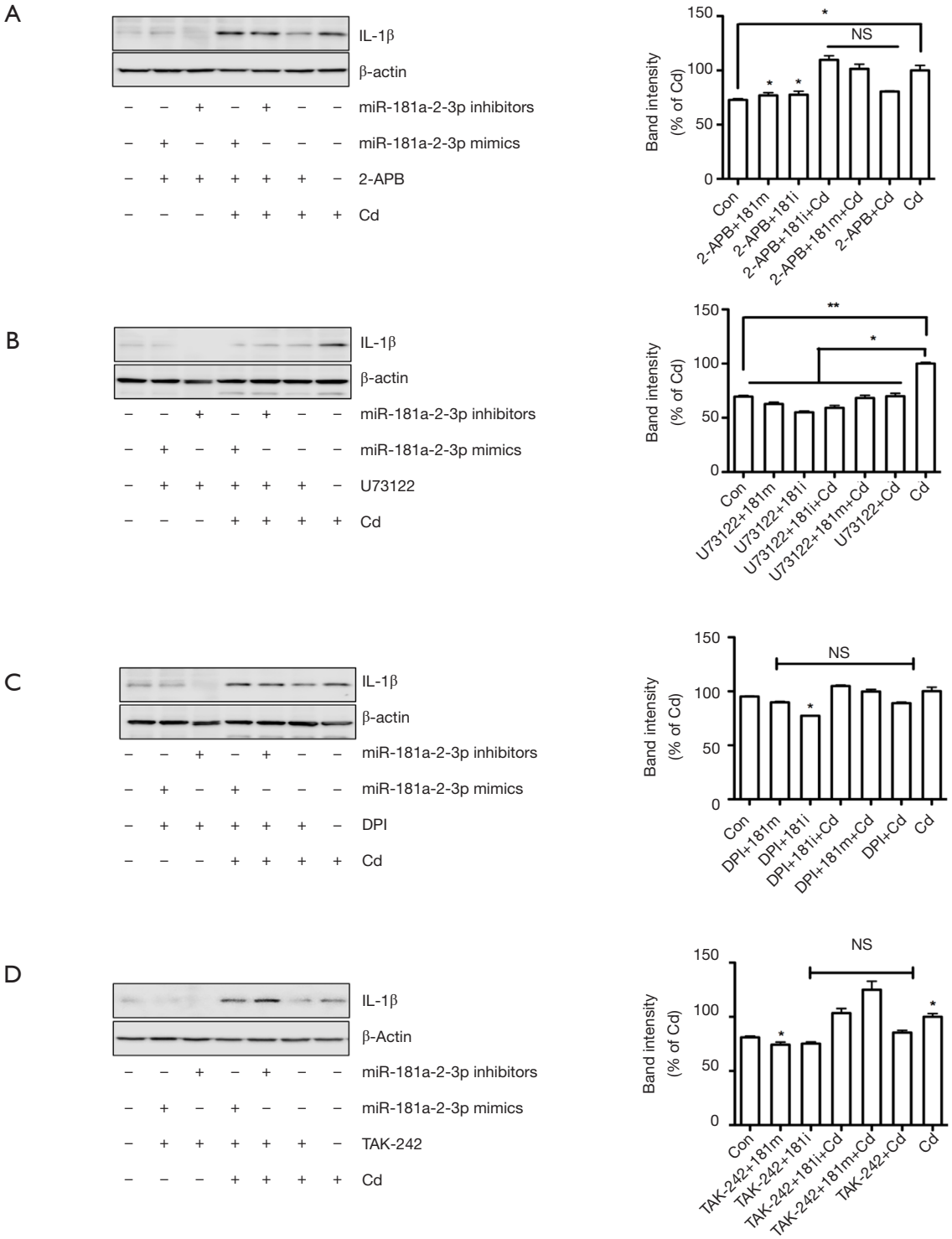

Figure 5 Effect of miR-181a-2-3p on multiple intracellular signaling pathways. BEAS-2B cells were pretreated with or without inflammatory signal downstream inhibitors (10 μM) for 1 h. Then, cells were transfected with/without miRNA 181a-2-3p inhibitor and then stimulated with 10 μM Cd for 24 h. 2-APB (A), U73122 (B), DPI (C), and TAK202 (D) were analyzed by immunoblotting, and intensities of bands were quantified and normalized with internal control β-actin by Image J software. All data shown are representative of at least three independent experiments. *, P<0.05; **, P<0.01 were considered significant. NS, not significant; Cd, cadmium; DPI, diphenyleneiodonium; 181m, 181 mimics; 181i, 181 inhibitor.

