A Doctoral Dissertation

Cyr61 synthesis is induced by Interleukin-6 and promotes migration and invasion of fibroblast-like synoviocytes in Rheumatoid arthritis

Department of Medicine

GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

Chang Min Choi

February 2023

류마티스 관절염 활막세포에서 인터루킨 6에 의한 Cyr61 발현 및 세포 이동과 침투 증가에 관한 연구

지도교수 김 진 석, 조 문 제

최 창 민

이 논문을 의학 박사학위 논문으로 제출함

2022년 6월

최창민의 의학 박사학위 논문을 인준함

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A thesis submitted in partial fulfillment of the requirement for degree of Doctor of Philosophy in Medicine

2023.02

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LIST OF ABBREVIATIONS

Cyr61, CCN1	Cysteine-rich 61
FLS	Fibroblast-like synoviocyte
RA	Rheumatoid arthritis
MMP	Matrix metalloproteinase
IL-6	Interleukin-6
ERK 1/2	Extracellular signal-related kinase 1/2
EGR 3	Early growth response 3
ECM	Extracellular matrix
mIL-6R	Membrane-bound IL-6 receptor
sIL-6R	Soluble IL-6 receptor
OA	Osteoarthritis
EMEM	Eagle's Minimum Essential Medium
DMEM	Dulbecco's Minimum Essential Medium
FBS	Foetal bovine serum
ATF3	Activating Transcription factor 3
NR4A1	Nuclear receptor subfamily 4 group A member 1
PBS	Phosphate-buffered saline
GAPDH	Glyceraldehyde 3'-phosphate dehydrogenase
siRNA	Small interfering RNA
DEGs	Differentially expressed genes

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ABSTRACT

Interleukin-6 (IL-6) is involved in fibroblast-like synoviocyte (FLS) activation and promotes pannus formation and bone and cartilage destruction in rheumatoid arthritis (RA). Cysteine-rich 61 (Cyr61) protein regulates cell proliferation, migration, and differentiation. The aim of this study was to investigate the role of Cyr61 in RA-FLS migration and invasion after IL-6 stimulation. In results, Cyr61 levels were elevated in FLSs from RA patients compared to those in osteoarthritis patients. Control and IL-6-treated FLSs showed differential gene expression. IL-6 stimulated protein synthesis of Cyr61, which was attenuated by the extracellular signal-related kinase 1/2 (ERK 1/2) inhibitor, PD98059, and knockdown of early growth response 3 (EGR3), but not of JUN. IL-6-induced Cyr61 protein synthesis increased expression of MMP2. Cyr61 promoted FLS migration and invasion in an autocrine manner. Knockdown of CYR61 and a neutralizing antibody attenuated Cyr61 synthesis and IL-6 induced FLS migration. Thus, by modulating the ERK/EGR3 pathway, IL-6 stimulated Cyr61 production and in turn increased invasiveness of FLS. Our data suggest that Cyr61 might be a potential target to prevent the progression of joint damage in RA.

Keywords; Cyr61, Interleukin-6, extracellular signal-regulated kinase, fibroblast-like synoviocyte, rheumatoid arthritis

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease that causes destruction of cartilage and bone and systemic inflammation via the interactions of different types of inflammatory cells [1, 2]. Fibroblast-like synoviocytes (FLSs) play an important role in the pathogenesis of RA and are major components of the hyperplastic pannus that invades cartilage and bone. These cells contribute to the local production of pro-inflammatory cytokines and enzymes that degrade the extracellular matrix (ECM) [3]. In addition, RA FLSs present a tumour-like phenotype with increased invasiveness into the extracellular matrix, which further exacerbates synovial hyperplasia and joint damage. These processes that are important for migration and invasion are mediated by cytoskeletal movement and the expression of adhesion proteins and proteolytic enzymes [4].

Cysteine-rich protein 61 (Cyr61 or CCN1) is an ECM component that belongs to the CCN family consisting of six members, CCN1–CCN6 [5]. Cyr61 mediates cell proliferation, adhesion, migration, and differentiation. The role of Cyr61 has been extensively studied in tumour biology and is also considered important for RA [6, 7]. Cyr61 is stimulated by interleukin-17 (IL-17) and in turn, promotes FLS proliferation, thus contributing to the hyperplasia of synoviocytes [8].

IL-6 is a pro-inflammatory cytokine that triggers host defence by sending out inflammatory signals when microbial infections or tissue damage occur. These responses are critical for the elimination of pathogens and regeneration of injured tissues. However, persistence of IL-6 stimulates the onset of inflammatory and auto-immune diseases such as diabetes, systemic lupus erythematosus, and RA [9]. IL-6 signals through binding to the membrane-bound IL-6 receptor (mIL-6R) via the classic signalling pathway or to the soluble IL-6 receptor (sIL-6R) via the trans-signalling pathway. After IL-6 binds to mIL-6R or sIL-6R, a cell surface glycoprotein called gp130 is recruited to form a receptor complex with IL-6 and IL-6R. This complex triggers downstream signalling and generates various biological responses [10]. IL-6 levels are elevated in the synovial fluid (SF) and sera of RA

patients, suggesting that IL-6 mediates many of the local and systemic effects of this disease. IL-6 is involved in FLS activation, osteoclast activation affecting pannus formation, and bone and cartilage destruction [11].

In this study, we investigated whether and how IL-6 stimulates the protein synthesis of Cyr61 and contributes to the invasion and migration of RA FLS. Further, we explored how Cyr61 affects FLSs in the development of RA.

2. MATERIALS AND METHODS

Isolation and culture of primary FLSs

Primary FLSs of RA patients (n=5) were obtained from Samsung Medical Center and primary synovial tissues of RA patients (n = 4) and osteoarthritis (OA) patients (n = 4) from Jeju National University Hospital for comparison. The Study was approved by the Institutional Review Board of Jeju National University. Informed consent in accordance with Declaration of Helsinki was obtained from all patients.

To isolate primary FLSs, primary synovial tissues were cut into pieces with operating scissors, digested in collagenase (Life Technologies, Grand Island, NY, USA) and dispase (Life Technologies, Grand Island, NY, USA) and dissolved in Eagle's Minimum Essential Medium (EMEM) (BioWhittaker, Inc., Walkersville, MD, USA). The medium containing the minced tissues was filtered through nylon mesh and centrifuged at 1500 rpm, 5 min, and 23 ℃. The cells were cultured in Dulbecco's MEM (DMEM) (BioWhittaker, Inc, Walkersville, MD, USA) supplemented with 10% foetal bovine serum (FBS) (Merck KGaA, Darmstadt, Germany) and primocin (InvivoGen, San Diego, CA, USA) in a humidified 5% CO_2 atmosphere. Cells from the 4^{th} to 8^{th} generations were used for experiments.

Reagents

IL-6, soluble IL-6 receptor, and CYR61 protein were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Monoclonal antibodies against human extracellular signal-regulated kinases 1/2 (ERK 1/2), phospho-ERK 1/2, Cyr61, beta-actin, and early growth response protein 3 (EGR3), as well as anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against c-Jun were purchased from Cell Signaling (Danvers, MA, USA).

Cyr61-neutralizing antibody was purchased from Novus Biologicals (Littleton, CO, USA).

Western blotting and culture supernatants

Cells were washed twice with phosphate-buffered saline (PBS) and lysed with radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Rockford, IL, USA) containing Proteinase Inhibitor (BioVision, Milpitas, CA, USA). Cell lysates were collected by scraping and centrifuged at 14,000 rpm for 15 min at 4 ℃. Protein concentrations were determined by using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts (25 μg) of proteins from all samples were electrophoresed on 10% sodium dodecyl sulphate-polyacrylamide gels and transferred to nitrocellulose membranes that were subsequently blocked for 1 h with 5% non-fat milk in tris (hydroxymethyl) aminomethane (Tris)-buffered saline (TBS) containing 0.1% Tween 20 (TBST). The membranes were incubated overnight at 4 ℃ with specific primary antibodies. After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h, followed by washing with PBS. The target proteins were examined with an enhanced chemiluminescence reagent (CYANAGEN, Bologna, Italy) and detected with autoradiography film.

To examine the effects of IL-6 on Cyr61 production in FLSs, FLSs were cultured with IL-6 for 24 h, and the culture supernatants were collected. Culture medium was concentrated onto Amicon Ultra 2 membranes (Merck KGaA, Darmstadt, Germany) at 4 ℃ according to the manufacturer's protocol. Cyr61 protein levels in the cell culture supernatants were determined by western blotting.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from FLSs using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesised by using a reverse transcriptase kit (Promega, Seoul,

Korea). PCR primers for human matrix metalloproteinase 1 (*MMP1*), *MMP2*, *CYR61*, glyceraldehyde 3'-phosphate dehydrogenase (*GAPDH*) were as follows: *MMP1* forward (5'-GGAGATCATCGGGACAACTC-3'),

MMP1 reverse (5'-ACCGGACTTCATATGTCG-3'), *MMP2* forward (5'-GAACACAGCCTTCTCCTCCT-3'), *MMP2* reverse (5'-CATCAAGGGCATTCAGGAGC-3'), *CYR61* forward (5'-TCCTCTGTGTCCCCAAGAAC-3'), *CYR61* reverse (5'-TCGAATCCCAGCTCCTTTACC-3'), *GAPDH* forward (5'-CCAAGGAGTAAGACCCCTGG-3'), *GAPDH* reverse (5'-TGGTTTGAGCACAGGGTACTT-3'). PCR products were loaded on a 1% agarose gel. Differences in band intensity were confirmed using ImageJ software (NIH, MD, USA) to analyse the relative levels in target RNAs.

Real time polymerase chain reaction

Total RNA extraction and cDNA synthesis were performed as previously described. Real time PCR was performed using SYBR Green Master mix (KAPA BIOSYSTEMS, Cape Town, South Africa) according to the manufacturer's instructions. The primers for human *MMP1, MMP2, CYR61, GAPDH* were as follows ; *MMP1* foward (5'- GGTAGAGCGTTCTAGGTGTATG-3'), *MMP1* reverse (5'-AACCCTCTGGCTAGAAGTAGTC-3'), *MMP2* forward (5'- AACCCTCTGGCTAGAAGTAGTC-3'), *MMP2* reverse (5'- CCTGTAGAGTTCACTCCTTACG-3'), *CYR61* forward (5'-GACCTGTGGAACTGGTATCTC-3'), *CYR61* reverse (5'- CCAGCGTAAGTAAACCTGAC-3'), *GAPDH* forward (5'- CACAAGAGGAAGAGAGAGACC-3'), *GAPDH* reverse (5'-CCTCTTCAAGGGGTCTACAT-3').

RNA interference (RNAi) for knockdown of gene expression

CYR61, *EGR3*, *C-JUN* small interfering RNAs (siRNAs) were designed and synthesised at Bioneer

Corp (Dajeon, Korea). Briefly, cells were seeded in 60 mm dishes at a density of 3×10^5 cells/dish and incubated overnight. After aspiration of the medium, a transfection mixture of siRNA oligonucleotides and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) in serum-free medium was added to the cells and incubated for 4 h. The medium was replaced with DMEM containing 10% FBS and incubated for an additional 24-h period.

Cell invasion and migration analysis

FLSs were seeded at a density of 10^5 cells/well in six-well plates. After 24 h of incubation when the cells were 70–80% confluent, the cell monolayers were scratched with a 200-μL pipet tip. Scratched monolayers were washed with PBS to remove detached cells. The bottom of the dish was marked for reference. The wound area was recorded during the 24-h period, and the images acquired for each sample were analysed quantitatively using ImageJ software. Wound closure rate was calculated as {(initial area – final area)/initial area} x 100.

For the invasion assay, transwells (Corning Incorporated, Corning, NY, USA) were used into which cells in serum-free medium were added to the upper chambers. The lower chambers of the transwells contained medium supplemented with 20% FBS and Cyr61 protein with or without anti-Cyr61 antibody. After 24 h of incubation, non-invaded cells remained above membranes of upper chamber were carefully removed with a cotton swab. Cells that had invaded into the underside of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet solution. The number of cells stained was calculated by visually counting three randomly chosen areas. All experiments were performed in triplicate.

Transcriptome analysis by RNA sequencing and analysis of RNA-seq data

Total RNA isolation was performed as described in the RT-PCR section. cDNA synthesis, sequencing,

and analysis of RNA-seq data were conducted by Cosmo Gentech. Co (Seoul, Korea). Differentially expressed genes (DEGs) were analysed by using online tools in Metascape (http://metascape.org). Functional enrichment was performed in cellular component (CC), molecular function (MF), Biological process (BP). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment was also performed.

Cell proliferation analysis

Cell proliferation analysis was performed via Electric Cell-substrate Impedance Sensing (ECIS). Electrode-stabilizing solution (200 uL) containing 10 mM L-cysteine (Applied Biosystems, Jordan Road Troy, NY, USA) was added to each well of ECIS Cultureware 8W10E+ Polyethylene terephthalate (PET) (Applied Biosystems), which was then kept at Room Temperature for 10 min. Later, the wells were washed with Distilled Water(DW) and the cell (3×10^3 cell/well) were seeded in each well. After 24 h, the cells were treated with IL-6/sIL-6R and Cyr61 protein and incubated at 37 ℃.

Immunohistochemistry (IHC)

Immunohistochemistry was performed on 4 μm thick sections obtained from TMA blocks. The tissues sections were stained with mouse monoclonal anti-IL-6 antibody at a dilution of 1:400 (Santa Cruz, CA, USA) and rabbit polyclonal anti-Cyr61 antibody at a dilution of 1:200 (Santa Cruz, CA, USA) using an automated immunostainer (Benchmark Ultra, Ventana Medical Systems Inc., Tucson, AZ, USA). Glandular cells in colon tissue served as positive control for Cyr61 and a subset of cells in lymph node served as positive control for IL-6. The primary antibody was omitted for negative control.

Statistical analysis

All experiments were repeated at least three times. Significance of differences was tested using analysis of variance, followed by Dunnett's test or Tukey's test (GraphPad Prism 8.0). p values < 0.05 were considered to be significant.

