

Slug suppression induces apoptosis via Puma transactivation in rheumatoid arthritis fibroblast-like synoviocytes treated with hydrogen peroxide

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Abbreviations: Ad-GFP, recombinant adenovirus expressing green fluorescent protein; Ad-p53, recombinant adenovirus expressing human wild-type p53 gene; FLS, fibroblast-like synoviocytes; RA, rheumatoid arthritis; ST, synovial tissue; WCL, whole cell lysates

Abstract

Inadequate apoptosis contributes to synovial hyperplasia in rheumatoid arthritis (RA). Recent study shows that low expression of Puma might be partially responsible for the decreased apoptosis of fibroblast-like synoviocytes (FLS). Slug, a highly conserved zinc finger transcriptional repressor, is known to antagonize apoptosis of hematopoietic progenitor cells by repressing Puma transactivation. In this study, we examined the expression and function of Slug in RA FLS. Slug mRNA expression was measured in the synovial tissue (ST) and FLS obtained from RA and osteoarthritis patients. Slug and Puma mRNA expression in FLS by apoptotic stimuli were measured by real-time PCR analysis. FLS were transfected with control siRNA or Slug siRNA. Apoptosis was quantified by trypan blue exclusion, DNA fragmentation and caspase-3

assay. RA ST expressed higher level of Slug mRNA compared with osteoarthritis ST. Slug was significantly induced by hydrogen peroxide (H₂O₂) but not by exogenous p53 in RA FLS. Puma induction by H₂O₂ stimulation was significantly higher in Slug siRNA-transfected FLS compared with control siRNA-transfected FLS. After H₂O₂ stimulation, viable cell number was significantly lower in Slug siRNA-transfected FLS compared with control siRNA-transfected FLS. Apoptosis enhancing effect of Slug siRNA was further confirmed by ELISA that detects cytoplasmic histone-associated DNA fragments and caspase-3 assay. These data demonstrate that Slug is overexpressed in RA ST and that suppression of Slug gene facilitates apoptosis of FLS by increasing Puma transactivation. Slug may therefore represent a potential therapeutic target in RA.

Keywords: apoptosis; Puma; rheumatoid arthritis; snail family transcription factors; synovial membrane

Introduction

Rheumatoid arthritis (RA) is a chronic joint disorder characterized by inflammation and proliferation of synovial tissue (ST) resulting in bone and cartilage destruction. Hyperplasia of the synovial intimal lining results from marked increase in macrophage-like and fibroblast-like synoviocytes (FLS) (Firestein, 2003). One explanation for the synovial proliferation is an imbalance between cell proliferation and apoptosis or programmed cell death. The amount of DNA fragmentation is significantly increased in rheumatoid synovium (Firestein *et al.*, 1995; Nakajima *et al.*, 1995), which is presumably due to the oxidative stress generated by chronic inflammation (Tak *et al.*, 2000). However, only low numbers of apoptotic cells are present in rheumatoid synovium (Nakajima *et al.*, 1995; Matsumoto *et al.*, 1996; Sugiyama *et al.*, 1996; Ceponis *et al.*, 1999), which suggests the discrepancy between apoptotic stimuli and real apoptotic cell death occurring in RA synovium. Therefore, impaired apoptosis may play an important role in the pathogenesis of RA.