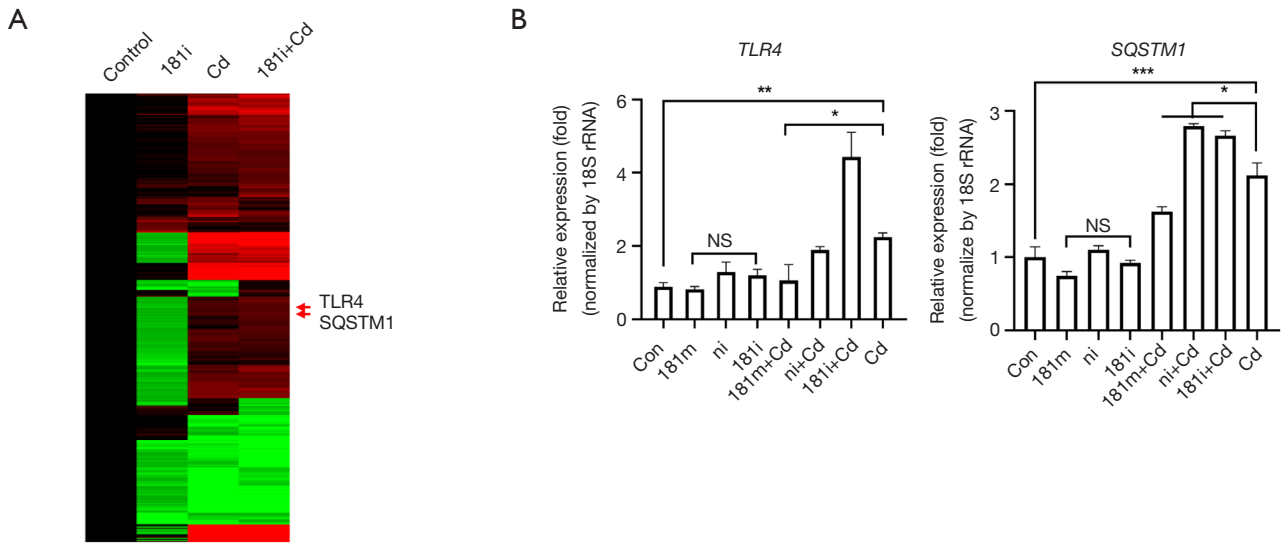

Figure 6 Profiling of global gene expression. The expression patterns for DEGs selected from mRNA profiling analysis on the four groups: control, Cd, miR-181a-2-3p knockdown, and miR-181a-2-3p knockdown treated with Cd. (A) Heat map of differentially expressed mRNAs on the four groups. Green indicates downregulated expression, and red indicates upregulated expression compared with a reference expression level. (B) Validation of miR-181a-2-3p mimics/inhibitors with Cd-treated cells using quantitative RT-PCR. *, P<0.05; **, P<0.01, and ***, P<0.001 were considered significant. NS, not significant; Cd, cadmium; 181m, 181 mimics; 181i, 181 inhibitor; ni, miRNA inhibitor negative control.

with calcium/calcineurin (*PPP3CA*, *PPP3CB*, *PPP3CC*, *PPP3R1*, *PPP3R2*) and NFATC (*NFATC1* to *NFATC4*) family of genes. As a result, knockdown of miR-181a-2-3p significantly enhances the expression levels of *PPPCB*, *PPPCC*, *PPP3R1*, *NFATC2*, or *PPP4R2* in Cd-exposed cells (*Figure S1*).This result indicated that the negative regulatory effect of miR-181a-2-3p on Cd-induced inflammation in human bronchial epithelial cells is partly mediated via intracellular calcium release-related signaling pathway.

