A Doctoral Dissertation

A Study of the function of the Cyr61 on Rheumatoid arthritis-fibroblast like synoviocyte and Skin dermal fibroblast

Eun-jeong Kwon

Department of Medicine

Medical School

Jeju National University

February, 2017

류마티스 관절염의 활막세포와 피부섬유아세포에서 Cyr61의 역할 규명에 관한 연구

지도교수 김진석, 박덕배

권 은 정

이 논문을 의학 박사학위 논문으로 제출함

2016년 12월

권은정의 의학 박사학위 논문을 인준함

심사위원장 조문제 ㊞

위 원 박덕배 ㊞

위 원 김진석 ㊞

위 원 최은경 ㊞

위 원 윤종현 ㊞

제주대학교 의학전문대학원

A Study of the function of the Cyr61 on Rheumatoid arthritis-fibroblast like synoviocyte and Skin dermal fibroblast

Eun-jeong Kwon

Supervised by professor **Jinseok Kim**

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

December, 2016

This thesis has been examined and approved

Moonjae Cho

Deok Bae Park

Jinseok Kim

Eun Kwang Choi

Chonghyeon Yoon

Date Approved

_____

Department of Medicine

MEDICAL SCHOOL

JEJU NATIONAL UNIVERSITY

ABSTRACT

Rheumatic disease is referred to musculoskeletal disease, which affect joint, muscle, connective tissue and ligament. Rheumatoid arthritis (RA) is chronic systemic inflammatory disorder of unknown etiology which is characterized by hyperproliferative fibroblast-like synoviocyte (FLS) and bone and cartilage erosion. Synovial fibroblast act as passive and active responder. Thus, it is important to understand action mechanism themselves.

Scleroderma is described by over-activation of fibroblasts which produce extracellular matrix and adhesive protein that are associated with wound healing, inflammation and angiogenesis.

Cyr61 is matricellular protein and it is associated with chronic inflammatory disease and activation of fibroblast. Therefore, we studied the association with Cyr61 and connective tissue related rheumatic disease.

Cyr61 protein was expressed in RA-FLS, and its intracellular expression and secretion levels were increased by TNF-α. Moreover, Cyr61 directly promoted RA-FLS migration and invasion. Rosiglitazone (RSG) significantly decreased TNF-α-induced Cyr61 expression. RSG decreased TNF-α-induced NF-κB activation and IκBα degradation. Furthermore, RSG inhibited the TNF-α-induced RA-FLS migration and invasion and decreased the Cyr61 treatment induced RA-FLS invasion. Finally, blocking Cyr61 significantly attenuated TNF-α-induced migration.

SIRT1 and Cyr61 were expressed in human dermal fibroblasts and the stimulation of TGF-β further induced expression SIRT1 and Cyr61. Treatment of resveratol (RSV), SIRT1 agonist or overexpression of SIRT1 also promoted expression of SIRT1 and Cyr61 in human dermal fibroblasts, whereas inhibition of SIRT1 activity by nicotinamide or knock down of SIRT1 down-regulated Cyr61 basal level as well as TGF-β or RSV-induced Cyr61expression. Blocking of ERK signaling by PD98509 reduced TGF-β or RSV-induced Cyr61 expression. TGF-β, RSV or SIRT1 vector enhanced β-catenin as well as Cyr61 expression. This stimulation was reduced by Wnt inhibitor, XAV939. RSV increased migration and nicotinamide attenuated RSV-induced migration of human dermal fibroblasts.

Furthermore, SIRT1 overexpression promoted cell migration whereas blocking Cyr61 attenuated SIRT1-stimulated migration of human dermal fibroblasts.

Consequently, activation of synovial fibroblast and skin-fibroblast is associated with Cyr61 expression and Cyr61 can be potential treatment target for rheumatic disease.

Key words: Rheumatoid arthritis, Scleroderma, Rheumatic disease, Cysteine rich angiogenic inducer 61, Peroxisome proliferator-activated receptor gamma

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Part Ⅰ

The PPARγ agonist rosiglitazone inhibits migration and invasion by downregulating Cyr61 in rheumatoid arthritis fibroblast-like synoviocytes

1. Introduction

Rheumatoid arthritis (RA) is chronic systemic inflammatory disorder of unknown etiology that is characterized by synovial fibroblast hyperplasia and a variable degree of cartilage and bone destruction. Conventional knowledge suggests that fibroblast-like synoviocytes (FLS) act as 'passive responders'. However, recent evidence suggests that rheumatoid arthritis FLS (RA-FLS) are key players in RA pathogenesis due to an aggressive phenotype without exogenous stimulation. FLS migrate and invade cartilage and bone with characteristics that are similar to malignant cells. Lefevre S. *et al.* demonstrated that human RA-FLS implanted in severe combined immunodeficient (SCID) mice migrate to distant and unaffected joints, leading to cartilage and bone destruction. Control of RA-FLS migration and invasion might be an important therapeutic target. However, the molecular mechanisms underlying uncontrolled RA-FLS activation are still incompletely understood.

Peroxisome proliferator activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear receptor family. PPARs were originally thought to control glucose and lipid homeostasis, but Cuzzocrea *et al*. recently showed another role for PPARs, especially PPARγ, as negative regulators of inflammatory responses. A role for PPARγ in inflammation has been studied in monocytes/macrophages, where ligands of this receptor inhibited macrophage activation and inflammatory cytokine production. Emerging evidence suggests that PPARγ agonists play important roles in regulating inflammation and joint destruction. PPARγ ligands not only inhibit IL-1β-induced MMP-1 expression but also modulate the expression of LPS-induced iNOS, COX-2 and proinflammatory cytokines in FLS.

Cysteine-rich angiogenic inducer 61 (Cyr61) is a matricellular protein of the CCN family, which consists of six distinct members (CCN1-6). One CCN family member, CCN1 or Cyr61, promotes cell survival, proliferation, and migration in a cell type and function-specific manner. Cyr61 is especially associated with diseases related to chronic inflammation, including RA, Crohn's disease and ulcerative colitis. Cyr61 was recently shown to be overexpressed in synovial tissue and FLS derived

from RA patients. In RA-FLS, Cyr61 has a critical role in IL-17-dependent proliferation and is involved in neutrophil infiltration. In contrast to its well-established expression site, the biological mechanisms and negative regulators of Cyr61 remain unknown.

In this study, we investigated the modulatory effect of PPARγ ligands on the motile and invasive characteristics of RA-FLS and identified a potential target protein of PPARγ ligands in RA.

2. Materials and Methods

2.1. Isolation and Culture of Synovial Fibroblasts

Synovial tissues were obtained from three patients undergoing total knee replacement surgery. RA diagnosis was based on the American College of Rheumatology 2010 for RA. Synovial fibroblasts were obtained via sequential tissue digestion of tissue with collagenase and dispase in MEM (BioWhittaker Inc., Walkersville, MD, USA) and filtration through a nylon mesh. The cells were washed twice with phosphate buffered saline (PBS) and cultured in DMEM (BioWhittaker Inc., Walkersville, MD, USA) supplemented with 10% FBS in a humidified 5% CO_2 atmosphere. Synovial cells were used for experiments at three to eight passages. The study was approved by the Institutional Review Board of our institution (IRB File No. 2014-04-017). Subjects gave informed consent in accordance with the Declaration of Helsinki.

2.2. Reagents

Rosiglitazone, a synthetic PPARγ ligand, was purchased from Sigma (St. Louis, USA). Monoclonal antibodies against human NFκB and IκBα were purchased from Cell Signaling Technologies (Danvers, MA, USA), and GAPDH and CYR61 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.3. RT-PCR

Total RNA was extracted from the cultured cells using TRIzol (Invitrogen, Carlsbad, CA) reagent according to the manufacturer's instructions. cDNA from equal amounts of total RNA was synthesized

using a reverse transcriptase kit (Promega, Seoul, Korea). PCR amplification was carried out according to the following program: 95°C for 2 min; 33 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; and final extension at 72°C for 5 min. PCR primers for human MMP1, MMP3, Cyr61 and GAPDH were designed as follows: MMP1 forward (5'-GGAGATCATCGGGACAACTC-3'), MMP1 reverse (5'-ACCGGACTTCATATGTCG-3'); MMP3 forward (5'-ATTCCATGGAGCCAGGCTTTC-3'), MMP3 reverse (5'-CATTTGGGTCAAACTCCAACTGTG-3'); Cyr61 forward (5'-TCCTCTGTGTCCCCAAGAAC-3'), Cyr61 reverse (5'-TCGAATCCCAGCTCCTTACC-3'); GAPDH forward (5'-CCAAGGAGTAAGACCCCTGG-3'), GAPDH reverse (5'-TGGTTGAGCACAGGGTACTT-3'). PCR products were separated on 1% agarose gels, and the images were scanned using a UV light box. Differences were confirmed using ImageJ software to analyze the relative ratio of changes in target RNA levels to those of the control.

2.4. Immunoblotting and Culture Supernatants

Synoviocytes were washed three times with PBS and lysed with RIPA buffer. Cell lysates were collected by scraping and centrifuged at 14,000 rpm for 15 min. The cell extract protein concentrations were determined using a BCA protein assay. In total, 30 μg of protein from each sample was electrophoresed on a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes, which were subsequently blocked for 1 h using 5% non-fat milk in TBS containing 0.1% Tween 20 (TBST). Then, the membranes were incubated at 4°C overnight with specific primary antibodies. Following washing and incubating with HRP-conjugated secondary antibodies, the bands were detected using ECL reagent.

We also examined the effects of RSG on CYR61 production in synoviocytes. Synoviocytes were cultured with RSG (120 μM) for 2 days, and the culture supernatants were collected. Culture medium was concentrated onto Amicon Ultra2 membranes (Millipore, Bedford, MA, USA) at 4°C. The CYR61 expression levels in synoviocyte culture media were determined by Western blot analysis.

Differences were confirmed using ImageJ software to analyze the relative ratio of changes in target protein levels to controls.

2.5. Wound Healing and Cell Invasion Assay

Synoviocytes were seeded at a density of 100,000 cells/well in 6-well plates. After 48 h of culture, the cells were 70-80% confluent. The cell monolayer was scraped in a straight line to create a scratch using a 200 μl pipet tip (initial scratch wound) [14,15]. Wounded monolayers were washed with PBS to remove detached cells. Reference points were made by marking the bottom of the dish with a pen. The wound width was recorded over 48 h following scratching. The data file showed the percentage of wound closure in the image at the time point. Percent Closure (%) = (initial scratch wound-wound gap at time)/initial scratch wound x 100. Cells that migrated into the empty area were stained with MTT solution. The images acquired for each sample were further analyzed quantitatively using ImageJ software.

For the in vitro invasion assay, collagen coated transwells (Corning Costar, USA) were used. The collagen (Sigma, USA) was diluted in serum free media and placed on the upper chamber of a 24-well transwell plate and incubated at 37°C for 2 h. After washing the upper chamber, the cells were kept in serum free medium. The lower chamber contained medium supplemented with 20% FBS and TNF-α. After 72 h of incubation, non-invasive cells on the upper membrane surface were carefully removed with a cotton swab. Cells that invaded onto the lower surface were fixed with 4% paraformaldehyde and stained with crystal violet solution. The number of invaded cells through the collagen was evaluated by visually counting three randomly chosen areas. All experiments were performed in duplicate.

