Brahma-related gene 1 induces apoptosis in a p53-dependent manner in human rheumatoid fibroblast-like synoviocyte MH7A

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Abstract
Blocked apoptosis and aggressive inflammatory responses occur in fibroblast-like synoviocyte (FLS) of rheumatoid arthritis (RA) patients. Although Brahma-related gene 1 (BRG1) is considered as a tumor suppressor, few research covers its role in RA. This study aims to reveal effects and potential mechanisms of BRG1 in human FLS cell line MH7A.

BRG1 expression in MH7A cells was altered by transfection of overexpression vectors or short hairpin RNAs (shRNAs). Cell viability and apoptosis were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry after transfection. Factors involved in inflammation and apoptosis were quantified by qPCR and Western blot. The interaction between BRG1 and p53 was assessed by immunoprecipitation (IP).

Results showed that BRG1 overexpression significantly suppressed MH7A cell viability and induced apoptosis (P < 0.01), and its knockdown had opposite effects. BRG1 reduced mRNA levels of matrix metallopeptidase 3, TIMP metallopeptidase inhibitor 2, cyclooxygenase 2, and interleukin 6, implying its suppressive effects on inflammation. BRG1 interacted with and promoted p53 knockdown (P < 0.05). B-cell chronic lymphocytic leukemia/lymphoma 2 was suppressed (P < 0.05), while cytochrome c, caspase 3 (CASP3) and CASP9 were activated (P < 0.01) by BRG1. However, the regulation on these factors was abrogated by p53 knockdown (P < 0.01).

These findings suggest that BRG1 may induce apoptosis and suppress inflammation in MH7A cells. Potential functional mechanisms involve the regulation of apoptotic factors by BRG1, which may depend on the recruitment and promotion of p53. This study provides the essential proof for applying BRG1 to the molecular therapy of RA.

Abbreviations: ANOVA = analysis of variance, BCL2 = B-cell chronic lymphocytic leukemia/lymphoma 2, BRG1 = Brahma-related gene 1, CASP3 = caspase 3, CASP9 = caspase 9, COX2 = cyclooxygenase 2, CYCS = cytochrome c, ECL = emitter-coupled logic, FBS = fetal bovine serum, FITC = fluorescein isothiocyanate, FLS = fibroblast-like synoviocyte, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, IgG = immunoglobulin G, IL6 = interleukin, IP = immunoprecipitation, MDM2 = murine double minute 2, MMPs = matrix metallopeptidases, MSCs = mesenchymal stem cells, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PB = phosphate-buffered saline, PI = propidium iodide, RA = rheumatoid arthritis, RIP = radioimmunoprecipitation assay, RPMI = Roswell Park Memorial Institute, shRNAs = short hairpin RNAs, SPSS = Statistic Package for Social Science, TUN = TEM = tissue inhibitor of metalloproteases.

Keywords: apoptosis, Brahma-related gene 1, fibroblast-like synoviocyte, p53, rheumatoid arthritis

1. Introduction
Rheumatoid arthritis (RA) is a common chronic disease affecting nearly 0.5% of the adults worldwide, especially in female and elderly patients. RA is characterized by the aggravated inflammation and hyperplasia in the fibroblast-like synoviocyte (FLS), which is a major constituent of the synovium. RA may eventually lead to systemic disorders including cardiovascular, pulmonary diseases, and tumor. Therapists are actively exploring potential targets for relieving the symptoms of RA, and have tried biological disease-modifying antirheumatic drugs such as tumor necrosis factor inhibitors in clinical experiments, nevertheless, the side-effect should not be neglected.

Significant changes happened in the FLS of RA patients, for instance, the elevated expression of disease-related cytokines and chemokines, among which matrix metallopeptidases (MMPs) and TIMP metallopeptidase inhibitors (TIMPs) are frequently reported in studies on RA. Besides, the uncontrolled proliferation of the FLS also exacerbates RA, thus researchers were striving to screen suitable proliferation inhibitors. Tumor protein p53, an important cell growth regulator, is emerging as a pivotal factor for controlling FLS apoptosis, proliferation, and invasion. Thereby factors modulating p53 offer promising alternatives or targets to suppress FLS proliferation and alleviating RA.

