Role of SNF5 in rheumatoid arthritis by upregulation of p16 and inactivation of JNK pathway

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Abstract: This study aims to explore the role and the possible underlying molecular mechanism of SNF5 in the pathogenesis of rheumatoid arthritis (RA). MH7A cells were respectively transfected with pc–SNF5 (pcDNA3.1 containing the Brg1 coding sequence), short hairpin RNA against SNF5 (shSNF5), and their negative controls (pcDNA3.1 and shNC). The alterations of SNF5 expression were assessed by qRT-PCR and western blot analysis. MTT assay, flow cytometry, and western blot analysis were performed to evaluate proliferation, apoptosis, and expression levels of p16 and JNK pathway associated proteins, respectively. Finally, the effect of SNF5 was verified in fibroblast-like synoviocytes (FLSs) obtained from a rat model with adjuvant-induced arthritis. Results showed that the expression of SNF5 was increased in the pc–SNF5 group (P < 0.05) while it was decreased in the shSNF5 group (P < 0.05). Afterwards, cell viability after transfection was reduced by SNF5 overexpression (P < 0.05, P < 0.01, or P < 0.001), whereas it was enhanced by SNF5 knockdown (P < 0.05 or P < 0.001). In terms of apoptosis, SNF5 overexpression promoted cell apoptosis (P < 0.01). The western blot analysis showed that the phosphorylated levels of proteins involved in the JNK pathway were downregulated by SNF5 overexpression while they were upregulated by SNF5 knockdown (P < 0.05, P < 0.01, or P < 0.001). However, the effect of SNF5 on the expression of p16 was the opposite. Finally, the effect of SNF5 was validated in murine FLSs. In conclusion, SNF5 suppresses proliferation and induces apoptosis of fibroblast-like cells through overexpression of p16 and suppression of the JNK pathway.

Key words: SNF5, SWI/sucrose nonfermentable, rheumatoid arthritis, p16, JNK pathway

1. Introduction
Eukaryotic cells respond to external stimuli through expression of a particular subset of genes in a coordinated fashion and modulation of an inactivated chromatin state (Sansam et al., 2005; Ramirez-Carrozzz et al., 2006; Clapier and Cairns, 2009; Euskirchen et al., 2011; Narlikar et al., 2013; Kadoch and Crabtree, 2015). The chromatin state is altered by several factors, including SWI/sucrose nonfermentable (SWI/SNF) which was first discovered in Saccharomyces cerevisiae (Clapier and Cairns, 2009; Narlikar et al., 2013). In mammals, the core complex of SWI/SNF consists of Brm/SNF2α, Brg1/SNF2β, SNF5/IN1/BAF47, and SWI3/BAF170/BAF155. Accumulating evidence demonstrated that SNF5 is essential for the chromatin remodeling function of SWI/SNF both in vivo and in vitro (Sansam et al., 2005; Ramirez-Carrozzz et al., 2006; Clapier and Cairns, 2009; Euskirchen et al., 2011; Narlikar et al., 2013; Kadoch and Crabtree, 2015).

Rheumatoid arthritis (RA), a chronic inflammatory condition, affects multiple joints and is characterized by inflammatory changes in the synovial tissue, hyperplasia and damage to the cartilage, and destruction of the underlying joint(s) (Choy and Panayi, 2001; Müllerladner et al., 2005). In RA, inflammatory cells are recruited and activated at the site of the affected joints. These inflammatory cells release large amounts of cytokines (inflammatory mediators), which facilitates extensive proliferation of the synovial fibroblasts, ultimately forming hyperplastic villous synovial tissues called pannus (Müllerladner et al., 2005). Pannus releases inflammatory mediators locally and is responsible for destruction of the affected joints. Hence, inhibition of pannus formation would suppress the progression of RA (Sherr and Roberts, 1995; Taniguchi et al., 1999; Nasu et al., 2001; Nonomura et al., 2001, 2006; Nishida et al., 2004; Choi et al., 2005; Murakami et al., 2012).

p16, a cyclin-dependent kinase inhibitor (CDKI), is encoded by the CDKN2A gene. CDKIs play an important role in cell cycle progression and upregulation of CDKIs causes cell cycle arrest (Sherr and Roberts, 1995). Studies have shown that adenoviral gene transfer of p16 led to the suppression of fibroblasts in human RA synovial tissues.
without induction of apoptosis and the forced expression of p16 ameliorated collagen-induced arthritis in mice (Nasu et al., 2001) and adjuvant-induced arthritis in rats (Nonomura et al., 2006).