3. RESULTS

1. Cyr61 expression is upregulated in the FLSs of RA patients induced by IL-6

As FLSs are involved in the pathogenesis of RA, and Cyr61 contributes to cell adhesion and migration, we first examined protein levels of Cyr61 in OA patients (n = 4) and RA patients (n = 4). We observed that Cyr61 protein levels were higher in FLSs from RA patients than in those from OA patients (Fig. 1A). As IL-6 is an important pro-inflammatory cytokine produced by lymphoid and non-lymphoid cells, such as T cells, B cells, monocytes, endothelial cells, and is found at high levels in the joint fluid. [9], [11] we analysed IL-6 and Cyr61 protein level in synovial tissues. The results showed that IL-6 and Cyr61 protein levels were higher in RA synovial tissues than in OA synovial tissues. (Figure. 1C) Next, We examined the effects of IL-6 on Cyr61 protein synthesis. Because FLSs do not express mIL-6Rs, we treated the FLSs with equal concentrations of IL-6 and sIL-6Rs. The results reveal ed that Cyr61 mRNA expression and protein synthesis were enhanced in a dose- and time-dependent manner after IL-6 stimulation (Fig. 1D, E, F, G). Cyr61 mRNA expression and protein levels peaked at 20 ng/mL of IL-6 at 2 h post-IL-6 addition, followed by a gradual decline.

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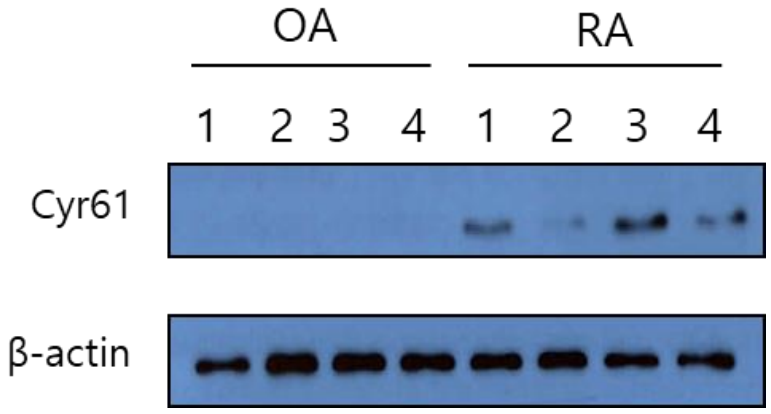

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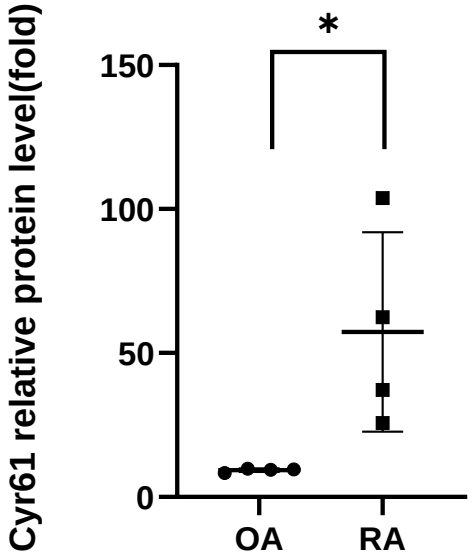

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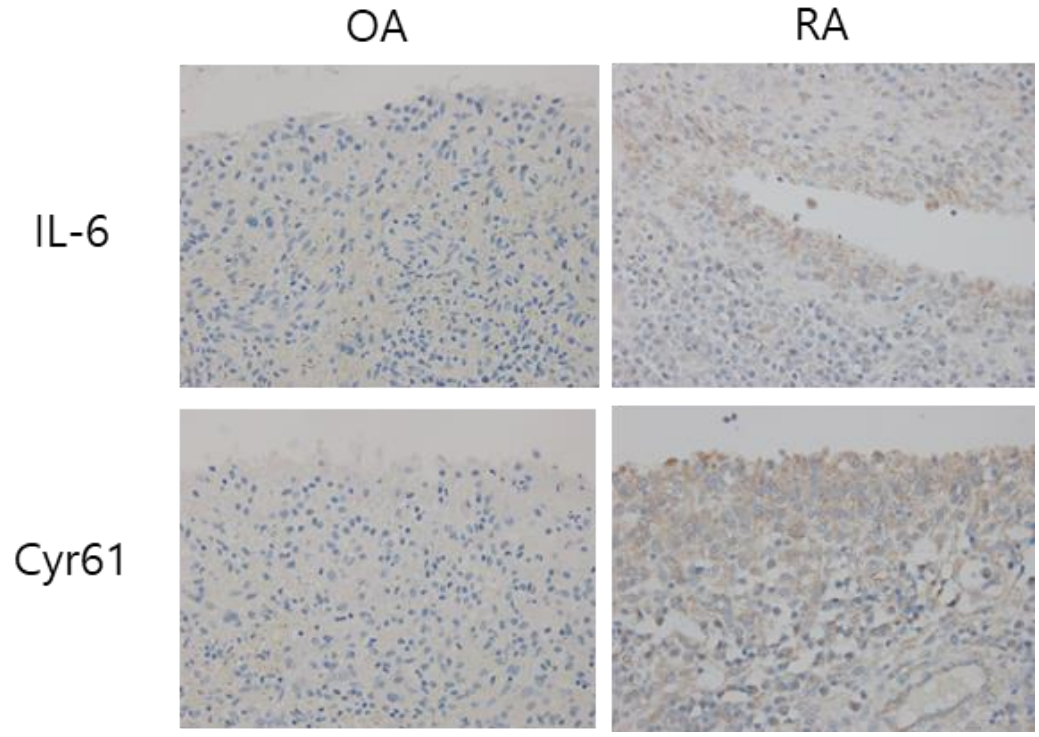

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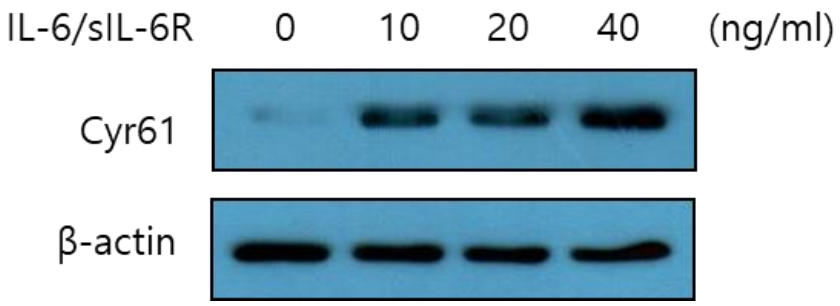

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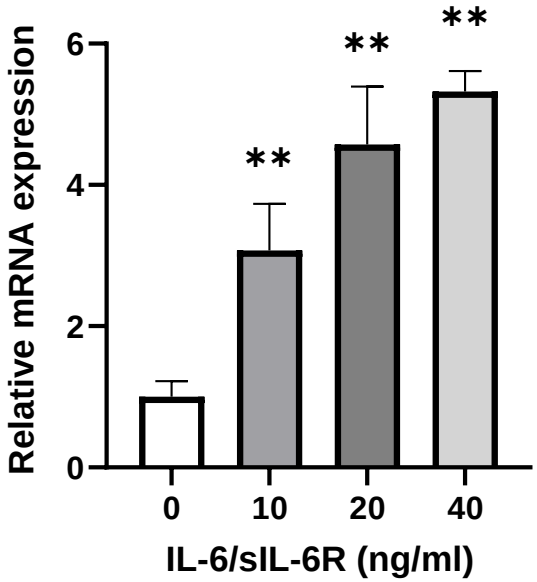

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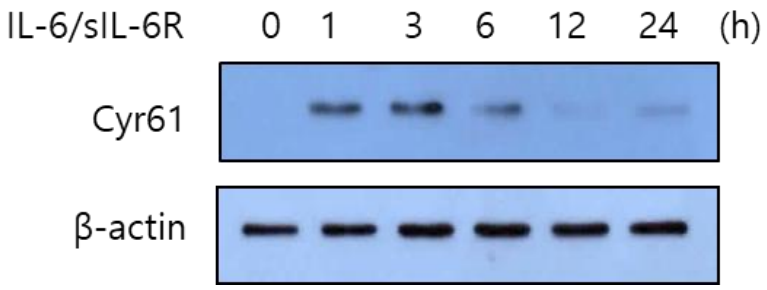

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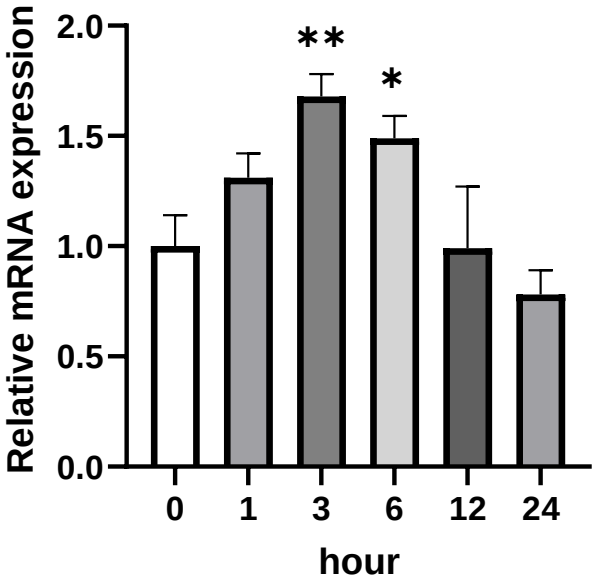

Fig. 1. Expression of IL-6 and Cyr61 in fibroblast-like synoviocytes (FLSs).

A B FLSs from osteoarthritis (OA; $n = 4$) or rheumatoid arthritis (RA, $n = 4$) patients. *$p < 0.05$ vs OA.

C synovial tissues from OA or RA patients. Original magnification x 400

D E RA FLSs stimulated by IL-6/sIL-6R for 2 h.

F, G FLSs stimulated by IL-6/s IL-6R (20 ng/mL) for the indicated periods.

D, E, F, G FLSs were incubated overnight in 1% FBS-containing medium before treatment with IL-6/sIL-6R.

A B D F Protein levels were determined by western blotting

E G The mRNA levels were determined through real time polymerase chain reaction. Values are means (± standard deviation) of at least three independent experiments.

*$p < 0.05$, **$p < 0.001$ vs untreated cells. IL-6, interleukin-6; sIL-6R, soluble IL-6 receptor; FBS, foetal bovine serum.

2. Regulation by IL-6 of expression of genes associated with migration and mitogen-activated protein kinase (MAPK) responses

To investigate the genome-wide effects of IL-6 on RA FLSs, transcriptome analysis by RNA-sequencing was performed using FLSs from 3 RA patients as a control group (group 1) and IL-6-treated FLSs from each patient (group 2). A multi-dimensional scaling (MDS) plot demonstrates the clustering of the transcriptomes of the three IL-6-treated samples, whereas no clustering was noted for the three untreated samples. These results indicate that gene expression increased in RA FLSs after IL-6 treatment (Fig. 2A). We identified a total of 315 DEGs, including 277 upregulated genes and 38 downregulated genes, by comparing group 1 and group 2 transcriptomes (Fig. 2B, C). Gene Ontology (GO) enrichment analysis using Metascape software revealed that these IL-6 induced DEGs were mainly involved in blood vessel development, tumour necrosis factor (TNF) signalling, proximal promoter sequence-specific DNA binding, and regulation of cell adhesion (Fig. 2D). Furthermore, the DEGs downregulated by IL-6 were mainly involved in Forkhead box O (FOXO)-mediated transcription (Fig. 2E).

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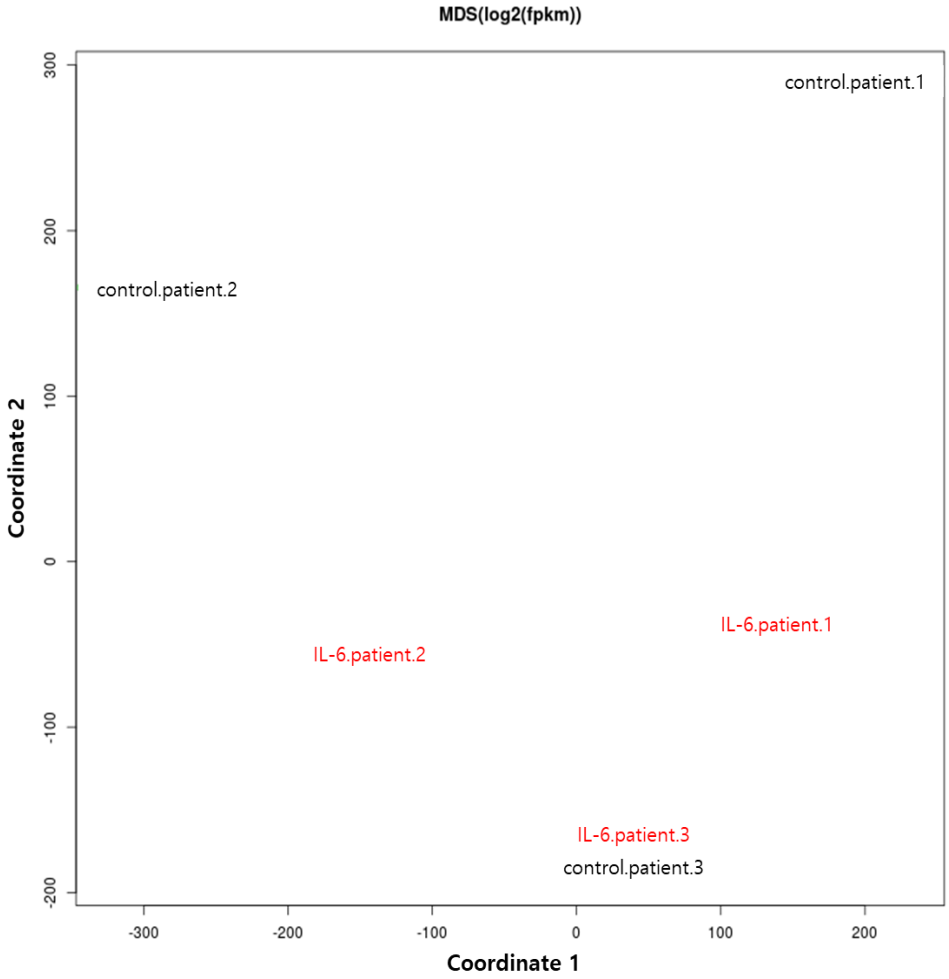