Brahma-related gene 1 (BRG1), a chromatin remodeling factor, encodes a subunit of the SWI/SNF complex and plays significant regulatory roles in the transcription of various
genes. It is revealed to be a tumor suppressor in some diseases, primary melanomas for instance. Importantly, BRG1 has been reported to interact with p53 to induce p21 level, thus executing the tumor suppressor function in breast cancer MCF7 and lung cancer H1299 cells. Given the pro-apoptotic nature of p53 in FLS, it stands a good chance that BRG1 may affect capacities of FLS. However, the role of BRG1 in FLS remains unclear.

This study aims to reveal the function of BRG1 in FLS and the potential mechanism. In human FLS cell line MH7A, BRG1 expression was altered by transfection with its overexpression vector or specific short hairpin RNA (shRNA), and then cell viability, apoptosis and expression of inflammatory factors were quantified. We also assessed whether p53 and its downstream apoptotic factors were involved in affecting MH7A apoptosis. These investigations will enrich the understanding of RA mechanisms and provide new therapeutic strategy for treating RA.

2. Materials and methods

2.1. Cell culture

Human rheumatoid FLS MH7A cells (Jennio Biological Technology, Guangzhou, China) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin (Gibco, Carlsbad, CA). The cells were incubated in humidified atmosphere with 5% (volume ratio) CO₂ at 37°C. The culture medium was changed every other day, and the cells were passaged when the confluence reached 80%.

2.2. Cell transfection

Overexpression vectors of BRG1 and shRNA vectors of BRG1 or p53 were transfected into MH7A cells, respectively, to alter expression levels of BRG1 or p53. The complete coding sequence of human BRG1 (GenBank U29175) was ligated to pcDNA3.1 overexpression vector (Thermo Scientific, Carlsbad, CA) and the correct clones were screened by sequencing. The shRNA vectors (sh-BRG1 and sh-p53) were synthesized by Ribobio (Guangzhou, China).

Cell transfection was conducted in 24-well plates with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. The cells (2 × 10^5/well) were precultured in antibiotic- and FBS-free medium to reach a confluence of 90%. In each well, 0.8 µg vectors together with the transfection complexes were added, and then the plates were incubated at 37°C. The medium was changed at 6 hours post-transfection, and thereafter, cells were collected at different time points for further analyses. Blank vectors were transfected as the control.

2.3. Cell viability assay

Cell viability changes after transfection were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method using MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Shanghai, China). Transfected cells were seeded in 96-well plates (2 × 10^3 cells/well), and then 10 µL MTT solution was added to each well. After incubation at 37°C for 4 hours, 100 µL Formazan solution was added and the plates were incubated until all crystals were dissolved. Optical density was measured at 570 nm by a microplate reader Multiskan Go (Thermo Scientific).

2.4. Cell apoptosis assay

Cell apoptosis was detected using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Kit (Biovision, Milpitas, CA) at 48 hours post-transfection. Cells (2 × 10^5) for each reaction were washed in phosphate-buffered saline (PBS) for 2 times, after which 100 µL Binding Buffer and 2 µL Annexin-V FITC (20 µg/µL) were added, and then the cells were incubated in the dark on ice for 15 minutes. After the incubation, 400 µL PBS and 1 µL propidium iodide (PI) were added and the cells were immediately analyzed by flow cytometry BD FACSCalibur (BD Biosciences, San Jose, CA). Cells in the lower right quadrant (FITC positive and PI negative) were considered to be apoptotic cells.