2.6. Statistical Analysis

All data were analyzed using SPSS v. 16.0 software (SPSS Inc., Chicago, IL, USA). Statistical

significance between experimental groups was determined using Student's *t*-test or Mann–Whitney U-test in SPSS software. Differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Effect of PPARγ Agonists on RA-FLS Migration and Invasion

Because the major detrimental characteristic of FLS is invasiveness, we first assessed the cancer-like high migration phenotypes of RA-FLS. We concurrently investigated the potential effects of PPARγ ligands on RA-FLS migration using scratch wound healing assays. Different concentrations of rosiglitazone (RSG), a synthetic PPARγ agonist, were added to TNF-α-stimulated RA-FLS. RSG significantly decreased TNF-α-induced FLS migration (Fig. 1a). The migration reduction was the most effective (49.95%) at a concentration of 120 μM.

The scratch wound healing assay demonstrated lateral movement, but three-dimensional infiltration requires different properties due to extracellular matrix penetration. Thus, we next studied the effect of RSG on FLS cell invasion using Boyden chamber assays. As demonstrated in Fig 1b, TNF-α-activated RA-FLS cells treated with 120 μM RSG exhibited significantly decreased invasion (42.18%). These findings strongly suggest that PPARγ activation inhibited migration and RA-FLS invasion.

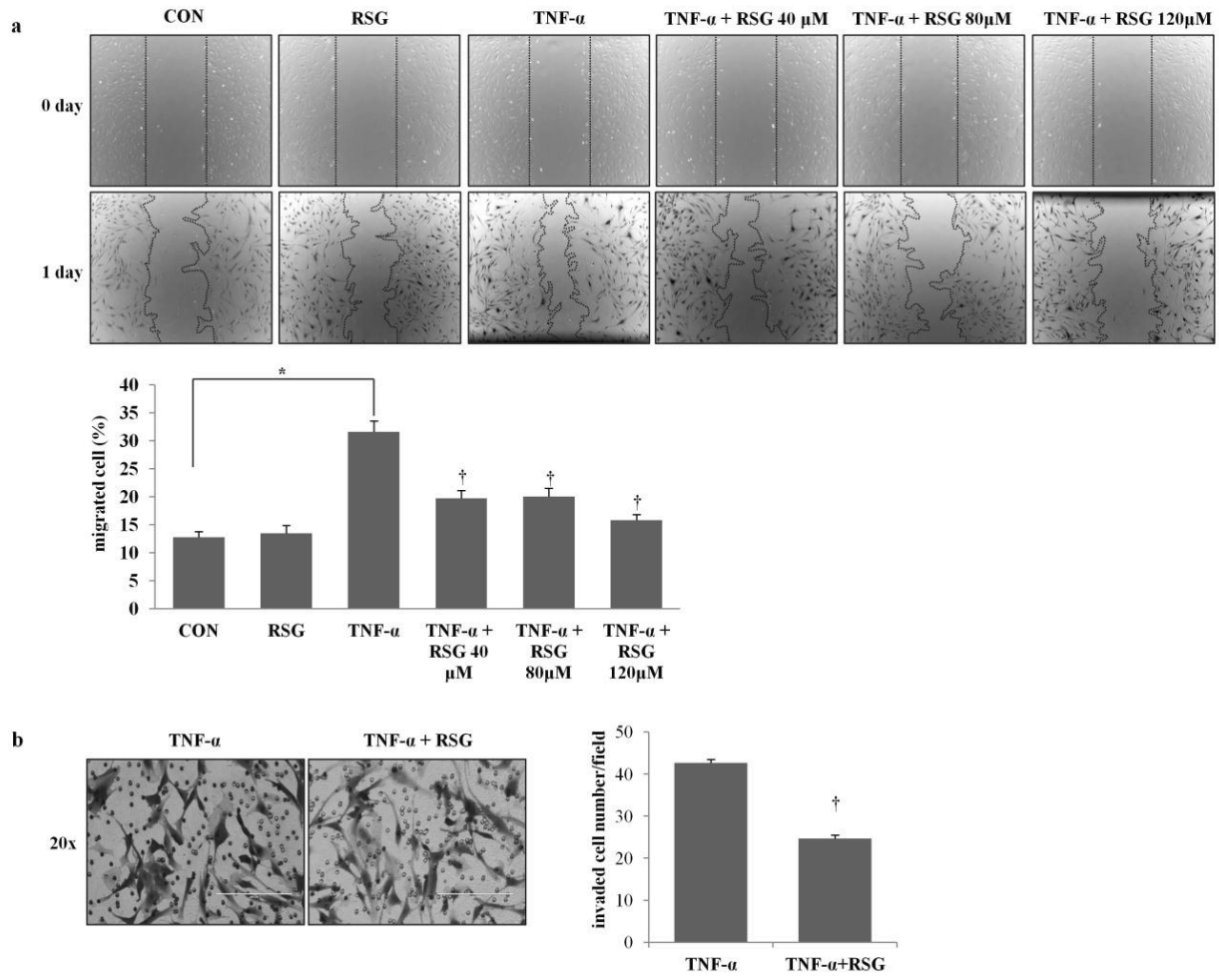

Figure 1. Rosiglitazone inhibited TNF-α-induced cell migration and invasion in RA-FLS. a RA-FLS were incubated with TNF-α (50 ng/ml) and RSG. Cell migration was analyzed for 0 h and 24 h. The data presented are the means +/- SEM for three different cell lines. **b** Invasion of TNF-α (50 ng/ml)-stimulated RA-FLS was analyzed in the presence or absence of RSG (120 μM) for 48 h. Representative images of transmembrane cells are shown in the left panel. The data presented are the mean number of transmembrane cells ± SEM per microscopic field of 3 independent experiments as quantified in the right panel. $p < 0.05$ was considered to be statistically significant *compared with control, †compared with the TNF-α group.

3.2. Effect of RSG on TNF-α-induced Cyr61 Expression

Cyr61 mediates inflammation and affects the cell environment in a variety of ways, especially cell migration and invasion. Thus, we examined changes in Cyr61 expression to gain insight into the mechanism of RSG-mediated RA-FLS migration and invasion.

Because TNF-α is a key cytokine in RA pathogenesis due to its role as a primary inducer of other cytokines such as IL-1, we hypothesized that RSG may block the TNF-α stimulated production of Cyr61 in RA-FLS. We confirmed that Cyr61 was expressed in RA-FLS and augmented by TNF-α in a time-dependent manner (Fig. 2a). Because Cyr61 is secreted as an extracellular matrix matricellular protein, we collected culture supernatants and measured the concentration of secreted Cyr61. In the same manner, intracellular (178.18%) and secreted (683.63%) Cyr61 levels were increased by TNF-α in RA-FLS (Fig. 2b).

The PPARγ agonist RSG affected cell migration and invasion. Thus, we asked whether the effect of RSG on RA-FLS invasiveness was due to the reduction of Cyr61 levels. As demonstrated by Western blot and RT-PCR in Fig. 2b and 2c, RSG treatment for 2 days decreased Cyr61 mRNA (32.76%) and protein levels (44.91%) in TNF-α-treated RA-FLS. MMPs are associated with cell migration and invasion. MMP3 levels were increased by Cyr61 in human oral squamous cell carcinoma cells [16]. To investigate changes in MMP expression, we detected TNF-α-induced RA-FLS mRNA synthesis. As demonstrated in Fig. 2d, Cyr61 increased MMP1 and MMP3 in RA-FLS. MMP1 and MMP3 were induced by TNF-α, which was significantly ameliorated by RSG treatment (Fig. 2e). Together, these results indicate that RSG treatment ameliorated TNF-α-induced increases of Cyr61 expression and secretion, and RSG treatment also downregulated MMP1 and MMP3 levels in RA-FLS.

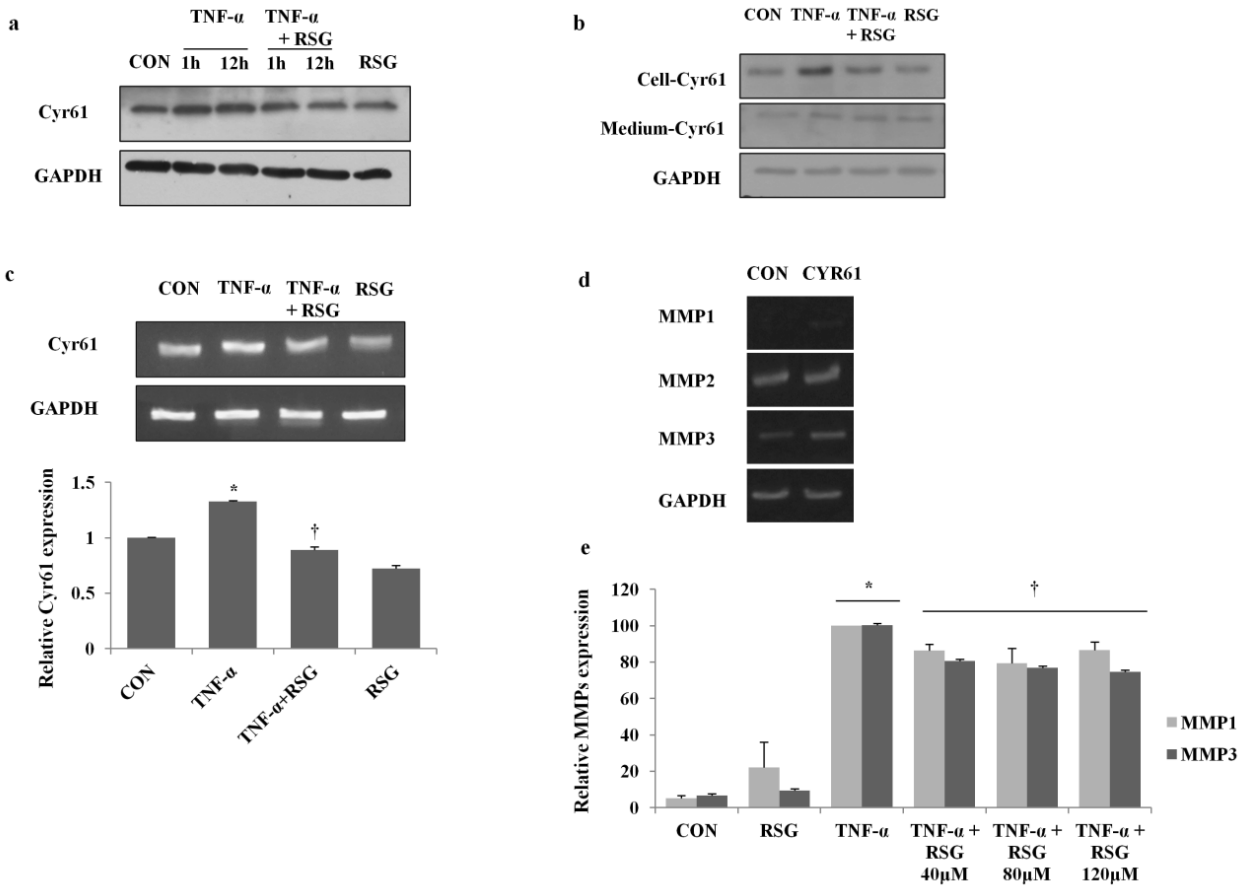

Figure 2. Cyr61 expression levels were enhanced by TNF-α treatment and decreased by Rosiglitazone treatment in RA-FLS. a RA-FLS were treated with TNF-α (50 ng/ml) and RSG (120 μM) for the indicated times. **b** RA-FLS were treated with TNF-α and RSG for 48 h. Cyr61 levels were determined by Western blot. **c** The Cyr61 mRNA expression levels were also changed by TNF-α or RSG treatment in RA-FLS**. d** MMP mRNA expression levels were changed by Cyr61 treatment in RA-FLS. **e** RSG treatment decreased TNF-α-induced MMP expression on RA-FLS. The values represent the means +/- (SEM) from three different cell lines. $p < 0.05$ was considered to be statistically significant. *compared with control, †compared with the TNF-α group.