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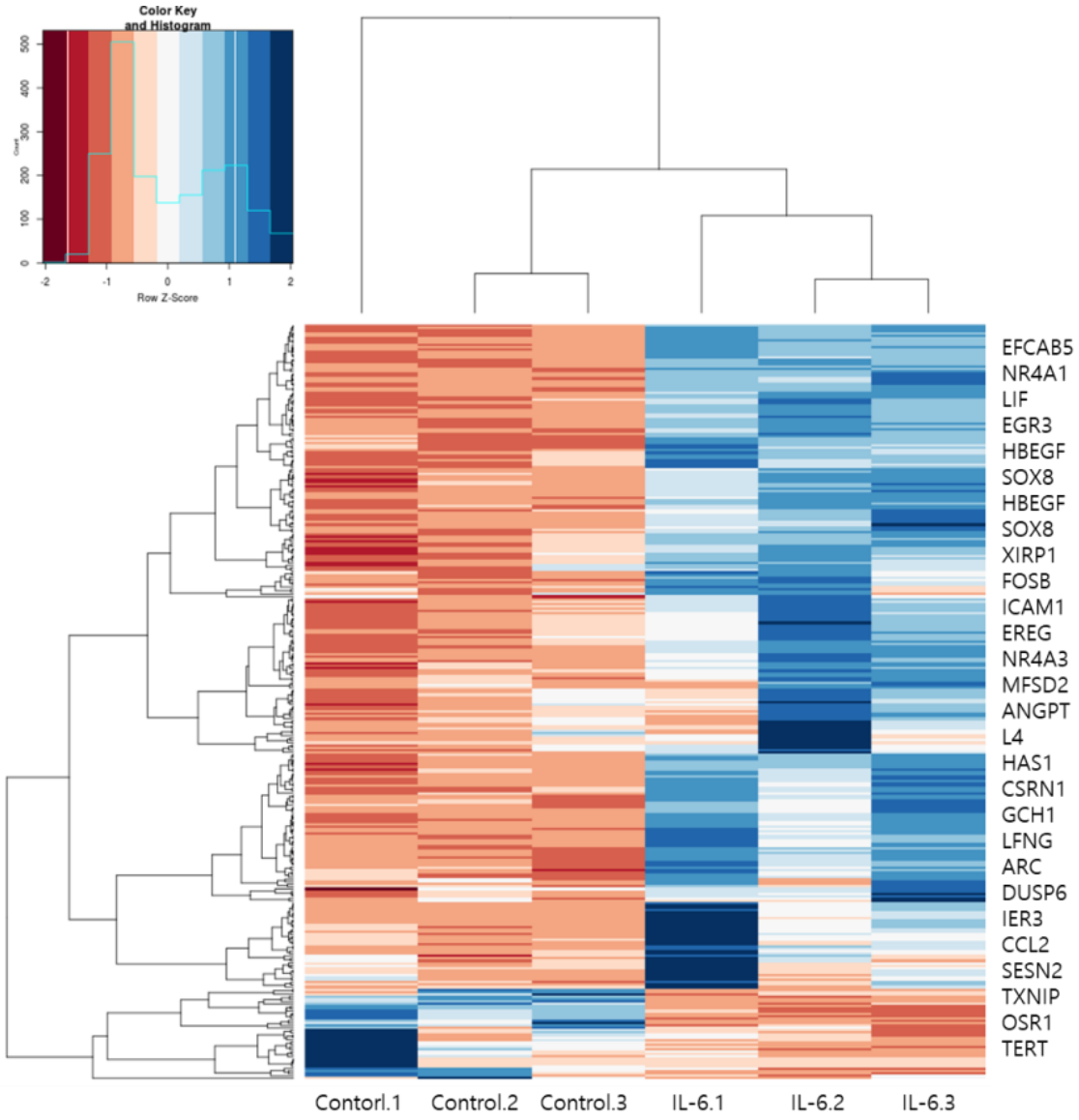

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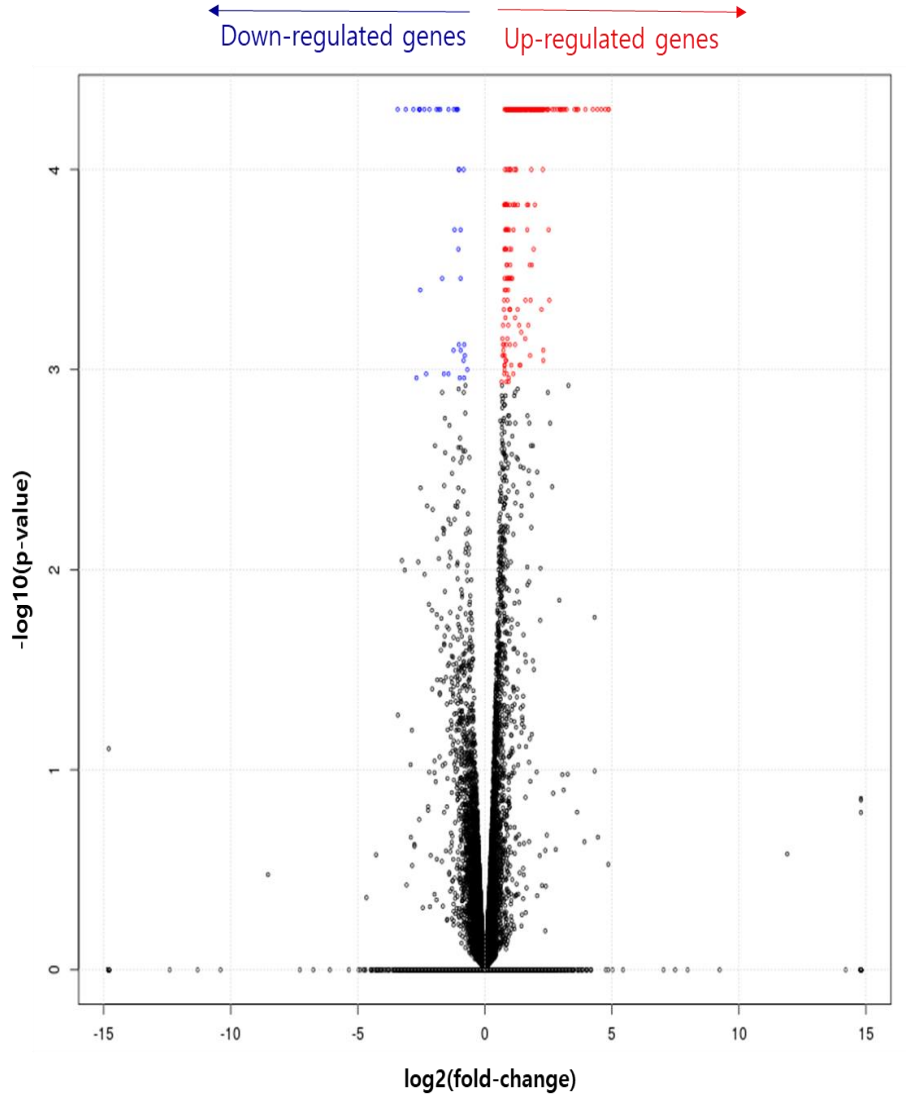

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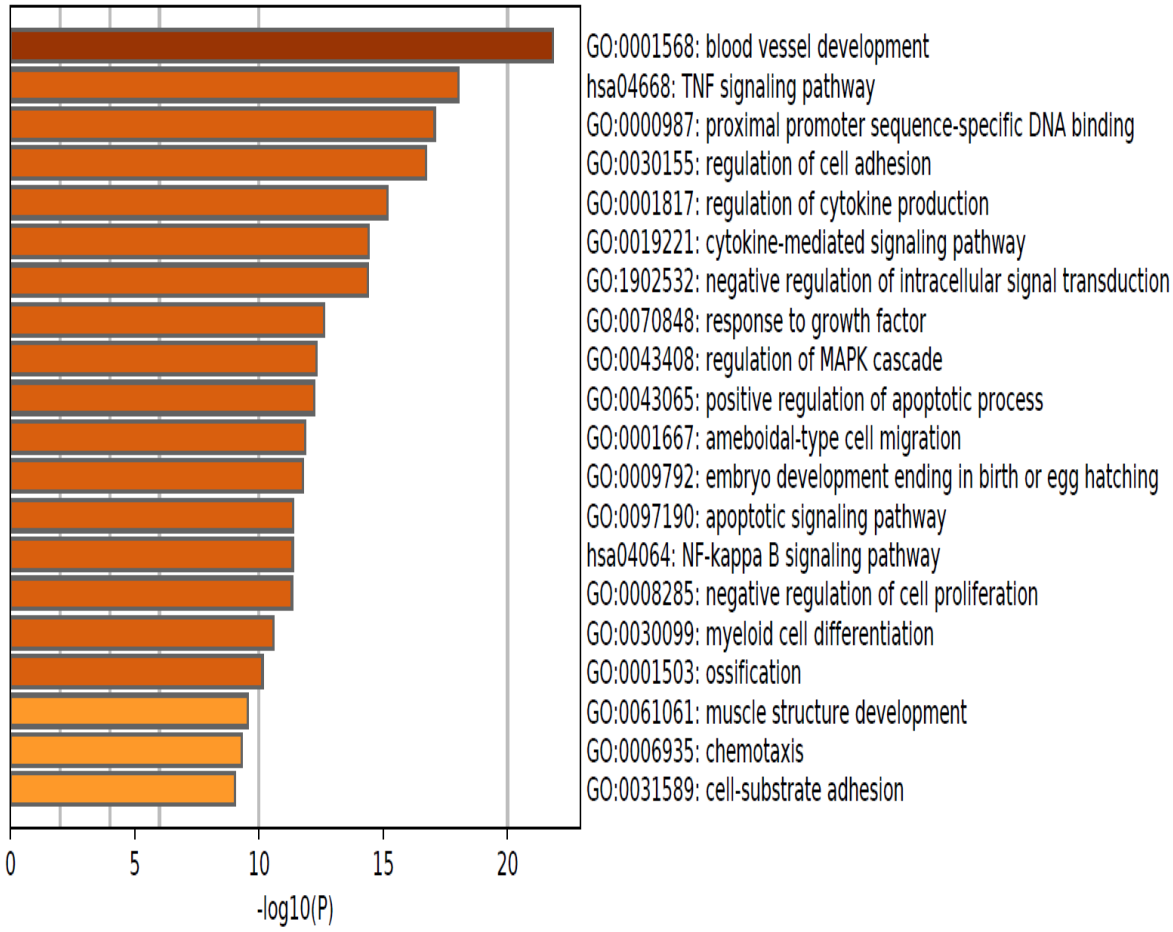

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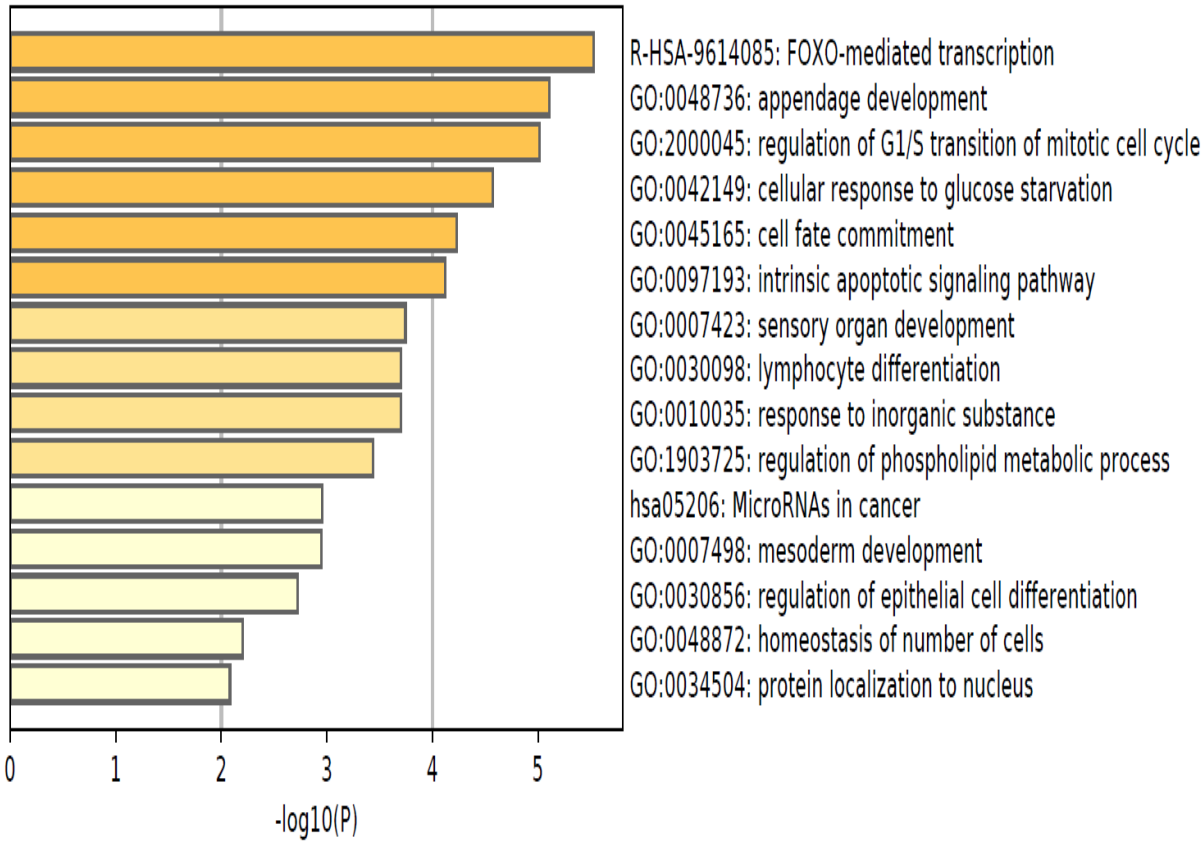


Fig. 2. RNA sequencing of fibroblast-like synoviocytes from 3 rheumatoid arthritis patients and interleukin-6 (IL-6)-treated samples.