Slug, which belongs to highly conserved Slug/Snail

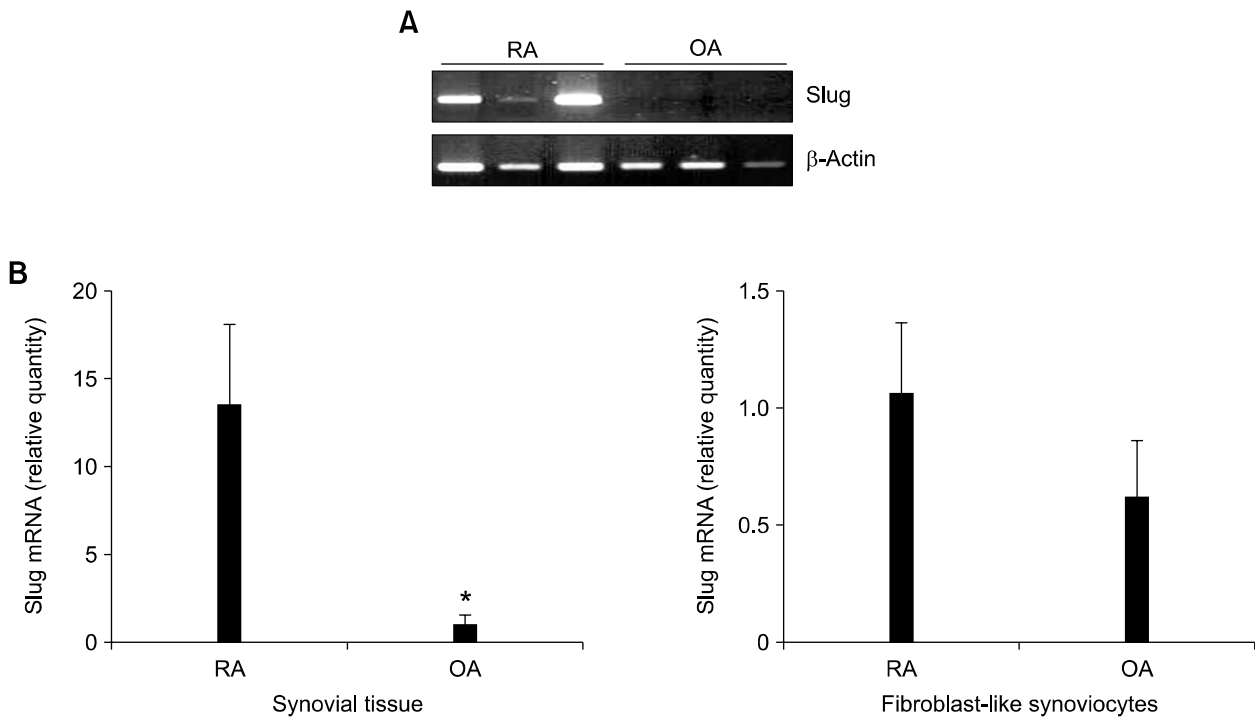


Figure 1. *Slug* is overexpressed in RA ST. (A) Total RNA was extracted from RA ($n = 3$) and osteoarthritis (OA) ($n = 3$) ST, and RT-PCR was performed to detect *Slug* mRNA expression. (B) Quantitative real-time PCR analysis was performed to quantify *Slug* mRNA expression in RA and osteoarthritis ST ($n = 6$ each) and FLS ($n = 3$ each). Data are mRNA level normalized to β -actin expression and shown as relative cell equivalents. * $P < 0.005$ compared with RA ST.

family, is a zinc finger transcriptional repressor. *Slug*/*Snail* protein family was found in diverse species from *C. elegans* to humans. In vertebrates, *Snail* and *Slug* transcription factors have been proposed to participate in mesoderm formation and neural crest cell migration (Hemavathy *et al.*, 2000). These proteins have also been implicated in carcinogenesis, invasiveness and metastasis in various cancers including ovarian cancer, breast cancer and melanoma (Gupta *et al.*, 2005; Kurrey *et al.*, 2005; Martin *et al.*, 2005; Perez-Mancera *et al.*, 2005). Hematopoietic stem cells are resistant to ionizing radiation-induced apoptosis. Wu *et al.* demonstrated that *Slug* is transcriptionally induced by p53 upon irradiation and then protects the damaged hematopoietic progenitor cells from apoptosis by directly repressing p53-mediated transcription of *Puma* (Wu *et al.*, 2005).

Puma is a Bcl-2 homology 3 (BH3)-only proapoptotic Bcl-2 family member and mediates p53-dependent and -independent apoptosis (Han *et al.*, 2001; Nakano and Vousden, 2001; Yu *et al.*, 2001, 2003). The interaction of *Puma* with anti-apoptotic Bcl-2 protein family members such as Bcl-xL induces mitochondrial dysfunction through Bax (Han *et al.*, 2001; Nakano and Vousden, 2001; Yu

et al., 2001). *Puma* is detected in both RA and osteoarthritis ST, but interestingly, its expression is low in RA synovial intimal lining, which is a major site of apoptotic stimuli and p53 expression. Exogenous p53 overexpression induced p21, but not *Puma* in FLS and this was associated with resistance of FLS to apoptosis. However, transfection of plasmid encoding *Puma* expression vector lead to rapid and profound apoptosis with caspase-3 activation in RA FLS (Cha *et al.*, 2006). Tissue- and cell type-specific selectivity of p53 in transactivation of downstream genes has been reported (Fei *et al.*, 2002). However, the mechanism in which p53 overexpression does not lead to *Puma* induction or apoptosis in FLS is unknown. *Slug*, which has been reported to repress *Puma* gene transcription in hematopoietic progenitor cells, might play a role in the down-regulation of *Puma* and impaired apoptosis of RA FLS. In this study, we investigated the expression and function of *Slug* in ST and cultured FLS in order to evaluate a potential strategy to induce apoptosis in synoviocytes. We now demonstrate that *Slug* gene is overexpressed in RA ST and that suppression of *Slug* facilitates apoptosis of RA FLS by increasing *Puma* gene expression.