3.3. Effect of RSG on the NFκB Signaling Pathway

Many inflammatory cytokines are regulated by NFκB signaling. To examine the potential antagonizing effect of RSG treatment on NFκB activation and Cyr61 expression, we incubated cells with TNF-α and RSG. RSG decreased TNF-α-induced NFκB nuclear translocation (36.39%) and IκBα degradation (101.63%) (Fig. 3). Inhibition of NFκB by parthenolide (PTN, 1 μM) decreased TNF-α-induced migration (86.83%) and invasion (76.42%) (Fig. 4a and 4b). Parthenolide also reduced TNF-α-induced Cyr61 expression (Fig. 4c).

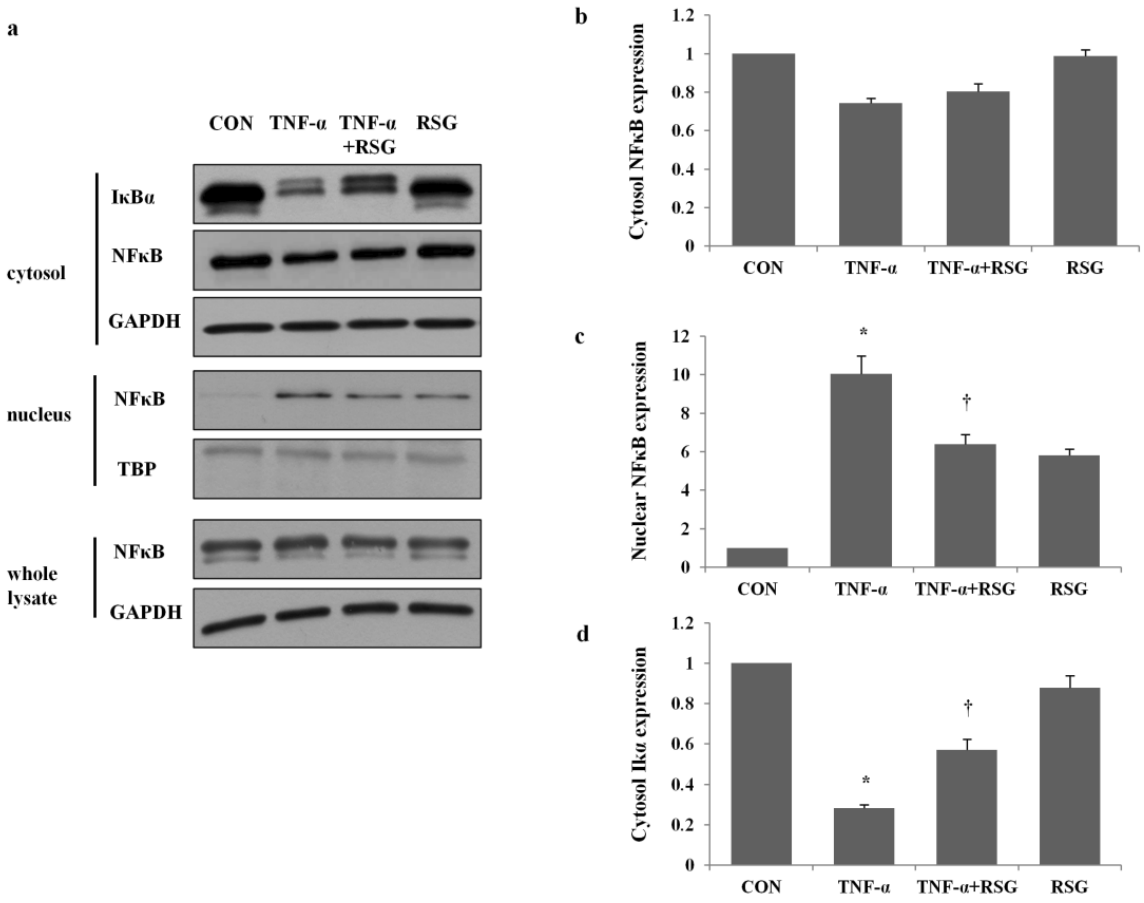

Figure 3. Rosiglitazone inhibited NFκB nuclear translocation in RA-FLS. a RA-FLS were treated with TNF-α (50 ng/ml) and RSG (120 μM) for 10 min. Cells were lysed, and then, NFκB and IκBα protein expression was detected in the cytosolic and nuclear fraction. **b** Cytosolic NFκB expression did not change significantly. **c** TNF-α-induced nuclear NFκB expression levels were decreased by RSG treatment. **d** TNF-α-induced cytosolic IκBα degradation was stabilized by RSG treatment. Values represent the means +/- (SEM) of three different cell lines. $p < 0.05$ was considered to be statistically significant *compared with control, †compared with the TNF-α group

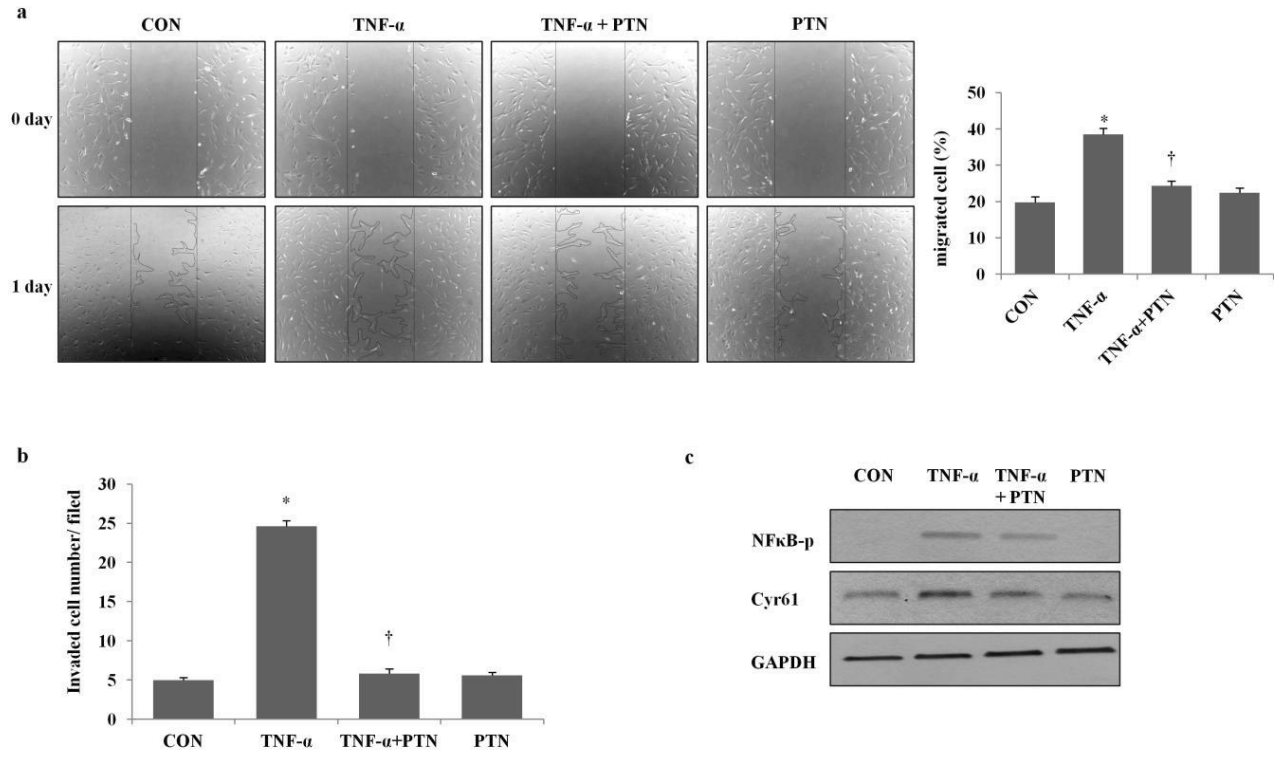

Figure 4. The NFκB pathway affected migration, invasion and Cyr61 expression in RA-FLS. a Cell migration was analyzed for 24 h after TNF-α (50 ng/ml) treatment using a wound repair assay with or without PTN (1 μM). The data presented are the means +/- SEM for three different cell lines. **b** Cell invasion was analyzed for 24 h after TNF-α treatment using collagen-coated transwells in the presence or absence of PTN (1 μM). **c** RA-FLS were treated with TNF-α and PTN (1 μM) for 24 h. $p < 0.05$ was considered to be statistically significant * compared with control, †compared with the Cyr61-treated group.

3.4. The Influence of Cyr61 on RA-FLS Migration and Invasion

We next investigated whether Cyr61 directly enhanced cell migration and invasion. The wound-healing assay demonstrated that recombinant Cyr61 (5 μg/ml) treatment increased migration (27.98%) compared with RA-FLS without Cyr61 (Fig. 5a and 5b). The Cyr61 blocking antibody reversed Cyr61-induced RA-FLS migration (33.15%) (Fig. 5a and 5b). Previously, studies revealed that Cyr61 overexpression in normal FLS increased cell invasion [17]. We also confirmed that invasion was greater in Cyr61-treated cells (58.19%) than in untreated cells (Fig. 5c,d). The number of invading cells was decreased (19.68%) by RSG treatment (Fig. 5c,d). These findings suggest that Cyr61 directly facilitated the migration and invasion of RA-FLS and that RSG can block Cyr61-induced RA-FLS invasion.