Mitogen-activated protein kinase (MAPK), one of the critical players in the pathogenesis of RA (Han et al., 1999; Tournier et al., 2000; Barr and Bogoyevitch, 2001; Han, 2001; Plecky and Anderson, 2001; Johnson and Lapadat, 2002), consists of three major families of MAPKs, namely c-Jun-N terminal kinase (JNK), extracellular regulating kinase (ERK), and p38 kinase (Han et al., 1999; Barr and Bogoyevitch, 2001; Johnson and Lapadat, 2002). MAPKs including JNKs are activated by phosphorylation of threonine and tyrosine residues modulated by MAPK kinases, which are in turn activated by MAPK kinase kinases (MKKs). There are two isoforms of MKKs, MKK4 and MKK7, of which MKK7 contributes to the pathogenesis of RA through development of fibroblast-like synoviocytes (FLSs) (Han et al., 1999; Tournier et al., 2000; Han, 2001).

Because of the increased incidence of toxicity and limited efficacy of the current drugs prescribed for RA (Roberts, 1998; Smolen and Steiner, 2003), the search for novel drugs continues and therein lies the importance of understanding the signaling pathways responsible in the pathogenesis RA. To the best of our knowledge, this is the first time to explore the role of SNF5 in the pathogenesis of RA along with an understanding of the possible underlying molecular mechanism.

2. Materials and methods

2.1. Cell culture

MH7A cells were obtained from the Riken Cell Bank (Ibaraki, Japan). Cells were cultured in RPMI-1640 (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (final concentration: 100 U/mL), and streptomycin (final concentration: 0.1 mg/mL) in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C (Nakayama et al., 2012).

2.2. Plasmids transfection

The SNF5 expression vector (pc-SNF5) was constructed by subcloning the full-length wild-type Brg1 coding sequence into pcDNA3.1 (+) and was confirmed by sequencing. The pcDNA3.1 (+) and was confirmed by sequencing. The empty pcDNA3.1 was transfected as a control (Xu et al., 2000; Han, 2001).

2.3. MTT assay

Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay according to standard methods. Each experiment was performed three times.

2.4. Apoptosis assay

Apoptosis analysis was performed to identify and quantify the apoptotic cells by using an annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). The MH7A cells (1 × 10⁵ cells/well) were seeded in a 6-well plate. Treated cells were washed twice with cold phosphate-buffered saline (PBS) and resuspended in binding buffer. The adherent and floating cells were combined and treated according to the manufacturer’s instructions and measured with a flow cytometer (Beckman Coulter, Miami, FL, USA) to differentiate apoptotic cells (annexin-V-positive and PI-negative) from necrotic cells (annexin-V- and PI-positive).

2.5. Quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was isolated from transfected cells by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and DNasel (Promega) (Gresh et al., 2005). Reverse transcription was performed by using Multiscribe Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA) and random hexamers or oligo(dT). The reverse transcription conditions were 10 min at 25 °C, 30 min at 48 °C, and a final step of 5 min at 95 °C. The sequences of the primers were as follows: SNF5 forward primer: 5′-TGG TGA AGA CGC CAG TGG A-3′, reverse primer: 5′-TGA AGA CGC CAG TGG A-3′; GAPDH forward primer: 5′-GCA AAC TG-3′, reverse primer: 5′-ATC TTG GCG AGG ATG TGC TTG TCT T-3′; MKK4 forward primer: 5′-CAG ACC ACG GCG UCA UCA UdT dT-3′ and antisense, 5′-AUG AUG ACG CGC CUG UCU GdT dT-3′; shSNF5 and its negative control shRNA (shNC) were synthesized by GenePharma Co. (Shanghai, China). Cell transfections were conducted using the Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Stable SNF5 transfection was generated by selection under G418 (GIBCO, Paisley, UK).