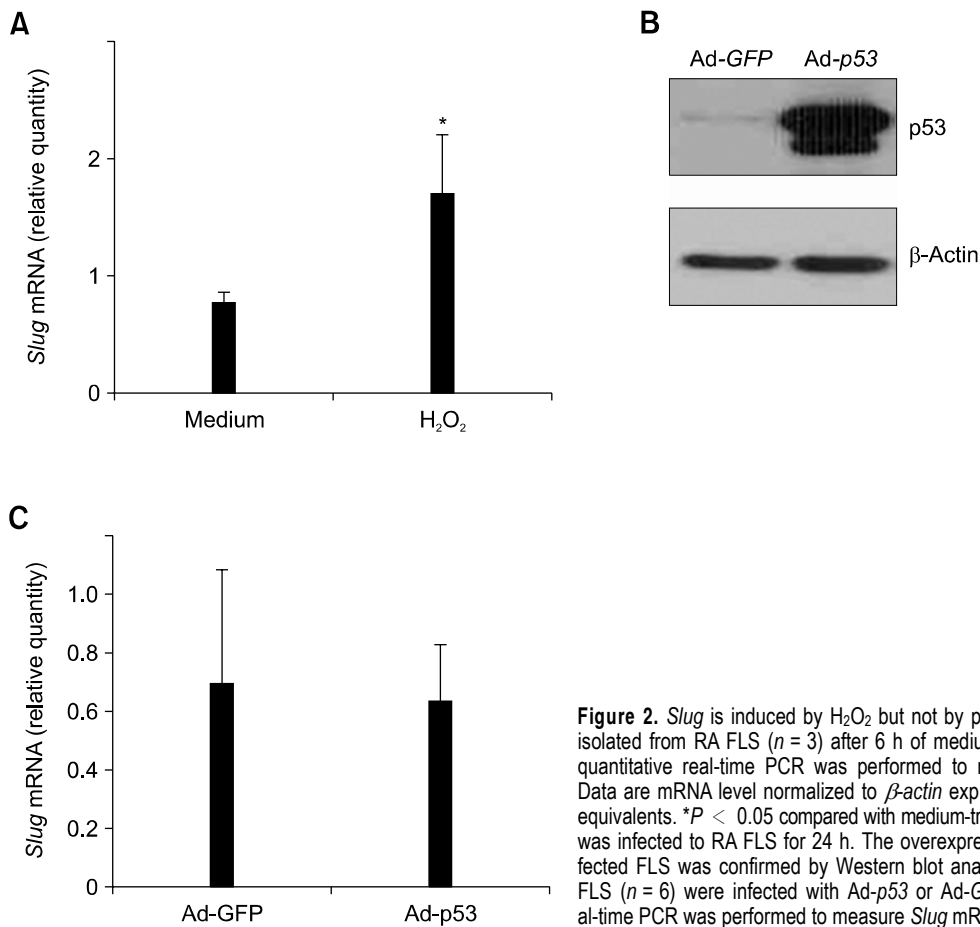


Figure 2. *Slug* is induced by H₂O₂ but not by p53 in RA FLS. (A) Total RNA was isolated from RA FLS ($n = 3$) after 6 h of medium or H₂O₂ (1 mM) treatment and quantitative real-time PCR was performed to measure *Slug* mRNA expression. Data are mRNA level normalized to β -actin expression and shown as relative cell equivalents. * $P < 0.05$ compared with medium-treated cells. (B) Ad-p53 or Ad-GFP was infected to RA FLS for 24 h. The overexpression of p53 protein in Ad-p53-infected FLS was confirmed by Western blot analysis (representative blot). (C) RA FLS ($n = 6$) were infected with Ad-p53 or Ad-GFP for 24 h, and quantitative real-time PCR was performed to measure *Slug* mRNA expression.

Results

Slug expression in RA ST and FLS

Initially, we performed studies to determine whether *Slug* is expressed in RA ST and FLS. As shown in Figure 1A, *Slug* mRNA was detected in RA ST but not in osteoarthritis ST on gel PCR ($n = 3$ each). Quantitative real-time PCR also showed significantly elevated *Slug* mRNA level in RA ST compared to osteoarthritis ST ($n = 6$ each, $P < 0.005$) (Figure 1B). *Slug* mRNA was also detected in FLS with a tendency of increased expression in RA FLS compared to osteoarthritis FLS ($n = 3$ each), but did not reach statistical significance ($P > 0.10$) (Figure 1B).

Next, we tried to examine whether apoptotic stimuli could induce *Slug* gene expression. Exposure of RA FLS to 1 mM H₂O₂ for 6 h resulted in significant increase of *Slug* mRNA ($n = 3$, 3.0 ± 0.8 fold induction, $P < 0.05$) (Figure 2A). To see the effect of p53 on *Slug* expression in FLS, Ad-p53 or Ad-GFP were infected for 24 h in RA FLS and marked increase in p53 protein expression in

Ad-p53-infected FLS was confirmed on Western blot analysis (Figure 2B). However, as shown in Figure 2C, Ad-p53-infected FLS showed no increase of *Slug* mRNA compared to Ad-GFP-infected FLS ($n = 6$, $P > 0.10$).