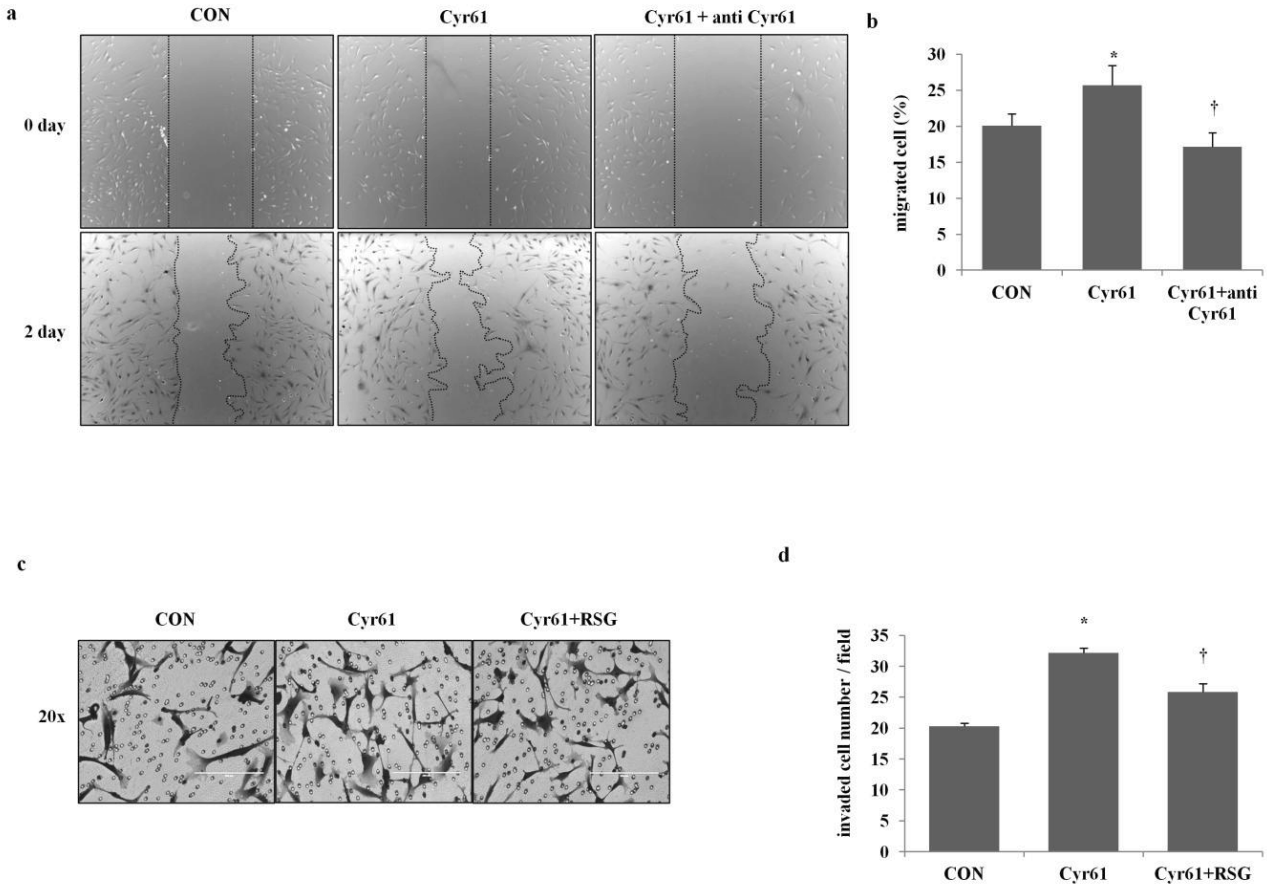

Figure 5. Cyr61 promoted RA-FLS migration and invasion compared with untreated cells. a Cell migration was analyzed for 48 h after Cyr61 (5 μg/ml) treatment using a wound repair assay. **b** The migrated distances of the Cyr61-treated group were significantly increased compared with the control group. The number of migrating cells in the neutralized Cyr61 group was lower than in the Cyr61-treated group. The data presented are the means +/- SEM for three different cell lines. **c** Cell invasion was analyzed for 48 h after Cyr61 (5 μg/ml) treatment using collagen-coated transwells in the presence or absence of RSG (120 μM). **d** Invaded cells from the Cyr61-treated group were significantly decreased compared with the control group. The number of invading cells in the Cyr61- and RSG-treated group was lower than the Cyr61-treated group. The number of invading cells was averaged from three 20x fields. $p < 0.05$ was considered to be statistically significant * compared with control, †compared with the Cyr61-treated group.

3.5. The Effect of Neutralizing Cyr61 on TNF-α-induced RA-FLS Migration

The previous figures demonstrated that RSG blocked TNF-α-induced Cyr61 expression and migration. Thus, we wanted to confirm whether TNF-α-enhanced RA-FLS migration decreased after neutralizing Cyr61. Blocking Cyr61 significantly attenuated TNF-α-induced migration of RA-FLS (54.93%) (Fig. 6). Therefore, Cyr61 is an important factor for TNF-α-induced RA-FLS migration.

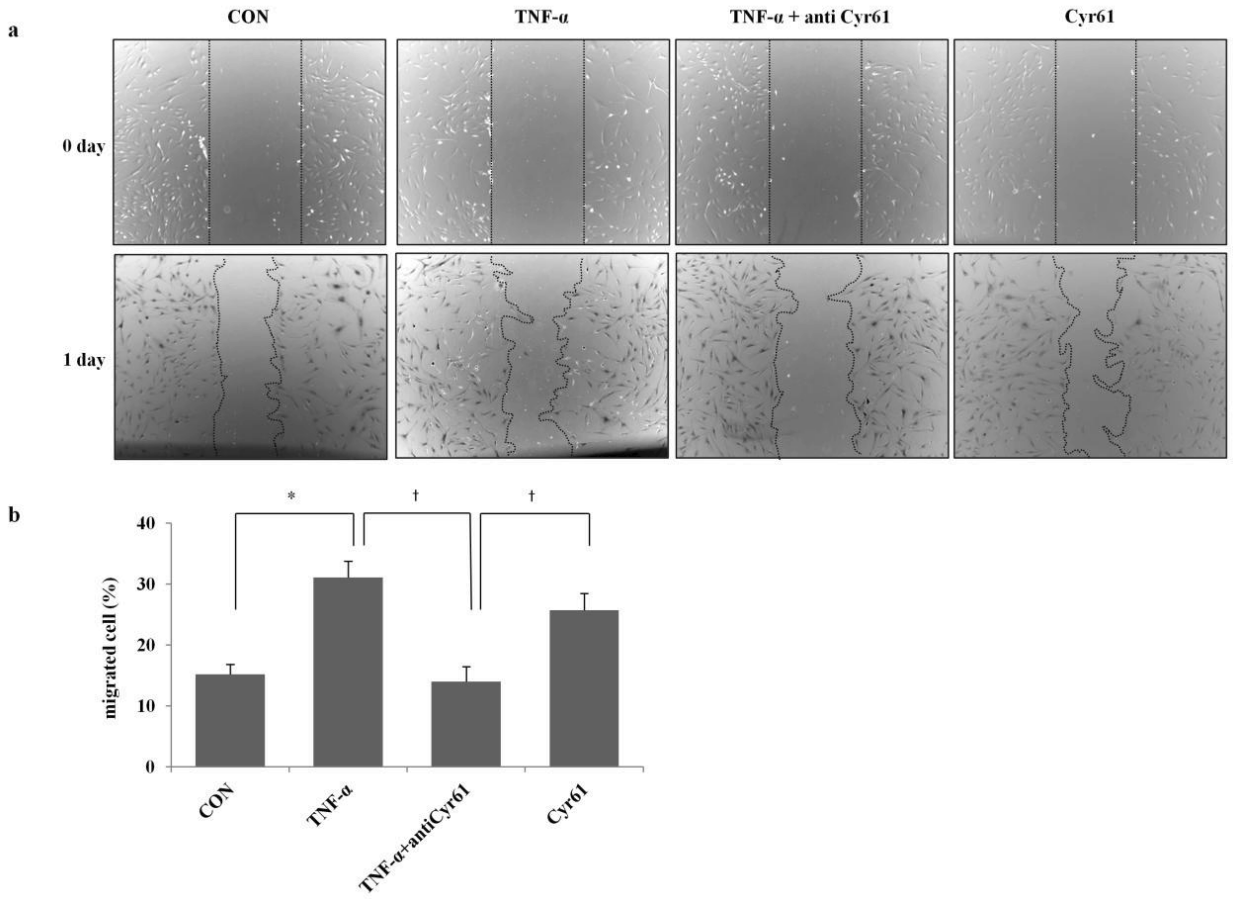

Figure 6. Blocking Cyr61 attenuated TNF-α-induced migration in RA-FLS. a Cell migration was analyzed for 24 h after TNF-α (50 ng/ml) treatment with Cyr61 blocking antibodies using a wound repair assay. The control group was treated with IgG. **b** Migrated distances of the TNF-α and anti-Cyr61 antibody-treated group were significantly decreased compared with the TNF-α or Cyr61-treated groups. The data presented are the means +/- SEM for three different cell lines. $p < 0.05$ was considered to be statistically significant *compared with control, †compared with the TNF-α-treated group or the Cyr61-treated group.

4. Discussion

In this study, we investigated the effect of the synthetic PPARγ activator RSG on TNF-α-induced migration and invasion in RA-FLS. RSG decreased TNF-α-enhanced migration and invasion in RA-FLS by downregulating Cyr61 expression and secretion-mediated NFκB inactivation.

In cancer research, PPARγ is thought to be a tumor suppressor gene that not only represses cell growth but also inhibits migration and invasion. In lung fibroblasts, RSG inhibits cell migration and proliferation. Treatment with ciglitazone resulted in decreased wound closure and chemotaxis of breast cancer cells. PPARγ ligands are anti-inflammatory agents in RA, and PPARγ activation reduced the inflammatory response without apoptosis by differentiating RA-FLS into adipocyte-like cells. Other groups demonstrated that 15d-PGJ2, a natural PPARγ agonist, reduced the expression of proinflammatory cytokines such as TNF-α, IL-1β and COX-2 as well as TNF-α-induced NFκB activation. However, we demonstrate another function for PPARγ agonists, as RSG decreased migration and invasion via inhibition of NFκB nuclear translocation by stabilizing IκBα. NFκB inactivation was concomitant with inhibiting Cyr61 expression. The Cyr61/CCN1 gene promoter has an AP-1 binding site. Mutating the AP-1 sites reduced the Cyr61/CCN1 gene promoter activity. Thus, AP-1 transcription factors regulate Cyr61/CCN1 promoter activity. NFκB and AP-1 transcription factors function cooperatively in the pancreatic tumor cell line MDAPanc-28. Thus, NFκB activation increased AP-1 activity. Moreover, the Cyr61 promoter region also contains shear-responsive elements (SSRE), which are the represented binding site of NFκB. NFκB modulation by RSG may affect the expression of its target gene Cyr61 directly or via AP-1. We confirmed that the NFκB inhibitor parthenolide inhibits TNF-α-induced migration, invasion, and Cyr61 expression in RA-FLS. Future studies are required to clarify which transcription factor regulates TNF-α-induced Cyr61 expression.

In a clinical study, pioglitazone reduced disease activity as measured by DAS28-CRP and insulin resistance in RA patients. Another study evaluated the combination therapy effect of pioglitazone and methotrexate in RA patients. These treatments decreased the proinflammatory cytokines TNF-α and

IL-1β and protected against reactive oxygen species, which cause tissue injury by increasing serum GSH and SOD levels. Moreover, a single pioglitazone treatment group also had a decreased arthritic index. These studies support PPARγ agonists as having possible benefits in RA treatment. Generally, the role of PPARγ in inflammation is to modulate proinflammatory cytokine release. However, our findings raise the possibility that the attenuation of RA disease activity by RSG may be associated with the Cyr61 level. Further studies are required to explore a correlation between Cyr61 serum levels and changes in disease activity in RA patients treated with RSG.

Several recent studies highlighted Cyr61 expression in RA. Cyr61 contributed to Th17 cell differentiation and IL-17 secretion, which enhanced RA-FLS proliferation. Zhu et al. reported that Cyr61-induced IL-8 production promoted neutrophil migration in RA. Cyr61 also promoted FLS cell proliferation and invasion via inhibition of apoptosis and regulation of MMP3 expression. In our study, we first demonstrated that RSG decreased Cyr61 mRNA and protein expression. Cyr61 is a human growth factor-inducible immediate early gene that rapidly responds to cellular stimuli. We demonstrated that short-term exposure of RA-FLS to RSG downregulated TNF-α-induced Cyr61 expression, which lasted for approximately 2 days. Furthermore, we observed that Cyr61-neutralizing antibodies significantly inhibited TNF-α-enhanced cell migration. Treatment with RSG suppressed TNF-α-induced Cyr61 expression. These results suggest that the inhibition of TNF-α-induced migration by RSG treatment may be mediated through downregulation of Cyr61 expression. These results suggest that RSG may be a useful future strategy for Cyr61 targeted therapy for RA treatment.

We demonstrated Cyr61-induced migration and invasion at 48 h, and these mobility patterns were sustained at 6 h, 24 h and 48 h (data not shown). Wounds did not completely close at 48 h of Cyr61 treatment. However, TNF-α-induced wound closure was faster than that of Cyr61 treatment alone. Therefore, TNF-α may affect cell mobility through a variety of mechanisms. Cyr61 may be one mechanism underlying TNF-α-induced cell movement.

MMP-3 is a crucial member of the MMP family because of its broad substrate specificity. It is important for connective tissue remodeling and correlates with inflammatory markers in human serum. Chen C.C. et al. demonstrated that Cyr61 enhanced MMP1 and MMP3 expression in a time and dose-

dependent manner. Chuang J.Y. et al. also demonstrated that Cyr61 increased human oral squamous cell carcinoma cell migration by upregulating MMP-3 expression. We demonstrated that TNF-α-induced Cyr61 expression also increased MMP1 and MMP3 mRNA expression and that RSG treatment reversed Cyr61-induced MMP1 and MMP3 expression.

