Knockdown of p54\textsuperscript{nrβ} inhibits migration, invasion and TNF-α release of human acute monocytic leukemia THP1 cells

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Abstract. 54 kDa nuclear RNA- and DNA-binding protein (p54\textsuperscript{nrβ}) which is also called non-POU domain-containing octamer-binding protein (NONO) is known to be multifunctional involved in many nuclear processes. It was shown that p54\textsuperscript{nrβ}/NONO was closely related to the occurrence of erythroleukemia. Whether p54\textsuperscript{nrβ}/NONO plays a role in progress of human acute monocytic leukemia remains unknown. In the present study, we examined the effects of p54\textsuperscript{nrβ} silencing on the biological characteristics of human acute monocytic leukemia THP1 cells. The results showed that p54\textsuperscript{nrβ} was strongly expressed in THP1 cells, and knockdown of p54\textsuperscript{nrβ} slightly promoted proliferation and strongly inhibited motility and invasion of THP1 cells. Moreover, knockdown of p54\textsuperscript{nrβ} strongly decreased the release of TNF-α from THP1 cells by inhibiting certain process of TNF-α secretion, specially for the release of TNF-α induced by lipopolysaccharide (LPS). Notably, the infection of negative control shRNA-containing lentiviruses promoted the migration and the release of TNF-α induced by LPS in THP1 cells. All the above results demonstrated that p54\textsuperscript{nrβ} is a powerful molecule involved in the regulation of cell motility and promotes the migration and invasion of THP1 cells, and it is more likely to be involved in the release of inflammatory mediators and the motility of inflammatory cells.

Introduction

p54\textsuperscript{nrβ}/NONO is known to be involved in a variety of biological events including pre-mRNA splicing, transcriptional regulation (1), nuclear retention of defective RNA (2,3), DNA unwinding and pairing (4), DNA damage repair (5,6). p54\textsuperscript{nrβ}/NONO was recently shown to be a component of a novel nuclear domain termed paraspeckles (7,8).

Paraspeckles are ribonucleoprotein bodies found in the interchromatin space of mammalian cell nuclei. The core paraspeckle proteins contain polypyrimidine tract-binding protein-associated splicing factor (PSF)/splicing factor proline/glutamine-rich (SFPQ), p54\textsuperscript{nrβ}/NONO and paraspeckle protein 1 (PSPC1). These proteins, together with the long non-protein-coding RNA NEAT1 (MEN-ε/β), associate to form paraspeckles (9,10). The core paraspeckle proteins have been shown to bind to both double and single-stranded DNA and RNA, and have been involved in numerous nucleus events including transcription and splicing. The function relevant to paraspeckles is the involvement of PSF/SFPQ and p54\textsuperscript{nrβ}/NONO in the nuclear retention of RNA, specifically preventing A to I hyperedited RNA from leaving the nucleus (11,12). Recent research hints at a more generic retention-release mechanism that exists for transcripts containing hyperedited inverted repeats in their 3’ UTR (12,13). Adenosine-to-inosine conversion (A-to-I editing) contributes to extensive transcriptome diversity (14). Disturbance in RNA editing has been implicated in various pathologic disorders, including cancer. Abnormal A-to-I editing was involved in cancer development (15).

Recent studies suggest that as a core paraspeckle protein and multifunctional protein, p54\textsuperscript{nrβ}/NONO may be implicated in tumor progress and metasstasis. Phosphorylated β-cell differentiation transcription factor HLXB9 promoted insulinoma cell proliferation through interaction with NONO protein (16). Expression of p54\textsuperscript{nrβ} was increased in breast cancer with estrogen receptor (17). The protooncogene Spi-1/PU.1 is involved in the erythroleukemic process via impeding the binding of p54\textsuperscript{nrβ} to RNA and alters the splicing process (18). Acute myeloid leukemia (AML) is a type of heterogeneous disease derived from haematopoietic stem cells. Whether p54\textsuperscript{nrβ}/NONO plays a role in the progress of human acute monocytic leukemia remains unknown. In the present study, we examined the effects of p54\textsuperscript{nrβ} silencing on the proliferation, migration, invasion and TNF-α release of human acute monocytic leukemia THP1 cells.

