GREM1 Is a Key Regulator of Synoviocyte Hyperplasia and Invasiveness

Eun-Jin Han, Seung-Ah Yoo, Gi-Myo Kim, Daehee Hwang, Chul-Soo Cho, Sungyong You, and Wan-Uk Kim

ABSTRACT. ***Objective.*** To investigate the expression of Gremlin 1 (GREM1), an antagonist of bone morphogenetic protein, in rheumatoid arthritis (RA) synovia and its involvement in the hyperplasia and invasiveness of fibroblast-like synoviocytes of RA (RA-FLS).

Methods. Computational analysis was introduced to identify FLS-predominant regulators. GREM1 expression was examined by immunohistochemistry, real-time PCR, and ELISA. FLS proliferation and apoptosis were determined using tetrazolium-based colorimetric assay and APOPercentage assay, respectively. FLS migration and invasion were evaluated by wound migration and Matrigel invasion assay, respectively. Expressions of Bax, Bcl2, pErk1/2, and pAkt were detected by Western blot analysis.

Results. Through global transcriptome profiling, we identified a *GREM1* gene predominantly expressed in RA-FLS. Indeed, the GREM1 expression was higher in synovia, synovial fluids, and FLS of patients with RA than in those of patients with osteoarthritis, and its levels correlated well with proinflammatory cytokine concentrations. Knockdown of GREM1 transcripts using short interfering RNA (siRNA) reduced the proliferation and survival of RA-FLS along with downregulation of pErk1/2, pAkt, and Bcl2 expressions, whereas it induced Bax expression. Conversely, the addition of recombinant GREM1 to RA-FLS showed the opposite results. Moreover, GREM1 siRNA decreased the migratory and invasive capacity of RA-FLS, whereas exogenous GREM1 increased it. The GREM1-induced FLS survival, migration, and invasion were completely blocked by neutralizing antibodies to $\alpha_v\beta_3$ integrin on RA-FLS, suggesting that $\alpha_v\beta_3$ integrin mediates the antiapoptotic and promigratory effects of GREM1.

Conclusion. GREM1 is highly expressed in RA joints, and functions as a regulator of survival, proliferation, migration, and invasion of RA-FLS. (J Rheumatol First Release February 1 2016; doi:10.3899/jrheum.150523)

Key Indexing Terms:
FIBROBLAST-LIKE SYNOVIOCYTES
GREMLIN 1
RHEUMATOID ARTHRITIS
$\alpha_v\beta_3$ INTEGRIN

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation and progressive joint destruction by activation of various inflammatory cells[1]. Particularly, fibroblast-like synoviocytes (FLS), the major cells in the synovial lining layer, directly participate in chronic inflammation through the secretion of inflammatory cytokines and matrix-degrading enzymes[2]. In addition, they have highly migratory and invasive features to the bone and cartilage after detaching from synovia, which leads to further joint destruction[1,2,3]. Moreover, FLS of patients with RA (RA-FLS) harbor somatic mutations of several oncogenes or tumor suppressor genes, such as *H-Ras* and *p53*, and proliferate abnormally despite the presence of cell death receptors and ligands[4,5,6].

From the POSTECH-CATHOLIC Biomedical Engineering Institute, and Department of Internal Medicine, Catholic University of Korea, Seoul; Center for Systems Biology of Plant Senescence and Life History, Institute for Basic Science, Daegu Gyeongbuk Institute of Science and Technology (DGIST), Daegu, Korea; Department of Surgery and Biomedical Sciences, Cancer Biology Program, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, California, USA.

Supported by grants from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare and Family Affairs (No. HI14C3417) and the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2014R1A2A1A11049812, 2014R1A6A3A04054066, and 2015R1A3A2032927).

E.J. Han, PhD Candidate, POSTECH-CATHOLIC Biomedical Engineering Institute, Catholic University of Korea; S.A. Yoo, PhD, POSTECH-CATHOLIC Biomedical Engineering Institute, Catholic University of Korea; G.M. Kim, PhD Candidate, POSTECH-CATHOLIC Biomedical Engineering Institute, Catholic University of Korea; D. Hwang, PhD, Center for Systems Biology of Plant Senescence and Life History, Institute for Basic Science, DGIST; C.S. Cho, MD, PhD, POSTECH-CATHOLIC Biomedical Engineering Institute, Catholic University of Korea; S. You, PhD, Department of Surgery and Biomedical Sciences, Cancer Biology Program, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center; W.U. Kim, MD, PhD, POSTECH-CATHOLIC Biomedical Engineering Institute, and Department of Internal Medicine, Catholic University of Korea.

Address correspondence to Dr. W.U. Kim, Department of Internal Medicine, Division of Rheumatology, Catholic University of Korea, School of Medicine, Seoul, 137-701, Korea. E-mail: wan725@catholic.ac.kr. Or: Dr. S. You, Department of Surgery and Biomedical Sciences, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, California 90048, USA. E-mail: Sungyong.You@cshs.org

Accepted for publication October 13, 2015.

Gremlin 1 (GREM1) is a 20.7 kDa protein belonging to the cerberus and dan subfamily of bone morphogenetic protein (BMP) antagonists[7]. This protein forms heterodimers with BMP-2, BMP-4, and BMP-7, and inhibits the interaction of BMP with its receptor on the cell surface[8,9,10]. Inhibition of BMP by GREM1 in mice allows the expression of the fibroblast-growth factor and sonic hedgehog, which are necessary for proper limb development[11]. In several studies, GREM1 was shown to be important for the proliferation, migration, and invasion of various cancer cells[12,13,14]. In addition, GREM1 exerts a proangiogenic activity in the endothelium of human lung tissues[15]. Despite the progress in the understanding of GREM1 in the development and cancer, little is known about GREM1 expression in RA and its involvement in FLS biology and RA pathogenesis.

Here, we identified a *GREM1* gene predominantly expressed in RA-FLS through a global transcriptome profiling of synovium and FLS. We also validated that GREM1 was highly expressed in synoviums, synovial fluids (SF), and FLS of patients with RA as compared with those of patients with osteoarthritis (OA). *GREM1* knockdown by short interfering RNA (siRNA) decreased the proliferation and survival of RA-FLS. In parallel, *GREM1* siRNA decreased the expressions of pAkt, pErk1/2, and Bcl2 in RA-FLS, while it increased Bax expression. Conversely, the addition of recombinant GREM1 to RA-FLS exhibited the exact opposite results. Additionally, *GREM1* siRNA reduced the migratory and invasive capacity of RA-FLS, whereas recombinant GREM1 induced it. The GREM1-induced FLS survival, migration, and invasion were completely blocked by neutralizing antibodies to $\alpha_v\beta_3$ integrin on RA-FLS. GREM1 is highly expressed in RA joints and promotes survival, proliferation, migration, and invasion of RA-FLS through its functional receptor $\alpha_v\beta_3$ integrin. These results suggest that GREM1 is crucial for abnormal proliferation and invasiveness of RA-FLS and could therefore be a potential target for anti-FLS therapy.

MATERIALS AND METHODS

Transcriptome data analysis. Gene expression data collected from FLS (GSE7669 and GSE4061) and synovial tissues (GSE12021) were obtained from the Gene Expression Omnibus[16]. Each of the datasets was normalized using the quantile method[17]. Differentially expressed genes (DEG) in RA versus OA, RA versus normal control, or RA versus common reference mRNA (CRM) samples were determined using the integrative hypothesis testing method, as previously reported[18,19]. DEG were selected as genes with false discovery rate < 0.05 and fold change ≥ 1.5. As a result, 17 genes were identified as commonly upregulated genes in all 3 comparisons.

Selection of potential regulators predominantly expressed in RA-FLS. Out of the 17 genes using gene ontology annotation, 13 genes were found to have regulatory activities (e.g., transcriptional regulators and/or signaling molecules). We further performed a selection of RA-FLS–predominant genes by comparison of expression levels of 13 regulators in RA-FLS and FLS of patients with OA (OA-FLS) with those of 28 normal tissues or 60 cancer cell lines. For this, the expression dataset for FLS (GSE7669) was combined with 28 normal tissue expression datasets obtained from the BioGPS database[20] and the NCI60 cancer cell line (a panel of 60 diverse human cancer cell lines used by the U.S. National Cancer Institute) gene expression dataset[21], separately, and then the expression levels of 13 regulators were compared with the mean expression levels of 28 normal tissues or NCI60 cell lines using the 1-sided Student t test.

Immunohistochemical analysis for GREM1. For the immunohistochemistry of GREM1, 5 μm sections of paraffin-embedded synovia of patients with RA and OA were treated with pepsin for 30 min and blocked with bovine serum albumin (BSA) for 30 min at room temperature. Tissue sections were then incubated with an anti-GREM1 antibody (1:100; Santa Cruz Biotechnology) overnight at 4°C. Nonspecific mouse immunoglobulin (Sigma-Aldrich) was used for a negative control. Each slide was washed 3 times in Tris-buffered saline (TBS) and incubated with biotinylated anti-mouse IgG (Vector Laboratories) in a humid chamber for 30 min. The GREM1-positive cells were detected by adding peroxidase-conjugated streptavidin (Vector Laboratories), followed by 3′3-Diamino-benzidine tetrahydrochloride (Vector Laboratories).