Effect of *Slug* suppression on *Puma* expression in RA FLS

Because *Slug* is known as a transcriptional repressor of *Puma*, we next investigated the effect of *Slug* suppression on *Puma* expression in RA FLS. RA FLS were transfected with control or *Slug* siRNA for 3 days and the knocked-down effect was confirmed by RT-PCR (Figure 3A). H₂O₂ stimulation (1 mM for 6 h) did not significantly increase *Puma* expression in control siRNA-transfected FLS ($n = 3$, 1.6 ± 0.2 fold increase, $P > 0.10$). However, in *Slug* siRNA-transfected FLS, H₂O₂ stimulation significantly induced *Puma* mRNA expression on quantitative real-time PCR experiments ($n = 3$, 4.7 ± 1.6 fold increase, $P < 0.05$) (Figure 3B). Without H₂O₂ treatment, increase of *Puma* mRNA by *Slug*

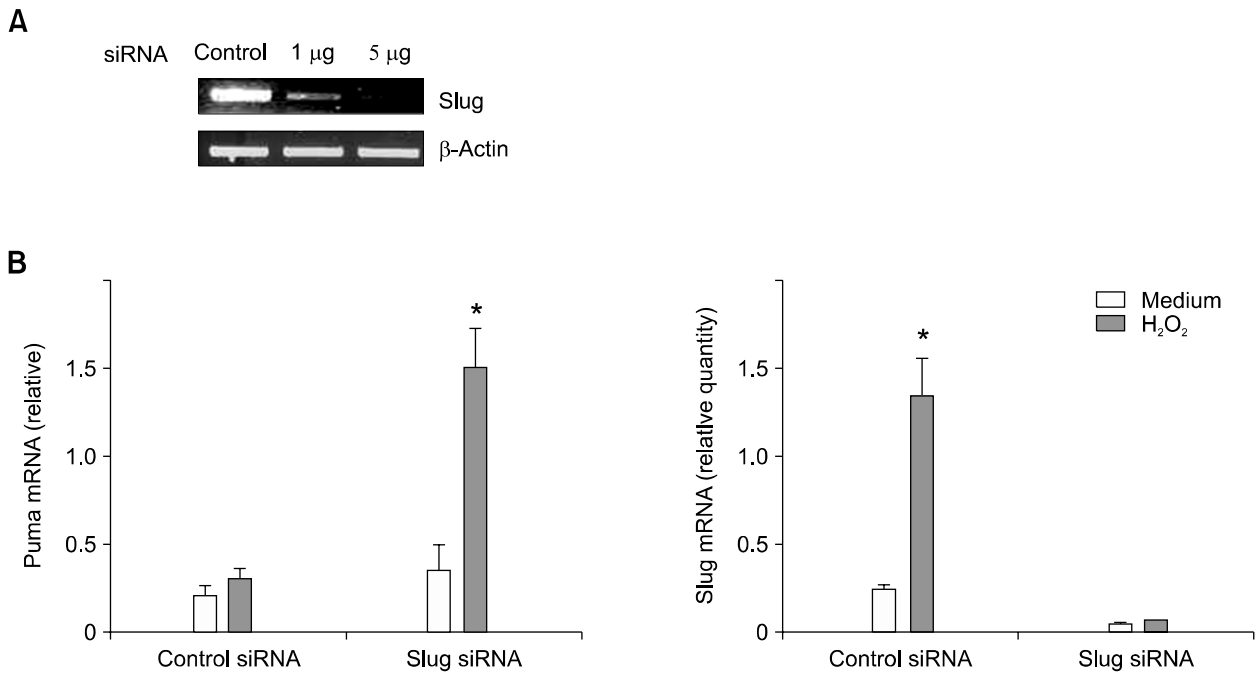


Figure 3. *Slug* knockdown increases H₂O₂-induced *Puma* expression in RA FLS. (A) Cultured FLS were transfected with 1 or 5 μg of *Slug* siRNA or scrambled nonsilence control siRNA as described in Methods. FLS were then incubated for 3 days, and RT-PCR was performed. Maximum knockdown effect was observed with 5 μg of *Slug* siRNA transfection. (B) RA FLS ($n = 3$) were transfected with 5 μg of control or *Slug* siRNA for 3 days, and then treated with medium or 1 mM H₂O₂ for 6 h. Total RNA was collected, and *Puma* mRNA was measured by quantitative real-time PCR. *Slug* mRNA level was measured in the same RNA samples using quantitative real-time PCR. Note the *Slug* suppression effect of *Slug* siRNA in both medium and H₂O₂-treated cells. * $P < 0.05$ compared with medium-treated cells.

siRNA transfection was minimal. In the same samples, we could find that the effect of significant induction of *Slug* by H₂O₂ in control siRNA-transfected FLS disappeared in *Slug* siRNA-transfected FLS with suppressed expression of *Slug* mRNA irrespective of H₂O₂ stimulation (Figure 3B).