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Materials and methods

Materials. Lipopolysaccharide (LPS) from *E. coli*, puro- 
mycin, Brefeldin A, propidium iodide (PI), p54nrb/NONO and 
β-actin antibodies, horseradish peroxidase (HRP)-conjugated 
secondary antibodies were purchased from Sigma-Aldrich (St. 
Louis, MO, USA). Proliferating cell nuclear antigen 
(PCNA) and TNF-α antibodies were purchased from Cell 
Signaling Technology Inc. (Beverly, MA, USA). RPMI-1640 
and fetal bovine serum (FBS) were purchased from Gibco- 
Life Technologies (Carlsbad, CA, USA). Matrigel and 8 µm 
pore-sized Transwell chambers were purchased from BD 
Biosciences (San Jose, CA, USA). RT-PCR.

Cell cultures and establishment of p54nrb silencing THP1 cell 
line. THP1 cells were obtained from the Department of 
Biochemistry (Guangdong Medical University, Guang- 
dong, China). THP1 cells were maintained in RPMI-1640 media 
supplemented with 15% FBS and penicillin (100 U/ml) and 
streptomycin (10 µg/ml). p54nrb-silencing THP1 cell line and 
negative control THP1 cell line were established in our labora- 
tory. The cell lines were established as follow: packaged 
growing THP1 cells were infected with p54nrb -specific 
lentiviruses or negative control shRNA lentiviral 
vector Lv3 (H1/GFP&Puro) (sense sequence 5'-UUCUCCG 
CAUUCAUATT-3') or with negative control shRNA- 
expressing GFP lentiviral vector L v3 (H1/ 
GFP&Puro-NONO) (sense sequence 5'-GGCGAAGUCUU 
CGACGTTTGTTTGG-3'). For negative control, 0.5 µg/ml puromycin 
was added to the medium, cells were grown at 37˚C in a 
CO2 incubator for 14 days. The efficiency of p54nrb 
silencing was determined 
Western blot analysis. THP1, THP1-p54nrb-si and 
THP1-NC cells (5x10² cells/well), respectively were seeded in 
6-well culture plates. To test the protein expression of PCNA 
and TNF-α in THP1, THP1-p54nrb-si and THP1-NC cells, the cells 
were collected for western blot analysis. To test the effect of 
p54nrb silencing on expression of TNF-α induced by LPS, the 
experiments were divided into three groups: i) blank group 
untreated; ii) LPS treatment group treated with 5 µg/ml LPS 
for 6 h; iii) BFA and LPS treatment group treated with 5 µg/ 
ml LPS for 6 h and simultaneously protein secretion inhibitor 
Brefeldin A (10 µg/ml) for last 5 h. Western blot analyses 
were performed as follow: the cells were collected and lysed 
with RIPA lyses buffer. Total proteins were subjected to 
SDS-PAGE, and then transferred onto polyvinylidene fluo- 
ride (PVDF) membranes. The membranes were incubated 
with 5% skimmed milk in TBST for 2 h at room temperature. 
After washing with TBST, the membranes were incubated 
overnight at 4˚C with the first antibodies against human p54nrb 
(1:800), PCNA (1:1,000), tumor necrosis factor α 
(TNF-α) (1:1,000) or β-actin (1:1,000), followed by incuba- 
tion with an HRP-conjugated secondary antibodies at room 
temperature for 1 h. Enhanced chemiluminescence (ECL) 
was used to detect the results. The expression of β-actin 
was used as loading control. The relative grey scale intensity was 
quantified using ImageJ2x software. Data are representative 
of three independent experiments.

Cell proliferation assay. Cell proliferation was assessed by 
the Cell Counting kit-8 (CCK-8) assay, in accordance with 
the manufacturer's instructions. Briefly, THP1, THP1-p54nrb-si 
and THP1-NC cells (2x10⁴ cells/well) were seeded in 96-well 
culture plates and cultured for either 48 or 72 h. After 
incubation for 30 min. The cell cycle distributions 
were then analyzed by flow cytometry and the percentage of 
cells in G1/G0, S or G2/M phase was calculated using ModFit 
LT software. Data represent the mean value derived from 
triplicate experiments.