Double immunofluorescence staining of RA synovia. Seven-μm sections of cryo-embedded synovia of patients with RA were incubated in acetone for 30 min at –20°C and blocked with BSA for 30 min at room temperature. Tissue sections were then incubated with anti-CD55 antibodies (1:200; Santa Cruz Biotechnology) plus anti-GREM1 antibodies (Santa Cruz Biotechnology) or anti-CD68 antibodies (1:50; Invitrogen) plus anti-GREM1 antibodies (Santa Cruz Biotechnology) overnight at 4°C. Each slide was washed 3 times in PBS and incubated with anti-mouse Alexa 488 for CD55 or CD68 (Invitrogen) and anti-goat Cy3 for GREM1 (Abm) in a dark humid chamber for 40 min. The slides were fixed with DAPI mounting solution.

Isolation and culture of FLS. FLS were isolated from the synovial tissues of patients with RA or OA, as described previously[22]. Cells from passage 3 to 6 were used throughout the experiments. Our study protocol was approved by the Institutional Review Board of the Catholic Medical Center (VC14TIMI0206).

ELISA. The levels of BMP-2, interleukin (IL) 1β, IL-6, and transforming growth factor (TGF) β in the SF of patients with RA and OA were measured with an ELISA kit (R&D) according to the manufacturer's instructions. The amount of GREM1 was also measured in the SF and culture supernatants of RA-FLS using an ELISA kit (USCN Life Science Inc.).

RNA isolation and real-time PCR. Total mRNA was isolated from the FLS of patients with RA and OA using an RNease Kit (Quiagen) and reverse transcribed to complementary DNA (cDNA). Quantitative real-time PCR was performed in the StepOnePlus Real-Time PCR System (Applied Biosystems) using SYBR Premix (Applied Biosystems) according to the manufacturer's instructions. GAPDH was used as an internal control for PCR amplification. Transcript levels were calculated relative to controls and expressed as –ΔΔCt.

Transfection of GREM1 siRNA. For the downregulation of *GREM1* transcripts, FLS were transfected with 50 nM *GREM1* siRNA (Dharmacon) using DharmaFECT transfection reagent (Dharmacon) in Opti-MEM medium (Invitrogen) according to the manufacturer's instructions. After transfection, FLS were cultured at 37°C for 4 h and then placed again in culture medium for FLS. After 48 h, *GREM1* mRNA expression levels in FLS were determined by real-time PCR using GREM1 primers (Bioneer).

Western blot analysis. FLS were lysed in lysis buffer for 15 min at 4°C. The concentration of lysate was measured by the Bradford assay (Bio-Rad). The membrane was blocked by 5% skim milk in TBS containing 0.25% Tween 20 and incubated overnight at 4°C with antibodies to pAkt (1:1000; Cell Signaling Technology), Akt (1:1000; Cell Signaling Technology), pErk (1:1000; Cell Signaling Technology), total Erk (1:1000; Santa Cruz Biotechnology), Bax (1:1000; Santa Cruz Biotechnology), Bcl2 (1:1000; Santa Cruz Biotechnology), and β-actin (1:10000; Abm). Membranes were then visualized using an enhanced chemo-luminescent technique.

Cell proliferation assay. After the addition of recombinant human GREM1 (rhGREM1; R&D), the FLS proliferation rate was determined using a 5-bromo-2′-deoxyuridine (BrdU) incorporation assay kit (Roche Life

Science) according to the manufacturer's instructions. In RA-FLS transfected with *GREM1* siRNA, cell proliferation was also assessed by tetrazolium (MTT) assay after stimulation of tumor necrosis factor (TNF)-α or TGF-β for 72 h as previously described[23].

Cell viability. Cell viability was determined by MTT assay as described previously[23,24].

Detection of apoptosis. FLS apoptosis levels were determined in the presence of rhGREM1 (R&D) or *GREM1* siRNA using an APOPercentage apoptosis assay kit (Biocolor Ltd) as described previously[23]. Photo images of APOPercentage dye-labeled cells, which stained pink under a light microscope, were used to quantify the extent of apoptosis. In some experiments, FLS apoptosis was assessed in the presence of BMP-2 (100 ng/ml; R&D), a GREM1 antagonist[24].

Wound migration assay. Wound migration was assessed as previously described[25]. Briefly, FLS were seeded to 80% confluence on 6 well plates, scratched with pipette tips, and then treated with 200 ng/ml of rhGREM1 in the presence or absence of BMP-2 (R&D) for 12 h. In other sets of experiments, the migration of *GREM1*-deficient RA-FLS was also assessed 12 h after transfection with *GREM1* siRNA, when cell viability was intact. The migrated FLS were quantitated by counting the cells that moved beyond the reference line.

Matrigel invasion assay. The BD BioCoat Matrigel Invasion Chamber (Becton Dickinson) was used to assess FLS invasion, according to the manufacturer's instructions. Briefly, after the transfection of *GREM1* siRNA or the addition of rhGREM1, RA-FLS were allowed to migrate in Matrigel invasion chamber for the indicated times. The noninvading cells were removed afterward by scrubbing with a cotton-tipped swab, and the cells on the lower surface of the membrane were stained with Diff-Quik stain (Baxter Diagnostics). For quantification, cells were counted in 8 random fields.

Flow cytometry analysis for $\alpha_v\beta_3$ integrin. RA-FLS were incubated with anti-$\alpha_v\beta_3$ integrin antibody as the primary antibody (Chemicon international) at 4°C for 30 min. The cells were then washed in PBS with 0.5% BSA and labeled with phycoerythrin-conjugated goat anti-mouse IgG (BD Biosciences) at 4°C for 30 min. The expression of $\alpha_v\beta_3$ integrin on FLS was detected using a FACSCanto flow cytometer (BD Biosciences).

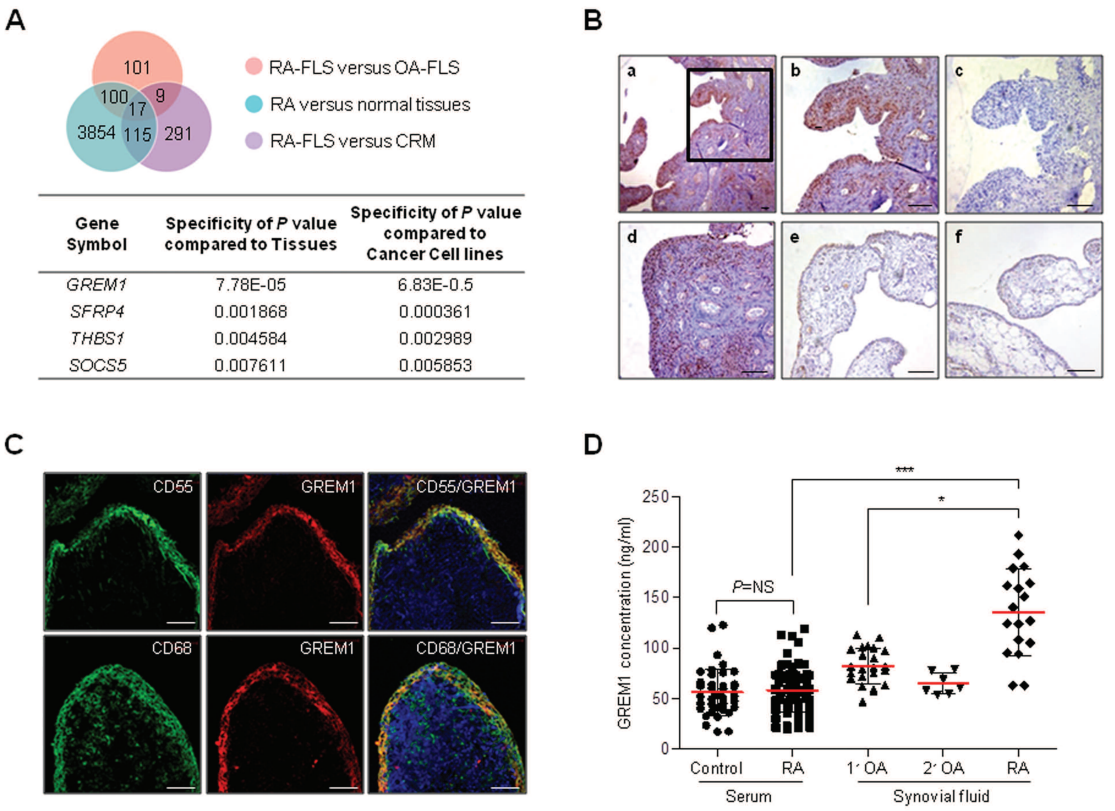
Statistical analysis. Data are expressed as the mean ± SD. Comparisons of the numerical data between groups were performed by the paired or unpaired Mann-Whitney U test. P values < 0.05 were considered statistically significant.