This study has several potential limitations. First, the sample size was small, and we observed Cyr61 expression changes only in the cell system. Therefore, we will study tissue as well as serum from RA patients who had been treated with RSG. Second, we did not confirm how RSG directly modulates Cyr61 expression. In further studies, we will investigate the gene regulation and molecular events underlying the transcriptional regulation of Cyr61 by RSG.

In summary, we demonstrated that the PPARγ activator RSG suppressed malignant tumor cell-like behavior in RA-FLS after TNF-α stimulation. First, RSG inhibited TNF-α-enhanced Cyr61 expression at the mRNA and protein level and decreased MMP-1 and MMP-3 expression in RA-FLS. Second, decreased RSG-mediated Cyr61 expression was associated with the modulation of NF-κB activity. Finally, RSG reversed TNF-α-induced RA-FLS migration distance and invasion and also decreased Cyr61-treated RA-FLS invasion. Our results demonstrate that PPARγ agonists may have beneficial effects on RA-FLS migration and invasion via Cyr61 downregulation. Therefore, PPARγ agonists could be useful for treating patients with RA.

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Part Ⅱ

SIRT-1 regulates TGF-β induced dermal fibroblast migration via modulation of Cyr61 expression

1. Introduction

Fibroblasts are the most common cell type of the connective tissues in human body. Fibroblasts synthesize structural proteins of the extracellular matrix, adhesive proteins and participate in wound healing, inflammation, and angiogenesis. Fibroblasts can be activated by many chemical mediators; transforming growth factor-β (TGF-β), in particular, has been characterized as a key mediator of fibroblast activation. Activation of fibroblasts is an initial step in the process that causes fibrosis. A key feature of activated fibroblasts is increased migration; uncontrolled activation of fibroblasts leads, in turn, to pathological conditions such as systemic sclerosis, kidney fibrosis and idiopathic pulmonary fibrosis.

Sirtuin is a NAD-dependent histone deacetylase with many cellular substrates. The human genome encodes seven different sirtuins (SIRT1-7). Among these sirtuins, SIRT1 is the most extensively characterized and is categorized as a class Ⅲ histone deacetylase that targets histone and non-histone proteins. SIRT1 has pleiotropic actions on inflammation, apoptosis, metabolism and tumorigenicity. However, the role of SIRT1 on cell migration is controversial. For example, in endothelial progenitor cells and oral cancer cells, SIRT1 increased cell migration and epithelial-to-mesenchymal transition (EMT). However, Wei J. et al. showed SIRT1 has an anti-fibrotic effect and resveratrol (RSV), a SIRT1 agonist, reduced TGF-β induced fibroblast migration. Furthermore, in pulmonary fibrosis RSV was suggested as a potential treatment molecule. The effect of SIRT1 on migration seems to be cell type-specific and context-dependent. Therefore, we have established the role of SIRT1 in human dermal fibroblast migration.

CCN proteins are a family of proteins named after the first three members discovered: cystein-rich protein 61 (Cyr61, known as CCN1), connective tissue growth factor (CTGF, known as CCN2) and nephroblastoma overexpressed protein (Nov, known as CCN3). CCN proteins are matricellular proteins with important roles in inflammation, wound healing, and angiogenesis. The CCN family consists of six distinct members (CCN1-6). CCN2 correlates with the severity of fibrosis and results

from abnormal fibroblast activation. CCN2 is overexpressed in lung fibroblasts isolated from patients with interstitial lung disease and systemic sclerosis. Anti-CCN2 antibodies reduce bleomycin-induced lung fibrosis. Recently, CCN1 (Cyr61) has also been found expressed in fibroblasts of bleomycin-induced scleroderma. CCN1 and CCN2 are known to stimulate cell migration or chemotaxis of dermal fibroblast.

In this study, we identified the role of SIRT1 and investigated the interplay between Cyr61 and SIRT1 on the migration of human dermal fibroblasts.

2. Material s and Methods

2.1. Isolation and culture of human dermal fibroblast

Human circumcised foreskins were obtained from donors, aged 6-12 years at the Jeju National University, Korea. The study was approved by the Institutional Review Board of our institution (Jeju National University, Korea). Subjects gave informed consent in accordance with the Declaration of Helsinki. Fibroblasts were obtained via sequential tissue digestion with collagenase and dispase in MEM (BioWhittaker Inc., Walkersville, MD, USA) and filtration through a nylon mesh. The cells were washed 2 times with phosphate buffered saline (PBS) and cultured in DMEM (BioWhittaker Inc., Walkersville, MD, USA) supplemented with 10% FBS in a humidified 5% CO2atmosphere. Fibroblast cells at passages three to sixteen were used for the experiments.

2.2. Reagents

Resveratrol, nicotinamide, and XAV939 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal antibodies against human ERK, phospho-ERK, phospho-smad2 and phospho-smad3 were purchased from Cell Signaling (Danvers, MA, USA), and antibodies against GAPDH, CYR61 and total smad2/3 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-mouse and anti-rabbit secondary antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.3. RT-PCR

Total RNA was extracted from the cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA from equal amounts of total RNA was synthesized

using a reverse transcriptase kit (Promega, Seoul, Korea). PCR primers for human SIRT1, and GAPDH were as follows: SIRT1 forward (5'-aacctgttccagcgtgtcta-3'), SIRT1 reverse (5'-gttctaactggagctggggt-3'); GAPDH forward (5'-ccaaggagtaagacccctgg-3'), GAPDH reverse (5'-tggttgagcacagggtactt-3'). PCR products were separated on a 1% agarose gel. Differences were confirmed using the Image J software (NIH, MD, USA) to analyze the changes in the levels of target RNA relative to the control.

2.4. Immunoblotting

Human dermal fibroblasts were washed three times with PBS and lysed with RIPA buffer. Cell lysates were collected by scraping and centrifuged at 15,000 rpm for 15 min. The protein concentrations of the cell extract were determined using a BCA protein assay. 30 μg of proteins from each sample was electrophoresed on a 6% or 10% SDS-PAGE gel and transferred onto nitrocellulose membranes, which were subsequently blocked for 1 h with 5% non-fat milk in TBS containing 0.1% Tween 20 (TBST). Then, the membranes were incubated at 4℃ overnight with specific primary antibodies. After washing and incubation with HRP-conjugated secondary antibodies, the bands were detected using the ECL reagent. Differences in protein expression were confirmed using the Image J software to analyze the changes in the levels of target RNA relative to the control.

2.5. MTT assay

Human dermal fibroblasts were seeded onto 96-well plates: 200 μl of a cell suspension with a density of 2 × 104 cells/ml was seeded in each well. Cells were treated with RSV for 24 h. The medium was aspirated and then the MTT solution (100 μl of a 0.5 mg/ml solution; AMRESCO) was added to each well and incubated for 37 °C for 4 h. Subsequently, the MTT solution was gently removed and replaced with 100 μl of DMSO and incubated for 10 min while shaking to dissolve the

precipitate. The absorbance of the samples was measured at 570 nm in a spectrophotometer.

2.6. Overexpression and knockdown of SIRT1

pcDNA3.1-Myc-SIRT1 and pcDNA3.1-Myc-Control were provided by Dr. Han-Geuk Seo (Department of Animal Biotechnology, Konkuk University, Seoul, Korea). The cDNA encoding human SIRT1 (GenBank accession no. AF083106) was cloned into the pcDNA3.1/Myc vector. The cells were seeded in 6-well dishes. After growing to ~70% confluence, cells were transfected with 2.5 μg of pcDNA3.1-Myc-SIRT1or pcDNA3.1-Myc-Control vector using Lipofectamine 2000 (Invitrogen, USA). After 24 h, the level of SIRT1 and Cyr61 expression were determined by western blotting. To silence SIRT1 expression, human dermal fibroblasts were transfected with 20 nM of control or SIRT1-specific small interfering RNAs (Bioneer, Daejeon, Korea) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For the determination of protein expression, lysates were collected 24 h after transfection.

2.7. Wound healing and cell invasion assay

Human dermal fibroblasts were seeded at a density of 100,000 cells/well in 6-well plates. After 24 h of culture, the cells were 70-80% confluent. A straight line was scraped in the cell monolayer to create a ‘‘scratch’’ using a 200-μl pipet tip. Wounded monolayers were washed with PBS to remove detached cells. Marks were made with a pen on the outside of dish bottom as reference points. The wound width was recorded during the 48 h period following scratching. The images acquired for each sample were analyzed quantitatively using the Image J software. The data show the percentage of wound closure in an image, at a time point. Percent Closure (%) = (Initial scratch wound-Scratch wound at indicated time) / Initial scratch wound x 100.

2.8. Statistical analysis

All the data were analyzed using the SPSS v. 16.0 software (SPSS Inc., Chicago, Il, USA). Statistical significance between experimental groups was determined using a Student's t-test or Mann–Whitney U-test in the SPSS software. Differences were considered to be significant when $P < 0.05$.

3. Results

3.1. TGF-β-induced cell migration and SIRT1 expression

TGF-β time-dependently increased migration in human dermal fibroblast (Fig. 7a). Next, we investigated the expression level of SIRT1 protein; for this purpose, human dermal fibroblasts from foreskin were treated with TGF-β (20 ng/ml). SIRT1 was expressed at low level in resting human dermal fibroblasts. However, activated human dermal fibroblasts, stimulated with TGF-β for 2-48 hours, showed increased SIRT1 expression. After treatment with TGF-β for 48 h, SIRT1 was maximally expressed: its expression level was 6.5-fold higher than resting human dermal fibroblasts (Fig. 7b-d).