Group 1 (control_patient 1, control_patient 2, control_patient 3), Group 2 (IL-6_patient 1, IL-6_patient 2, IL-6_patient 3).

A Multi-dimensional scaling (MDS) plot showing Pearson correlation coefficient between log2(fragments per kilobase of exon per million fragments mapped; FPKM) of genes.

B Heat map of differentially expressed genes. Gene expression expressed as FPKM. Expression normalised by z-score transformation before visualization with heatmap..

C Volcano plot of differentially expressed genes. The most upregulated genes are towards the right and the statistically significant genes are in red, the most downregulated genes are towards the left and the statistically significant genes are in blue, and the most statistically significant genes are towards the top.

B, C Cutoff q-value < 0.05

D Top 20 Gene Ontology (GO) enrichment results after IL-6 treatment. Upregulated genes in 2B were subjected to GO analysis.

E Downregulated genes in 2B were subjected to GO analysis.

Top 20 GO categories are shown; (-log10 (P) values were calculated by Metascape software); has: *Homo sapiens*.

3. IL-6-induced Cyr61 production depends on ERK 1/2–EGR3 pathway

To explore which signalling pathways are responsible for IL-6-induced synthesis of Cyr61 protein, we used known inhibitors of several pathways, including LY294002 (an inhibitor of phosphoinositide 3-kinase (PI-3K) activation), SB203580 (an inhibitor of p38 MAPK), PD98059 (an inhibitor of ERK1/2), AG490 (an inhibitor of Janus-activated kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3)). IL-6-stimulated synthesis of Cyr61 protein was markedly decreased in the presence of the ERK 1/2 inhibitor. In contrast, other inhibitors showed little effect on IL-6-induced Cyr61 protein levels (Fig. 3A) Thus, these results indicate a dominant role for the non-canonical ERK 1/2 pathway in the regulation of Cyr61 protein synthesis by IL-6.

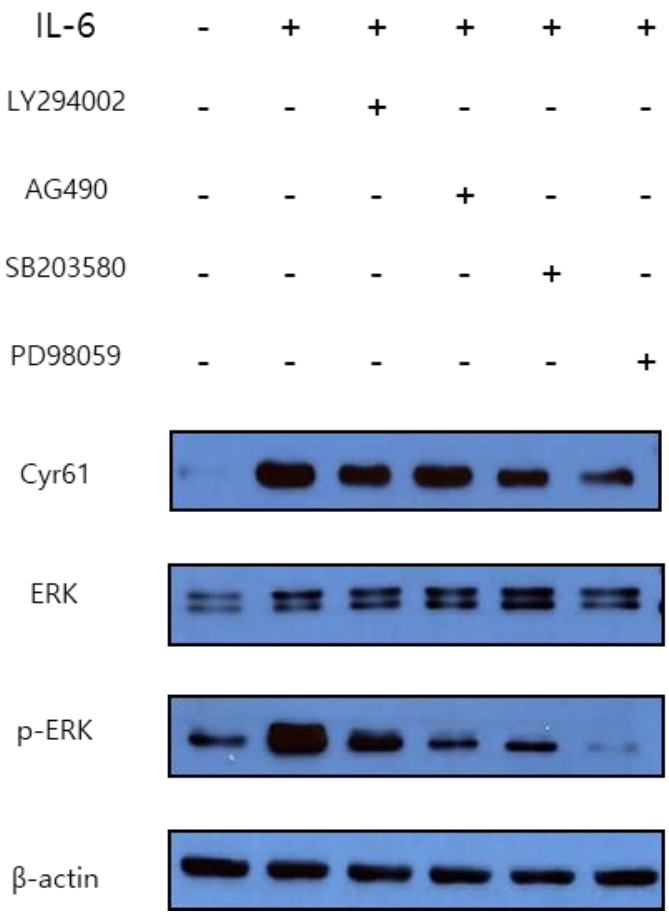
After analysis of RNA-sequencing, we found several transcriptional factors that might be involved in Cyr61 transcriptional regulation such as Nuclear receptor subfamily 4 group A member 1 (NR4A1), Activating Transcription factor 3 (ATF3), EGR3, and c-Jun. We examined these factors to determine their effects on Cyr61 protein synthesis.

In response to cytokines, growth factors, and oxidative stress, c-Jun binds to the promoters of target genes to modulate their expression in cell proliferation and inflammation in different types of cells.[12] [13] In this study, we confirmed that IL-6 stimulation increased c-Jun protein synthesis (Fig. 3B). We also found that the ERK 1/2 inhibitor PD98059 abrogated IL-6-induced Cyr61 protein synthesis (Fig. 3C, E). However, c-Jun protein synthesis was not affected by the ERK 1/2 inhibitor (Fig. 3C), and the knockdown of *c-Jun* expression using siRNA did not affect Cyr61 protein synthesis (Fig. 3D). Moreover, other transcription factors such as NR4A1 and ATF3 did not affect Cyr61 protein synthesis (Fig. 3E).

Interestingly, we found that EGR3 protein synthesis was regulated by ERK 1/2 (Fig. 3F). To examine the effect of EGR3 on Cyr61 protein synthesis, we knocked down *EGR3* expression using siRNA and observed a decrease in Cyr61 protein levels (Fig. 3G). These results indicate that EGR3 modulated Cyr61 protein synthesis through ERK 1/2 after IL-6 stimulation.

Sirtuin 1(SIRT1) is a deacetylase that regulates oxidative stress, aging, migration and invasion processes. It can affect a variety of substrate using enzymatic reactions. To explore a role for SIRT1 in IL-6 induced Cyr61 protein synthesis, we measured the level of Cyr61 protein synthesis using siRNA of SIRT1. Knockdown of *SIRT1* expression decreased the Cyr61 protein synthesis. (Fig. 3H).

A



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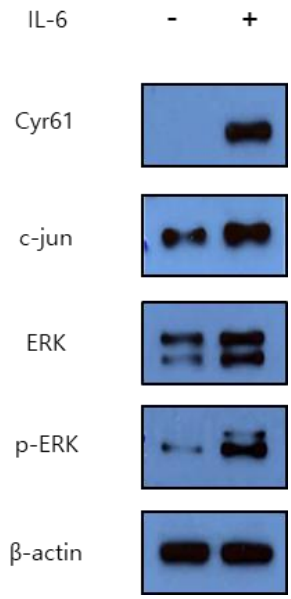

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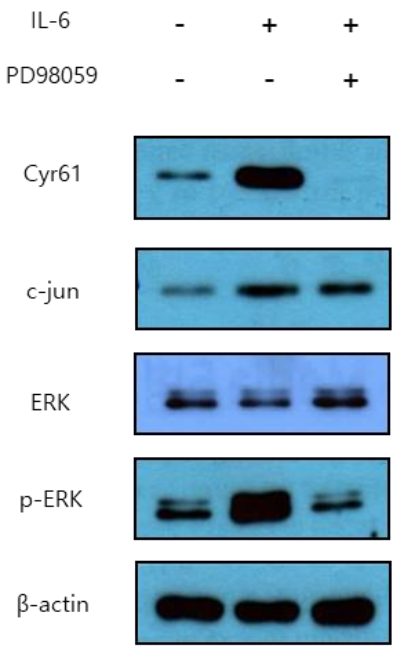

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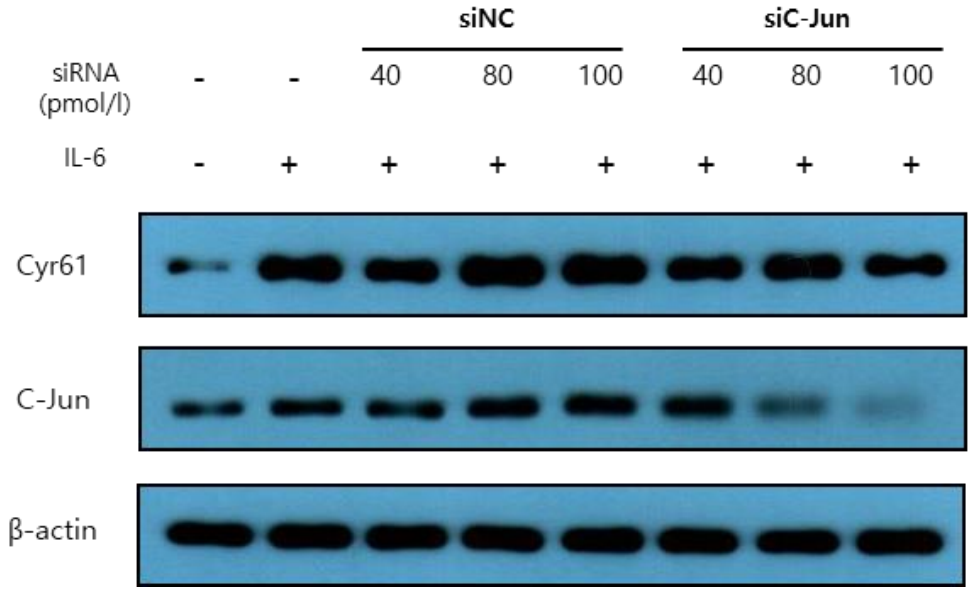

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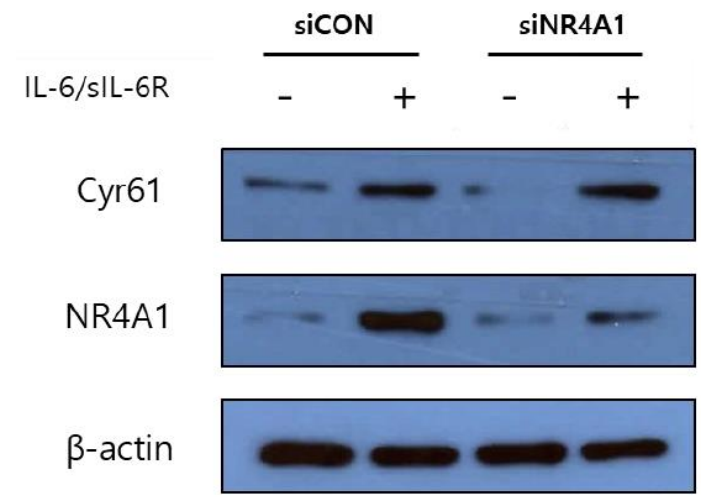

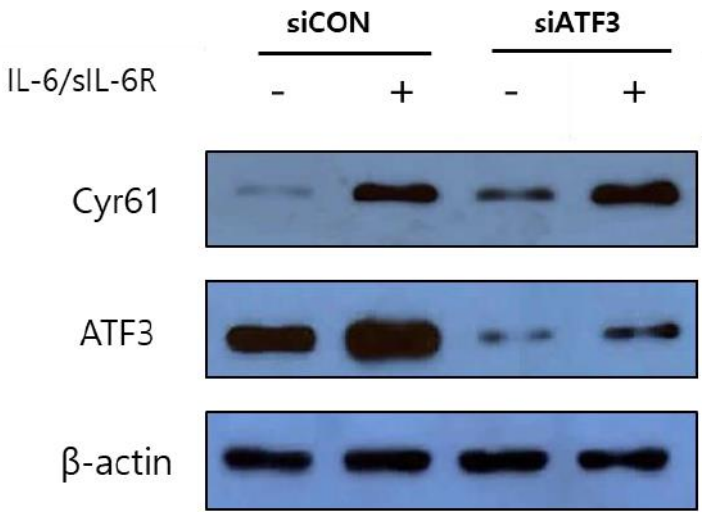

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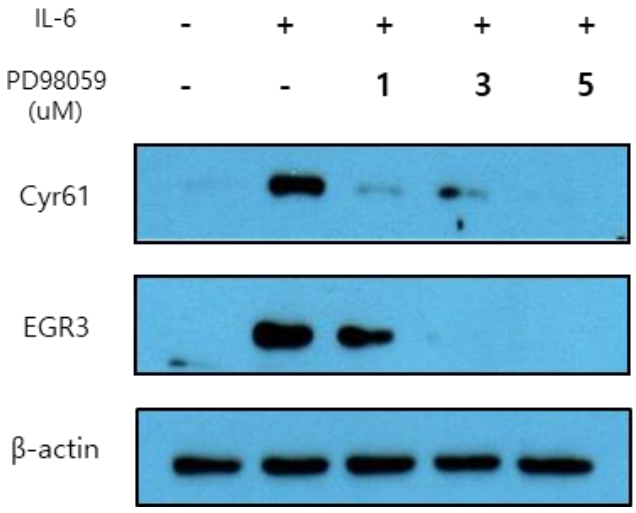

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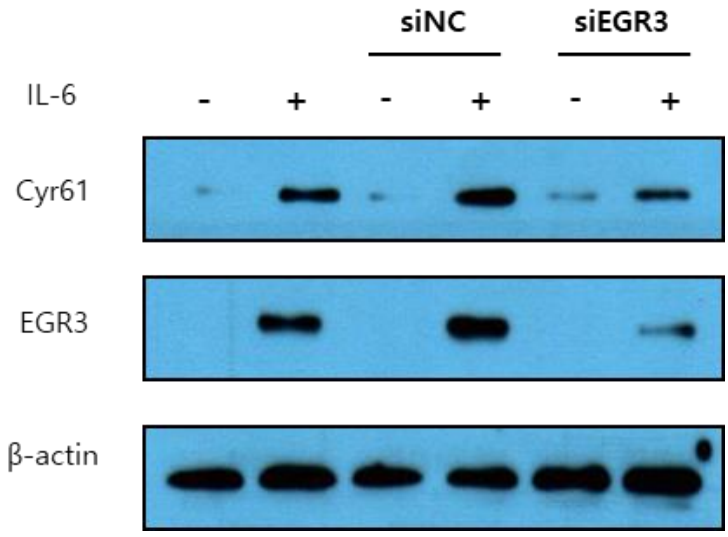

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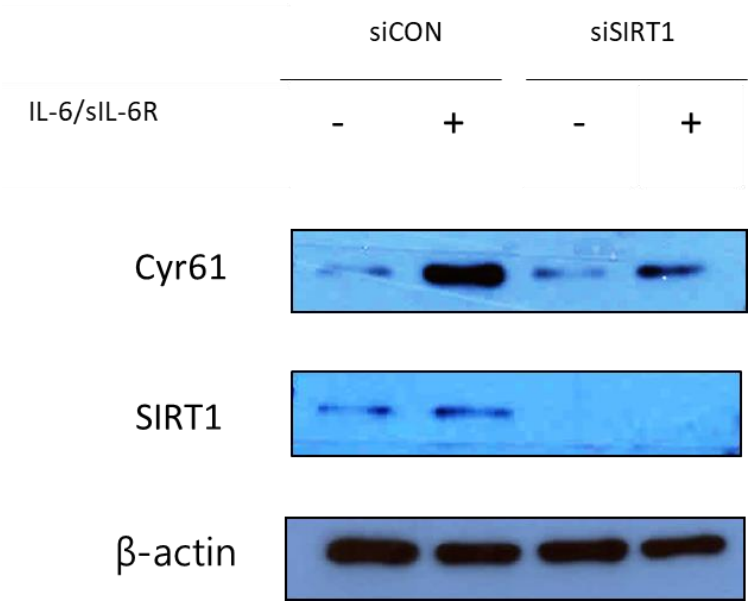

Fig. 3. Signaling pathways involved in IL-6-regulated protein synthesis of Cyr61 in rheumatoid arthritis-fibroblast-like synoviocytes (RA-FLSs).