RESULTS

Identification of GREM1 gene by profiling the global gene expression of RA-FLS. Disease-associated genes have been shown to be frequently upregulated in a certain disease condition, and their expression reflects tissue origin[26,27]. Thus, we hypothesized that key genes might be highly expressed under RA conditions and also that their expression levels should be predominant in the synovia. To assess this hypothesis, we first collected 3 gene expression datasets derived from studies for FLS (GSE7669 and GSE4061) and synovial tissues (GSE12021)[28,29,30]. Three sets of upregulated genes, including 227 genes (RA-FLS vs OA-FLS), 4086 genes (RA vs normal synovial tissues), and 432 genes (RA-FLS vs CRM samples), were identified by integrative hypothesis testing (Details under Materials and Methods; Figure 1A). We then focused on the 17 genes that are commonly upregulated in all 3 comparisons. Among the 17 genes, we further selected 13 genes with regulatory activities (e.g., transcriptional regulators and/or signaling molecules) using gene ontology annotation (Supplementary Table 1; all supplementary material available from www.ribjd.com/base/data/data01.php?com). Interestingly, the list of 13 genes included well-known regulators involving RA pathogenesis. For example, *CDH11* is highly expressed in synovial cells at the pannus region in rheumatoid synovitis and induces the invasive property of FLS[31]. *FAS* and *TNC* are expressed at the areas of inflammation and tissue damage in RA synovia[32,33]. These results suggest that others of the 13 genes, albeit not studied in RA pathogenesis, can be essential for pathologic actions of RA-FLS. Thus, we subsequently tried to select the genes specific for RA-FLS by comparison of expression levels of 13 potential regulators with transcriptome profiles of 28 normal tissues or 60 cancer cell lines. As a result, we identified 4 genes — *GREM1*, *SFRP4*, *THBS1*, and *SOCS5* — out of the 13 regulators predominantly expressed in RA-FLS over 28 normal tissues and 60 cancer cell lines (Figure 1A and Supplementary Figure 1). Interestingly, *GREM1* gene showed the greatest statistical significance in terms of specificity (Figure 1A), and its involvement in RA pathogenesis has never been studied. Taken together, this result proposed that GREM1 could be a novel regulator with predominant expression in RA-FLS.

Increased expression of GREM1 in FLS and SF of patients with RA. To validate our postulation, we first performed immunohistochemical staining for GREM1 in the synovial tissues of patients with RA and OA. The high level of GREM1 protein expression was observed in the synovial tissues of patients with RA (Figure 1B: a, b, and d) as compared with those of OA (Figure 1B: e and f), particularly in the lining layer. To assess the GREM1-expressing cells in the RA synovium, double immunofluorescence staining was performed using anti-CD55 antibody (green), which was specific to FLS in the synovia[2], and anti-GREM1 antibody (red). The result showed that the superimposition of CD55+ staining on GREM1 staining (yellow) highlighted the lining layer of RA synovia. As a control, anti-CD68 antibody (green), which stained macrophage lineage[2], was done, showing that the cells positive for GREM1 were distinct from CD68+ cells in the synovia and the colocalization of GREM1 with CD68 was lacking in the sublining layer. This result indicated that GREM1 was expressed in FLS in the synovial lining region (Figure 1C).

GREM1 is a secreted glycoprotein. Thus, we measured the concentration of secreted GREM1 in the SF obtained from patients with RA and OA by ELISA. While the concentration of GREM1 was not elevated in the sera of patients with RA (n = 80) as compared with those of non-RA controls (n = 20), it was significantly higher in the SF of patients with active RA (n = 19) than in those of the patients with primary (n = 25) and secondary OA (n = 8; Figure 1D). The SF of secondary OA indicated noninflammatory joint fluids (white blood cell count/mm^3 < 1000) of patients with RA. These results indicated that abnormal GREM1 response was specific to RA joints, the actual sites of chronic inflammation



Gene Symbol	Specificity of *P* value compared to Tissues	Specificity of *P* value compared to Cancer Cell lines
GREM1	7.78E-05	6.83E-0.5
SFRP4	0.001868	0.000361
THBS1	0.004584	0.002989
SOCS5	0.007611	0.005853

Figure 1. Elevated GREM1 expression in RA joints. (A) Transcriptome analysis of FLS and synovial tissues. Venn diagram depicts the number of overlapping genes with significant overexpression in 3 RA transcriptome datasets. (B) Immunohistochemical staining of synovia of patients with RA (a, b, c, and d) and OA (e and f) using anti-GREM1 antibody (a, b, d, e, and f) and isotype control (c). Rectangular area in (a) is magnified to (b) and (c). Bars = 200 μm. (C) Double immune-fluorescent staining of an RA synovium (n = 3) using anti-CD55 antibody plus anti-GREM1 antibody (upper panel) or anti-CD68 antibody+ and anti-GREM1 antibody (lower panel). Bars = 100 μm. (D) Concentration of GREM1 in the sera of non-RA controls (n = 20), sera of patients with RA (n = 80), and synovial fluids of patients RA (n = 19), primary (1′) OA (n = 25), and secondary (2′) OA (n = 8) as determined by ELISA. Secondary OA indicates noninflammatory joint fluids (white blood cell count/mm^3 < 1000) of patients with RA. Data show the mean ± SD. * P < 0.05. *** P < 0.0001. GREM1: Gremlin 1; RA: rheumatoid arthritis; FLS: fibroblast-like synoviocytes; OA: osteoarthritis; CRM: common reference mRNA; SFRP4: secreted frizzled-related protein 4; THBS1: thrombospondin 1; SOCS5: suppressor of cytokine signaling 5.

with synovial hyperplasia. Collectively, RA-FLS in the synovial lining layer were a major cellular component expressing the GREM1 protein, and they may secrete soluble GREM1 into the SF of patients with RA.

Proinflammatory cytokines induced GREM1 expression. Figure 1 shows that FLS are a major cell type expressing GREM1 protein. Based on this finding, we examined the basal expression levels of GREM1 in FLS of patients with RA and OA. Real-time PCR analysis revealed that the GREM1 mRNA expression level was more than 3-fold higher in RA-FLS (n = 6) than in OA-FLS (n = 6; Figure 2A). Proinflammatory cytokines, including IL-1β, TNF-α, TGF-β, and hypoxic stimulus CoCl2, increased the *GREM1* mRNA expression in RA-FLS, but this was not noted with OA-FLS (Figure 2B). In contrast, IL-4, an antiinflammatory cytokine, decreased *GREM1* mRNA expression in RA-FLS (Figure 2B). Moreover, as determined in the culture supernatants of RA-FLS by ELISA, treatment of proinflammatory cytokines resulted in an increase in the secretion of the GREM1 protein (Figure 2C). Additionally, GREM1 concentrations in the SF of patients with RA (n = 27) correlated well with levels of IL-1β, IL-6, and TGF-β in the same SF (Figure 2D). Taken together, these results suggest that not only is GREM1 constitutively overexpressed in RA-FLS, but its levels can also be further increased by proinflammatory cytokines and hypoxia.

GREM1 promoted FLS proliferation and survival. RA-FLS, similar to cancer cells, abnormally proliferate and are resistant to apoptotic death, resulting in the formation of a hyperplastic pannus[1,2]. Moreover, GREM1 controls the proliferation of various cancer cells[12,13,14]. Therefore, we investigated the contribution of GREM1 to FLS proliferation and survival using siRNA against *GREM1* transcripts as well as using rhGREM1. As seen in Figure 3A, we first confirmed that the GREM1 mRNA expression was markedly reduced by transfection of RA-FLS with GREM1 siRNA for 48 h. We then tested whether GREM1 regulates FLS proliferation in