SQSTM1 and TLR4 are potential targets of miR-181a-2-3p

To further identify the potential targets of miR-181a-2-3p contributing to enhancement of the inflammatory response in Cd-treated bronchial epithelial cells, the expression profile of Cd-treated BEAS-2B cells followed by knockdown of miR-181a-2-3p was compared with that of Cd-treated BEAS-2B cells using cDNA microarray. *Figure 6A* shows a heat map of differentially expressed mRNAs between groups. A total of 128 genes (39 upregulated and 89 downregulated) were altered specifically by miR-181a-2-3p knockdown in Cd-treated cells (–2≤ fc ≤2; *Figure S2A*). To investigate the biological relevance of these genes, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes analyses were performed. The main GO categories that included 128 genes were regulation of endocytosis, cell motion, neuron projection development, regulation of response to external stimuli, and apoptosis (*Figure S2B*). Interactions network analysis revealed that a set of genes, including *sequestosome 1* (*SQSTM1*) and *TRL4*, are involved in the neurotrophin receptor-interacting factor, p75 neurotrophin receptor, and NF-κB signaling pathway (*Figure S2C*). We further verified that the expression levels of *SQSTM1* and *TRL4* genes were elevated by miR-181a-2-3p knockdown in Cd-treated cells compared with nontreated ones (*Figure 6B*). These data suggested that *SQSTM1* and *TRL4* could be potential targets of miR-181a-2-3p in Cd-treated bronchial epithelial cells.

Discussion

Numerous studies have recognized miRNAs as major drivers or modifiers in the development and progression of various pulmonary diseases. Thus, elucidating alterations in expression of specific miRNAs and their circulation in blood can be developed to therapeutic targets and biomarkers for pulmonary disease. Although there are limitations on serum miRNA profiling methods and platforms, profiling of serum

miRNAs has several advantages. It not only offers new biomarkers for prediction and early detection of COPD but also provides insights into mechanisms of pathogenesis or airway epithelium damage. In our study, we newly identified downregulation of miR-181a-2-3p in lung tissues and serum of COPD patients and demonstrated its negative regulatory role in promoting inflammatory responses and inflammasome activation in human bronchial epithelial cells. These results suggested that miR-181a-2-3p may serve as a new marker in individuals with inflammatory responses and could be a potential target for the development of anti-inflammatory drugs.

miR-181a-2-3p belongs to the miR-181 family, including six members: miR-181a-1/2, miR-181b-1/2, miR-181c, and miR-181d, which are encoded by three independent sequences located on three separate chromosomes (33). The miR-181a-1 and miR-181a-2 clusters locating at chromosomes 1p32.1 and 9q33.3 have a high sequence similarity, which leads to an identical mature miRNA. The miR-181a-2-3p cluster has been reported to be involved in various biological processes, such as development, differentiation, cellular metabolism, and homeostasis (34-36). Detectable miR-181a generally was downregulated in tissues of COPD patients compared with controls and identified as a cytokine-responsive miRNA regulating cellular responses to inflammation (37-39). However, the precise molecular mechanisms by which it exerts this effect have not been fully elucidated.

Aberrant activation of inflammatory responses can lead to cell death and tissue damage caused by activation of multiprotein complexes called inflammasomes, which is constant secretion of cytokines, such as IL-1β. Previous results showed that cigarette smoking induces IL-1β release in the human lung (40). In addition, elevated levels of IL-1α and IL-1β are found in the lungs of COPD patients (30,41), and their secretion is amplified in lungs during disease exacerbations. Several indirect lines of evidence link inflammasome-dependent cytokines to disease pathology of COPD (42,43), but a direct role for the inflammasomes has yet to be clearly shown. Although the effect of miR-181a on inflammatory responses has been reported (37,39), their molecular mechanisms underlying the inflammasome activation have not yet been studied. To our knowledge, ours is the first investigation of the regulatory effect of a miR-181a-2-3p on inflammasome activation. Interestingly, miR-181a-2-3p overexpression attenuated NLRP3, NLRC4, and AIM2 inflammasome-mediated IL-1β production. Unlike miR-181a-2-3p overexpression, its knockdown enhanced NLRP3 inflammasome activation, but did not affect NLRC4, and AIM2 inflammasome-mediated IL-1β production. Thus, understanding the function of miR-181a-2-3p in regulating inflammatory response and inflammasome activation will prompt the development of miRNA-based therapy for COPD.