Materials. Lipopolysaccharide (LPS) from *E. coli*, pu- 
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overnight at 4˚C with the first antibodies against human p54nrb 
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temperature for 1 h. Enhanced chemiluminescence (ECL) 
was used to detect the results. The expression of β-actin 
was used as loading control. The relative grey scale intensity was 
quantified using ImageJ2x software. Data are representative 
of three independent experiments.
Cell invasion and motility assay. In vitro invasion assay was performed by using Matrigel-coated Transwell chambers. Pore-sized polycarbonate membranes (8 µm) were coated with Matrigel (100 µg/cm²) and incubated overnight. THP1, THP1-p54nrb-si or THP1-NC cells (1x10^6 cells) suspended in PRMI-1640 medium supplemented with 1% BSA were respectively seeded in upper chamber which was placed over the lower chamber. The PRMI-1640 medium supplemented with 15% FBS in lower chamber was used as a chemoattractant. Invasion was allowed to proceed for 28 h, then the invaded cells in lower chamber were collected and counted. Transwell motility assay was performed similar to the above invasion assay, with the exception that Transwell insert was not coated with Matrigel. Migration was allowed to proceed for 19 h, then the migrated cells in the lower chamber were collected and counted. All the experiments were repeated three times.

TNF-α release and content assay. THP1, THP1-p54nrb-si and THP1-NC cells (5x10^5 cells/well) were seeded in 6-well culture plates, then 5 µg/ml LPS was added per well and incubated for 0, 3, 6 and 9 h. The cells were subsequently centrifuged at 2,000 rpm for 15 min, then TNF-α-containing supernatants were collected and measured with Human TNF-α Immunoassay Valukine™ ELISA kit, in accordance with the manufacturer’s instruction. The assay was conducted in triplicate for each sample and three parallel experiments were performed.

Statistical analysis. The SPSS version 16.0 software package and GraphPad Prism were used for the statistical analysis and data plotting. The data were expressed as mean ± SD. ANOVA was carried out followed by the Student-Newman-Keuls and LSD tests. P<0.05 was considered to indicate a statistically significant result.

Results

Expression of p54nrb mRNA and protein in THP1, THP1-p54nrb-si and THP1-NC cell lines. To test the interference efficiency of p54nrb-specific shRNA-containing lentiviruses, THP1 cells, THP1-p54nrb-si and THP1-NC cells were harvested. The expression levels of p54nrb mRNA were assessed by semi-quantitative PCR following reverse transcription of the RNA. β-actin was used as loading control. Western blotting was used to detected p54nrb protein expression in THP1, THP1-p54nrb-si and THP1-NC cells. β-actin was used as loading control. The gray values of p54nrb mRNA were determined by ImageJ2x software and normalized with the gray value of β-actin. Data are presented from three repeated test. ***P<0.01 vs. wild-type and negative control THP1 cells.

Knockdown of p54nrb promotes THP1 cell proliferation. The effect of p54nrb silencing on THP1 cell cycle distribution was analyzed by flow cytometric assay. As shown in Fig. 2A and B, THP1-p54nrb-si cells showed a decrease of cells in G0/G1 phase, and an increase of cells in the S and G2/M phases compared with wild-type or negative control THP1 cells.
Knockdown of p54nrb inhibits migration, invasion and TNF-α release of THP1 cells.

To detect the effect of p54nrb silencing on THP1 cell proliferation, CCK-8 assay was performed. As shown in Fig. 2A, the percentage of cells in G0/G1, S, or G2/M phase significantly increased in p54nrb-silencing THP1 cells compared with wild-type or negative control THP1 cells. Moreover, expression of PCNA, a marker of cell proliferation, was increased in p54nrb-silencing THP1 cells compared with wild-type or negative control THP1 cells (Fig. 2D and E). These data demonstrated that p54nrb silencing slightly promoted the proliferation of THP1 cells.

Knockdown of p54nrb inhibits the invasion and motility of THP1 cells. To detect the effect of p54nrb silencing on THP1 cell motility, Transwell assay was performed. As shown in Fig. 3A and C, the migration of THP1-p54nrb-si cells was significantly reduced compared with wild-type and negative control THP1 cells. Similar effect was observed for the invasion assay. Matrigel-coated Transwell chambers were used to measure the effect of p54nrb silencing on THP1 cell invasion. The result showed that the invasion of THP1-p54nrb-si cells was significantly reduced compared with wild-type and negative control THP1 cells (Fig. 3B and D). Above results showed that knockdown of p54nrb significantly inhibited the invasion and migration of THP1 cells. The result also displayed that THP1-NC cells infected negative control shRNA lentiviruses displayed stronger migration than wild-type THP1 cells (Fig. 3A and C).