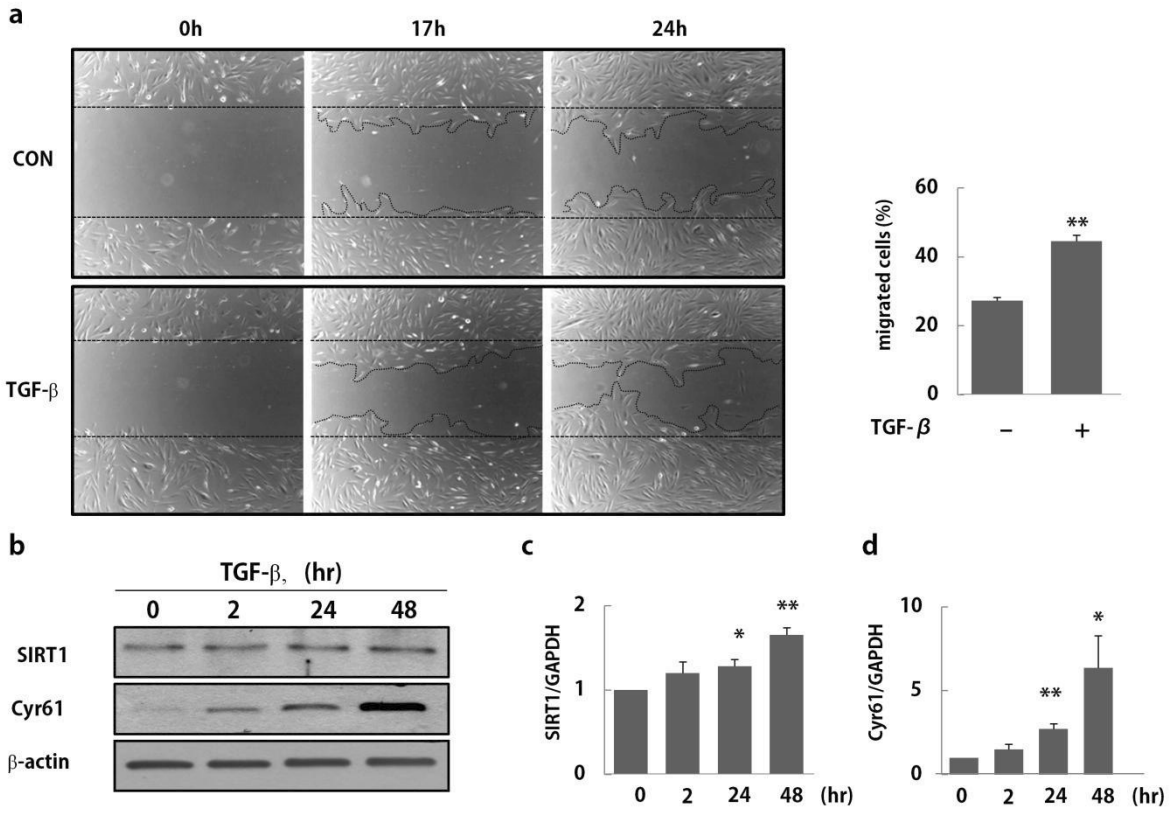

Figure 7. Effect of TGF-β induced cell migration and cell protein expression in human dermal fibroblast. a Left, migration of human dermal fibroblasts was measured by scratch wound healing assay. Cells were incubated with TGF-β (20 ng/ml) for 17 hours and 24 additional hours after a scratch was created using pipette tip. Right, representative graphs are shown from 6 independent experiments. The distance of migration, in TGF-β-treated or control cells, was measured relative to the control for 24 hours, as described in Materials and Methods. Values are represented as the mean +/- SEM. ** = $p < 0.01$. **b** Human dermal fibroblasts were treated with TGF-β (20 ng/ml) for the indicated times and were collected for western blot analysis. (c-d) Representative graphs are shown from 3 independent experiments. Expression level of SIRT1 and Cyr61 protein is presented as the mean +/- SEM in a time-dependent manner. * = $p < 0.05$, **=$p<0.01$.

3.2. Increased SIRT1 stimulates Cyr61 expression

In Figure 7, Cyr61 showed a low basal expression level, and stimulation with TGF-β increased Cyr61 expression time-dependently. Because TGF-β-induced Sirt1 expression was concomitant with the increase in Cyr61 expression, we investigated the modulatory effect of SIRT1 on Cyr61 expression. Confluent human dermal fibroblasts were incubated with RSV, a known activator of SIRT1. First, RSV did not affect proliferation on human dermal fibroblasts for 24 hours (Fig. 8a). Treatment of human dermal fibroblasts with RSV caused a modest induction of SIRT1 mRNA (Fig. 8b). On protein level, however, stimulation with RSV caused a significant dose-dependent increase in SIRT1 and Cyr61 expression (Fig. 8c-e). Treatment with 60 μM RSV resulted in a 3.7-fold increase of SIRT1. We next performed SIRT1-overexpression experiments to confirm the relationship between Sirt1 and Cyr61. As expected, SIRT1 overexpression in cells was associated with a 2.25-fold increase of Cyr61 expression (Fig. 8f-h). These results suggest that the expression of SIRT1 in human dermal fibroblasts leads to the induction of Cyr61 expression.

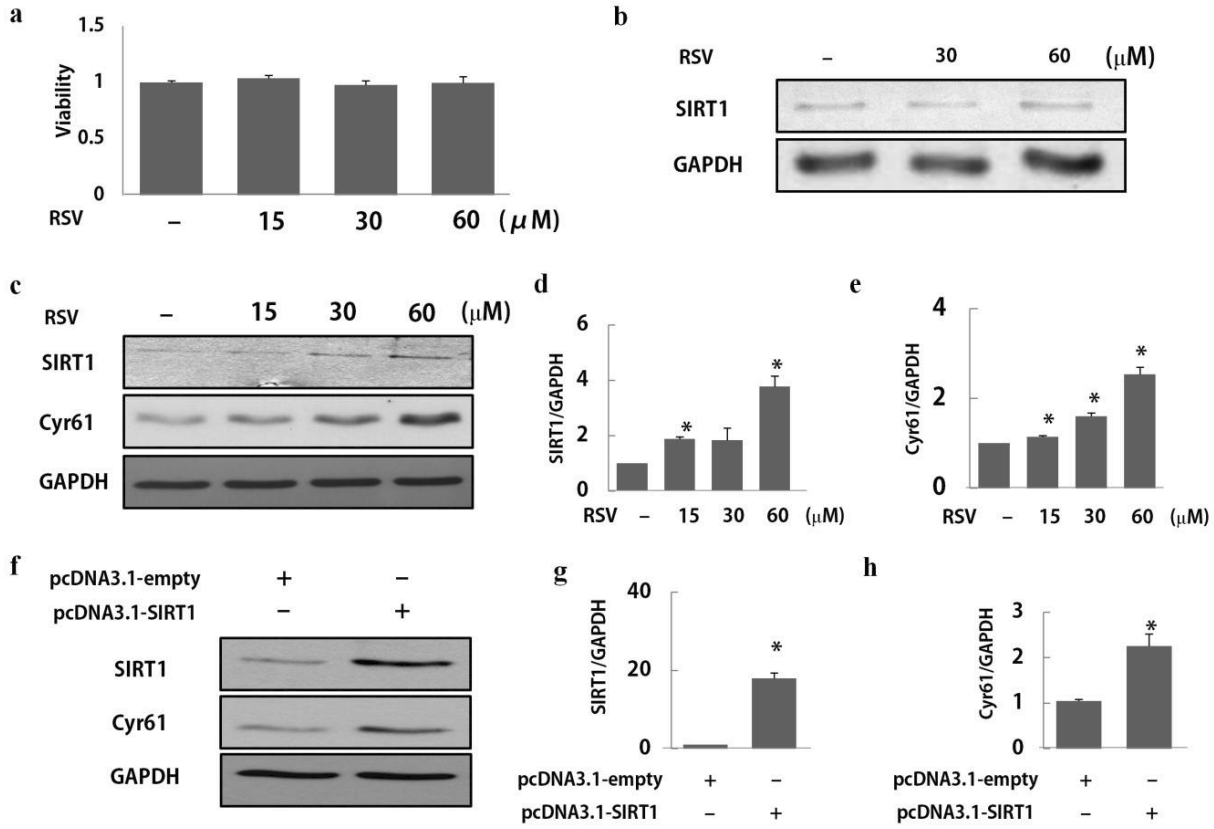

Figure 8. Effect of SIRT1 on Cyr61 expression in human dermal fibroblast. a MTT assay to study the cytotoxicity associated with various concentrations of resveratrol (RSV) for 24 hours. **b** RSV was applied at the concentration of 0, 30, or 60 μM for 24 hours and SIRT1 gene expression was measured by RT-PCR. **c-e** RSV was Cells were treated with RSV (0, 15, 30, or 60 μM) for 24 hours and the protein expression of SIRT1 and Cyr61 were analyzed by western blot. **f-h** Human dermal fibroblasts were transfected with 2.5 μg of pcDNA3.1-control or SIRT1-vector, using Lipofectamine, for 24 hours. Cells were harvested and the level of SIRT1 and Cyr61 expression was measured by western blot. Values are represented as the mean +/- SEM. * = $p < 0.05$.

3.3. Inhibition of SIRT1 activity reduces Cyr61 expression

The previous figures demonstrated that SIRT1 enhanced Cyr61 expression. Thus, we analyzed whether downregulation of SIRT1 expression may affect Cyr61 expression. Despite low levels of expression, both SIRT1 and Cyr61 were expressed in resting human dermal fibroblasts. Silencing of SIRT1 decreased Cyr61 basal expression (Fig. 9a). To further confirm these results, we tested the effect of nicotinamide, a noncompetitive inhibitor of SIRT1, on Cyr61 expression. Cells were pretreated with nicotinamide (50 mM) for 30 min prior to stimulation with TGF-β (20 ng/ml) for 24 hours (Fig. 9b). The results showed that nicotinamide reduced TGF-β-induced (2.06-fold) (Fig. 9c) as well as RSV-induced SIRT1 and Cyr61 expression (1.92-fold) (Fig. 9d).

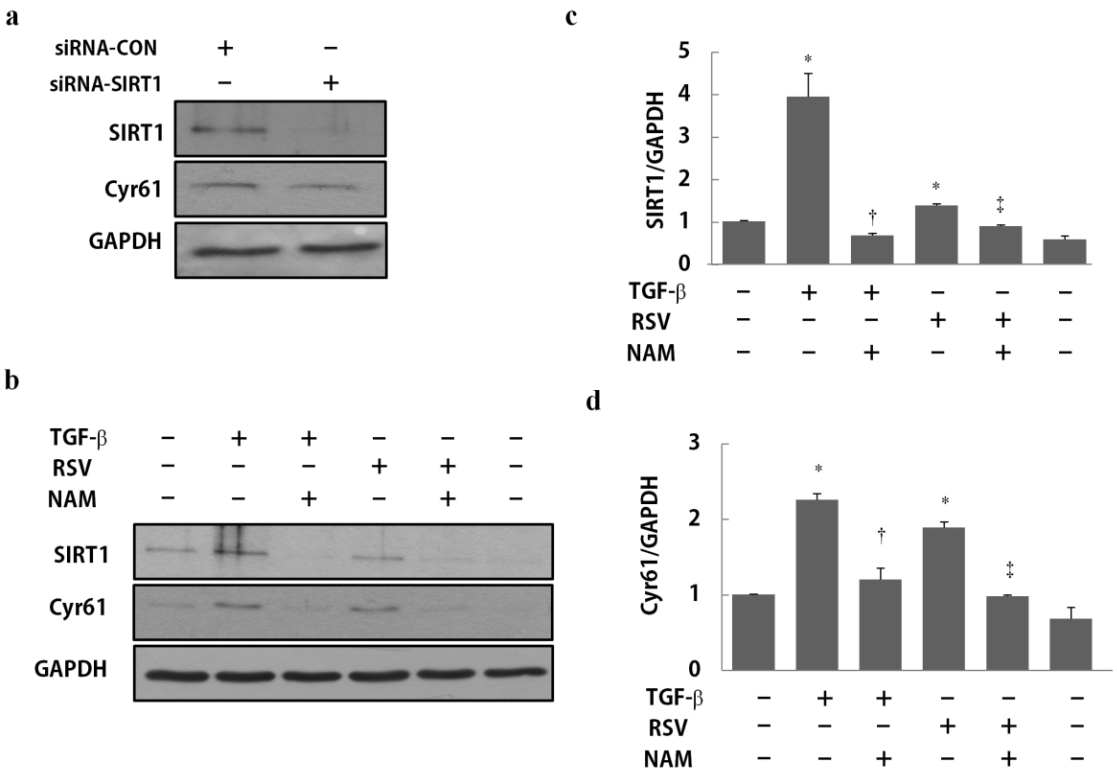

Figure 9. Blocking of SIRT1 reduces Cyr61 expression in human dermal fibroblast. a Cells were cultured at 70% confluency in culture dish and transfected with siRNA-control or SIRT1 (20 nM), using Lipofectamine, for 24 hours. **b-d** Human dermal fibroblasts were treated with nicotinamide (50 mM) for 1 h before stimulation with TGF-β (20 ng/ml) or RSV (60 μM) for 24 hours and expression levels of SIRT1 and Cyr61 were measured by western blot. The intensity of expression was quantified using the Image J software. Representative pictures are shown from 3 independent experiments. Values are represented as the mean +/- SEM. * = $p < 0.05$ vs. control group, †= $p < 0.05$ vs. TGF-β group, ‡= $p < 0.05$ vs. RSV group.