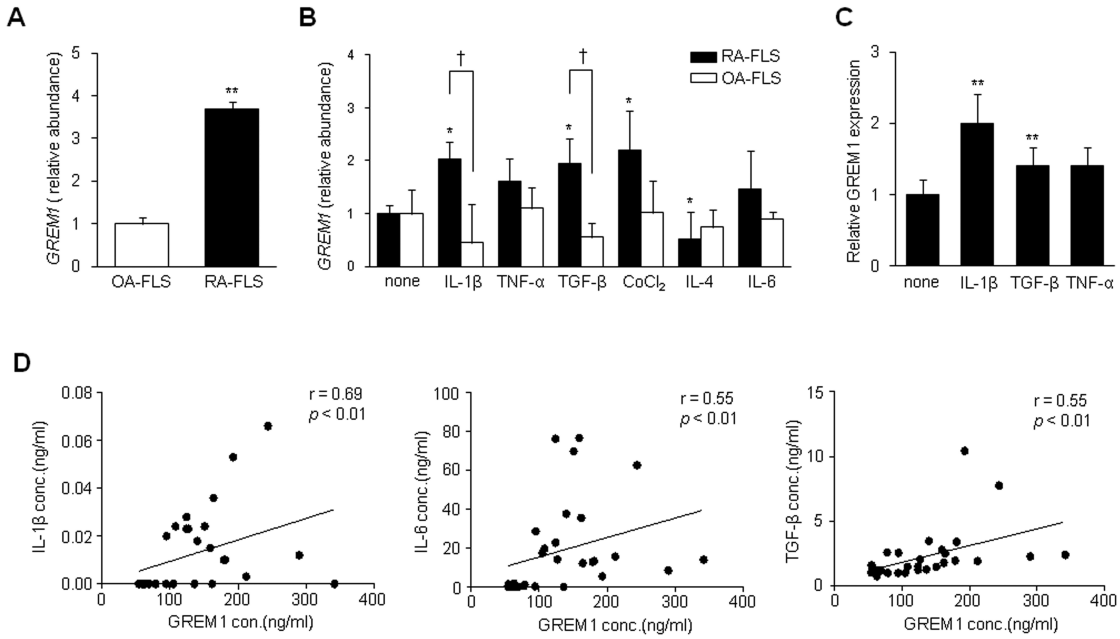


Figure 2. Association of GREM1 expression with proinflammatory cytokines. (A) Basal expression of *GREM1* mRNA in RA-FLS (n = 8) versus OA-FLS (n = 8). FLS (6×10^5) were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented 10% FBS for 24 h. The mRNA level was measured by real-time PCR. GAPDH was used as an internal control for PCR amplification. ** $p < 0.01$ versus OA-FLS. (B) Stimulated expression of *GREM1* mRNA in RA-FLS (n = 6) and OA-FLS (n = 6). FLS (6×10^5) were cultured in DMEM supplemented with 1% FBS and stimulated with IL-1β (10 ng/ml), TNF-α (10 ng/ml), TGF-β (10 ng/ml), IL-4 (10 ng/ml), IL-6 (10 ng/ml), and CoCl2 (200 μM) for 12 h. The mRNA level was determined by real-time PCR. GAPDH was used as an internal control for PCR amplification. * $P < 0.05$ versus unstimulated RA-FLS. † $P < 0.05$. (C) GREM1 protein expression in RA-FLS. RA-FLS (1×10^4) were treated with IL-1β (10 ng/ml), TNF-α (10 ng/ml), and TGF-β (10 ng/ml) for 24 h, and then GREM1 concentrations in the supernatants were measured by ELISA. Data are the relative values to basal level of GREM1 secreted by unstimulated RA-FLS. Mean level of GREM1 produced by unstimulated RA-FLS (none) was 1.9 ± 0.8 ng/ml. ** $p < 0.01$ versus unstimulated cells. (D) Correlation of GREM1 and proinflammatory cytokine concentrations (conc.) in the SF of patients with RA, which were measured by ELISA. Spearman rank correlation coefficient (r) and its level of significance (p) were computed by SPSS software. GREM1: Gremlin 1; RA: rheumatoid arthritis; FLS: fibroblast-like synoviocytes; OA: osteoarthritis; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; IL-1β: interleukin 1β; TNF-α: tumor necrosis factor-α; TGF-β: transforming growth factor β.

response to proinflammatory cytokines. The result showed that GREM1 siRNA completely abrogated TNF-α–induced and TGF-β–induced RA-FLS proliferation (Figure 3B). To the contrary, proliferation of RA-FLS was increased by the treatment of rhGREM1 for 5 days, as determined by the BrdU incorporation assay (Figure 3C), suggesting that GREM1 promotes FLS proliferation. A signaling pathway critical for FLS proliferation, such as pAkt and pErk1/2, has been described in RA[34]. As shown in Figure 3D, transfection of GREM1 siRNA decreased the levels of pAkt and pErk1/2, whereas exogenous rhGREM1 increased it. The induction of pAkt and pErk1/2 in RA-FLS occurred as early as 5 min and remained high up to 30 min after treatment with rhGREM1, supporting the notion that GREM1 is an important mediator of FLS proliferation. However, the expression levels of Akt and total Erk were altered by neither GREM1 siRNA nor rhGREM1.

The activation of pErk1/2 maintains mitochondrial integrity through the upregulation of antiapoptotic Bcl2 expression or through the inhibition of proapoptotic Bax expression[35,36]. Thus, we tested whether GREM1 controlled FLS survival and apoptosis-related molecules, Bcl2 and Bax. As seen in Figure 4A, the number of apoptotic cells markedly increased in GREM1-deficient RA-FLS as compared with control siRNA-transfected RA-FLS, as determined by the APOPercentage assay. Moreover, *GREM1* knockdown using siRNA decreased the expression of Bcl2 in RA-FLS, but increased the Bax expression (Figure 4C). On the contrary, the rhGREM1 addition significantly inhibited starvation-induced apoptosis of RA-FLS (Figure 4B). In parallel, rhGREM1 addition induced Bcl2 expression in RA-FLS, while reducing the Bax expression (Figure 4C). Additionally, *GREM1* siRNA significantly decreased the viability of RA-FLS after 48 h and 72 h of transfection, as determined by the MTT assay (Figure 4D). Together, these results suggest that GREM1 confers apoptotic resistance to RA-FLS, contributing to synovial hyperplasia in RA.

GREM1 increased FLS migration and invasion. In addition

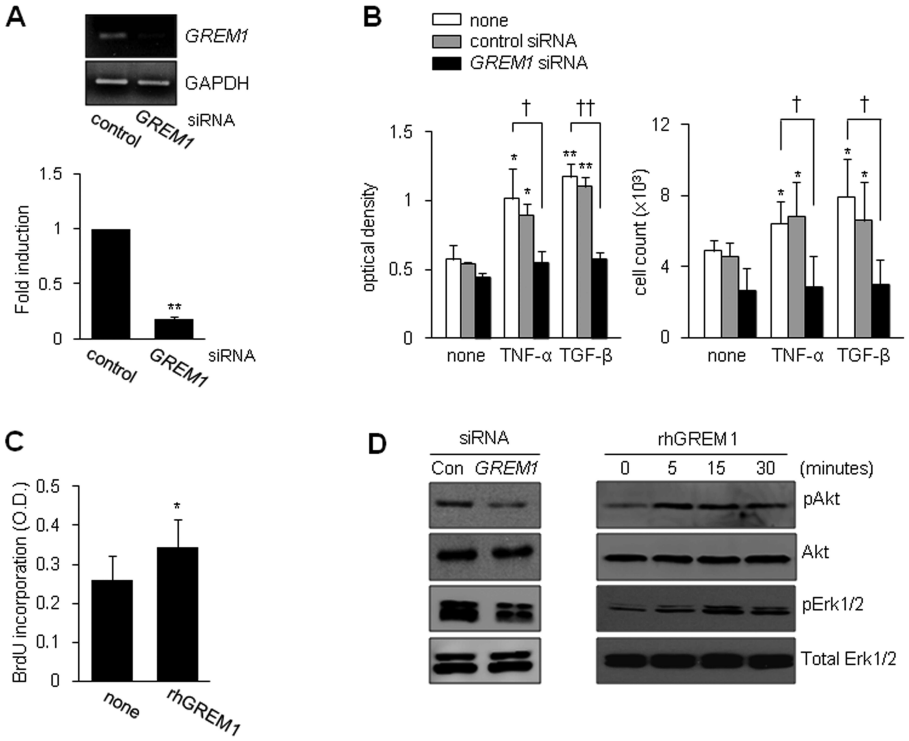


Figure 3. Effects of GREM1 on FLS proliferation. (A) Downregulation of GREM1 mRNA expression in RA-FLS by siRNA. Levels of GREM1 mRNA were measured by reverse transcription PCR (upper panel) or real-time PCR (lower panel). The bar plot in the lower panel represents the relative abundance of GREM1 mRNA expression normalized by GAPDH mRNA level. ** $P < 0.01$ versus control (scrambled) siRNA-transfected RA-FLS. (B) FLS proliferative responses to TNF-α and TGF-β. Twenty-four h after transfection with GREM1 siRNA, RA-FLS (1×10^4 cells) were treated with TNF-α (10 ng/ml) or TGF-β (10 ng/ml) for 72 h. Optical density was measured by tetrazolium assay (left). Under the same culture conditions, manual cell counts were also performed by trypan blue exclusion to identify viable cells (right). Data are the mean ± SD of 3 independent experiments in duplicate. * $P < 0.05$ and ** $P < 0.01$ versus unstimulated cells. † $P < 0.05$. †† $P < 0.01$. (C) FLS proliferation in response to human recombinant GREM1 (rhGREM1). RA-FLS were cultured in DMEM supplemented with 1% FBS, stimulated with rhGREM1 (200 ng/ml) for 5 days, and subjected to BrdU incorporation assay. Data are the mean ± SD of 3 independent experiments in duplicate and expressed as optical density (O.D.). * $P < 0.05$ versus unstimulated cells. (D) GREM1 control of pAkt and pErk1/2 expressions in RA-FLS. In the left panel, the cells were transfected with GREM1 siRNA for 48 h. In the right panel, the cells were cultured with rhGREM1 (200 ng/ml) in DMEM supplemented with 0.5% FBS for the indicated times. Expressions of pAkt, Akt, pErk1/2, and total Erk were determined by Western blot analysis. Data represent 3 independent experiments with similar results. GREM1: Gremlin 1; FLS: fibroblast-like synoviocytes; RA: rheumatoid arthritis; siRNA: short interfering RNA; TNF-α: tumor necrosis factor-α; TGF-β: transforming growth factor β; DMEM: Dulbecco modified Eagle's medium; FBS: fetal bovine serum.

to abnormal proliferation and apoptotic resistance, increased invasiveness and migration were unique features of RA-FLS and were essential to joint destruction[1,31]. Thus, we investigated whether GREM1 regulated migration and invasion of RA-FLS. As shown in Figure 5A, under nonlethal time conditions (after 12 h), the knockdown of *GREM1* transcripts suppressed the wound migration of RA-FLS. Further, invasion of RA-FLS in a Matrigel chamber was also significantly blocked by transfection with *GREM1* siRNA (Figure 5C). Conversely, rhGREM1 (200 ng/ml) treatment significantly increased FLS migration and invasion as compared with untreated cells (Figures 5B and 5D). Collectively, these data indicated that GREM1 was a positive regulator of the migration and invasion of RA-FLS.