To summarize, there were four groups: the pcDNA3.1 group (MH7A cells transfected with the empty pcDNA3.1), pc-SNF5 group (MH7A cells transfected with pc-SNF to overexpress SNF5), shNC group (MH7A cells transfected with shNC), and shSNF5 group (MH7A cells transfected with shSNF5 to silence SNF5).

2.6. Western blot analysis

Proteins were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel System and proteins were electrophoresed according to the manufacturer’s instructions. Primary antibodies against SNF5 (ab192864), total JNK (t-JNK, ab208035), phosphorylated JNK (p-JNK, ab76572), total c-JUN (t-c-JUN, ab119944), and p16 (ab51243) were purchased from Abcam (Shanghai, China). Primary antibodies against total MKK4 (t-MKK4, 9152), phosphorylated MKK4 (p-MKK4,
9155), and phosphorylated c-JUN (p-c-JUN, 2993) were purchased from Cell Signaling Technology (Beverly, MA, USA). GAPDH antibody was purchased from Sigma (G9545, St. Louis, MO, USA). Each primary antibody was incubated with the membrane at 4 °C overnight, followed by washing and incubation with secondary antibodies marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) carried blots and antibodies were transferred into the Bio-Rad ChemiDoc XRS system, and then 200 µL of Immobilon Western Chemiluminescent HRP Substrate (Millipore) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab Software (Bio-Rad, Shanghai, China).

2.7. Validation in primary cells from a rat model with adjuvant-induced arthritis

Male Sprague-Dawley rats (180–220 g) were purchased from the Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). A rat model with arthritis was induced by an intradermal injection of Freund's complete adjuvant supplemented with 10 mg/mL Mycobacterium butyricum (Wei et al., 2013) in both the right hind paw and the tail of the rats. Twenty-four days after immunization, synovial tissues were carefully dissected and cut into pieces, followed by treatment of 0.2% type II collagenase (Chondrex, Redmond, WA, USA) at 37 °C for 2 h. The cells were then washed and resuspended with RPMI 1640 containing 10% FBS in a humidified incubator at 37 °C with 5% CO₂. After washing and passaging, the primary FLSs were obtained and transfected with pcDNA3.1, pc-SNF5, shNC, or shSNF5 (the target sequence was replaced by the Brg1 gene of rat). Thereafter, cell viability and apoptosis were respectively measured by MTT assay and flow cytometry.

2.8. Statistical analysis

All the experiments were repeated three times. The results are presented as mean ± SD. Statistical analyses were performed using GraphPad statistical software (GraphPad, San Diego, CA, USA). One-way or two-way analysis of variance (ANOVA) was done to compare the results obtained from different groups. P < 0.05 was considered statistically significant.

3. Results

3.1. Expression of SNF5 in different groups of MH7A cells

The expression of SNF5 in all four groups mentioned above was assessed through measurement of the specific mRNA and protein levels of SNF5. Western blot analysis revealed a significant increase (P < 0.001) in the SNF5 expression in the pc-SNF5 group compared with the pcDNA3.1 group, whereas a significant decrease (P < 0.001) was seen in the SNF5 expression in the shSNF5 group in comparison with that in the shNC group. SNF5 expression levels were comparable (no significant difference, P > 0.05) between the two control groups (pcDNA3.1 and shNC) (Figures 1A and 1B).

3.2. Effects of SNF5 on cell proliferation

When compared to the respective controls, cell viability analysis of MH7A cells in different groups revealed that cell viability was significantly reduced at 2 days, 3 days, and 4 days after transfection in the pc-SNF5 group (P < 0.05, P < 0.01, or P < 0.001), whereas in the shSNF5 group, cell viability was increased significantly at 2 days, 3 days, and 4 days after transfection (P < 0.05 or P < 0.001) (Figure 2).