Under Cd stress, multiple inflammatory signal transduction pathways may get activated. Cd has been shown to be involved in a number of calcium-dependent pathways (44). Recent studies have implicated elevations in cytosolic Ca^{2+} concentration in NLRP3 inflammasome activation in response to several stimuli (45,46). Although it remains unclear whether the miR-181a-2-3p regulates inflammation via calcium signaling in COPD, there is an accumulating evidence for a significant association of calcium flux with miR-181 family members. In mice, miR-181a has been shown to enhance calcium flux and Erk phosphorylation in T cell (47). Moreover, it has been reported that thapsigargin-induced intracellular Ca^{2+} release suppressed pre-miR-181a levels through acetylcholinesterase (AChE), protein kinase C (PKC) and protein kinase A (PKA) cascade(s) (48). In another study, it has been demonstrated that miR-181c can directly target the mitochondrial encoded-gene, *mt-COX1*, which leads to increased $\Delta\Psi_m$ and could be associated to increased $[Ca^{2+}]_m$ influx and ROS production (49). Although biological studies for its direct role in calcium flux is limited, miR-181a-2-3p has been shown to regulates calcium/calcineurin signaling.

Transcriptomic analysis revealed that *TRL4* and *SQSTM1* are potential targets of miR-181a-2-3p for elevating inflammatory response in Cd-treated bronchial epithelial cells. TLRs are the first-line sensors that trigger inflammatory cascades (50,51), which leads to upregulation of pro-inflammatory molecules that further amplify the inflammatory response and cell apoptosis. Preclinical studies illustrated that genetic *TLR4* knockout was associated with improved outcomes after COPD (36,52). Evidence is now emerging indicating that TLR activation probably affects the expression of miR-181a-2-3p in airway inflammation. However, our results showed that miR-181a-2-3p knockdown did not have any effect on IL-1β secretion after TLR4 inhibitor treatment. In addition, we found no change in the degradation or phosphorylation of IκB αβ, or in the phosphorylation of p65 (data not shown), which indicates that miR-181a-2-3p expression dose not regulate their upstream signaling mediators. These results suggested that miR-181a-2-3p regulation is likely to be complex and redundant or may not be involved in this process.

SQSTM1 is known as an ubiquitin-binding adapter protein and promotes tumorigenesis due to autophagy dysfunction and NF-κB activation in Cd-treated BEAS-2B cells (53). Moreover, transcript level of SQSTM1 is significantly increased in COPD-emphysema lungs compared with normal nonsmokers as well as cigarette smoke extract-treated BEAS-2B cells (54). These results suggested that upregulation of SQSTM1 by knockdown of miR-181a-2-3p in Cd-treated BEAS-2B cells may be implicated with the development and progress of COPD.

In conclusion, our study demonstrated that miR-181a-2-3p is downregulated in the lung tissues and serum of COPD patients compared with normal subjects. Downregulation of miR-181a-2-3p in airway cells enhanced inflammatory responses in Cd-treated airway cells. These findings provided an unprecedented biological function of miR-181a-2-3p in human bronchial epithelial cells and their potential target genes (*TRL4* and *SQSTM1*), highlighting its pathological implications related with airway inflammation and inflammasome activation in the COPD.

Acknowledgments

Funding: This study was supported by grants from the National Research Foundation of Korea (NRF) (2017R1A2B4003790) and by a grant from the Global PhD Fellowship Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2018H1A2A1063441).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Institutional Review Board of Kangwon National University Hospital (2012-06-007). All participants provided written informed consent.

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Cite this article as: Kim J, Kim DY, Heo HR, Choi SS, Hong SH, Kim WJ. Role of miRNA-181a-2-3p in cadmium-induced inflammatory responses of human bronchial epithelial cells. J Thorac Dis 2019;11(7):3055-3069. doi: 10.21037/jtd.2019.07.55