$\alpha_v\beta_3$ integrin mediated GREM1-induced increase in FLS survival and migration. GREM1 is a well-known antagonist of BMP[7], so we investigated whether GREM1 action on FLS originates from its antagonism to BMP. We focused particularly on BMP-2 among the BMP family, since BMP-2 is dominantly expressed in the RA synovium and promotes FLS apoptosis[24]. As shown in Supplementary Figure 2A (www.ribjd.com/base/data/data01.php?com), cotreatment of rhGREM1 blocked the BMP-2–induced apoptosis of RA-FLS, suggesting that GREM1 antagonizes proapoptotic

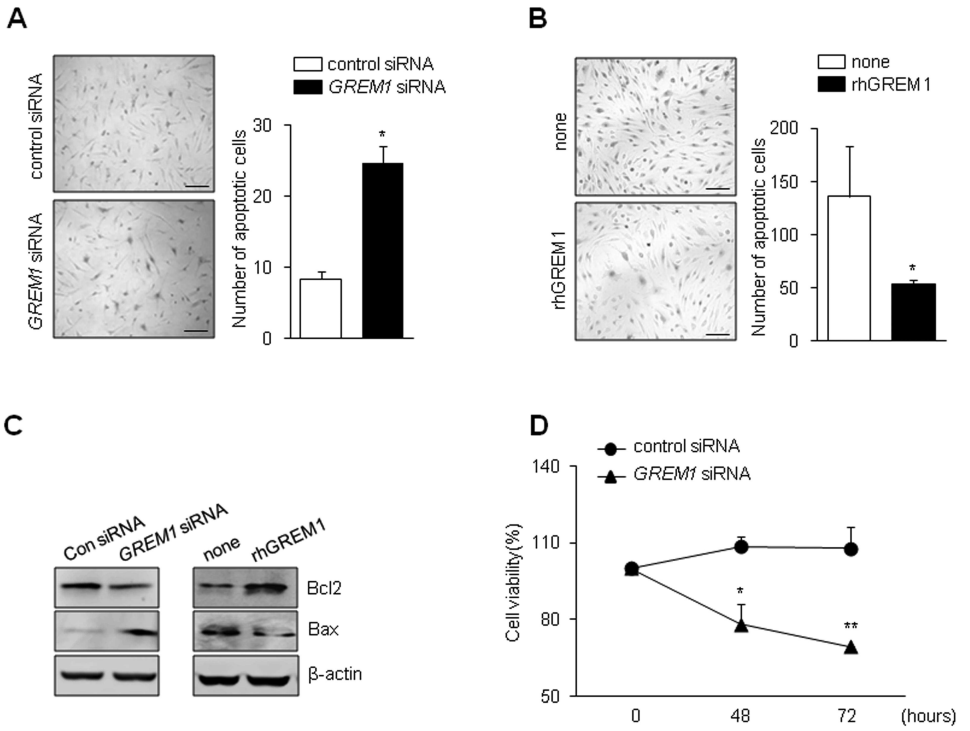


Figure 4. GREM1 regulation of FLS survival. (A) Apoptosis of RA-FLS by GREM1 siRNA. RA-FLS (n = 3) were transfected with GREM1 siRNA or control (scrambled) siRNA and then cultured in DMEM supplemented with 2% FBS for 48 h. The extent of apoptosis was determined by APOPercentage apoptosis assay. Apoptotic cells were stained. Bar plot on the right shows the number of apoptotic cells. Data are expressed as the mean ± SD of 3 independent experiments in duplicate. * $P < 0.05$ versus control siRNA-transfected cells. Bars = 200 μm. (B) GREM1 protection of starvation-induced apoptosis. Degrees of apoptosis were assessed by APOPercentage apoptosis assay. RA-FLS (n = 3) were cultured in the absence (upper panel) or presence (lower panel) of rhGREM1 (200 ng/ml) for 12 h. Data show the mean ± SD of 3 independent experiments in duplicate. * $P < 0.05$ versus unstimulated cells. Bars = 200 μm. (C) GREM regulation of Bcl2 and Bax expressions. RA-FLS (1×10^5 cells) were transfected with GREM1 siRNA for 48 h (left) or stimulated with 200 ng/ml of GREM1 for 24 h (right) in DMEM supplemented with 0.5% FBS. The expressions of Bcl2 and Bax were determined by Western blot analysis. The results are the representative of 3 independent experiments. Con siRNA: control siRNA. (D) GREM1 siRNA induction of FLS death. The FLS (n = 3) were cultured in DMEM supplemented with 1% FBS for the indicated times. Cell viability was determined by MTT assay. Data are normalized with respect to control siRNA transfected cells. * $P < 0.05$. ** $P < 0.005$. Values are the mean ± SD of 3 independent experiments in triplicate. GREM1: Gremlin 1; FLS: fibroblast-like synoviocytes; RA: rheumatoid arthritis; siRNA: short interfering RNA; DMEM: Dulbecco modified Eagle's medium; FBS: fetal bovine serum; MTT: tetrazolium.

activity of BMP-2. However, the GREM1-induced increase in FLS migration and invasion were rarely affected by BMP-2 (data not shown). Paradoxically, the addition of BMP alone increased FLS migration and invasion (data not shown), indicating that GREM1 antagonism to BMP-2 was not a major mechanism responsible for all pathologic actions of GREM1.

We demonstrated that RA-FLS expressed 3 vascular endothelial growth factor receptors — Flt-1, KDR, and neuropilin 1, of which neuropilin 1 is the major one[37]. In endothelial cells, GREM1 acts as a noncanonical KDR ligand, triggering a potent angiogenic response in a BMP-independent manner[38]. A report has also demonstrated that GREM1 stimulated the formation of KDR/$\alpha_v\beta_3$ integrin complexes in endothelial cells, and that anti-β3 antibodies blocked GREM1-induced angiogenesis[39]. Thus, we tested whether the effect of GREM1 on RA-FLS was mediated by the KDR or $\alpha_v\beta_3$ integrin. We first confirmed that the $\alpha_v\beta_3$ integrin was highly expressed on RA-FLS, and its levels were further instigated by the stimulation of IL-1β and TNF-α (Supplementary Figure 3). We also found that the rhGREM1-induced increase in FLS survival was completely abrogated by treatment with neutralizing antibodies to the $\alpha_v\beta_3$ integrin (Figure 6A). Moreover, GREM1-induced FLS migration and invasion were completely blocked by antibodies to the $\alpha_v\beta_3$ integrin, but not by antibodies to KDR, Flt-1, and neuropilin 1 (Figures 6B and 6C). Notably, we found that anti-$\alpha_v\beta_3$ antibody reduced the phosphorylation of Akt, but not pErk, in RA-FLS in the presence of rhGREM1 (Figure 6D), indicating that GREM1 induced the phosphory-