Knockdown of p54nrb inhibits the release of TNF-α induced by LPS from THP1 cells. The effect of p54nrb knockdown on the release of TNF-α induced by LPS from THP1 cells was measured with Human TNF-α Immunoassay Valukine™ ELISA kit. As shown in Fig. 4A, the content of TNF-α in supernatant of THP1-p54nrb-si cells with or without LPS treatment significantly decreased compared with THP1 or THP1-NC cells, which indicated that knockdown of p54nrb significantly inhibited the release of TNF-α from THP1 cells, especially for the release of TNF-α induced by LPS. Moreover, we...
found that content of TNF-α in supernatant of THP1-NC cells treated with LPS was remarkably higher than that of wild-type THP1 cells, which indicated that infection of negative control lentiviruses promoted the release of TNF-α induced by LPS from THP1 cells.

In order to explore the mechanism involving in reduction of TNF-α release in p54 nrb silencing THP1 cells, we tested the expression of TNF-α protein in THP1, THP1-p54 nrb-si and THP1-NC cells. Western blot analysis showed that the protein level of TNF-α was similar in THP1, THP1-p54 nrb-si and THP1-NC cells without treatment. After LPS treatment, the protein level of TNF-α in THP1-p54 nrb-si cells was significantly higher than that of THP1 and THP1-NC cells. TNF-α can be released from THP1 cells when THP1 cells are stimulated by LPS. Therefore, the protein level of TNF-α was monitored after different THP1 cells were simultaneously treated with LPS and protein secretion inhibitor Brefeldin A. As shown in Fig. 4B, the protein level of TNF-α was similar in THP1-p54 nrb-si and THP1-NC cells upon simultaneously treated with LPS and Brefeldin A. The results demonstrated that knockdown of p54 nrb decreased the content of TNF-α in culture supernatant by inhibiting certain process of TNF-α secretion from THP1-p54 nrb-si cells, instead of effecting the TNF-α protein expression.

Discussion

AML is a heterogeneous disease derived from haematopoietic stem cells. To identify new genes involved in tumor progress is important for the diagnosis and treatment of AML (19). In the present study, we analyzed the effects of p54 nrb silencing on the biological characteristics of human acute monocytic leukemia THP1 cells.

Cell cycle and cell proliferation assay showed that knockdown of p54 nrb slightly promoted proliferation, and western blotting displayed that knockdown of p54 nrb increased the expression of PCNA protein that is a marker of cell proliferation (20). The above results indicated that knockdown of p54 nrb slightly promoted the proliferation of THP1 cells. As a core paraspeckle protein and multifunctional protein, p54 nrb/NONO was reported to be implicated in tumor progress and metastasis. Previous research demonstrated that p54 nrb played diverse roles in cell proliferation. Knockdown of p54 nrb in melanoma cell lines led to reduced proliferation rates (21).
β-cell differentiation transcription factor HLXB9 promoted insulinoma cell proliferation through interaction with NONO protein (16). Spi-1/PU.1 blocks the differentiation of proerythroblast and promotes their malignant transformation in the Friend erythroleukemia, and Spi-1/PU.1 might be involved in leukemogenesis via impeding the binding of p54nrb to RNA and alters the splicing process (18). NONO bound to the p16-Ink4A cell cycle checkpoint gene and potentiated its circadian activation. Loss of NONO abolished this activation and circadian expression of p16-Ink4A and eliminated ciricadian cell cycle gating. Fibroblasts from NONO gene-trapped mice showed increased proliferation and decreased senescence (22). Our results displayed that knockdown of p54nrb slightly promoted proliferation of THP1 cells, human umbilical vein endothelial cells (HUVEC). Various research demonstrated that p54nrb promotes or inhibits cell proliferation in different tumor cells. These alterations may be responsible for functional diversity of p54nrb protein including mRNA splicing and transcription. As a core paraspeckle protein, p54nrb/NONO involved in the nuclear retention of RNA, specifically for A to I hyper-edited RNA (11,12). The common dysregulation of A-to-I editing in human cancers may contribute to the altered transcriptional proteins.