Supplementary

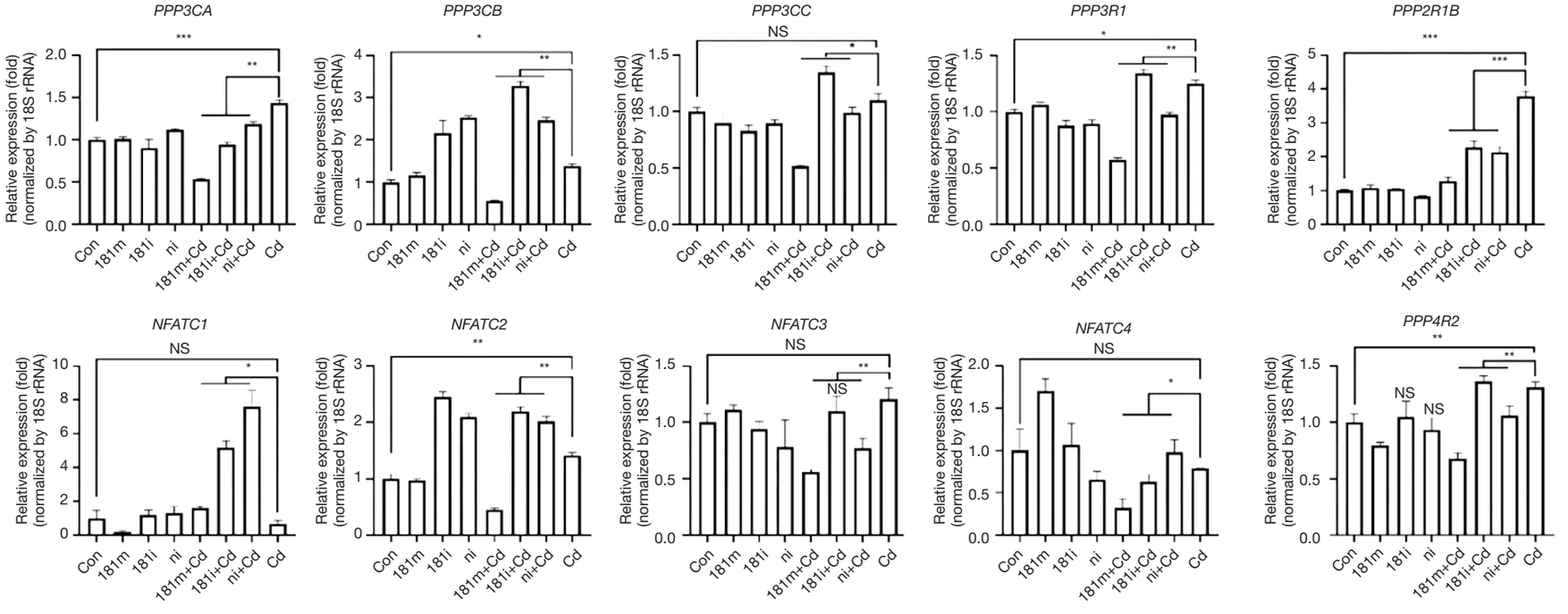
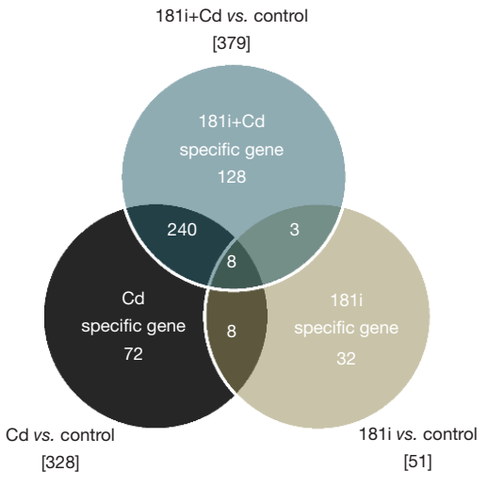


Figure S1 Expression profiles of miR-181a-2-3p target genes. Quantitative RT-PCR analysis of the expression of different genes in Cd-treated cells, transfected with miR-181a-2-3p mimics/inhibitors. *, P<0.05; **, P<0.01, and ***, P<0.001 were considered significant. NS, not significant; Cd, cadmium; 181m, 181 mimics; 181i, 181 inhibitor; ni, miRNA inhibitor negative control.

A



B

181i+Cd specific gene [128]

Term	Count	P value	Gene
Regulation of endocytosis	4	0.001306	*CDH13, ITGA2, NEDD4L, PTENP1*
Cell motion	8	0.001331	*CDH13, KIF5C, ITGA2, SEMA3AGAS1, TPM4, SLIT2, PTENP1*
Positive regulation of response to external stimulus	4	0.001501	*CDH13, ITGA2, TLR4, SLIT2*
Neuron projection development	6	0.002057	*KIF5C, SEMA3A, GAS1, SLIT2, PTENP1, NGF*
Positive regulation of cellular component organization	5	0.003812	*ITGA2, NEDD4L, EIF5A2, SLIT2, NGF*
Positive regulation of apoptosis	7	0.003948	*SSTR3, SQSTM1, TLR4, STK17A, PTENP1, BTK, NGF*