2.5. Immunoprecipitation (IP)

IP was performed to detect the interaction between BRG1 and p53 proteins in MH7A cells at 48 hours post-transfection using Pierce Classic IP Kit (Thermo Scientific) according to the manufacturer’s instruction. Briefly, cells were washed in cold PBS for 2 times and incubated in cold lysis buffer on ice for 5 minutes, after which they were centrifuged and the supernatant was collected. Anti-BRG1 or anti-p53 antibodies (ab110641, ab31333, Abcam, Cambridge, UK) were incubated with magnetic beads for 1 hours at room temperature and then the beads were collected and mixed with the cell lysate. Proteins on the beads were eluted and detected with anti-p53 or anti-BRG1 antibodies, respectively, based on Western blot procedures.

2.6. Western blot

The protein sample of cells was extracted with radioimmuno-precipitation assay (RIPA) Lysis Buffer (Beyotime) according to the manufacturer’s instruction at 48 hours post-transfection, and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins on the gel were blotted to a polyvinylidene fluoride membrane, which was then blocked in 5% skim milk for 2 hours. The blot was incubated in the specific primary antibodies anti-BRG1, p53, proto-oncogene MDM2 (ab178938), cytochrome c (CYCS, ab133504), B-cell chronic lymphocytic leukemia (CLL)/lymphoma 2 (BCL2, ab32124), pro-caspase 3 (pro-CASP3, ab32150), cleaved CASP9 (ab32042), pro-CASP9 (ab69514), and cleaved CASP9 (ab2324, 1:1000, Abcam) overnight at 4°C. Anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (ab9485) was used as an internal control. Then the membrane was washed in PBS for 3 times (5 minutes each) and incubated in goat anti-rabbit secondary antibodies (house radish peroxidase-conjugated, 1:2000, and ab6721) for 1 hour at room temperature. Positive signals were developed by ECL (Emitter-Coupled Logic) plus Western Blotting Substrate (Thermo Scientific) and quantified by ImageJ 1.49 (National Institutes of Health, Bethesda, MD).

2.7. Quantitative polymerase chain reaction (qPCR)

The quantification of mRNAs in MH7A cells were conducted at 48 hours post-transfection by qPCR after total RNA extraction and reverse transcription. RNA was extracted with TRizol (Invitrogen) and purified by DNase I (Invitrogen), and then
reverse transcription was conducted using 1 μg RNA for each sample under the catalysis of SuperScript III Reverse Transcriptase (Invitrogen). qPCR was performed on QuantStudio 6 Flex Realtime PCR system (Applied Biosystems, Carlsbad, CA) with specific primers for BRG1 (Fw: 5′-GCTCA AGGCC ATCGA GGAG-3′ and Rv: 5′-GGTGA AGACC GACTG CAAGA-3′), MMP3 (Fw: 5′-ACAAAG GGATCA CAAACA GGAG-3′ and Rv: 5′-CATTG ACAGAG CACAC A-3′), TIMP2 (5′-ACATT TATGGA CCAACA CTATG AA-3′ and Rv: 5′-TCAGG CCCTTG AGAC ATCTT TA-3′), cyclooxygenase 2 (COX2, 5′-TTTCCA TGTGAT CAGAG CAGGC AGAT-3′ and Rv: 5′-GATG CATGT GACCA TAGAC TGCT-3′), interleukin 6 (IL6, 5′-CTCTCA CCTCTC CAAACA AAGAT-3′ and Rv: 5′-CGCTC AGACAA TTCTCC ATGG-3′) or p53 (5′-ACGAG GTTGA CGACGG TTCCC TG-3′ and Rv: 5′-CCGCT GTGATC TACGG C GCCG TC-3′) in each reaction system. Data were calculated with the 2−ΔΔCt method normalized by GAPDH (Fw: 5′-GAAGG TGAAG GTCGG AGTC-3′ and Rv: 5′-CCTCA CCCTC CAACA AAGAT-3′).

### 2.8. Statistical analysis

All the experiments were repeated in triplicate. Results were expressed as mean ± standard deviation. Statistical Package for Social Science (SPSS) 20 was used to analyze the data by one-way analysis of variance (ANOVA) and Student’s t test. P < 0.05 was considered to be significant.