A C F Cells were pretreated with inhibitors for 2 h before IL-6 (20 ng/mL) stimulation for 2 h. LY294002 (10 μM): PI3K/AKT inhibitor, AG490 (50 μM): JAK2/STAT3 inhibitor, SB203580 (10 μM): p38 MAPK inhibitor, PD98059 (1 μM): ERK inhibitor.

D G H RA-FLSs transfected with either small interfering RNA (c-Jun or EGR3, SIRT1) or siNC (control) (20 pmol/L) stimulated by IL-6 (20 ng/mL) for 2 h. Data are representative of at least three independent experiments.

E Transcription factors not involved in IL-6 induced Cyr61 protein synthesis. RA-FLSs transfected with either small interfering RNA (NR4A1 or ATF3) or siNC (control) (20 pmol/L) stimulated by IL-6 (20 ng/mL) for 2 h.

(A–H) Protein levels were determined by western blotting. FLSs were incubated overnight in 1% FBS-containing medium before treatment with IL-6/sIL-6R.

4. IL-6 induced increase in autocrine Cyr61 protein production from FLSs

Given that Cyr61 is an ECM component, we collected culture supernatants and measured the concentrations of secreted Cyr61. Cyr61 protein levels were increased by IL-6 in a time- and dose-dependent manner as shown in Fig. 1 (Fig. 4A, B). We then examined whether Cyr61 protein had an autocrine effect on the FLSs. The results show that Cyr61 protein synthesis was increased by Cyr61 in the supernatant medium (Fig. 4C). Moreover, Cyr61 protein increased IL-6 mRNA level (Fig. 4D). Because MMPs are associated with joint destruction, cell migration, and invasion [14], we assessed the effects of IL-6 and Cyr61 protein on MMP protein synthesis. As demonstrated in Fig. 4E F and G H, both IL-6 and Cyr61 increased *MMP2* mRNA levels. However, neither IL-6 nor Cyr61 protein affected *MMP1, MMP3* expression. These results indicate that the induction of the expression of the *MMP* genes was partly dependent on IL-6 and Cyr61 protein.

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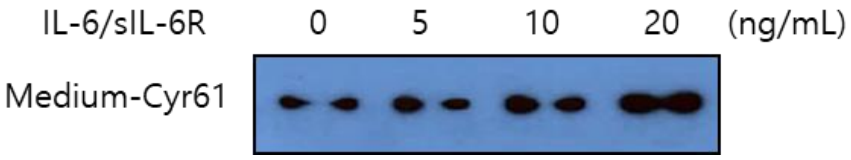

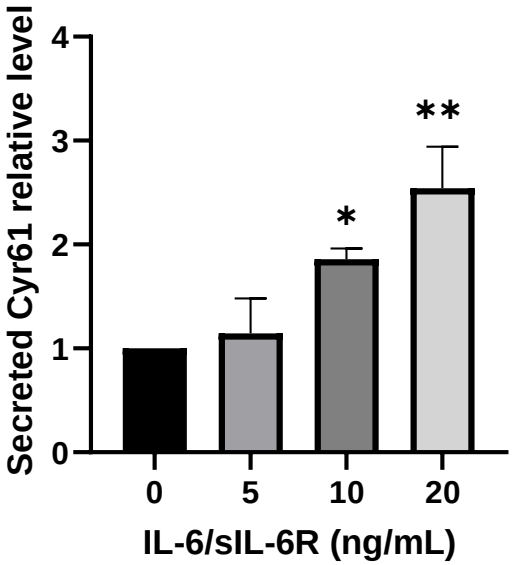

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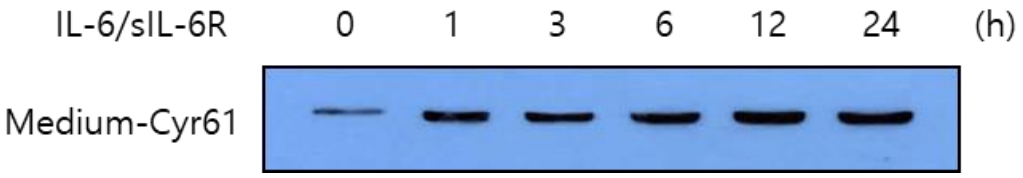

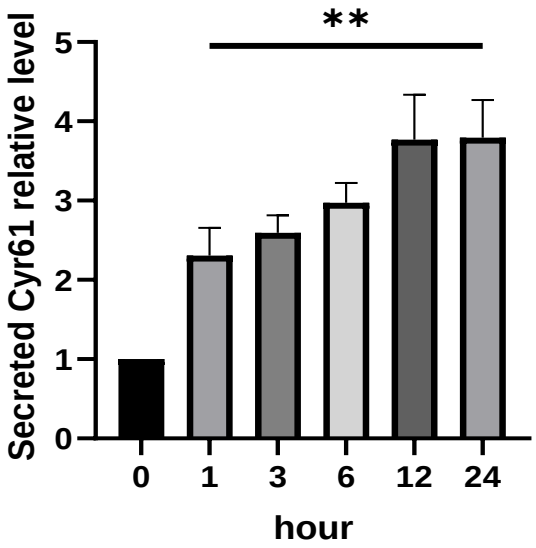

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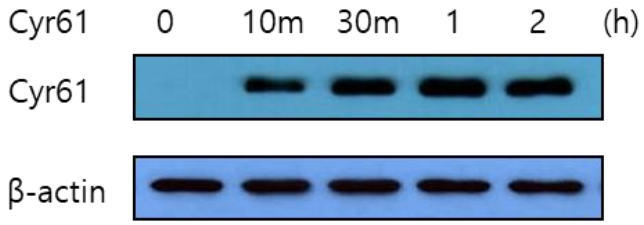

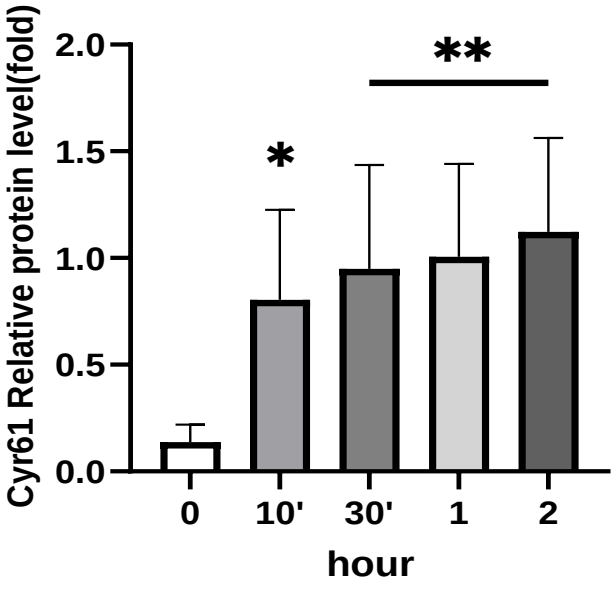

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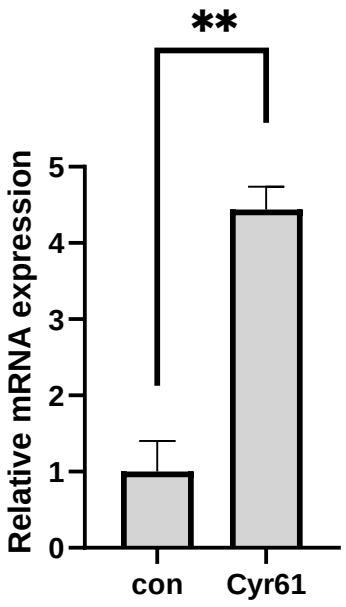

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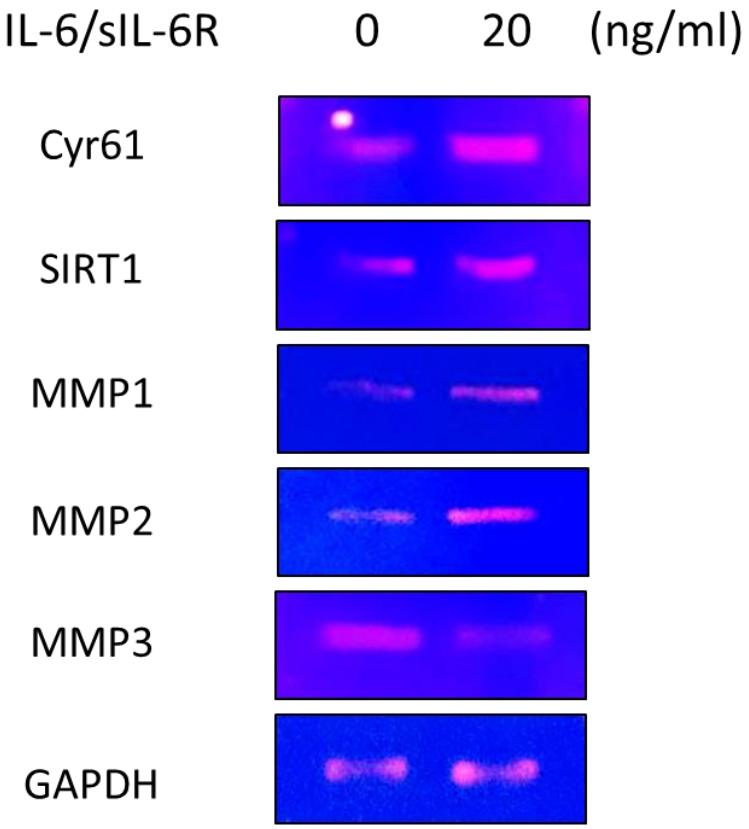

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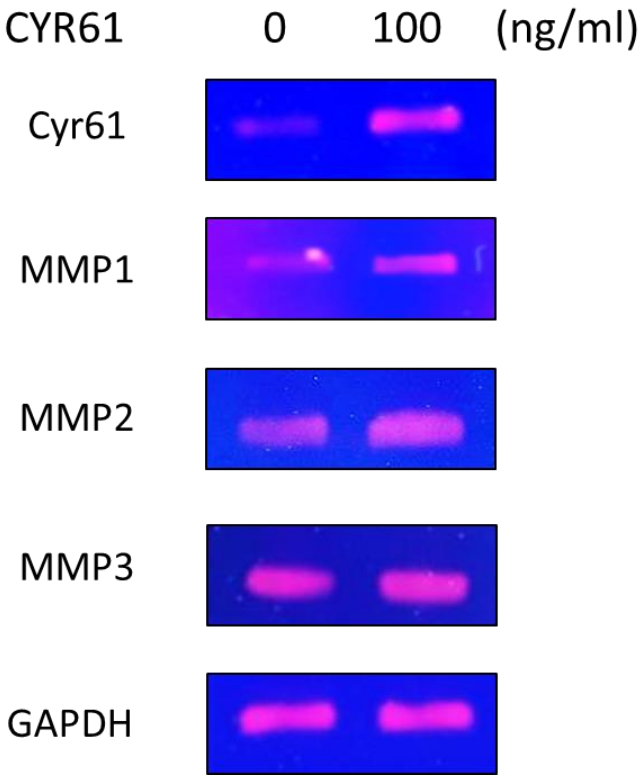

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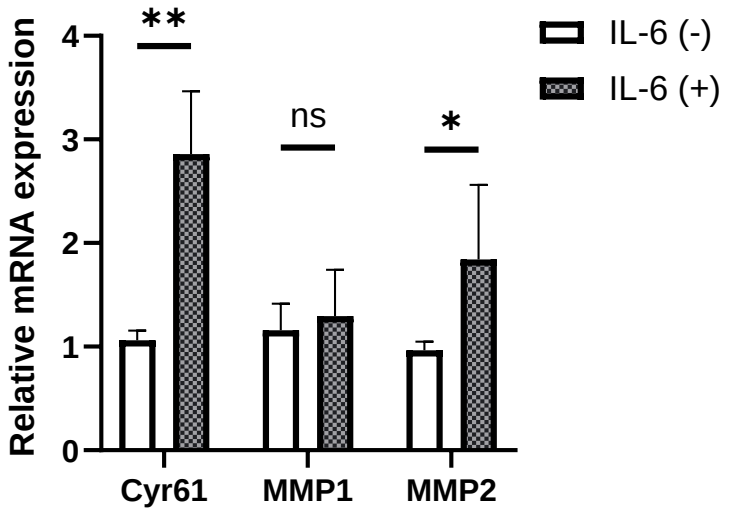

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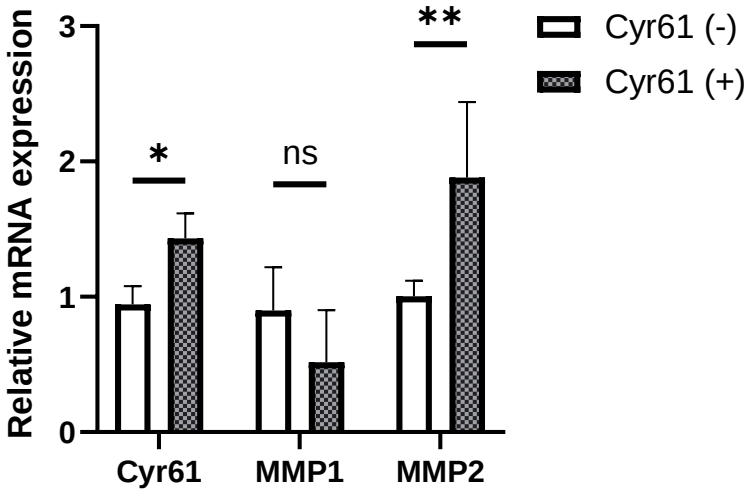

Fig. 4. Cyr61 secretion was induced by IL-6.