C

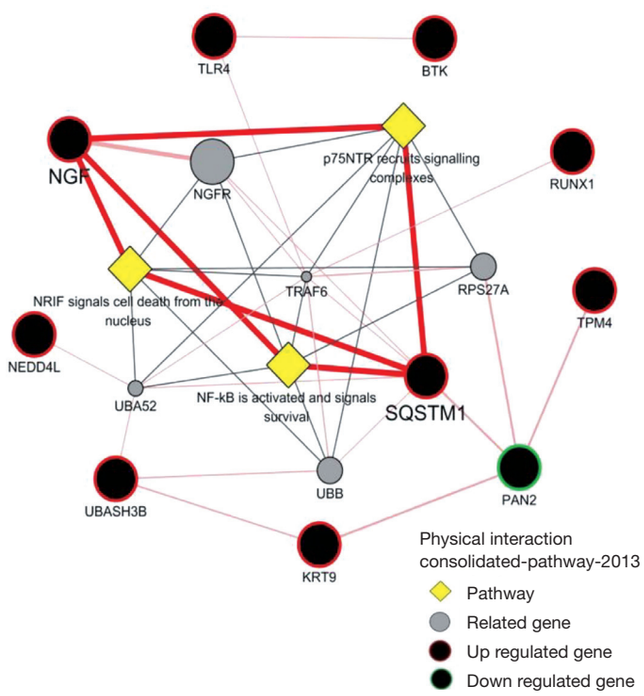


Figure S2 Interaction map of miR-181a-2-3p and its target mRNAs using cDNA microarray. Target genes were differentially expressed in normal and miR-181a-2-3p knockdown BEAS-2B cells treated with Cd. (A) Venn diagram of the overlap of mRNA profiles in comparative analyses between different groups. (B) GO term enrichment analysis for biological processes of miR-181a-2-3p knockdown treated with Cd. (C) Interaction network of specific target genes in miR181a-2-3p knockdown cells treated with Cd. Cd, cadmium; 181i, 181 inhibitor.

Table S1 Primer sequences used for quantitative RT-PCR

Gene	Primer	Sequence 5' to 3'	Product size (bp)	Tm (℃)
IL-1α	F	ATC AGT ACC TCA CGG CTG CT	179	60.5
	R	TGG GTA TCT CAG GCA TCT CC		
IL-1β	F	CTG TCC TGC GTG TTG AAA GA	161	58.4
	R	TTC TGC TTG AGA GGT GCT GA		
IL-6	F	TAC CCC CAG GAG AAG ATT CC	164	60.5
	R	TTT TCT GCC AGT GCC TCT TT		
IL-8	F	GTG CAG TTT TGC CAA GGA GT	186	60.5
	R	CTC TGC ACC CAG TTT TCC TT		
TNFα	F	AAC CTC CTC TCT GCC ATC AA	162	58.4
	R	CCA AAG TAG ACC TGC CCA GA		
COX2	F	TGA GCA TCTACG GTT TGC TG	159	58.4
	R	TGC TTG TCT GGA ACA ACT GC		
SQSTM1	F	GAG TTC CAG CAC AGA GGA GA	210	58.4
	R	AAG ACA GAT GGG TCC AGT CA		
TLR4	F	GAC AAC CAG CCT AAA GTA TT	206	62.1
	R	TGC CAT TGA AAG CAA CTC TG		
PPP3CA	F	GCG CAT CTT ATG AAG GAG GGA	115	61.3
	R	TGA CTG GCG CAT CAA TAT CCA		
PPP3CB	F	CCC CAA CAC ATC GCT TGA CAT	140	61.3
	R	GGC AGC ACC CTC ATT GAT AAT TC		
PPP3CC	F	ACC GCG TCA TCA AAG CTG T	126	57.3
	R	CTT CCA GTC GTC CTT CCT TTA C		
PPP3R1	F	CCT TTG GAA ATG TGC TCA CAC T	137	60.3
	R	GGA TTC TGT TGT AAC TCA GGC AG		
PPP3R2	F	GCA GAG CAC GGA CAG CTA TC	213	62.5
	R	GGG CTT TCT CCA CGA AAA TGA		
PPP4R2	F	ACG AAG GCC CTG TAA GTA GT	129	58.4
	R	CTT CTG TGG CTT CGT CAT CA		
NAFTC1	F	GCA GAG CAC GGA CAG CTA TC	101	62.5
	R	GGG CTT TCT CCA CGA AAA TGA		
NAFTC2	F	GCT GGT TCC GGT GTA CTC G	172	61.7
	R	AGA GAC CAC TCG AAT CTG CCA		
NAFTC3	F	GCT CGA CTT CAA ACT CGT CTT	96	59.4
	R	GAT GCA CAA TCA TCT GGC TCA		
NAFTC4	F	CTT CTC CGA TGC CTC TGA CG	173	62.5
	R	CGG GGC TTG GAC CAT ACA G		
18S rRNA	F	GTA ACC CGT TGA ACC CCA TT	152	58.4
	R	CCA TCC AAT CGG TAG TAG CG		