### 3. Results

#### 3.1. BRG1 promotes apoptosis and inhibits inflammatory factors in MH7A cells

BRG1 was overexpressed or knocked down in MH7A cells to assess its effects. BRG1 mRNA was significantly up-regulated by the overexpression vector (P < 0.01, Fig. 1A) and markedly down-regulated by its specific shRNA (P < 0.001). Besides, its protein level showed the similar changes in the transfected cells (Fig. 1B), suggesting the successful alteration of BRG1 level in MH7A cells. Thus, these transfected cells were valid for the following analyses.

Cell viability was assessed by MTT assay in the transfected cells, and results showed that MH7A cell viability was significantly suppressed by BRG1 overexpression when detected at 1, 2, and 3 days post-transfection (P < 0.01, Fig. 2A), but was obviously elevated by BRG1 knockdown at 2 and 3 days post-transfection (P < 0.05), suggesting BRG1 might inhibit MH7A cell viability. Then cell apoptosis was detected by flow cytometry at 48 hours post-transfection. BRG1 overexpression obviously elevated MH7A cell apoptosis (P < 0.01, Fig. 2B), and correspondingly, its knockdown suppressed cell apoptosis (P < 0.001). Thus BRG1 might promote MH7A cell apoptosis.

Since aggravated inflammation is a major feature of RA, inflammatory factors including MMP3, TIMP2, COX2, and IL6 were also detected in the transfected cells. qPCR indicated that BRG1 overexpression led to significant mRNA reductions in all the four factors (P < 0.01 or P < 0.001, Fig. 2C), implying the anti-inflammatory effect of BRG1 in MH7A cells.

#### 3.2. BRG1 interacts with and promotes p53

Based on established studies that BRG1 is a pivotal modulator of p53,15 the relationship between BRG1 and p53 was analyzed in MH7A cells in this study. Results showed that BRG1 overexpression induced the up-regulation of both p53 mRNA (P < 0.05, Fig. 3A) and protein levels. Thus p53 was positively regulated by BRG1.

Next we performed IP in MH7A cell lysates to test the possibility that BRG1 interacted with p53. When using beads incubated with the anti-p53 antibody, BRG1 could be detected in the immunoprecipitates (Fig. 3C), suggesting that p53 could interact with BRG1 in the cell lysate. Similar results were detected when using beads incubated with the anti-BRG1 antibody. Meanwhile, the positive control (input) and the negative control (immunoglobulin G, IgG) showed expected results. Taken together, BRG1 could interact with p53 in MH7A cells.

#### 3.3. BRG1 regulates apoptotic factors in a p53-dependent manner

We further detected the expression changes of p53 downstream apoptotic factors in order to reveal possible mechanisms of BRG1 in regulating MH7A cell apoptosis. Western blot results indicated that BRG1 overexpression up-regulated p53 protein level, as well as a series of downstream apoptotic factors, including CYCS and the proportion of cleaved CASP3 and cleaved CASP9 (Fig. 4A). The anti-apoptotic BCL2 was suppressed. Significant differences were detected between groups (P < 0.05, 0.01, or 0.001, Fig. 4B).

However, when p53 was knocked down by the shRNA, the regulation of BRG1 on these factors were abrogated (P < 0.05 or...
suggesting the necessity of p53 in the regulation of these apoptotic factors by BRG1.

4. Discussion

As a key modulator of gene expression in multiple diseases, BRG1 is rarely studied in the pathogenesis of RA or the capacities of FLS. Through detection in cell viability, apoptosis and factor levels after transfection, this study found that BRG1 overexpression could repress viability and promote apoptosis of human FLS MH7A cells. BRG1 not only inhibited the expression of inflammatory factors MMP3, TIMP2, COX2, and IL6, but also interacted with p53 protein as revealed by IP. Cytosates were immunoprecipitated with anti-p53 antibody (IP-p53) or anti-BRG1 antibody (IP-BRG1) and then BRG1 or p53 was detected by Western blot. Input, cell lysates without IP process is set as a positive control. IgG, IP with anti-immunoglobulin G (IgG) is set as a negative control. BRG1 = Brahma-related gene 1, IP = immunoprecipitation, MDM2 = murine double minute 2.