A B Extracellular protein levels of Cyr61 in culture supernatants of IL-6-treated RA-FLSs measured by western blotting; IL-6 (20 ng/mL).

C Cyr61 protein synthesis stimulated by extracellular Cyr61 (100 ng/mL) for indicated time periods.

D The mRNA level of IL-6 stimulated by Cyr61 protein over 2 h as determined via real time polymerase chain reaction. Cyr61 protein (100 ng/mL).

E F G H The mRNA levels of *Cyr61, SIRT1, MMP1, 2, 3* and *GAPDH* induced by IL-6 and extracellular Cyr61 protein.

E F The mRNA levels were determined by reverse-transcription polymerase chain reaction. **G H** The mRNA levels were determined through real time polymerase chain reaction. Data are representative of at least three independent experiments. * $p < 0.05$, ** $p < 0.001$.

5. IL-6 stimulated RA-FLS migration and invasion through Cyr61 protein secretion

Both IL-6 and Cyr61 protein enhanced FLS migration compared to the control group (Fig. 5A). Because Cyr61 is associated cell proliferation, we determined whether IL-6 and Cyr61 stimulate RA-FLS proliferation through analysis of cell proliferation. IL-6 and Cyr61 protein promoted FLS proliferation compared to the control group. (Fig. 5B). As shown in Fig. 4, IL-6 increased levels of the secreted Cyr61 protein and promoted Cyr61 protein synthesis. After confirming the IL-6-induced Cyr61 protein synthesis was blocked by the anti-Cyr61 antibody, decreasing the Cyr61 protein level (Fig. 5C), we observed an attenuation of IL-6-induced and Cyr61-induced increase in FLS migration by the anti-Cyr61 antibody (Fig. 5D, E), which was confirmed by the use of siRNA to knock down *CYR61* (Fig. 5F, G). Furthermore, knockdown of *CYR61* could not affect MMP3 expression (Fig. 5F). The invasion data from the transwell assays also indicate that FLS invasiveness was increased by Cyr61 treatment compared to the control group (Fig. 5H) and was reduced by the neutralizing antibody (Fig. 5I).

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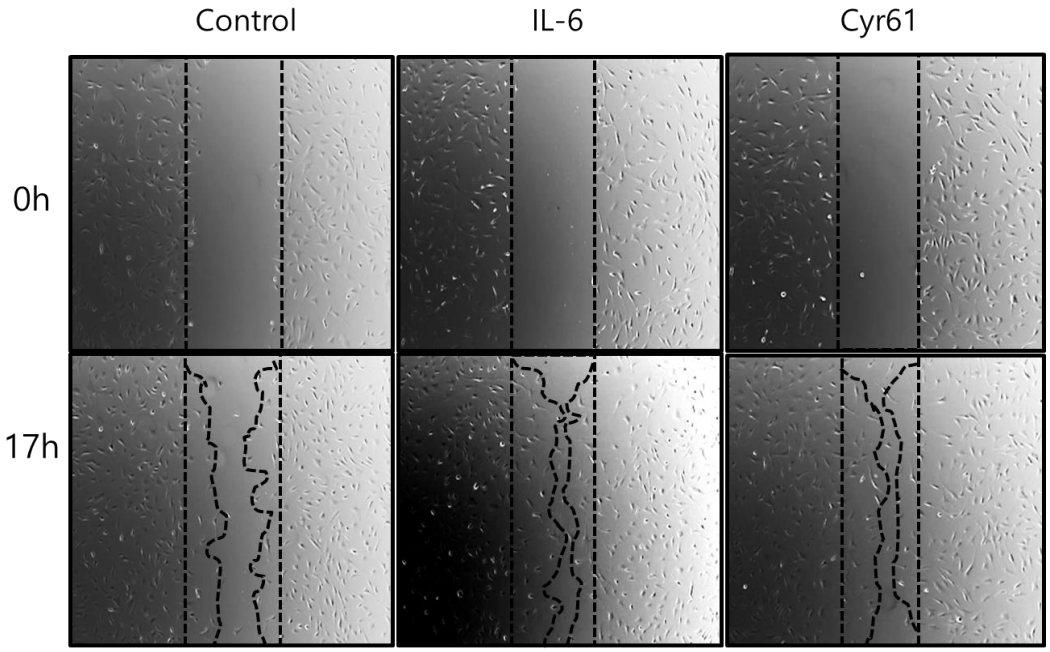

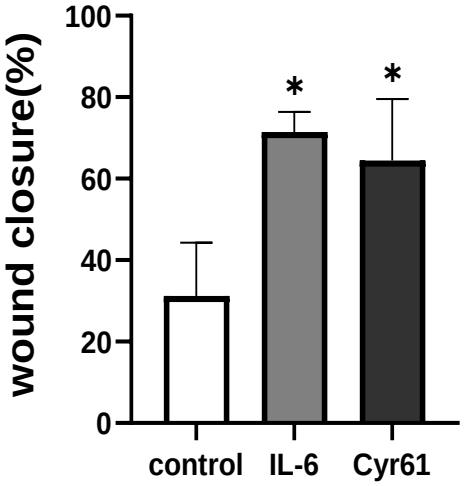

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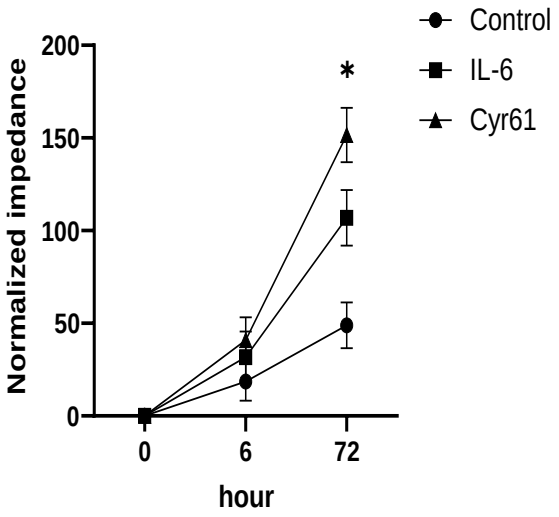

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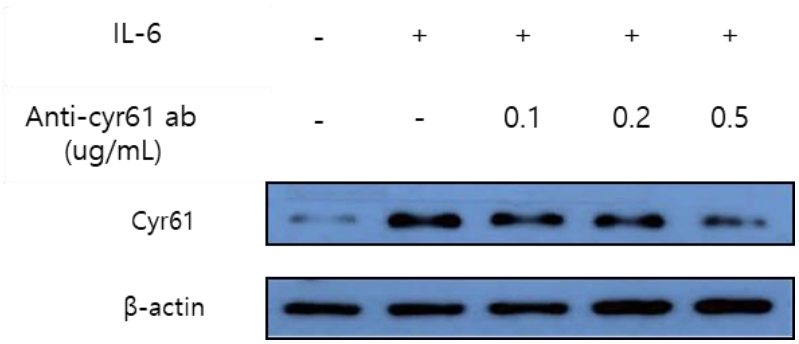

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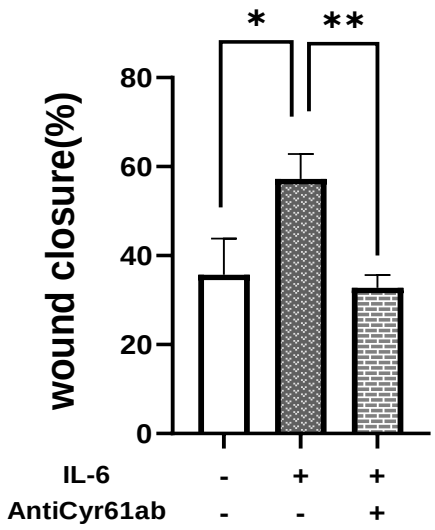

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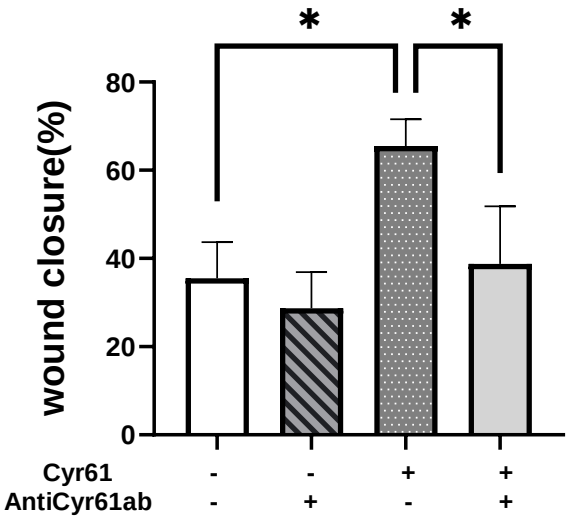

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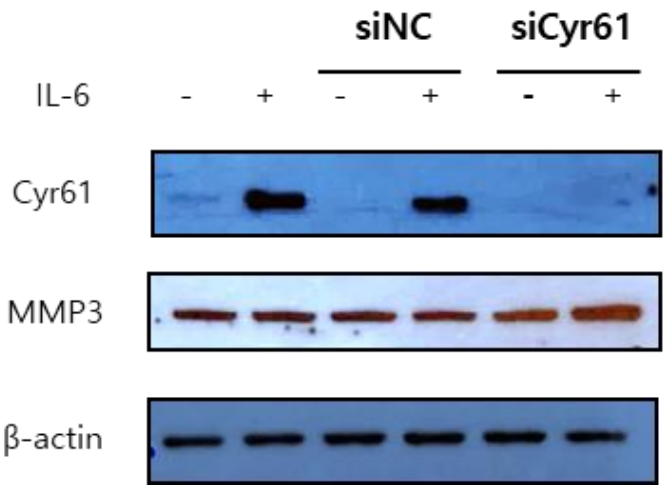

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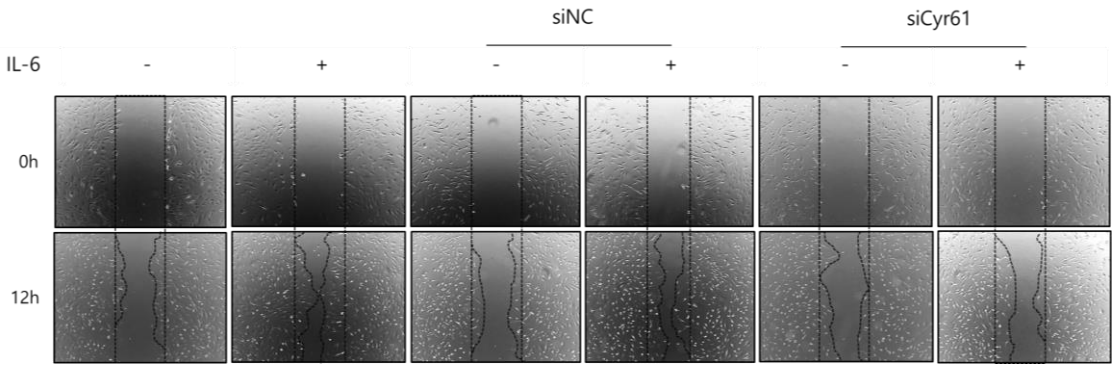

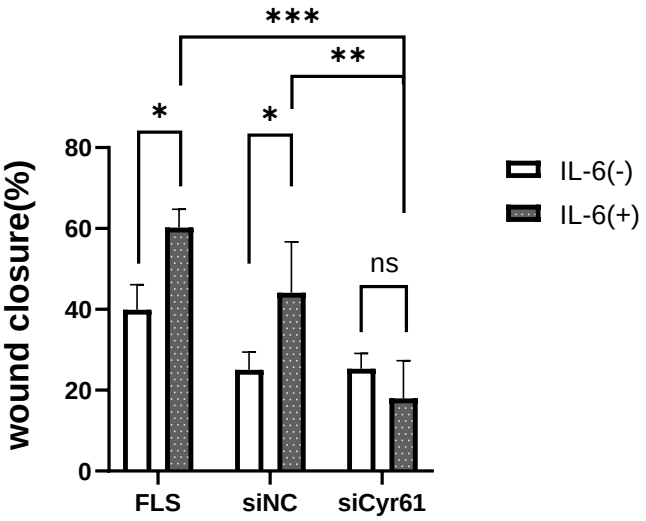

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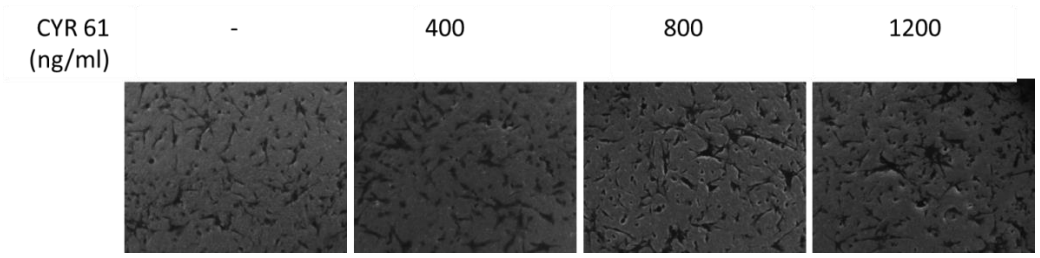