Effect of *Slug* suppression on FLS apoptosis

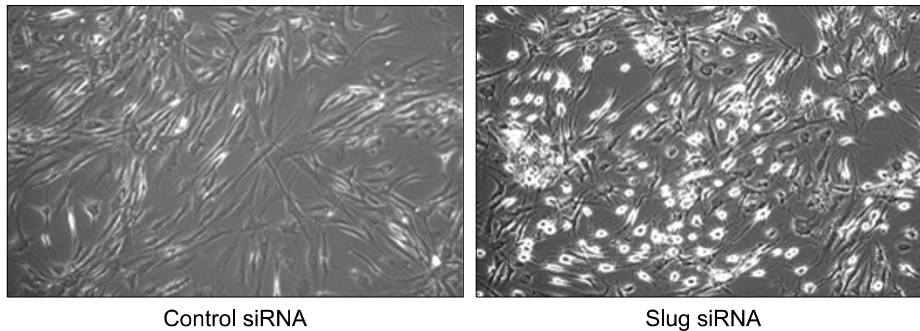
Because we found that *Slug* suppression increased *Puma* expression in RA FLS, we next tried to examine whether *Slug* suppression could induce apoptosis of RA FLS. RA FLS were transfected with control or *Slug* siRNA for 3 days and then stimulated with 0.5 mM H₂O₂ for 6 h. Cell morphology was observed on phase-contrast microscopy. As shown in Figure 4A, dead cells, which are characterized by nonadherent round appearance, were abundant in *Slug* siRNA-transfected FLS compared to control siRNA-transfected FLS. We next evaluated the effect of *Slug* suppression on cell death using trypan blue exclusion ($n = 4$). *Slug* siRNA transfection resulted in significantly decreased viable cell number after 9 h of 0.5 mM H₂O₂ stimulation compared to control siRNA transfection ($6.9 \times 10^4 \pm 2.6 \times 10^4$ vs $11.1 \times 10^4 \pm 3.1 \times$

10^4 , $P < 0.05$) (Figure 4B). Enhanced apoptosis of RA FLS by *Slug* suppression was confirmed by ELISA detecting fragmented DNA in cytoplasm. RA FLS transfected with *Slug* siRNA showed 6.7 ± 2.1 fold induction of DNA fragmentation compared to 2.1 ± 0.6 fold induction of DNA fragmentation in control siRNA-transfected FLS by H₂O₂ (0.5 mM for 6 h) ($n = 5$, $P < 0.05$) (Figure 5A). To confirm the activation of apoptosis pathway by *Slug* suppression, caspase-3 activity was measured after treatment of 0.5 mM H₂O₂ for 6 h in *Slug* siRNA- and control siRNA-transfected FLS. As shown in Figure 5B, caspase-3 activity in response to H₂O₂ treatment was significantly elevated in *Slug* siRNA-transfected FLS compared to control siRNA-transfected FLS ($n = 3$, $P < 0.05$).

Discussion

Tumor-like proliferation of synoviocytes plays a key role in the progression of RA, and inadequate apoptosis of FLS contributes to this process (Firestein, 2003). Therefore, facilitating apoptosis in these cells is a reasonable strategy in the treatment of RA. In this report, we demonstrated

A



Control siRNA

Slug siRNA

B

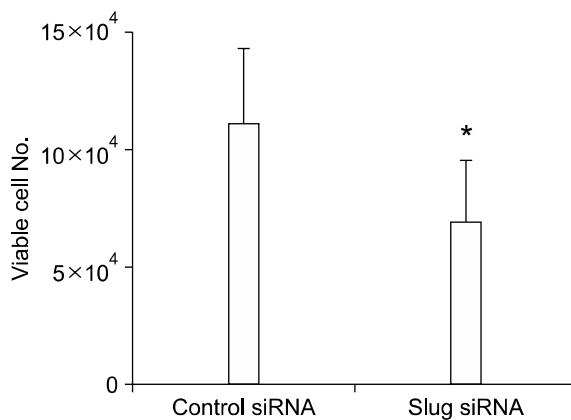


Figure 4. *Slug* knockdown facilitates H_2O_2 -induced FLS death. (A) RA FLS were transfected with control or *Slug* siRNA for 3 days and then stimulated with 0.5 mM H_2O_2 . After 6 h, cell morphology was observed under phase-contrast microscopy (magnification 50 \times). (B) RA FLS were transfected with control or *Slug* siRNA for 3 days and then treated with 0.5 mM H_2O_2 . After 9 h, viable cell number was counted by trypan blue exclusion as described in Methods. * $P < 0.05$ compared with control siRNA-transfected cells.

for the first time that suppressing *Slug* could efficiently enhance apoptosis in RA FLS.

In the present study, *Slug* was detected in ST and FLS, and was transcriptionally more activated in RA ST compared to osteoarthritis ST. This result suggests that increased activation of *Slug* in RA synovium might contribute to impaired apoptosis. *Slug* was first identified as a transcription factor whose expression during embryonic development has been associated with mesoderm and migratory neural crest cells undergoing epithelial mesenchymal transformation (Hemavathy *et al.*, 2000). Subsequently, evidence is accumulating that *Slug* regulates tumor carcinogenesis, metastasis and invasiveness in adult tissues. For example, *Slug*-overexpressing mice developed mesenchymal cancers, which were mainly leukemias (Perez-Mancera *et al.*, 2005). Aberrant expression of *Slug* in breast adenocarcinomas protected against apoptosis induced by genotoxic stress and resulted in phenotypic alterations, including loss of cell adhesion and acquisition of invasive growth (Kajita *et al.*, 2004). Induction of *Slug* by autocrine production of stem cell factor and c-Kit activation resulted in multidrug resistance in malignant mesothelioma cells (Catalano *et al.*, 2004). *Slug*