3.3. Effects of SNF5 on cell apoptosis

Results of flow cytometry showed a maximum and significant increase (P < 0.01) in the number of apoptotic cells in the pc-SNF5 group (SNF5 overexpressed) compared with the pcDNA3.1 group; the number of

Figure 1. Alteration of SNF5 expression in transfected cells. MH7A cells were respectively transfected with pcDNA3.1, pc-SNF5, shNC, or shSNF5. SNF5 expressions at both mRNA (A) and protein (B) levels were then measured by qRT-PCR and western blot analysis, respectively. Data presented are the means of three independent experiments. Error bars indicate SD. *: Significant difference compared with pcDNA3.1 group; ***: P < 0.001; #: significant difference compared with shNC group; ##: P < 0.01. pc-SNF5, pcDNA3.1 containing Brg1 coding sequence; shSNF5, short hairpin RNA against SNF5; shNC, negative control of shSNF5; qRT-PCR, quantitative reverse-transcription PCR.
apoptotic cells in the shSNF5 group and the shNC group was not significantly different (P > 0.05) (Figures 3A and 3B).

3.4. Effects of SNF5 on the expressions of p16 and JNK pathway associated proteins

Western blot analysis revealed a significant decrease (P < 0.05, P < 0.01, or P < 0.001) in the phosphorylated levels of the JNK pathway associated proteins, namely MKK4, JNK, and c-Jun, in the pc-SNF5 group when compared to the pcDNA3.1 group. However, the p16 expression of the same cells in the pc-SNF5 group was significantly increased (P < 0.001) when compared to the pcDNA3.1 group (Figure 4). Again, the effect of SNF5 knockdown on phosphorylated levels of JNK pathway associated proteins and the expression level of p16 was opposite to SNF5 overexpression when compared to the shNC group (P < 0.05, P < 0.01, or P < 0.001) (Figure 4).

3.5. Effects of SNF5 expressions on proliferation and apoptosis of primary cells from a rat model with adjuvant-induced arthritis

As with MH7A cells, the cell viability was markedly reduced at 2 days, 3 days, and 4 days after transfection in the pc-SNF5 group (P < 0.05 or P < 0.001), whereas in the shSNF5 group, cell viability was increased significantly at apoptotic cells in the shSNF5 group and the shNC group was not significantly different (P > 0.05) (Figures 3A and 3B).

Figure 2. Comparison of cell viability at different time intervals among transfected cells. MH7A cells were respectively transfected with pcDNA3.1, pc-SNF, shNC, or shSNF5. Cell viability was then measured by MTT assay. Data presented are the means of three independent experiments. Error bars indicate SD. *: Significant difference compared with pcDNA3.1 group, #: P < 0.05, **: P < 0.01, ***: P < 0.001; #: significant difference compared with shNC group; P < 0.05, ###: P < 0.001. pc-SNF5, pcDNA3.1 containing Brg1 coding sequence; shSNF5, short hairpin RNA against SNF5; shNC, negative control of shSNF5; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Figure 3. Comparison of cell apoptosis among transfected cells. (A) Percentage of apoptotic cells in the transfected cells. (B) Comparison of apoptotic cells (in percentage) in the transfected cells. MH7A cells were respectively transfected with pcDNA3.1, pc-SNF, shNC, or shSNF5. Cell apoptosis was then assessed by flow cytometry. Data presented are the means of three independent experiments. Error bars indicate SD. *: Significant difference compared with pcDNA3.1 group, **: P < 0.01. pc-SNF5, pcDNA3.1 containing Brg1 coding sequence; shSNF5, short hairpin RNA against SNF5; shNC, negative control of shSNF5.
2 days, 3 days, and 4 days after transfection (P < 0.05, P < 0.01, or P < 0.001) (Figure 5A). Meanwhile, cell apoptosis was remarkably promoted by SNF5 overexpression (P < 0.001), as seen in Figure 5B.