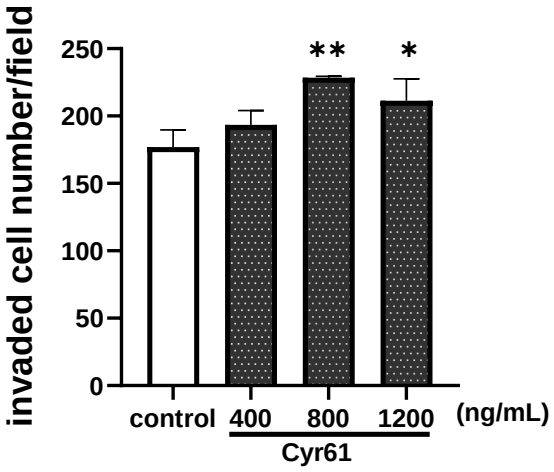

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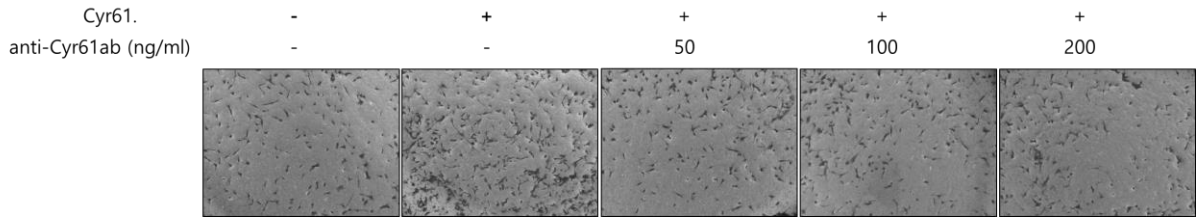


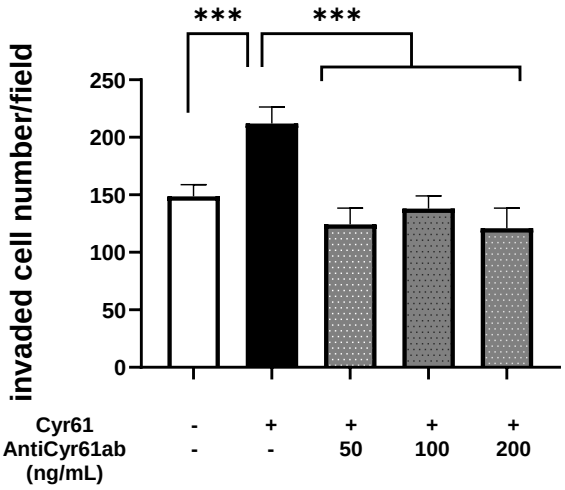


Fig. 5. Migration and invasion of rheumatoid arthritis-fibroblast-like synoviocytes (RA-FLSs) promoted by IL-6 and Cyr61 secretion.

A D E G Wound-closure over 17 h.

B ECIS proliferation analysis over 72h

C F Western blotting for Cyr61 protein detection.

H I Cyr61-stimulated invasion of RA-FLSs in transwells over 24 h ± antiCyr61 ab.

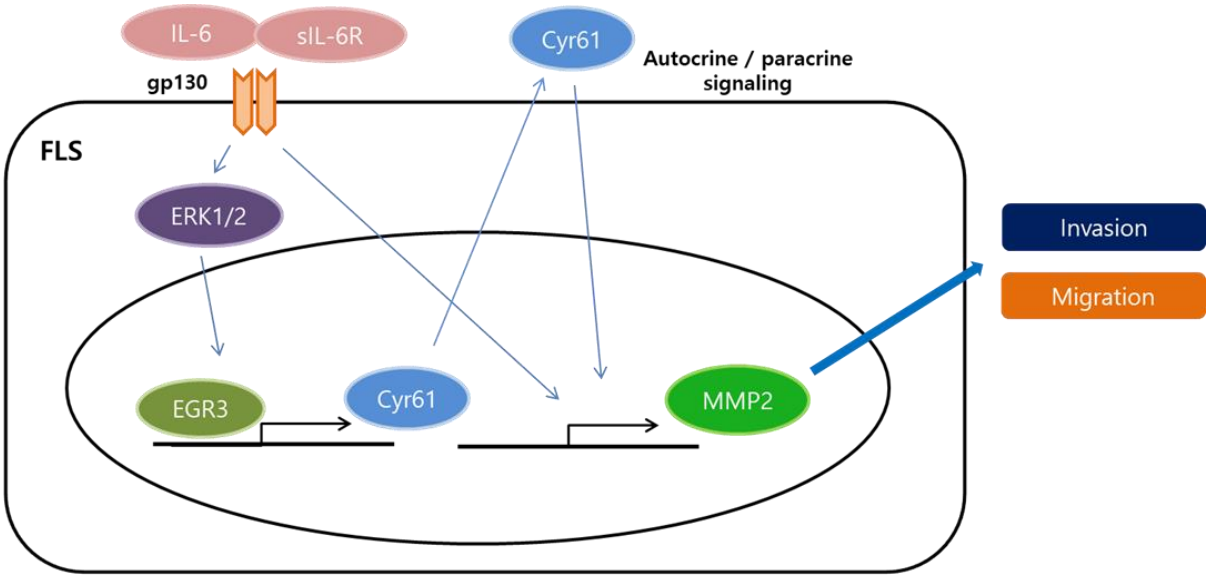
F G transfection with 20 pmol/L of small-interfering Cyr61 RNA (siCyr61) or siNC (negative control). IL-6 for 17 h (A, D, G: 200 ng/mL); IL-6 for 2h (C, F : 20 ng/mL); IL-6 and Cyr61 protein for 72h (B: IL-6: 200ng/ml, Cyr61 protein:100 ng/mL) Cyr61 protein (A, B, E: 100 ng/mL; I: 800 ng/mL); antiCyr61 antibody (ab) for 2 h (D: 100 ng/mL; E: 50 ng/mL, I: 50, 100, 200 ng/mL) before IL-6 and Cyr61 protein treatment.

H Original magnification x 10. Values are means (± standard deviation) of at least three independent experiments.

*$p < 0.05$, **$p < 0.001$.

Fig. 6. A schematic model for IL-6-stimulated *Cyr61* expression and its role in FLS migration and invasion.

IL-6 and soluble IL-6 receptor complex stimulate *Cyr61* expression via the ERK/ EGR3 signalling pathway. Secreted Cyr61 protein activates FLSs in an autocrine or paracrine manner and the resulting increase in extracellular Cyr61 protein enhances FLS migration and invasion. IL-6, interleukin-6; FLS, fibroblast-like synoviocyte; ERK, extracellular signal-regulated kinase; EGR3, early growth response 3.



4. DISCUSSION

IL-6 plays a critical role in the development of RA [10],[15] and has been found to stimulate vascular endothelial growth factor production by vascular endothelial cells in affected joints, leading to joint swelling, synovial growth, and accumulation of synovial fluid [15]. Several studies showing that Cyr61 increases invasion and angiogenesis in several types of tumours led us to hypothesize that Cyr61 might be functionally linked to IL-6 [16],[17],[18]. Furthermore, pro-inflammatory cytokines, such as IL-17, have been recently shown to affect the expression of *CYR61* in FLSs [19]. Our results reveal that *CYR61* expression and protein synthesis in FLSs from RA patients were enhanced by IL-6, and that both Cyr61 and IL-6 enhanced migration and invasion of RA-FLSs.

Joint destruction with synovitis is a characteristic of affected joints in RA. Pannus formation results in direct contact of FLSs with bone and cartilage tissues, leading to cartilage and bone destruction. The pannus invades cartilage, the surface of which is covered by FLSs, after which the local invasion of the cartilage matrix by the pannus starts [20]. We suspected that elevated levels of IL-6 in RA synovium might affect the FLS genotype and accelerate pannus formation. A transcriptome analysis was performed to identify genetic alterations of RA-FLSs after IL-6 treatment. In our study, we identified 315 DEGs between the RA samples and IL-6 treated RA samples, including 277 upregulated genes and 38 downregulated genes. A GO enrichment analysis revealed that the upregulated genes were mainly involved in blood vessel development, regulation of cell adhesion, and chemotaxis. These GO terms represented angiogenesis and regulation of epithelial cell migration that are associated with a cancer-like phenotype. In addition to TNF and the nuclear factor-kappa B (NF-κB) signalling pathway related to the inflammatory response, IL-6-induced FLS gene expression also regulates the MAPK signalling cascade, which is critical in the invasion and angiogenesis of cells. Our results suggest that increased IL-6 levels promote FLS phenotype transition to tumour-like patterns that are responsible for the vicious cycle of cytokine and chemokine production. These findings are consistent with the finding that IL-6 stimulates tumour-like

proliferation of FLSs in RA [21] and indicate that tumour progression mechanisms could account for pannus formation and function.

The 38 downregulated DEGs found in our study are most closely associated with FOXO-mediated transcription. FOXO transcription factors regulate many cellular processes, including cell survival, apoptosis, and resistance to oxidative stress. In particular, FOXO proteins regulate bone cell survival, cell cycle, proliferation, and also participate in network control among different kinds of bone cells [22]. Given that our results show that IL-6 downregulated FOXO-mediated transcription in FLSs, further research on RA pathogenesis is warranted.

IL-6 acts through the JAK/STAT, MAPK, and PI-3K/AKT pathways [23]. It binds to plasma membrane receptor complexes or to soluble receptor complexes to trigger its association with the signal-transducing gp130 [24]. Signal transduction involves activation of JAK kinases, leading to the activation of transcription factors of the STAT family, particularly STAT3 [25]. To determine which pathway is responsible for promoting the expression of *CYR61*, we used known inhibitors of several pathways. Although the canonical pathway of IL-6 is reported to be the JAK/STAT pathway, our results suggest that IL-6 altered Cyr61 protein synthesis mainly through the ERK 1/2 pathway. Although IL-6 and IL-6R are known to be important targets for RA, our results suggest that targeting IL-6 specific downstream signalling proteins could also be beneficial for RA therapy.

Based on our DEG results, we selected candidates such as c-jun, NR4A1, ATF3, and EGR3 to determine whether they regulated IL-6-induced Cyr61 protein synthesis. Previous studies have suggested that the effect of IL-6 on target gene transcription may involve c-jun [26]. Hence, we examined whether c-jun was involved in the IL-6-induced increase in Cyr61 protein synthesis and found that knocking down *JUN* expression did not affect IL-6-induced Cyr61 protein synthesis. NR4A1 and ATF3 also did not show any significant effects.

Interestingly, we found that EGR3 modulated IL-6-induced Cyr61 protein synthesis through the ERK 1/2 pathway. EGR3 is a member of the early growth response (EGR) gene family of

transcription factors that regulates a wide range of biological processes in response to growth factors, cytokines, and mechanical forces. In human foreskin fibroblasts lacking EGR3, transforming growth factor-beta 2 (TGF-β2) induction of the fibrotic genes collagen alpha 1 (*COL1A1*), alpha-smooth muscle actin *(ACTA2)*, *TGFB1*, connective tissue growth factor (*CTGF)*, and plasminogen activator inhibitor-1 *(SERPINE1)* was significantly abrogated [27]. Furthermore, in human T cells, EGR4 and EGR3 interact with NF-κB to control the transcription of genes encoding inflammatory cytokines such as IL-2 and TNF-α, as well as intercellular adhesion molecule 1 [28]. Thus, EGR3 contributes to production of Cyr61 as a fibrotic or inflammatory mediator. Further studies are required to clarify the binding site for EGR3 on the *CYR61* gene.

SIRT1 is NAD-dependent deacetylase associated with regulation of oxidative stress, apoptosis, DNA repair. Synovial hyperplasia is a characteristic of RA progression. So, we hypothesized that SIRT1 affect IL-6-induced Cyr61 protein synthesis. We confirmed that *SIRT1* knockdown decreased Cyr61 protein synthesis induced by IL-6. However, SIRT1 functions with protein substrate for its effect, it requires further study which transcription factors interact with.

As a secreted ECM protein, Cyr61 is considered to mediate cell proliferation, adhesion, migration, and act on pro-inflammatory molecules that induce the production of several cytokines and chemokines [29]. Moreover, activation of MMPs is essential for cells to migrate, through the rearrangement of ECM to facilitate cell migration [14]. Given its autocrine and paracrine features, increased secretion of Cyr61 by FLSs after IL-6 stimulation were found to promote proliferation and stimulate the expression of *MMP2*, suggesting a role of Cyr61 in the activation of FLS migration and invasion. These results support the hypothesis that expression of *CYR61* leads to hyperplasia and increased angiogenesis and invasion in joints in RA, leading to greater degrees of synovial inflammation and cartilage erosion. We confirmed that Cyr61 promote to IL-6 expression, thereby forming a positive feedback loop. Thus, specifically how IL-6 and Cyr61 create a positive feedback system requires further study. We also observed that IL-6 and Cyr61 led to an increase in FLS proliferation. However, in this study, we focused on migration and invasion of FLS. The mechanism

of effect of IL-6 and Cyr61 on FLS proliferation requires further study.

To summarize, we report here that protein synthesis of Cyr61 was enhanced in the FLSs of RA patients compared to those from OA patients. Interestingly, IL-6 stimulated the expression of *CYR61* through the ERK/EGR3 pathway in RA-FLSs and modulated the expression of genes associated with angiogenesis, cell migration, and chemotaxis. Cyr61 was produced and secreted into the ECM environment and acted on the FLSs in an autocrine/paracrine manner. *MMP2* expression was consequently enhanced and contributed to FLS migration and invasion. As a result, RA FLS migration and invasion were stimulated due to an enhancement of *CYR61* expression (Fig. 6).