down-regulation by RNA interference facilitated apoptosis and inhibited invasive growth in neuroblastoma cells (Vitali *et al.*, 2008). *Slug* is also known to be expressed and induced in nonmalignant cells such as keratinocytes by ultraviolet radiation (Hudson *et al.*, 2007). Our study further demonstrated that *Slug* is inducible by genotoxic stimuli in RA FLS. *Slug* suppression by RNA interference markedly enhanced H_2O_2 -induced apoptosis in RA FLS, and this was mediated by increased caspase-3 activation. H_2O_2 -induced cell necrosis could also have contributed to FLS death in our experiment (Byun *et al.*, 2008), but obvious influence of *Slug* suppression on FLS apoptosis has been demonstrated by apoptosis specific ELISA and caspase-3 activation assay. Because experiments comparing RA FLS with osteoarthritis FLS were not performed, we don't know whether these results are specific to RA FLS and it is unclear whether these *Slug* mediated apoptotic regulation is also operating in osteoarthritis or normal FLS. However, these results suggest that *Slug* plays an important role in the regulation of FLS apoptosis.

In response to DNA damage, the tumor suppressor p53 can either elicit apoptosis via *Puma*

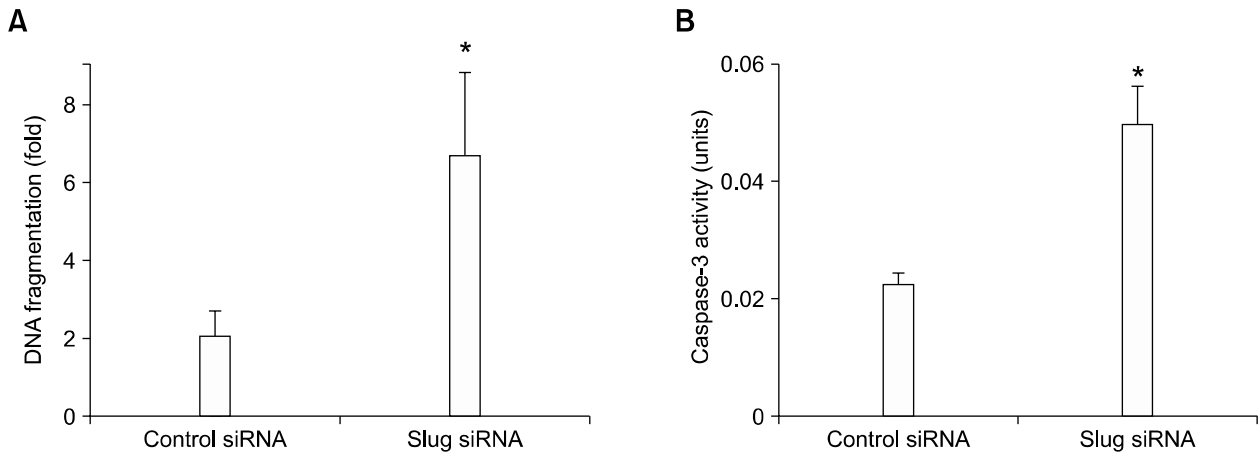


Figure 5. *Slug* knockdown enhances H_2O_2 -induced FLS apoptosis and caspase-3 activation. (A) RA FLS ($n = 5$) were transfected with control or *Slug* siRNA for 3 days and then stimulated with medium or 0.5 mM H_2O_2 . After 6 h, DNA fragmentation was measured by ELISA. The magnitude of induction of DNA fragmentation in H_2O_2 -treated FLS is shown relative to control value (medium-treated FLS). * $P < 0.05$ compared with control siRNA-transfected cells. (B) RA FLS ($n = 3$) transfected with control or *Slug* siRNA were treated with 0.5 mM H_2O_2 for 6 h. Caspase-3 activity was measured using ApoAlert Caspase-3 assay. * $P < 0.05$ compared with control siRNA-transfected cells.

transactivation or activate p21 pathway, resulting in cell-cycle arrest and DNA repair (Vousden and Lu, 2002). However, how this critical step is differentially regulated in specific cell types remains largely unknown. In FLS, overexpression of p53 did not induce apoptosis via *Puma* transactivation, but induced p21 (Cha *et al.*, 2006), and the mechanism for this selective transactivation of downstream molecules could not be explained. *Slug* was proposed to regulate the activation of p53 downstream molecules in hematopoietic progenitor cells. Once activated by DNA damage, p53 induces molecules including *Puma* and *Slug*. *Slug* then shifts the fate of damaged myeloid progenitor cells toward arrest and survival by selectively repressing *Puma* gene transactivation (Wu *et al.*, 2005; Zilfou *et al.*, 2005). Our present findings also demonstrate that *Slug* is involved in *Puma* gene regulation. *Puma* transactivation by H_2O_2 was minimal in control FLS, and this was compatible with the previous findings (Cha *et al.*, 2006). On the other hand, we found that when *Slug* was knocked-down by RNA interference, *Puma* gene transactivation was significantly increased by H_2O_2 stimulation. Without H_2O_2 treatment, *Puma* induction by *Slug* knock-down was minimal, which suggests that influence of *Slug* on *Puma* expression is restricted on the condition of the presence of extracellular apoptotic stimuli such as H_2O_2 . Contrary to previous findings, however, *Slug* was not induced by p53 in FLS in our study. Exogenous overexpression of p53 did not increase transcriptional activation of *Slug* in FLS, and this finding was discordant with the results observed in hematopoietic progenitor cells as mentioned above (Wu *et al.*, 2005). These

results imply that, in FLS, *Slug* can be induced by genotoxic stimuli such as H_2O_2 via p53-independent mechanism, which then leads to the suppression of proapoptotic gene transcription.