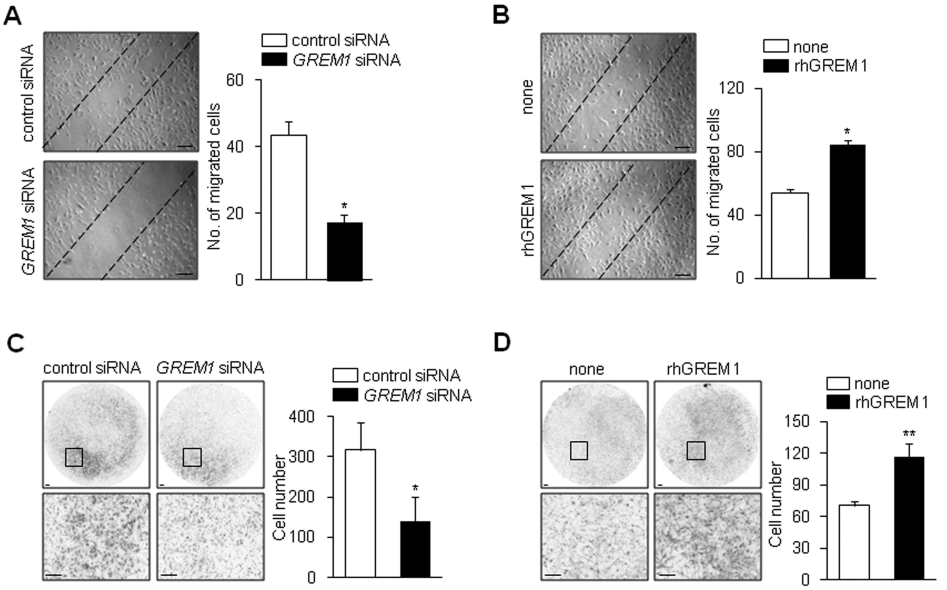


Figure 5. GREM1 control of migration and invasion of RA-FLS. (A) GREM1 siRNA suppression of wound migration of RA-FLS, as indicated by images on the phase-contrast microscopy (left) and by the number of manually counted migrated cells (right). After 12 h of transfection with GREM1 siRNA or control siRNA, RA-FLS (1×10^5 cells) were wounded with the tip of a micropipette and incubated in DMEM containing 1% FBS for 12 h. Cells migrating beyond the reference line were photographed and counted. Original magnification × 100. Data show the mean ± SD of 3 independent experiments. * $P < 0.05$ versus control siRNA. Bars = 200 μm. (B) GREM1 induction of wound migration of FLS. The RA-FLS (n = 3) were cultured with rhGREM1 (200 ng/ml) in DMEM containing 0.5% FBS for 12 h. Data show the mean ± SD of 3 independent experiments. * $P < 0.05$ versus untreated cells. Bars = 200 μm. (C) GREM1 siRNA suppression of FLS invasion. After 24 h of transfection with GREM1 siRNA or control siRNA, RA-FLS (5×10^5 cells) were incubated in a Matrigel invasion chamber for 12 h. Invasive cells were stained using a Diff-Quik kit, and the number of stained cells was counted. Representative photo images are shown on the left, where the rectangular area in the upper panel is magnified to the lower panel. The mean ± SD of 3 independent experiments is presented on the right. * $P < 0.05$ versus control siRNA. Bar = 200 μm. (D) GREM1 induction of FLS invasion. The RA-FLS (5×10^5 cells) were incubated in a Matrigel invasion chamber for 12 hours. The upper chamber contained DMEM supplemented with 0.1% FBS plus rhGREM1 (200 ng/ml), and the lower chamber contained serum-free medium. Bar = 200 μm. Data are expressed as the mean ± SD. * $P < 0.05$ and ** $P < 0.01$ versus untreated cells. GREM1: Gremlin 1; RA: rheumatoid arthritis; FLS: fibroblast-like synoviocytes; siRNA: short interfering RNA; DMEM: Dulbecco modified Eagle's medium; FBS: fetal bovine serum.

lation of Akt through the $\alpha_v\beta_3$ integrin. These results, together with previous reports[38,39], suggested that GREM1-induced FLS survival, migration, and invasion were mainly dependent on the $\alpha_v\beta_3$ integrin.

DISCUSSION

A key issue in developing a systems approach to diseases is to extract disease-relevant information by integrating global datasets and to apply it to clinical settings[40]. The identification of cell-specific key molecules is especially essential in understanding a particular pathophysiology in complex tissue microenvironments or in discovering novel targets for personalized therapeutic approaches[41,42]. Here, we introduced a computational approach through the integration of transcriptome profiles to identify FLS-predominant regulators. Through an unbiased analysis of RA transcriptomes in FLS and synovial tissues, as well as through the integration with transcriptome profiles of various normal tissues and cancer cell lines, we successfully identified 4 genes – *GREM1*, *SFPR4*, *THBS1*, and *SOCS5* – that were predominantly overexpressed in RA-FLS with regulatory activity. In particular, GREM1 had the greatest statistical significance, and its expression and function were validated in our patients' samples. These data suggest that our systems approach can help clinicians unveil a novel mechanism underlying disease pathogenesis and identify new targets for RA. The involvement of the other 3 genes in FLS pathology can be similarly determined.

In our present study, we demonstrated first that GREM1 was found in the SF, FLS, and synovial tissues of patients with RA at high levels, and its expression in RA-FLS was induced by proinflammatory cytokines, including IL-1β, TNF-α, and TGF-β, but not by antiinflammatory cytokine IL-4. Moreover, GREM1 concentrations in SF correlated well with IL-1β, IL-6, and TGF-β levels, suggesting that the

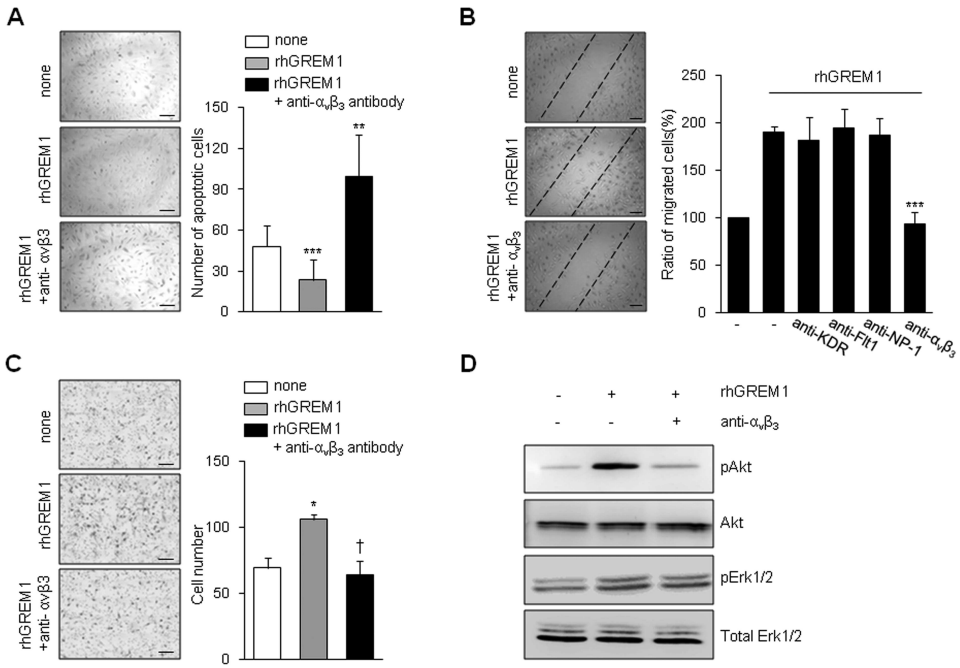


Figure 6. Involvement of $\alpha_v\beta_3$ integrin in GREM1-induced FLS survival, migration, and invasion. (A) Dependency of GREM1-induced FLS survival on $\alpha_v\beta_3$ integrin. RA-FLS (n = 3) were cultured with rhGREM1 (200 ng/ml) in the absence or presence of anti-$\alpha_v\beta_3$ integrin antibody (10 μg/ml) for 24 h. Degrees of apoptosis were assessed by APOPercentage apoptosis assay. Data show the mean ± SD of 3 independent experiments in duplicate. ** $P < 0.01$ and *** $P < 0.001$ versus unstimulated cells. Bars = 200 μm. (B) Effect of antibodies to VEGF receptors or anti-$\alpha_v\beta_3$ integrin antibody on the wound migration of synoviocytes. RA-FLS (n = 3, 1×10^5 cells) were incubated in DMEM containing 0.5% FBS with 200 ng/ml of rhGREM1 in the absence or presence of 10 μg/ml of anti-VEGF receptor antibodies [including anti-Flt-1 antibody, antineuropilin 1 antibody (anti-NP-1), and anti-KDR antibody] or anti-$\alpha_v\beta_3$ integrin antibody (10 μg/ml) for 12 h. Cells migrating beyond the reference line were photographed and counted. Data show the mean ± SD of 3 independent experiments and are presented as a ratio (%) relative to unstimulated conditions. * $P < 0.001$ versus rhGREM1 only. Bars = 200 μm. (C) Dependency of GREM1-induced FLS invasion on $\alpha_v\beta_3$ integrin. RA-FLS (5×10^5 cells) were incubated in a Matrigel invasion chamber for 12 h. The upper chamber contained DMEM supplemented with 0.1% FBS plus rhGREM1 (200 ng/ml) in the absence or presence of anti-$\alpha_v\beta_3$ integrin antibody (10 μg/ml), and the lower chamber contained serum-free medium. Data are expressed as the mean ± SD. * $P < 0.05$ versus untreated cells. † $P < 0.05$ versus rhGREM1 only. Bar = 200 μm. (D) Phosphorylation of Akt and Erk1/2 in RA-FLS treated with the anti-$\alpha_v\beta_3$ integrin antibody. The cells were precultured with the anti-$\alpha_v\beta_3$ integrin antibody (10 μg/ml) for 1 h and then incubated with rhGREM1 (200 ng/ml) in DMEM supplemented with 0.5% FBS for 15 min. Expressions of pAkt, total Akt, pErk1/2, and total Erk were determined by Western blot analysis. Data are the representative of 3 independent experiments with similar results. GREM1: Gremlin 1; FLS: fibroblast-like synoviocytes; RA: rheumatoid arthritis; siRNA: short interfering RNA; VEGF: vascular endothelial growth factor; DMEM: Dulbecco modified Eagle's medium; FBS: fetal bovine serum.

inflammatory milieu contributes to high GREM1 expression in RA joints. Interestingly, GREM1 expression in OA-FLS was not changed by proinflammatory stimuli, suggesting that cytokine induction of GREM1 might be specific for RA-FLS. Our data further indicated that GREM1 is involved in FLS proliferation.