Cell motility and invasion assay showed that knockdown of p54nrb significantly inhibited the motility and invasion of THP1 cells. p54nrb/NONO shows diverse roles in cell proliferation in different tumor cells and research, but its role in migration was very consistent and obvious. Knockdown of p54nrb strongly decreased the motility and invasion of THP1, HUVEC, SKBR-3 and CNE2 cells (partial data not shown). All these studies showed that p54nrb is a powerful molecule involving in the regulation of cell motility. To date, only a few reports on the mechanism of p54nrb participating in cell motility have been reported. Fibroblast growth factor 1 (FGF1) functions as a modifier of endothelial cell migration and proliferation. Heterogeneous nuclear ribonucleoprotein M (hnRNPM) and p54nrb present in protein complexes bound to the FGF1 promoter and to the mRNA internal ribosome entry site. Knockdown of either p54nrb or hnRNPM blocks endogenous FGF1 induction and myotube formation (28). Angiopoietin-1 (Ang1) regulates angiogenesis as a ligand of Tie 2 receptor tyrosine kinase. p54nrb was the tyrosine phosphorylated protein in Ang-1 induced signaling pathway in HUVEC cells. p54nrb was validated as a molecule involved in cell migration of HUVEC cells, which can be supported by the results obtained from microarray analysis: overexpression of p54nrb significantly upregulated the genes involved in cell motility and structural functions in HUVEC cells (data not shown) (27). In vivo, NONO gene-trapped mice showed defective wound repair. Considering the above results, we speculate that p54nrb might be a key molecule involving in cell motility via effecting the expression or function of certain cytoskeleton proteins.

THP1 cells are often used as an inflammatory cell model in the study of inflammation and some leukemia is relative to infection. Therefore, we also analyzed the effect of p54nrb silencing on the release and expression of inflammatory mediator TNF-α in THP1 cells. The results indicated that knockdown of p54nrb had no effect on the expression of TNF-α protein, but significantly inhibited the release of TNF-α from THP1 cells, especially for the release of TNF-α induced by LPS, the mechanism of which is still obscure. The secretion of TNF-α from THP1 cells is an exocytosis process which depends on microtubules (29-31). Furthermore, knockdown of p54nrb blocks endogenous FGF1 induction and myotube formation (28). Overexpression of p54nrb significantly upregulated the genes involved in cell motility and structural functions in HUVEC cells (27). Thus, we speculate that knockdown of p54nrb might inhibit the release of TNF-α from THP1 cells via effecting the exocytosis of TNF-α which depends on cytoskeleton protein microtubules. Cell movement and organelle transport all depends on the activities of cytoskeleton proteins. There may

Figure 4. Effect of p54nrb silencing on the release and expression of TNF-α induced by LPS in THP1 cells. (A) Knockdown of p54nrb inhibited the release of TNF-α induced by LPS from THP1 cells. THP1, THP1-p54nrb-si and THP1-NC cells respectively were incubated with 5 µg/ml LPS for 0, 3, 6 and 9 h, then the supernatants were collected and the content of TNF-α in supernatant was measured. The assay was conducted in triplicate for each sample and three parallel experiments were performed. "P<0.001 vs. wild-type and +LPS (## P<0.01 vs. wild-type THP1 cells; •P<0.05 vs. wild-type and negative control THP1 cells. (B) Effect of p54nrb silencing on expression of TNF-α induced by LPS in THP1 cells. THP1, THP1-p54nrb-si and THP1-NC cells, respectively were seeded in 6-well culture plates. Experiments were divided into three groups: i) blank group untreated; ii) LPS treatment group treated with 5 µg/ml LPS for 6 h; iii) BFA and LPS treatment group treated with 5 µg/ml LPS for 6 h and simultaneously protein secretion inhibitor Brefeldin A (10 µg/ml) for the last 5 h. The protein expression of TNF-α or p54nrb in cells was examined by western blotting. β-actin was used as loading control.

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be some connection between p54\textsuperscript{nr}b silencing inhibiting the motility of THP1 cells and the release of TNF-\textalpha{} from THP1 cells. Moreover, the infection of negative control shRNA-containing lentiviruses promoted the migration and the release of TNF-\textalpha{} induced by LPS in THP1 cells. We speculate that increase of the migration and the release of TNF- \textalpha{} in THP1 cells infected by control vector lentiviruses is a defensive reaction of THP1 cells to viruses.

Based on the present study, it is suggest that p54\textsuperscript{nr}b slightly inhibited THP1 cell proliferation, but significantly promoted migration, invasion and release of TNF-\textalpha{}. Our and other recent studies indicate that p54\textsuperscript{nr}b may be a powerful molecule involving in the regulation of cell motility, and it may promote metastasis, invasion of tumor cells and angiogenesis. p54\textsuperscript{nr}b is more likely to promote the release of inflammatory mediators and the motility of inflammatory cells. The occurrence of many tumors is closely related to infection and inflammation (32), and we also speculate involvement in tumor processes and inflammation.

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