Although *Slug* has been observed to be induced by p53 in hematopoietic stem cells, this finding has not been reproduced in other cell types. In early vertebrate mesoderm, *Slug* was reported to be induced by NF- κ B, which, in turn, was activated by Bcl-xL (Zhang *et al.*, 2006). *Slug* was induced by stem cell factor/*c-Kit* pathway in multidrug-resistant malignant mesothelioma cells (Catalano *et al.*, 2004). Ultraviolet radiation-induced *Slug* elevation in keratinocytes was mediated, at least in part, through the ERK and p38 MAPK cascades (Hudson *et al.*, 2007). The mechanism in which *Slug* is induced by H_2O_2 in FLS remains to be illuminated in the future, but p53 does not seem to be an important mediator from our result. Despite the fact that p53 has been reported to be overexpressed in RA synovium, the role of p53 in RA FLS still remains uncertain. In fact, p53 may not play an important role in FLS apoptosis. Adenoviral gene transfer of p53 did not induce apoptosis of FLS (Cha *et al.*, 2006). A recent study indicated that p53 not only was found to be important in inducing *Puma* in nucleus, but also was found to be necessary in cytoplasm to mediate the downstream apoptotic effect of *Puma* (Chipuk *et al.*, 2005). However, in FLS, p53 was not required in *Puma*-mediated apoptosis (You *et al.*, 2006). *Puma* efficiently induced apoptosis in both control and p53-deficient FLS (You *et al.*, 2006). Furthermore, adenoviral gene transfer of *Puma* caused massive apoptosis of malignant

glioma cells regardless of p53 status (Ito *et al.*, 2005). These inconsistent findings imply that apoptosis regulating molecules including p53, Slug, and Puma may be interacting differentially according to tissue-specific cell types. The FLS-specific apoptotic pathway needs to be elucidated in order to establish apoptosis as a therapeutic target in RA.

In conclusion, the present study demonstrated that *Slug* was expressed and induced by genotoxic stimuli in RA FLS. Suppression of *Slug* augmented H₂O₂-induced *Puma* gene transactivation and facilitated apoptosis of FLS. *Slug* may therefore represent a novel therapeutic target in RA.

Methods

ST samples and primary synoviocytes culture

The experimental protocol was approved by the local ethics committee and a signed consent form was obtained from each patient. ST samples were obtained from patients with osteoarthritis and RA at the time of joint replacement. Patients with RA were diagnosed according to the standards of the American College of Rheumatology (Arnett *et al.*, 1988). The ST samples were either processed for cell culture or snap frozen and stored at -80°C. ST was minced, digested overnight with 5 mg/ml type IV collagenase (Sigma, St. Louis, MO) and 150 µg/ml type I DNase (Sigma), and separated from undigested tissue by unit gravity sedimentation. After collecting the suspended cells into fresh tubes, the cells were harvested by centrifugation at 500 × *g* for 10 min. The pellet was washed twice with DMEM (Life Technologies, NY) containing 10% FCS. Resuspended cells were plated at a concentration of 2 × 10⁵/ml in a total volume of 1 ml/200 mm² into T-25 culture flasks. After overnight incubation, non-adherent cells were removed by replacing fresh culture medium, and attached cells were cultured in DMEM with 10% FCS and 50 units/ml penicillin, 50 µg/ml streptomycin, and 0.025 µg/ml amphotericin B until 90% confluent growth. Primary cultured cells were passaged three to four times over several weeks for subsequent experiments.

RNA isolation and RT-PCR

Total RNA was isolated from ST or cultured FLS using TRIZOL reagent that contained phenol and guanidine isothionate in a monophasic solution following the manufacturer's recommendations (Invitrogen Carlsbad, CA). 1 µg of pooled RNA was reverse transcribed in a total reaction volume of 20 µl containing 0.5 µg random hexamer primer, 20 units RNasin[®] ribonuclease inhibitor and superscript[™] II reverse transcriptase (Invitrogen, CA). The PCR amplification of the cDNA aliquots was performed by adding 2.5 mM deoxyribonucleoside triphosphate (dNTPs), 2.5 U Taq DNA polymerase (Boehringer Mannheim, Germany) and 0.25 µM each of the sense and anti-sense