It is well known that IL-1β, TNF-α, and TGF-β levels are greater in the sera, SF, and synovial tissues of patients with RA than in those of patients with OA[1,2,3,43]. Also, it has been reported that the expression level of the IL-1β receptor is also higher in the synovial membrane of patients with RA than in that of patients with OA[44]. TGF-β receptor is highly expressed in RA-FLS as compared with OA-FLS[45]. Therefore, RA-FLS may have a greater opportunity to be stimulated by these cytokines than OA-FLS. Moreover, the IL-1β–induced or TNF-α–induced nuclear factor-κB (NF-κB) pathway and the TGF-β–induced Smad pathway are more activated in the synovia of patients with RA than those of patients with OA[2]. We believe that cell responsiveness to IL-1β, TNF-α, and TGF-β is generally increased in RA-FLS, which leads to a higher expression of GREM1 in RA-FLS than in OA-FLS upon IL-1β or TGF-β stimulation. Under these circumstances, proinflammatory cytokines induce GREM1 expression in RA-FLS, and such an increased level of GREM1, in turn, could lead to synovial hyperplasia, which facilitates the further secretion of cytokines and thus estab-

lishes a vicious cycle (Supplementary Figure 4, www.ribjd.com/base/data/data01.php?com).

It remains undetermined how GREM1 expression is regulated by inflammatory stimuli. IL-1β is well known to regulate the expression of target genes through the activator protein 1 and nuclear factor-κB pathway[46]. Therefore, we presume that the interaction of these transcription factors with the promoter sites of GREM1 gene might be involved in the upregulation of GREM1 expression upon cytokine stimulation. However, the basal expression levels of GREM1 mRNA were also higher in RA-FLS than in OA-FLS, which suggests that elevated GREM1 expression is intrinsic to RA-FLS. Imprinted anomalies of RA-FLS have been identified, which are associated with altered microRNA expression and DNA methylation[47]. Therefore, it is possible that epigenetic dysregulation may contribute to the increased GREM1 expression in RA-FLS at the basal state.

We also showed that GREM1 is essential to FLS survival. GREM1 knockdown resulted in increased apoptotic death of RA-FLS with Bcl2 downregulation and Bax upregulation. Conversely, the addition of exogenous rhGREM1 to RA-FLS showed the opposite result, suggesting that secreted GREM1 by RA-FLS confers apoptotic resistance to FLS themselves in an autocrine/paracrine manner. Additionally, GREM1 siRNA decreased pErk1/2 and pAkt activities, while rhGREM1 increased such activities. Since pErk1/2 is an upstream regulator of Bcl2 and Bax[35,36], GREM1 promotes FLS survival by regulating Bcl2/Bax expression, possibly through the activation of pErk1/2. Together, our data suggest that overproduced GREM1 within RA joints protects RA-FLS from apoptosis, thereby maintaining a unique hyperplastic phenotype.

From the hyperplastic synovia, RA-FLS can be detached, and then they migrate to and invade adjacent bone and cartilage[1,2,44]. At the reattached sites, RA-FLS destroy cartilage and bone by releasing matrix degrading enzymes. In our study, similar to results in cancer cells[14], rhGREM1 increased the migration and invasion of RA-FLS. Conversely, siRNA-mediated elimination of GREM1 transcripts impaired these processes, suggesting that GREM1 is a key regulator of FLS migration and invasion. Considering that FLS migration and invasion are critical for joint destruction[1,2], these results suggest that a specific blockade of GREM1 activity may delay bone and cartilage destruction by intervening in these processes. Interestingly, it has been reported that skeletal overexpression of GREM1 impairs bone formation and causes osteopenia. Thus, we expect that GREM1 inhibitors can induce bone remodeling in a way that increases bone formation by osteoblasts as well as decrease bone destruction by blocking the promigratory and invasive activity of FLS.

BMP are currently considered pleiotropic cytokines that influence the proliferation, growth, differentiation, and apoptosis of different cell types[45,46,48,49]. BMP-2 promotes apoptosis of RA-FLS and osteoblasts[24,48,50,51], and its receptors are expressed on RA-FLS[52]. Here, we found that RA-FLS were exposed to the skewed production of GREM1 over BMP-2, as evidenced by the ratio of GREM1 over BMP-2 in the SF, which was significantly higher in patients with RA than in patients with OA (Supplementary Figure 2B). However, in contrast to FLS survival, cotreatment of BMP-2 did not block GREM1-induced FLS migration and invasion (data not shown), suggesting that the effects of GREM1 on FLS biology are only partially because of the GREM1 inhibition of BMP-2 (Supplementary Figure 4).

It has been reported that GREM1 directly binds to KDR and $\alpha_v\beta_3$ integrin heterodimer in endothelial cells[39]. However, antibodies to KDR did not affect GREM1-induced RA-FLS migration in our study, which may be because of the very low expression of KDR in RA-FLS[22]. Instead, blocking experiments using anti-$\alpha_v\beta_3$ antibody demonstrated that $\alpha_v\beta_3$ integrin, like endothelial cells, predominantly mediates the GREM1-induced increase in FLS survival, migration, and invasion. The $\alpha_v\beta_3$ integrin can induce activation of pAkt and pErk in various cell types[53,54]. Interestingly, we found that the anti-$\alpha_v\beta_3$ antibody nearly completely abrogated the recombinant GREM1-induced increase in pAkt expression but not pErk expression in RA-FLS. These data indicate that secreted GREM1 increases the phosphorylation of Akt through $\alpha_v\beta_3$ integrin and also suggest that GREM1-induced pErk activation is mediated possibly through different receptors from $\alpha_v\beta_3$ integrin. Taken together, although we failed to identify the direct binding partner(s) of GREM1 on RA-FLS, GREM1 may play proliferative, prosurvival, and promigratory roles through its functional receptor $\alpha_v\beta_3$ integrin (Supplementary Figure 4).

Through global transcriptome profiling, we identified a *GREM1* gene that was predominantly overexpressed in RA-FLS. In fact, GREM1 expression was elevated in synovia, SF, and FLS of patients with RA, and correlated with proinflammatory cytokine concentration. We also demonstrated the functional involvement of GREM1 in promoting the hyperplasia, migration, and invasiveness of RA-FLS, the key pathologic features of invasive pannus in RA. In this respect, GREM1 may be an effective therapeutic target for RA capable of demoting hyperplastic synovitis.

ACKNOWLEDGMENT

The authors thank all members of the Institute of Bone and Joint Diseases at the Catholic University of Korea.

REFERENCES

1. Firestein GS. Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? Arthritis Rheum 1996;39:1781-90.
2. Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev 2010;233:233-55.
3. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med 2011;365:2205-19.
4. Kobayashi T, Okamoto K, Kobata T, Hasunuma T, Kato T, Hamada