Table S2 The genes predicted to be down-regulated by miR-181a-2-3p in seven miR-databases

Gene	FC	P value	Relative of expression levels
SFTPC	–0.655767365	3.61E–09	12199.90149
BANF1	–0.460164268	3.36E–14	63.73298496
DYNLRB1	–0.432892943	2.60E–15	146.1455458
TMED3	–0.388070092	9.23E–08	23.74731223
TMEM150A	–0.357928322	2.15E–08	9.946943966
MTX2	–0.351826053	3.71E–10	17.39302484w
POLE4	–0.350029087	2.81E–08	36.00007791
C11orf73	–0.33690663	4.64E–10	14.19046089
CLK4	–0.317810059	1.68E–07	16.32098212
MAP2K5	–0.299291122	1.40E–08	6.535417212
MRPL1	–0.289296308	4.84E–08	13.29145089
RNF220	–0.287288146	4.17E–09	10.75798231
BCCIP	–0.226971026	1.05E–07	25.99758884

FC, fold change.

Table S3 The genes predicted to be up-regulated by miR-181a-2-3p in seven miR-databases

Gene	FC	P value	Relative of expression levels
BCCIP	0.141359679	2.13E–08	30.87231165
YME1L1	0.14681086	1.24E–07	31.3662725
SYNCRIP	0.152373877	1.99E–09	13.2009573
TBC1D14	0.175317547	8.04E–08	6.08602146
ZFYVE26	0.177817115	3.91E–08	7.613957172
KDM5B	0.180621772	1.56E–15	24.675775
MBTPS1	0.190016724	4.42E–07	10.00839376
NFAT5	0.198041404	4.68E–10	13.62896533
TAOK3	0.210266949	1.17E–07	4.626780497
KIAA2026	0.210603089	3.45E–10	3.837854399
SYNJ1	0.218088367	2.58E–07	21.38963896
ZDHHC20	0.218521887	1.81E–09	9.928368397
SNX18	0.220512031	5.61E–11	5.534562161
CAMSAP1	0.223960612	8.10E–11	9.109183396
SETD2	0.231841659	4.28E–09	44.55153945
TACC1	0.232335673	6.99E–08	14.41540211
GOLGA4	0.234400668	2.03E–07	7.844815734
SMG1	0.246868909	3.20E–07	5.789424095
JHDM1D	0.250957948	6.56E–08	7.371499332
STAG1	0.258121637	4.35E–09	10.32410087
PIK3C2A	0.274096929	1.11E–07	7.167154236
TAOK1	0.280821312	1.30E–11	7.478640816
MED1	0.286434652	4.12E–10	8.941804783
MAPK8	0.30084885	9.84E–10	5.967073227
RLIM	0.301335706	1.20E–10	13.72146289
SETD7	0.141359679	2.13E–08	17.96669999
PPP4R2	0.317638925	1.15E–07	30.87231165
CRK	0.323066169	9.06E–16	27.2747069
ADRBK2	0.33185984	5.10E–13	7.138990696
KIAA1549	0.345497662	3.85E–08	0.769423816
KDM5A	0.362528523	1.49E–12	5.729807498
TNRC6B	0.3728883	8.88E–19	4.348090137
VPS13B	0.382865278	2.72E–12	2.740252005
NCOR1	0.384445394	5.15E–10	11.26056239
FBXO48	0.405376008	2.24E–10	0.866248945
C9orf41	0.472268719	1.75E–11	3.66602759
ANTXR2	0.501711831	6.45E–12	12.54870428
TCP11L2	0.508754542	1.32E–10	9.555533445
DOCK5	0.744290207	3.32E–17	4.198296448

FC, fold change.