primers. The following sense and anti-sense primers for *Slug* and *β-actin* were used (all written in 5'→3' direction): *Slug* sense TCCTCCATCTGACACCTCCTC, anti-sense CCCCCGTGTGAGTTCTAATGT; *β-actin* sense TACGTTGCTATCCAGGCTGTG, anti-sense AGTACTTGCGCTCAGGAGGAG. The levels of *Slug*, *Puma*, and *β-actin* mRNA expression were measured by real-time RT-PCR using an ABI PRISM 7000 sequence detector system (Applied Biosystems, Foster City, CA). The real-time RT-PCR amplification was performed using the pre-developed Assay-on-demand gene expression set for *Slug* gene (Assay ID Hs00161904_m1, Gene bank accession number NM_003068, Applied Biosystems), *Puma* gene (Assay ID Hs00248075_m1, Gene bank accession number NM_014417, Applied Biosystems) and human *ACTB* (*β-actin*) for the endogenous control (VIC/MGB Probe, Applied Biosystems) in combination with the TaqMan[®] Universal PCR Master Mix. Quantitation of *Slug* and *Puma* mRNA expression was calculated using the absolute method provided by the manufacturer (Applied Biosystems). Analysis was performed using the ABI PRISM 7000 Sequence Detection software (Applied Biosystems, Foster City, CA). The expression level of selected genes in unknown samples was calculated as ratio of selected gene mRNA versus *β-actin*. Quantitation of selected gene mRNA and *β-actin* were performed using standard curves made from known serial dilution of rheumatoid FLS. A negative control without template was included in each experiment.

Synoviocytes transfections

Recombinant adenovirus expressing human wild-type p53 gene (Ad-p53) and recombinant adenovirus expressing green fluorescent protein (Ad-GFP) were purchased from VECTOR BIOLABS (Philadelphia, PA). Cells cultured in DMEM supplemented with 1% FCS for 24 h were infected with Ad-p53 or Ad-GFP at an approximate multiplicity of infection of 100 for 24 h. Small interference RNA (siRNA) for *Slug* and control siRNA were purchased from Dharmacon (Lafayette, CO). Transient transfection of FLS was performed using the Amaxa Human Dermal Fibroblast Nucleofector[™] kit (NHDF-adult; Amaxa Biosystems, Gaithersburg, MD), according to the manufacturer's protocols. Briefly, siRNA (1 µg) was added to 5 × 10⁵ FLS suspended in 100 µl of Nucleofector[™] solution. The mixture was then transferred into the electroporation cuvette and subjected to electroporation using the U-23 program, according to the manufacturer's instructions. Immediately after electroporation, the cells were suspended in 500 µl of cell culture medium, and transferred into culture dishes or plates. The transfected cells were then grown until the knocked-down effect of *Slug* siRNA was evident by RT-PCR.

Western blot analysis

Whole cell lysates (WCL) were prepared using RIPA buffer that contained 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% NP40, 0.1% SDS, and 10 mM sodium deoxycholate. The concentration of WCL was determined using a Bio-RAD protein assay kit (Bio-Rad, Richmond, CA). Next, 15 µl of WCL were separated by electrophoresis on a 10% acrylamide gel using a constant voltage of 80 V for 2 h.

The separated proteins were transferred electrophoretically onto nitrocellulose membranes that had been preblocked for 4 h at room temperature in TTBS (20 mM Tris-Cl, pH 8.0, 500 mM NaCl, 0.05% (v/v) Tween-20) containing 1% skim milk powder. The membranes were incubated overnight at 4°C with 1:1000-diluted antibodies to p53 (mouse monoclonal anti-p53 antibody, Santacruz, CA) and β -actin (mouse monoclonal anti- β -actin antibody, Sigma, St. Louis, MO) in TTBS. The membranes were washed with TTBS three times for 15min, and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit antibody (1:5000, Jackson Immunoresearch, West Grove, PA). Proteins were detected using an ECLTM Western blot detection system (Amersham Life Science, Buckinghamshire, UK).

Cell viability and apoptosis assays

Three days after siRNA transfection, FLS were stimulated with 0.5 mM H₂O₂ for 9 h. Both nonadherent and adherent FLS were suspended in 0.4% trypan blue in PBS (pH 7.4), and counted using Hemacytometer (Horsham, PA). The cells that excluded the blue dye and had a well-defined cellular outline were scored as live. Total number of live cells was calculated and averaged over at least three times. FLS transfected with siRNA were plated in 96-well plastic plates (2 × 10⁴ cells/well) and incubated with media or 0.5 mM H₂O₂ for 6 h. After incubation, apoptosis was determined using a Cell Death Detection ELISA^{PLUS} kit (Roche, Mannheim, Germany) according to the manufacturer's instruction. It is based on a quantitative sandwich-enzyme immunoassay using mouse monoclonal antibodies against DNA and histones that allow for the specific, quantitative determination of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes). Each measurement was done in duplicate, and the results are presented as the fold induction compared with control samples (media-treated cells).

Caspase-3 activation assay

FLS transfected with control or *Slug* siRNA were plated in six-well plastic plates (2 × 10⁵ cells/well) and incubated with media or 0.5 mM H₂O₂ for 6 h. Caspase-3 activation was measured using the ApoAlert Caspase-3 Colorimetric Assay kit (Clontech, CA, US) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean ± SEM. The statistical significance of experimental results was calculated by paired Student's *t*-test or Mann-Whitney *U*-test. *P* values less than 0.05 were considered significant.

Acknowledgements

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