Conclusions

Our findings suggest that IL-6 regulated Cyr61 is a key player in FLS migration and invasion and eventually contributes to joint destruction in RA. Therefore, Cyr61 could be a potential therapeutic target for anti-IL-6 treatment of RA

5. REFERENCE

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6. 국문 초록

연구배경

류마티스 관절염은 만성 염증 반응으로, 관절 및 뼈의 파괴 뿐만 아니라 폐, 혈관 등 몸 전체로도 진행되는 자가면역 질환이다. 류마티스 관절염을 일으키는 원인은 명확하게 밝혀져 있지 않지만, 유전적 소인을 가지고 있는 환자에게 흡연 혹은 이물질 등의 환경적 요인이 작용하여 자가항체가 생성되고, 이를 정상적인 수준으로 조절해야 하는 면역체계의 이상으로 발생하는 것으로 알려져 있다.

섬유아세포 유사 활막세포 (이하 활막세포)는 활막표층에 존재하며 류마티스 관절염의 진행 과정에서, 비정상적으로 증식하고 신생혈관을 생성하면서 파누스(pannus)를 형성한다. 파누스는 관절 내 조직을 침윤하여 파괴하는 데 관여하고, 류마티스 관절염의 특징적인 조직학적 소견을 나타낸다.

염증 매개 물질 중 Interleukin-6 (IL-6), Tumor Necrosis Factor (TNF)-α, Interleukin-17 (IL-17) 등이 류마티스 관절염 악화에 관여한다고 알려져 있다. 특히, IL-6는 전염증 사이토카인으로, 지역적 손상으로부터 나온 염증 신호를 혈액을 통해 전신으로 내보내 숙주 방어를 일으키는 역할을 한다. 세균 감염이나 조직 손상이 일어날 때 급성기 면역반응, 혈청학적 반응을 유도한다. 하지만, 류마티스 관절염 환자의 혈청에서 IL-6가 증가되어 있고, 질병의 중증도나 관절의 손상 정도와 양의 상관관계가 있다고 보고되고 있으며, IL-

6의 지속적인 높은 발현은 결국 만성적인 염증질환으로 이어진다.

Cysteine-rich protein 61 (Cyr61 혹은 CCN1)은 세포에서 분비되는 세포 외 기질 단백질로, 세포의 부착(adhesion), 증식(proliferation), 이동(migration)과 혈관생성(angiogenesis) 등을 조절하는 것으로 알려져 있다. 특히, 대장암, 유방암 등 몇몇의 암의 병의 진행 과정에서 Cyr61이 가지고 있는 특징으로 인해 종양세포의 증식과 전이에 중요한 역할을 한다고 알려져 있고, Cyr61를 억제함으로써 증식이나 침투 과정이 저해되는 연구결과가 보고되었다. 그런데, 최근에 다양한 연구 결과에서 Cyr61이 류마티스 관절염의 진행 과정에서 활막세포의 증식을 촉진하고 전염증성 물질로서 중요하게 작용하는 것으로 알려지고 있다.

따라서, 류마티스 관절염 조직 내에 증가되어 있는 IL-6에 의해 Cyr61의 조절이 정상적으로 이루어지지 않는다면, 이 기전을 밝히는 것은 발병요인의 분자적 측면에서 중요하다고 사료되었다. 또한, Cyr61를 통해 활막세포의 이동 및 침투 변화로 인해 관절 조직 파괴에 어떠한 영향을 미치는지 이번 연구를 통해 알아보고자 하였다.

연구방법 및 재료

골관절염 환자와 류마티스 관절염 환자 각각의 활막세포에서 Cyr61 단백질의 발현은 Western blotting 방법으로 평가하였다. 또한, 활막조직에서는 면역조직화학염색을 이용해 IL-6와 Cyr61을 비교하였다.

IL-6에 의한 Cyr61 발현 유도는 활막세포를 1% FBS을 포함한 배지에서 starvation 시키고 IL-6를 처리한 뒤 Western blotting과 real-time PCR을 통해 평가하였다. 모든 실험에서 IL-6는 항상 soluble IL-6 receptor와 동시에 처리하였다.

IL-6가 류마티스 관절염 환자의 섬유아세포 유사 활막세포에 미치는 유전자 발현 변화는, 3명의 환자의 세포군과 이 3명의 환자 세포에 각각 IL-6를 처리한 군을 코스모진텍 (Cosmo Gentech.. Co)을 통해 분석·의뢰하였다.

유전자의 발현은 FPKM (fragments per kilobase of exon per million fragments mapped)으로 나타내고, $\log_2$FPKM 값들 사이의 Pearson correlation coefficient 을 통해 유전자 발현의 상관관계를 평가하고, Multi-dimensional scaling (MDS) plot으로 나타내었다.

FPKM은 z-score transformation을 통해 정규화한 뒤, IL-6 처리 전후의 유의미한 유전자 발현 변화, 즉 differentially expressed genes (DEGs) 들은 Heat map, volcano plot을 통해 비교하여 나타내었다. IL-6에 의해 발현이 유의미하게 증가 혹은 감소한 유전자들을 각각 Metascape software (http://metascape.org) 를 이용해 gene ontology를 평가하였다.

IL-6에 의해 증가된 Cyr61 발현 기전을 밝히기 위해, IL-6가 관여하는 몇 가지 경로를 차단제를 사용하여 각각 비교하였다. PI3K/AKT 차단제 LY294002, JAK2/STAT3 차단제 AG490, p38 MAPK 차단제 SB203580, ERK 차단제 PD98059을 각각 IL-6와 함께 사용하였다. 또한, c-Jun, NR4A1, ATF3과 EGR3의 유전자의 기능을 억제하기 위해 small interfering RNA (siRNA)을 사용하였고, 대조군으로 small interfering Negative Control으로 비교하였다. 그리고 위의 실험들은 western blotting을 통해 단백질 수준에서 평가하였다.

Cyr61 단백질의 세포외분비를 평가하기 위해, IL-6을 처리한 활막세포가 있는 배지를 western blotting을 이용해 평가하였다. 또한, IL-6 및 Cyr61 단백질의 자가분비에 의해 유도되는 Cyr61, MMP1, MMP2, MMP3에 대한 mRNA 합성을 평가하기 위해 Reverse transcription-PCR과 Real-time PCR을 사용하였다.

활막세포의 이동과 침투를 cell migration assay와 transwell assay를 사용하여 평가하였다. 또한, IL-6와 Cyr61의 자극이 활막세포의 증식에 미치는 영향을 Electric Cell-substrate Impedance Sensing (ECIS) 을 통해 평가하였다.

연구결과

골관절염 환자에 비해 류마티스 관절염 환자의 활막세포에서 더 높은 Cyr61 단백질 발현을 나타내었다. 활막조직에서도 류마티스 관절염 환자 군에서 IL-6와 Cyr61가 더 높은 발현을 나타내었다. 또한, IL-6에 의해 Cyr61의 mRNA 및 단백질 발현이 증가됨을 알 수 있었다. 골관절염에 비해 류마티스 관절염에서 IL-6와 Cyr61가 병인 기전에 관여한다는 사실과, IL-6가 Cyr61 발현에 영향을 미치는 것을 알 수 있었다.

RNA Sequencing 을 통해, 3명의 류마티스 관절염 환자 활막세포에 IL-6를 처리하였을 때가 처리하지 않았을 때보다 유전자들의 발현의 상관관계가 더 높아짐을 알 수 있었다. 유의미하게 발현이 변화된 유전자 중 277는 발현이 증가되었고, 38개는 감소되었다. 발현이 유의미하게 증가된 유전자들을 이용하여 Gene ontology를 찾아보았을 때, 유전자들이 관여하는 기능에서 신생혈관의 발달 및 cell adhesion, MAPK 신호 전달 체계의 조절에 관련된 기능들이 활성화된다는 것을 알 수 있었다. 이는 IL-6에 의해 발현이 증가된 유전자들의 기능들이 세포의 이동 및 침투와 밀접한 관련이 있음을 알 수 있었다.

IL-6가 관여하는 4가지 세포 전달 경로 저해제를 처리하였을 때, IL-6에 의해 증가된 Cyr61 발현이 ERK 1/2 저해제인 PD98059 (1uM)에 의해 가장 저해되었다. 이를 통해, IL-6에 의해 유도된 Cyr61 발현 조절에 ERK 1/2 경로가 주로 관여한다는 것을 알 수 있었다.

앞서, RNA Sequencing 후, 유의미하게 증가된 유전자들 중 전사인자로서 기능을 하며 Cyr61 발현을 조절할 것이라고 예상 되는 몇 가지 후보 유전자들을 대상으로 Cyr61 발현에 관여하는지 실험했다. 그 중 c-jun은 IL-6에 의해 발현이 증가되었지만, ERK 1/2 inhibitor에 의해 저해되지 않았다. 또한, siRNA을 통해 c-jun을 knockdown 시켰을 때 역시 Cyr61 발현이 감소되지 않았다. 하지만, EGR3가 Cyr61와 함께 IL-6에 의해 발현이 증가되며, ERK 1/2 inhibitor에 의해 저해되는 것을 확인할 수 있었다. 또한, EGR3를 knockdown 시켰을 때, IL-6에 의해 유도된 Cyr61 발현이 감소했다. 이를 통해, IL-6에 의해 유도된 Cyr61 발현에 EGR3가 ERK 1/2 와 함께 관여하는 것을 알 수 있었다.

IL-6를 세포에 처리하였을 때, 배지에서 Cyr61 단백질이 증가되어 있는 것을 western blotting을 통해 확인했다. 그리고 Cyr61 단백질을 세포에 처리했을 때, 세포에서 Cyr61 발현이 역시 증가하는 것을 알 수 있었다. 즉, IL-6에 의해 Cyr61 생산과 세포 외 분비가 증가하며, 분비된 Cyr61에 의해 다시 Cyr61 발현이 증가한다는 것을 알 수 있었다.

한편, IL-6와 Cyr61를 처리했을 때 MMP2 유전자 발현이 증가하는 것을 확인했다. 이 효소들의 활성화로 세포 외 기질을 분해함으로써 세포의 이동과 침투가 활성화 되는 것을 예상해 볼 수 있었다.

IL-6와 Cyr61를 각각 처리했을 때, 대조군에 비해 활막세포의 이동이 유의미하게

증가하는 것을 확인했다. 또한, 세포의 증식 역시 증가하는 것을 알 수 있었다. IL-6와 Cyr61 단백질의 처리로 증가되었던 세포의 이동은 항 Cyr61 항체에 의해 감소되는 것을 알 수 있었다. 세포의 침투에서도 역시 같은 결과를 나타내었다. Cyr61 발현 자체를 siRNA을 통해 저해 했을 때, IL-6의 자극에도 세포의 이동이 증가하지 않는다는 것을 확인했다. IL-6와 Cyr61가 활막세포의 이동과 침투, 증식을 증가하며, Cyr61의 발현 및 단백질을 저해함으로써 이를 감소시킬 수 있다는 것을 알 수 있었다.

연구고찰

IL-6는 활막조직의 성장, 관절 부종 및 염증을 일으키며 류마티스 관절염을 악화하는 인자로 알려져 있다. 또한, Cyr61은 몇 가지 암 세포의 침투와 혈관생성에 중요한 역할을 한다는 연구가 보고된 것을 통해 IL-6와 Cyr61가 연관성이 있을 것으로 가정하였다. IL-6가 활막세포에 작용하였을 때 발현이 증가된 유전자들의 기능들이 세포의 이동에 관여하는 MAPK 경로 조절과 세포의 부착, 혈관생성 등인 것을 통해 활막세포가 파누스를 형성하고 관절 조직을 파괴함에 있어 IL-6에 의한 Cyr61 발현이 중요한 역할을 한다는 것을 알 수 있다.

IL-6는 JAK/STAT 경로에 관한 연구가 많이 있었지만, 본 연구를 통해 활막세포에서 IL-6는 ERK 1/2와 EGR3를 활성화시켜 Cyr61의 발현을 증가시킨다는 것을 알아냈다. 이는, ERK에 의한 경로 역시 류마티스 관절염 병인 기전에서 중요함을 알 수 있다. 또한, 이번 연구에서 EGR3가 Cyr61의 발현 조절에 영향을 미친다는 것을 알 수 있었지만, EGR3가 Cyr61의 전사인자로서 작용하는 DNA 위치 등에 관한 연구는 앞으로 더 필요할 것으로 생각한다.

Cyr61은 분비 단백질로, 다시 세포를 자극하며 자가분비 혹은 주변분비를 함으로써 Cyr61 발현을 증가시키는 양성 피드백을 형성한다. 또한, IL-6와 Cyr61 자극에 의한 MMP2의 발현은 세포외기질 분해 및 재배열을 통해 세포의 이동을 촉진할 수 있다는 데

의미가 있다. 이는 본 연구에서 세포의 이동과 침투가 증가한 결과에 대한 원리를 설명해준다.

Cyr61 단백질의 자가분비를 통한 양성 피드백을 형성한다는 것을 밝혀냈지만, Cyr61이 IL-6를 유도하는 기전에 대해선 추가적인 연구가 필요할 것으로 생각한다. 또한, 활막세포의 증식 기전 역시 실험이 앞으로 더 필요할 것으로 생각한다.

본 연구는 류마티스 관절염 환자의 관절 조직에서 증가되어 있는 IL-6가 ERK/EGR3 경로를 통해 Cyr61발현을 일으키는 기전을 밝히고, 이 단백질에 의해 활막세포가 이동 및 침투가 증가해 관절조직의 파괴에 기여한다는 것을 알아냈다. 또한, Cyr61을 저해함으로써 이러한 작용이 억제되는 것을 확인함을 통해, Cyr61이 류마티스 관절염 치료를 위한 타겟 후보 물질이 될 수 있다는 데 의의가 있다.

ACKNWOLEGEMENT