Some studies maintain that BRG1 is a tumor suppressive factor that plays pro-apoptotic roles in cancer cells. Indeed, BRG1

Figure 2. Effects of Brahma-related gene 1 (BRG1) on viability, apoptosis, and inflammatory factor expression in rheumatoid fibroblast-like synoviocyte MH7A cells. MH7A cells were transfected with pcDNA3.1-BRG1 vector to overexpress BRG1 or short hairpin RNA (shRNA) vector of BRG1 (sh-BRG1) to knock down BRG1. Blank vectors pcDNA3.1 and sh-control were transfected in the corresponding control group. (A) BRG1 inhibits cell viability as indicated by optical density (OD) at 570 nm. MTT was performed to detect cell viability at 0, 1, 2, and 3 d post-transfection. (B) BRG1 increases percent of apoptotic cells as quantified by flow cytometry at 48 h post-transfection. (C) BRG1 suppresses the mRNA level of inflammatory factors matrix metalloproteinase 3 (MMP3), TIMP metalloproteinase inhibitor 2 (TIMP2), cyclooxygenase 2 (COX2), and interleukin 6 (IL6) as revealed by qPCR at 48 h post-transfection. P values are indicated. BRG1 = Brahma-related gene 1, COX2 = cyclooxygenase 2, IL6 = interleukin 6, MDM2 = matrix metalloproteinase 3, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, OD = optical density, qPCR = quantitative polymerase chain reaction, shRNA = short hairpin RNA, TIMP2 = TIMP metalloproteinase inhibitor 2.

Figure 3. Brahma-related gene 1 (BRG1) interacts with p53 and promotes p53 expression in rheumatoid fibroblast-like synoviocyte MH7A cells. MH7A cells were transfected with pcDNA3.1-BRG1 vector to overexpress BRG1. Blank vector pcDNA3.1 was transfected as a control. qPCR, immunoprecipitation (IP) and Western blot were performed at 48 h post-transfection, with GAPDH as an internal control. (A) BRG1 overexpression promotes p53 mRNA and inhibits proto-oncogene MDM2 mRNA as shown by qPCR results. P values are indicated. (B) BRG1 overexpression promotes protein levels of p53 and MDM2 as shown by Western blot. (C) BRG1 interacts with p53 protein as revealed by IP. Cell lysates were immunoprecipitated with anti-p53 antibody (IP-p53) or anti-BRG1 antibody (IP-BRG1) and then BRG1 or p53 was detected by Western blot. Input, cell lysates without IP process is set as a positive control. IgG, IP with anti-immunoglobulin G (IgG) is set as a negative control. BRG1 = Brahma-related gene 1, IP = immunoprecipitation, MDM2 = murine double minute 2.

Figure 4. Regulation of apoptotic factors by BRG1 depends on p53. Rheumatoid fibroblast-like synoviocyte MH7A cells were transfected with pcDNA3.1-BRG1 vector to overexpress BRG1, or the short hairpin RNA for p53 (sh-p53) to knockdown p53. Western blot was performed at 48 h post-transfection. (A) Western blot showing that apoptotic factors cytochrome c (CYCS), cleaved caspase 3 (cleaved-CASP3), cleaved-CASP9, and B-cell chronic lymphocytic leukemia/lymphoma 2 (BCL2) were regulated by BRG1, but the regulation was suppressed when p53 was knocked down. (B) Relative protein levels calculated based on the band density in Western blot results. P values are indicated. BCL2 = B-cell chronic lymphocytic leukemia/lymphoma 2, BRG1 = Brahma-related gene 1, cleaved-CASP3 = cleaved caspase 3, CYCS = cytochrome c.
overexpression induces cell cycle arrest and apoptosis in rat mesenchymal stem cells (MSCs), and accelerates apoptosis and inhibits proliferation in human aortic smooth muscle cells. Similarly in this study, up-regulation of BRG1 by the overexpression vector suppressed MH7A cell viability and promoted cell apoptosis, and knockdown of BRG1 by shRNA showed the opposite changes, suggesting that BRG1 has pro-apoptotic roles in FLS MH7A cells, which is consistent with former studies. This phenomenon implies the potential regulatory function of BRG1 in RA.