H, et al. Differential regulation of Fas-mediated apoptosis of rheumatoid synoviocytes by tumor necrosis factor alpha and basic fibroblast growth factor is associated with the expression of apoptosis-related molecules. Arthritis Rheum 2000;43:1106-14.
5. Roivainen A, Jalava J, Pirilä L, Yli-Jama T, Tiusanen H, Toivanen P. H-ras oncogene point mutations in arthritic synovium. Arthritis Rheum 1997;40:1636-43.
6. Yamanishi Y, Boyle DL, Green DR, Keystone EC, Connor A, Zollman S, et al. p53 tumor suppressor gene mutations in fibroblast-like synoviocytes from erosion synovium and non-erosion synovium in rheumatoid arthritis. Arthritis Res Ther 2005;7:R12-8.
7. Topol LZ, Marx M, Laugier D, Bogdanova NN, Boubnov NV, Clausen PA, et al. Identification of drm, a novel gene whose expression is suppressed in transformed cells and which can inhibit growth of normal but not transformed cells in culture. Mol Cell Biol 1997;17:4801-10.
8. Canalis E, Economides AN, Gazzerro E. Bone morphogenetic proteins, their antagonists, and the skeleton. Endocr Rev 2003;24:218-35.
9. Topol LZ, Bardot B, Zhang Q, Resau J, Huillard E, Marx M, et al. Biosynthesis, post-translation modification, and functional characterization of Drm/Gremlin. J Biol Chem 2000;275:8785-93.
10. Hsu DR, Economides AN, Wang X, Eimon PM, Harland RM. The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. Mol Cell 1998;1:673-83.
11. Michos O, Gonçalves A, Lopez-Rios J, Tiecke E, Naillat F, Beier K, et al. Reduction of BMP4 activity by gremlin 1 enables ureteric bud outgrowth and GDNF/WNT11 feedback signalling during kidney branching morphogenesis. Development 2007;134:2397-405.
12. Sneddon JB, Zhen HH, Montgomery K, van de Rijn M, Tward AD, West R, et al. Bone morphogenetic protein antagonist gremlin 1 is widely expressed by cancer-associated stromal cells and can promote tumor cell proliferation. Proc Natl Acad Sci U S A 2006;103:14842-7.
13. Namkoong H, Shin SM, Kim HK, Ha SA, Cho GW, Hur SY, et al. The bone morphogenetic protein antagonist gremlin 1 is overexpressed in human cancers and interacts with YWHAH protein. BMC Cancer 2006;6:74.
14. Kim M, Yoon S, Lee S, Ha SA, Kim HK, Kim JW, et al. Gremlin-1 induces BMP-independent tumor cell proliferation, migration, and invasion. PLoS One 2012;7:e35100.
15. Stabile H, Mitola S, Moroni E, Belleri M, Nicoli S, Coltrini D, et al. Bone morphogenic protein antagonist Drm/gremlin is a novel proangiogenic factor. Blood 2007;109:1834-40.
16. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 2002;30:207-10.
17. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003;19:185-93.
18. Lee HJ, Suk JE, Patrick C, Bae EJ, Cho JH, Rho S, et al. Direct transfer of alpha-synuclein from neuron to astroglia causes inflammatory responses in synucleinopathies. J Biol Chem 2010;285:9262-72.
19. Hwang D, Rust AG, Ramsey S, Smith JJ, Leslie DM, Weston AD, et al. A data integration methodology for systems biology. Proc Natl Acad Sci U S A 2005;102:17296-301.
20. Wu C, Orozco C, Boyer J, Leglise M, Goodale J, Batalov S, et al. BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources. Genome Biol 2009;10:R130.
21. Shankavaram UT, Reinhold WC, Nishizuka S, Major S, Morita D, Chary KK, et al. Transcript and protein expression profiles of the NCI-60 cancer cell panel: an integromic microarray study. Mol Cancer Ther 2007;6:820-32.
22. Yoo SA, Yoon HJ, Kim HS, Chae CB, De Falco S, Cho CS, et al. Role of placenta growth factor and its receptor flt-1 in rheumatoid inflammation: a link between angiogenesis and inflammation. Arthritis Rheum 2009;60:345-54.
23. Kong JS, Yoo SA, Kim HS, Kim HA, Yea K, Ryu SH, et al. Inhibition of synovial hyperplasia, rheumatoid T cell activation, and experimental arthritis in mice by sulforaphane, a naturally occurring isothiocyanate. Arthritis Rheum 2010;62:159-70.
24. Lories RJ, Derese I, Ceuppens JL, Luyten FP. Bone morphogenetic proteins 2 and 6, expressed in arthritic synovium, are regulated by proinflammatory cytokines and differentially modulate fibroblast-like synoviocyte apoptosis. Arthritis Rheum 2003;48:2807-18.
25. Lee MS, Yoo SA, Cho CS, Suh PG, Kim WU, Ryu SH. Serum amyloid A binding to formyl peptide receptor-like 1 induces synovial hyperplasia and angiogenesis. J Immunol 2006; 177:5585-94.
26. Lage K, Hansen NT, Karlberg EO, Eklund AC, Roque FS, Donahoe PK, et al. A large-scale analysis of tissue-specific pathology and gene expression of human disease genes and complexes. Proc Natl Acad Sci U S A 2008;105:20870-5.
27. Winter EE, Goodstadt L, Ponting CP. Elevated rates of protein secretion, evolution, and disease among tissue-specific genes. Genome Res 2004;14:54-61.
28. Geurts J, Vermeij EA, Pohlers D, Arntz OJ, Kinne RW, van den Berg WB, et al. A novel Saa3-promoter reporter distinguishes inflammatory subtypes in experimental arthritis and human synovial fibroblasts. Ann Rheum Dis 2011;70:1311-9.
29. Kasperkovitz PV, Timmer TC, Smeets TJ, Verbeet NL, Tak PP, van Baarsen LG, et al. Fibroblast-like synoviocytes derived from patients with rheumatoid arthritis show the imprint of synovial tissue heterogeneity: evidence of a link between an increased myofibroblast-like phenotype and high-inflammation synovitis. Arthritis Rheum 2005;52:430-41.
30. Huber R, Hummert C, Gausmann U, Pohlers D, Koczan D, Guthke R, et al. Identification of intra-group, inter-individual, and gene-specific variances in mRNA expression profiles in the rheumatoid arthritis synovial membrane. Arthritis Res Ther 2008;10:R98.
31. Lee DM, Kiener HP, Agarwal SK, Noss EH, Watts GF, Chisaka O, et al. Cadherin-11 in synovial lining formation and pathology in arthritis. Science 2007;315:1006-10.
32. Midwood K, Sacre S, Piccinini AM, Inglis J, Trebaul A, Chan E, et al. Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. Nat Med 2009;15:774-80.
33. Tu-Rapp H, Hammermuller A, Mix E, Kreutzer HJ, Goerlich R, Kohler H, et al. A proinflammatory role for Fas in joints of mice with collagen-induced arthritis. Arthritis Res Ther 2004;6:R404-14.
34. Zhang HG, Wang Y, Xie JF, Liang X, Liu D, Yang P, et al. Regulation of tumor necrosis factor alpha-mediated apoptosis of rheumatoid arthritis synovial fibroblasts by the protein kinase Akt. Arthritis Rheum 2001;44:1555-67.
35. Boucher MJ, Morisset J, Vachon PH, Reed JC, Lainé J, Rivard N. MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-X(L), and Mcl-1 and promotes survival of human pancreatic cancer cells. J Cell Biochem 2000;79:355-69.
36. Harada H, Quearry B, Ruiz-Vela A, Korsmeyer SJ. Survival factor-induced extracellular signal-regulated kinase phosphorylates BIM, inhibiting its association with BAX and proapoptotic activity. Proc Natl Acad Sci U S A 2004;101:15313-7.
37. Kim WU, Kang SS, Yoo SA, Hong KH, Bae DG, Lee MS, et al. Interaction of vascular endothelial growth factor 165 with neuropilin-1 protects rheumatoid synoviocytes from apoptotic death

by regulating Bcl-2 expression and Bax translocation. J Immunol 2006;177:5727-35.
38. Mitola S, Ravelli C, Moroni E, Salvi V, Leali D, Ballmer-Hofer K, et al. Gremlin is a novel agonist of the major proangiogenic receptor VEGFR2. Blood 2010;116:3677-80.
39. Ravelli C, Mitola S, Corsini M, Presta M. Involvement of αvβ3 integrin in gremlin-induced angiogenesis. Angiogenesis 2013;16:235-43.
40. Auffray C, Chen Z, Hood L. Systems medicine: the future of medical genomics and healthcare. Genome Med 2009;1:2.
41. Shen-Orr SS, Tibshirani R, Khatri P, Bodian DL, Staedtler F, Perry NM, et al. Cell type-specific gene expression differences in complex tissues. Nat Methods 2010;7:287-9.
42. Burmester GR, Feist E, Dörner T. Emerging cell and cytokine targets in rheumatoid arthritis. Nat Rev Rheumatol 2014;10:77-88.
43. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 2007;7:429-42.
44. Deleuran BW, Chu CQ, Field M, Brennan FM, Katsikis P, Feldmann M, et al. Localization of interleukin-1 alpha, type 1 interleukin-1 receptor and interleukin-1 receptor antagonist in the synovial membrane and cartilage/pannus junction in rheumatoid arthritis. Br J Rheumatol 1992;31:801-9.
45. Bira Y, Tani K, Nishioka Y, Miyata J, Sato K, Hayashi A, et al. Transforming growth factor beta stimulates rheumatoid synovial fibroblasts via the type II receptor. Mod Rheumatol 2005;15:108-13.
46. Liu FL, Chen CH, Chu SJ, Chen JH, Lai JH, Sytwu HK, et al. Interleukin (IL)-23 p19 expression induced by IL-1beta in human fibroblast-like synoviocytes with rheumatoid arthritis via active nuclear factor-kappaB and AP-1 dependent pathway. Rheumatology 2007;46:1266-73.
47. Bottini N, Firestein GS. Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors. Nat Rev Rheumatol 2013;9:24-33.
48. Neidhart M, Seemayer CA, Hummel KM, Michel BA, Gay RE, Gay S. Functional characterization of adherent synovial fluid cells in rheumatoid arthritis: destructive potential in vitro and in vivo. Arthritis Rheum 2003;48:1873-80.
49. Hogan BL. Bone morphogenetic proteins in development. Curr Opin Genet Dev 1996;6:432-8.
50. Wozney JM, Rosen V. Bone morphogenetic protein and bone morphogenetic protein gene family in bone formation and repair. Clin Orthop Relat Res 1998:26-37.
51. Hyzy SL, Olivares-Navarrete R, Schwartz Z, Boyan BD. BMP2 induces osteoblast apoptosis in a maturation state and noggin-dependent manner. J Cell Biochem 2012;113:3236-45.
52. Marinova-Mutafchieva L, Taylor P, Funa K, Maini RN, Zvaifler NJ. Mesenchymal cells expressing bone morphogenetic protein receptors are present in the rheumatoid arthritis joint. Arthritis Rheum 2000;43:2046-55.
53. Chetty C, Lakka SS, Bhoopathi P, Rao JS. MMP-2 alters VEGF expression via alphaVbeta3 integrin-mediated PI3K/AKT signaling in A549 lung cancer cells. Int J Cancer 2010;127:1081-95.
54. Shinderman-Maman E, Cohen K, Weingarten C, Nabriski D, Twito O, Baraf L, et al. The thyroid hormone-avβ3 integrin axis in ovarian cancer: regulation of gene transcription and MAPK-dependent proliferation. Oncogene 2015 Jul 13 (E-pub ahead of print).