3.4. SIRT1-dependent ERK phosphorylation regulates Cyr61 expression

The previous data indicated that SIRT1 was induced by TGF-β stimulation (Fig. 7b). Thus, we hypothesized that SIRT1 activates Cyr61 expression through the canonical TGF-β/SMAD signaling pathway. We evaluated changes in TGF-β dependent signaling. TGF-β stimulation for 2 h enhanced the phosphorylation of smad and ERK (Fig. 10a). RSV did not affect smad2/3 signaling, but influenced the time-dependent activation of ERK, instead (Fig. 10b, c). We evaluated whether the inhibition of ERK affected the TGF-β-induced Cyr61 expression. Cells were pretreated with PD98059 (10 μM) for 30 min prior to stimulation with TGF-β (20 ng/ml). In this experiment, blocking ERK phosphorylation reduced the expression of Cyr61 induced by TGF-β or RSV (Fig. 10 e, f). Taken together, these data indicate that expression of SIRT1, increased by TGF-β, modulates Cyr61 expression through EKR signaling.

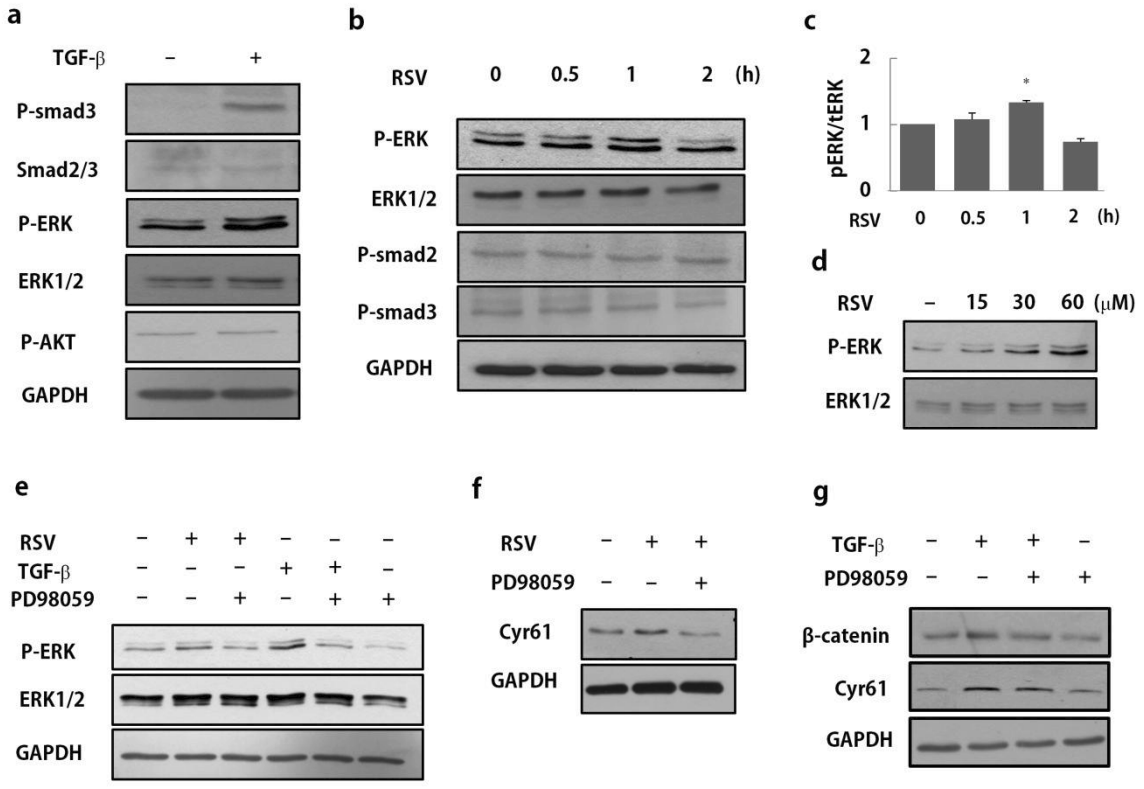

Figure 10. Effect of ERK phosphorylation on Cyr61 expression in human dermal fibroblast. a Human dermal fibroblasts were stimulated with TGF-β (20 ng/ml) for 2 hours. Western blot analysis was performed for smad, ERK, and AKT. **b** Human dermal fibroblasts were treated with RSV (60 μM) for the indicated time. Western blot analysis was performed for smad, and ERK. **c** Representative graphs are shown from 3 independent experiments. Band intensity of phosphorylated ERK was quantified using the Image J software. Values are represented as the mean +/- SEM. * = $p < 0.05$. **d** Human dermal fibroblasts were treated with RSV at the indicated concentration for 1 hour. **f-g** Cells were treated with PD98059 (10 μM) for 1 h before treatment with RSV (60 μM) or TGF-β (20 ng/ml) for 24 hours. Cyr61 expression was analyzed western blot.

3.5. SIRT1-dependent β-catenin modulation regulate Cyr61 expression

ERK inhibition by PD98059, decreased both β-catenin and Cyr61 expression on TGF-β-stimulated human dermal fibroblasts (Fig. 10g). We evaluated whether β-catenin affects SIRT1-mediated Cyr61 expression. The overexpression of SIRT1 induced β-catenin and Cyr61 expression and this stimulation was blocked by the Wnt inhibitor XAV939 (Fig. 11a,b). TGF-β- or RSV-induced Cyr61 expression was also inhibited by XAV939 (Figure 11c).

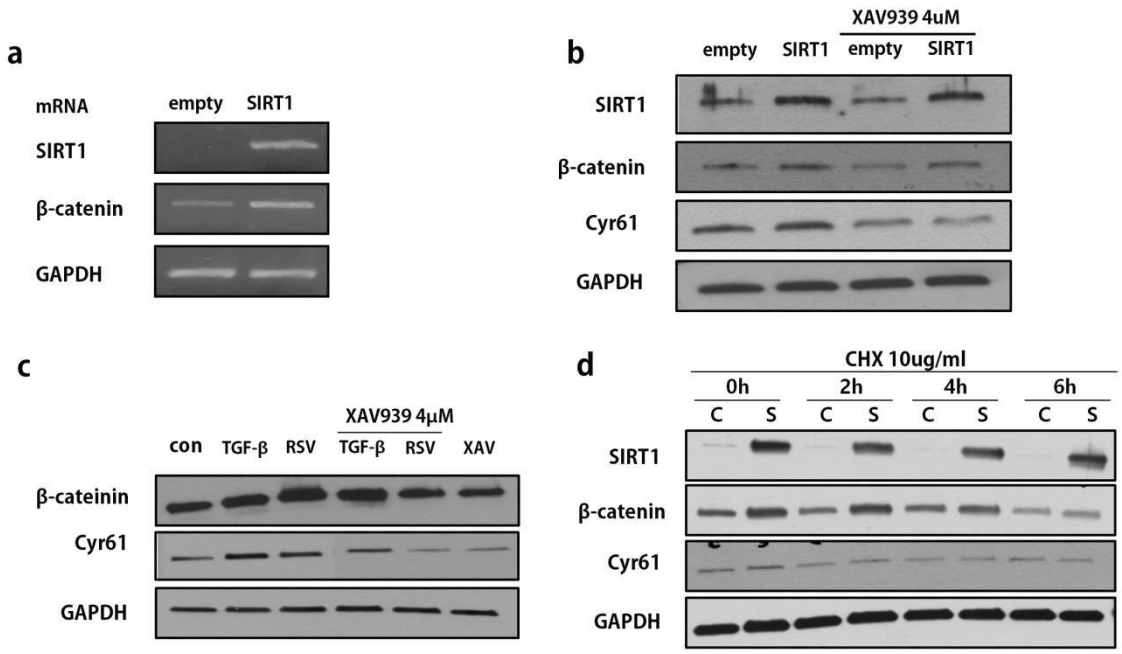

Figure 11. Effect of β-catenin modulation on Cyr61 expression in human dermal fibroblast. a Human dermal fibroblasts were transfected with 2.5 μg of pcDNA3.1-control or SIRT1 vector using Lipofectamine for 24 h. Gene expression of SIRT1 and β-catenin was measured by RT-PCR. **b** XAV939 (4μM) was treated after overexpression SIRT1. **c** XAV939 (4 μM) was treated for 1 h before RSV (60 μM) or TGF-β (20 ng/ml) for 24 hours. Level of SIRT1, β-catenin and Cyr61 were analyzed western blot.

3.6. SIRT1 activation stimulates human dermal fibroblasts migration

To further investigate the basis for the effect of SIRT1 expression on wound healing, we performed a scratch wound healing assay. RSV increased the migration of human dermal fibroblasts by 2.89-fold (Fig. 12a, b). In contrast, nicotinamide, a SIRT1 antagonist, attenuated the RSV-induced migration (2.17-fold). As expected, treatment with a Cyr61 neutralizing antibody directly activated the migration of human dermal fibroblasts (2.19-fold). To analyze the SIRT1-independent action of RSV, we verified if the overexpression of SIRT1 influences fibroblasts migration. As shown in Figure 12c , cells transfected with SIRT1 migrated more than control cells (1.81-fold), and this effect was blocked by treatment with a Cyr61 neutralizing antibody (1.29-fold). Therefore, RSV increased migration of human dermal fibroblasts via SIRT1-induced Cyr61 expression. Our data reveal a model in which SIRT1, increased by TGF-β, upregulates Cyr61, leading in turn to increased migration of human dermal fibroblast.