Because of the aggravated inflammatory responses in RA, factors involved in inflammation, MMP3, TIMP2, COX2, and IL6 were detected in the transfected MH7A cells, and results showed that all the four factors were significantly down-regulated by BRG1 overexpression. COX2 and IL6 are crucial inflammatory factors that are induced by stimuli and participate in inflammatory responses. MMPs are capable of degrading extracellular matrix proteins and involved in multiple pathological processes including RA. Moreover, TIMP2 is also closely related to RA progression, with its antibodies detected in a higher frequency in RA patients. Since BRG1 has been reported regulating various inflammatory factors, there is a possibility that BRG1 regulates MMP3, TIMP2, COX2, or IL6, which was supported by the qPCR results of this study. Thus, BRG1 inhibits these inflammatory factors in MH7A cells, which may imply its potential anti-inflammatory effects in FLS of RA patients.

BRG1 overexpression leads to cell cycle arrest and cell apoptosis in rat MSCs, which depends on its activating the p53 pathway. Research in cancer cells has indicated that both exogenous and endogenous BRG1 is able to interact with p53 and improve p53 activity to regulate downstream factors. Besides, some BRG1-associated proteins such as 58-kDa microspherule protein is also in the position to regulate the p53 pathway. In this study, p53 mRNA and protein levels were elevated by BRG1 overexpression, suggesting the promotive modulation by BRG1. Consistently, the expression of MDM2, which is an antagonist of p53, was suppressed by BRG1, further suggesting the promotive effects of BRG1 on p53. Moreover, IP results suggested that BRG1 could interact with p53, perhaps indirectly, thus it is possible that BRG1 induces p53 expression and recruits p53 to execute the regulation on MH7A cell apoptosis.

This study further tried to describe potential mechanisms of BRG1 in regulating MH7A apoptosis via detecting apoptotic factors related to p53. Overexpression of BRG1 elevated CYCS, activated CASP3 and CAS9P9, and inhibited BCL2, while these effects were abrogated when p53 was knocked down. Previous reports have shown that p53 is up-regulated while BCL2 is down-regulated to induce CYCS release from the mitochondrion, which further activates CASP9 and CASP3 to encourage cell apoptosis. The regulation of these factors by p53 may be directly or indirectly dependent on the transcription-regulatory function of p53. The modulated apoptotic factors by BRG1 and the BRG1-induced MH7A apoptosis found in this study imply one possible mechanism of BRG1 in regulating MH7A apoptosis. In addition, p53 knockdown impaired the effects of BRG1, suggesting that BRG1 regulates these apoptotic factors in a p53-dependent manner. Taken together, BRG1 overexpression may interact with p53 and promote p53 level. As an important transcription factor, p53 regulates the transcription of its targets, some of which are apoptotic genes, and further induces MH7A cell apoptosis (Fig. 5).

Since quite a few studies discovered the promotive roles of BRG1 in cell proliferation and inflammation, and regulatory disparities of BRG1 in affecting p53 and other related factors, it is reasonable to speculate that BRG1 may affect different predominant pathways in different cell types. In MH7A cells overexpressing BRG1, the recruitment of p53 may generate more pronounced effects than the regulation of other factors by BRG1. Multiple pathways involved in the BRG1 functional mechanism require more investigation, which will be necessary for the future therapeutic application of BRG1 in RA.

To sum up, this study indicates that BRG1 induces apoptosis and suppresses inflammatory factor expression in MH7A cells. The pro-apoptotic role of BRG1 may be related to its regulation on apoptotic factors, which relies on its recruitment and promotion of p53. These results provide fundamental evidence for the potential application of BRG1 in treating RA.

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