4. Discussion
SNF5 (alternatively known as Ini1/Baf47/Smarca1) as a tumor suppressor is involved in various human cancers (Sansam et al., 2005; Kadoch and Crabtree, 2015). Malignant rhabdoid tumors (MRTs), a very aggressive type of cancer that affects the pediatric population, occurs mainly in the brain, kidneys, and soft tissues and is induced by a specific biallelic mutation leading to the inactivation of the SNF5 gene. Both clinical data (Kusafuka et al., 2004) and animal experiments (in a murine model) supported this (Capsoni et al., 2015). SNF5 is extensively distributed in the central nervous system and in the mandibular area of the first branchial arch (Kusafuka et al., 2004). Roberts et al. concluded that homozygous silencing of the SNF5 gene led to the death of the mice during the embryonic period. Additionally, although heterozygous silencing does not kill the mice during the embryonic period and the mice appeared to be normal, within 5 weeks of age the heterozygous mice developed tumors consistent with MRTs (Roberts et al., 2000). Thus, the importance of SNF5 in the suppression of cancer is well established. However, the role of SNF5 in other biological processes is quite unexplored. Gresh et al. described that the normal expression of SNF5 is essential to liver development and glucose metabolism, and selective suppression of SNF5 in the liver can lead to death in the perinatal period (Gresh et al., 2005).
In this study, we also explored the possible alterations in the molecular mechanism following SNF5 suppression in RA, which led to the disease progression of RA. We found that the expression of SNF5 was significantly increased in MH7A human RA synovial cells transfected with pc-SNF5 in comparison with cells transfected with empty pcDNA3.1. Conversely, transfection of shSNF5 led to a decrease of SNF5 expression.

Cell proliferation, measured by MTT assay, revealed that overexpression of SNF5 (pc-SNF group) led to significant suppression while SNF5 knockdown (shSNF5 group) led to a significant stimulation of cell proliferation in the MH7A cells, strongly supporting the already established role of SNF5 as a tumor suppressor. Meantime, flow cytometric analysis also revealed that overexpression of SNF5 in cells transfected with pc-SNF5 led to a significant increase in the percentage of apoptotic cells, along with a nonsignificant difference between the shNC group and shSNF5 group. These findings suggested that RA disease progression was suppressed in the SNF5-overexpressing cells through increased expression of favorable p16.

The roles of p16 and proteins involved in the JNK pathway in pathogenesis have already been well discussed in several studies. Taniguchi et al. found that introduction of p16 in the synovial fibroblast cells with the help of adenoviral gene therapy led to the suppression of disease progression in an animal model of RA (Taniguchi et al., 1999). Murakami et al. concluded that p16, an inhibitor of cell cycle progression, showed antiinflammatory effects on rheumatoid synovial fibroblasts and its upregulation led to inhibition of inflammatory cytokine production from the macrophages (Murakami et al., 2012). Ultimately, these findings indicate a protective role of p16 in the suppression of the disease progression of RA. In our study, we found that the expression of p16 in SNF5-overexpressing cells (pc-SNF5 group) was significantly upregulated in comparison with the control (pcDNA3.1), and the expression of p16 in SNF5-knockdown cells (shSNF5 group) was significantly decreased in comparison with the control (shNC group). These findings suggested that RA disease progression was suppressed in the SNF5-overexpressing cells through increased expression of favorable p16.

The ultimate outcome of disease progression of RA is the destruction of the affected joints. Activation of the JNK pathway has already been implicated in the development of FLSs, which are mainly responsible for the joint destruction in RA. Han described the role of the JNK pathway in the pathogenesis of RA through the use of a novel JNK pathway inhibitor and JNK knockout rat model of adjuvant-induced arthritis (Han, 2001). It was found that the novel JNK pathway inhibitor SP600125 not only modestly decreased the paw edema in the rat model of adjuvant-induced arthritis but also suppressed the underlying joint destruction, as evidenced by radiographic findings (Han, 2001).

In this study, we found that the phosphorylated levels of JNK pathway associated proteins (M KK4, JNK, and c-Jun) were all decreased significantly in the MH7A cells with SNF5 overexpression (pc-SNF group) when compared to the control (pcDNA3.1 group). Meanwhile,
the phosphorylated levels of MKK4, JNK, and c-Jun were all increased markedly in cells with SNF5 knockdown (shSNF5 group) when compared to the control (shNC group).

Thus, we can conclude that SNF5, as a core component of the chromatin remodeling complex (SWI/SNF), is a known tumor suppressor and can also modify the course of RA through its suppressive effects on RA FLSs. The underlying molecular mechanism that promotes apoptosis and suppresses proliferation of RA FLSs was mediated through the upregulation of P16 and suppression of the JNK pathway. Moreover, the effect of SNF5 was validated in RA FLSs from a rat model of adjuvant-induced arthritis. Further studies are required to predict the therapeutic benefits of SNF5 as a novel drug target for the treatment of RA.

References


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