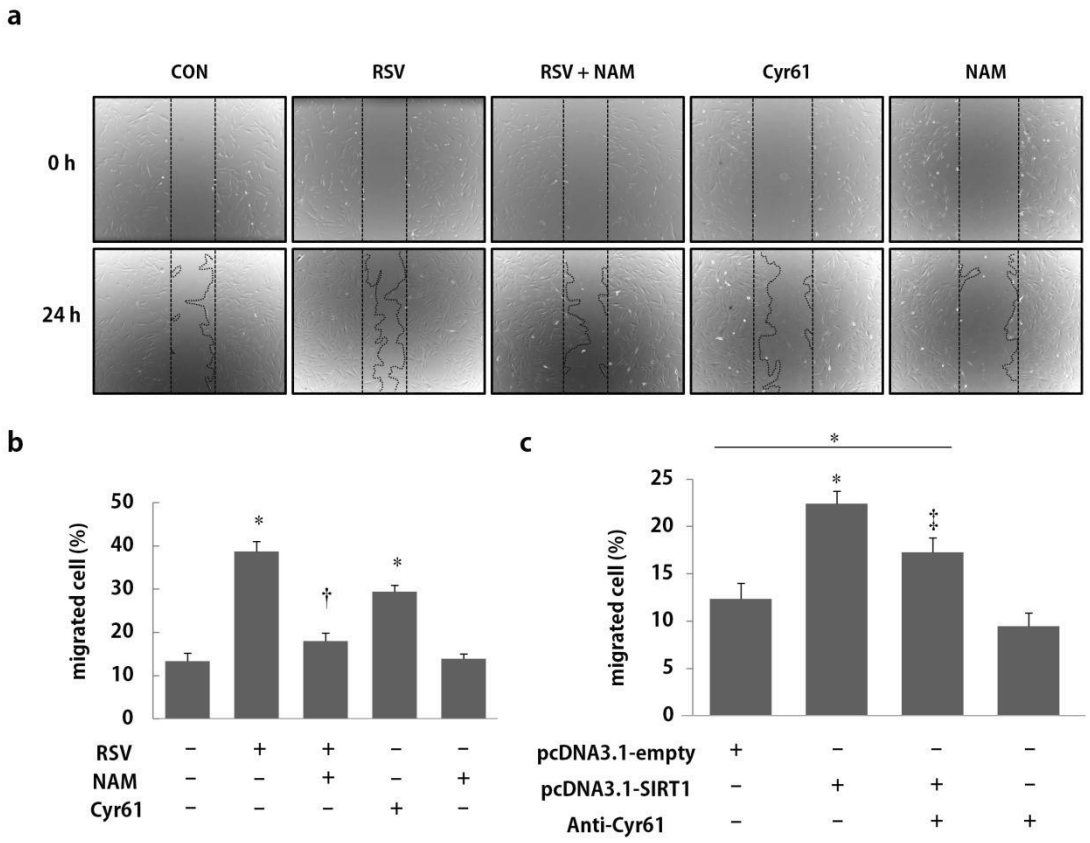

Figure 12. Effect of SIRT1- induced cell migration in human dermal fibroblast. a-b Human dermal fibroblasts were cultured till ~70% confluency. The cell monolayer was scratched with pipette tips and treated with RSV (60 μM), nicotinamide (50 mM), or Cyr61 peptide (100 ng/ml) for the indicated time. Representative graphs are shown from 6 independent experiments. The distance of migration of the cells was measured relative to the control, for 24 h as described in Materials and Methods. **c**) Human dermal fibroblasts were transfected with 2.5 μg of pcDNA3.1-control or SIRT1-vector, using Lipofectamine, for 24 hours. After the cell monolayer was scratched with pipette tips, cells were treated with a neutralizing antibody for Cyr61 for 24 hours. Representative graphs are shown from 6 independent experiments. The distance of migration of pcDNA3.1-control or SIRT1-vector transfected cells, with or without a blocking antibody for Cyr61, was measured relative to the control for 24 h as described in Materials and Methods. Values are represented as the mean +/- SEM. * = $p < 0.05$ vs. control group, †= $p < 0.05$ vs. resveratrol group, ‡= $p < 0.05$ vs. SIRT1-transfected group.

4. Discussion

Fibroblasts have an essential role in wound healing in response to tissue injury. However, continuous wound healing and migration lead to fibrosis. Therefore, it is important to understand the molecular mechanism of fibroblast activation and migration. In this study, we described the function of SIRT1 in dermal fibroblasts migration. We demonstrated that SIRT1 induces cell migration through the increased expression of Cyr61. We showed that the activation of SIRT1 with the natural polyphenol RSV caused increased migration and Cyr61 expression. Consistent with the pharmacologic study, overexpression of SIRT1 also caused the same effects in human dermal fibroblasts. On the other hand, we observed that knockdown of SIRT1 affects Cyr61 expression and nicotinamide, a SIRT1 antagonist, also decreases the expression of Cyr61 induced by TGF-β or RSV as well as the migration of dermal fibroblast induced by RSV. Furthermore, ERK signaling, part of the non-canonical TGF-β signaling pathway, was also involved in SIRT1-induced Cyr61 expression and migration, as indicated by the induction of ERK phosphorylation. This was confirmed by pretreatment of the cells with an ERK inhibitor (PD98059, 10 μM), which attenuated the effect of TGF-β and RSV.

TGF-β is a ubiquitously expressed cytokine. This mediator plays a critical role in processes such as proliferation, wound healing and synthesis of extracellular matrix. Especially, TGF-β is involved in all stages of wound healing. However, excessive stimulation of TGF- β leads to a persistent wound healing response, which results in tissue injuries. Therefore, TGF-β has both positive and negative effects in wound healing. Chronic inflammatory responses induced by TGF-β create an inflammatory environment and cause over-activation of fibroblasts and fibrosis.

Most studies regarding the association of SIRT1 with cell migration have focused on cancer cells. The regulation of SIRT1 on cell migration appears different in cell-dependent manner. SIRT1 activation results in increased cell migration in ovarian and prostate cancer and in fibrosarcoma. On the contrary, other groups show that SIRT1 or its pharmacologic activation by resveratrol down-regulates cell migration in colorectal and breast cancer, and in smooth muscle cells and retinal epithelial cells. Therefore, it remains unclear whether SIRT1 acts as an activator or inhibitor of cell

migration. Especially, the action of the SIRT1 in systemic sclerosis is ambiguous. Wei J. et al. demonstrated that SIRT1 attenuated skin fibrosis in an experimental animal model. However, Zerr P. et al. showed that fibroblast specific knockout of SIRT1 ameliorated experimental fibrosis. These contradictory opinions appear to result from different fibrotic animal model and disease activity. Thus, the function of SIRT1 on fibrosis might depend on the stages of fibroblasts. Our group explored the mechanism for the migratory effects of SIRT1 in human dermal fibroblasts. Induction of SIRT1 expression by RSV or SIRT1 overexpression increased the migration of cells. These results suggested that SIRT1 might participate in skin dermal fibrosis. Future studies are required to explore the action of SIRT1 on skin fibrosis in animal models as well as patients.

To exclude the effect of the growth rate on cell migration, using a MTT assay our group showed that treatment with RSV for 24 hours does not affect proliferation. SIRT1 induces the migration but not the proliferation of human dermal fibroblasts.

A previous study shows that SIRT1 acts as an important regulator of Wnt signaling: loss of function of SIRT1 leads to a decrease in Dishevelled proteins; inhibition of SIRT1 results in changes in gene expression of Wnt target genes; and Wnt-stimulated cell migration is inhibited by a SIRT1 inhibitor. It has been shown that a TCF/LEF consensus- binding element (TBE) exists in the target genes of Wnt/β-catenin. Li Z. et al. identified two TBEs in the human Cyr61 promoter region. There is many research about Wnt/β-catenin signaling in fibrogenesis. Human tissue samples from idiopathic pulmonary fibrosis and systemic sclerosis show activated β-catenin signaling. Accumulation of β-catenin, resulting from activation of Wnt signaling, contributed to the migratory activities in fibroblast and fibrosis. In present study, we show that SIRT1 modulated both β-catenin and Cyr61 protein expression. In further studies, we will continue investigating the transcriptional regulation in the SIRT-1-Wnt/β-catenin-Cyr61 axis.

The MAPK and the PI3K/AKT signaling pathways have been implicated in cell migration. Indeed, TGF-β activates Smads, PI3K/AKT or MAPK to regulate migration. Our data show that TGF-β induces migration and SIRT1 expression; therefore, we investigated the relation between TGF-β and SIRT1. TGF-β activates the smad and ERK pathways. RSV affects only the ERK signaling. Blocking

of TGF-β- or RSV-activated ERK results in decreased expression of Cyr61, suggesting that SIRT1 triggers ERK-mediated Cyr61 expression. Additionally, an ERK inhibitor, PD98059, also attenuated the expression of β-catenin induced by TGFβ. However, further study is required to explore the correlation between β-catenin and ERK signaling in Cyr61 expression.

Various cellular mediators cooperate in tissue repair and fibrogenesis. Especially, TGF-β, endothelin-1 (ET-1), CCN2 and platelet derived growth factor (PDGF) are believed to be responsible for scleroderma. Recently, Chen et al. reported that Cyr61/CCN1, a member of the CCN family, is also implicated in tissue repair and fibrogenesis, and has similar function to CCN2. Fibroblast-specific Cyr61/CCN1-deficient mice are resistant to bleomycin-induced skin fibrosis. Accordingly, Cyr61 is also emerging as an important cellular mediator for scleroderma. In this report, SIRT1 induced Cyr61 expression and migration, induced by SIRT1-overexpression was blocked by neutralization of Cyr61. Therefore, SIRT1 might be a regulator of Cyr61 expression and action. These findings indicate that SIRT1 is a potential target in dermal fibroblast activation and fibrosis.

Our results support the hypothesis that SIRT1 is an important factor for the migration of dermal fibroblasts via TGF-β and RSV. Increased SIRT1 stimulated the expression of Cyr61 and had an effect, through the ERK and the Wnt/β-catenin signaling, on cell migration. Thus, SIRT1-induced Cyr61 production is very important for human dermal fibroblasts migration. Further studies address the transcriptional regulation of the Cyr61 gene by SIRT1.

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국문 초록

류마티스 질환은 관절, 결합조직 그리고 인대를 침범하는 질환으로 근골격계를 침범하는 질환이다. 대표적인 질환으로 류마티스 관절염은 전신 만성 염증성 질환으로 병인은 잘 알려져 있지는 않으나 관절 내 활막 섬유 모세포의 증식과 연골과 뼈의 파괴를 특징으로 하고 있다. 활막섬유모세포는 염증성 사이토카인에 대한 수동적 응답자로 활동하는 것 뿐 아니라 류마티스 관절염의 진행의 주요 세포로서 작용하기 때문에 활막세포의 자체 활성화 기전을 이해 하는 것이 중요하다.

또 다른 질환으로 전신경화증에서 피부를 침범하는 피부 경화증이 있다. 피부섬유모세포는 세포외기질과 섬유부착단백질을 생산함으로써 창상치유, 염증반응과 신생혈관과정에 관여한다. 하지만 이러한 과정이 과도하게 진행될 경우 섬유증을 일으킨다.

Cyr61은 모세포 단백질로 세포외로 분비되는 물질이다. 이는 만성 염증질환과 섬유모세포의 활성과 관계가 있다는 여러 보고에 따라 결합조직과 관련된 류마티스 질환에서 어떠한 관계가 있는지 알아 보고자 하였다. 따라서 본 연구는 part1에서 류마티스 관절염에서 활막섬유모세포의 활성과 Cyr61의 역할을 알아보고 Cyr61의 발현을 낮추는 물질을 찾아 보고자 하였고, part2에서 피부섬유모세포의 활성과 Cyr61의 관계를 알아보고자 하였다.

활막 섬유모세포에서 TNF-α의 자극은 Cyr61의 발현을 증가 시켰으며 이로 인해 활막

섬유모세포의 이동성과 침투성이 증가되었다. Peroxisome proliferator-activated receptor gamma (PPARγ)에 대한 효능제인 rosiglitazone의 처리는 TNF-α에 의해 증가한 Cyr61을 감소시켜 세포이동성을 감소시켰다. 따라서 류마티스 관절염의 활막 섬유모세포는 염증성 사이토카인에 의한 Cyr61 증가로 인하여 활성화 되며 PPARγ는 Cyr61의 발현을 감소시킴으로써 위의 과정을 억제 할 수 있다는 것을 확인하였다.

피부섬유모세포에서 TGF-β의 자극은 SIRT1과 Cyr61의 발현을 증가 시켰으며 SIRT1의 발현저해는 TGF-β자극에도 불구하고 Cyr61의 발현이 감소됨을 볼 수 있었다. SIRT1으로 인한 Cyr61의 발현은 ERK과 β-catenin의 신호전달을 통해 증가하는 것을 볼 수 있었고 이를 통해 피부섬유모세포의 이동성이 증가하는 것을 볼 수 있었다.

결론적으로, 본 연구는 활막 섬유모세포와 피부섬유모세포의 활성이 Cyr61의 발현의 증가와 관련이 있다는 것을 밝히면서 류마티스 관절염과 피부경화증의 치료적 목표를 제시한다고 할 수 있다.

감사의 글

제주대학교 의학전문대학원의 첫 MDphD과정의 학생으로서 공부할 수 있었던 것은 저에게 크나큰 영광이었습니다. 그 동안 많은 분들의 도움과 격려로 지금 이 자리까지 올 수 있었습니다. 이 글을 빌어 많은 분들께 감사의 마음을 전합니다.

지난 시간 동안 저에게 많은 가르침을 주시고 아낌없는 사랑으로 지도해주신 김진석 교수님께 진심으로 감사 드리며, 존경한다는 말을 전하고 싶습니다. 그리고 연구의 암흑기에서 헤매고 있을 때 마다 최전선에서 많은 조언을 해주신 조문제 교수님께 감사 드립니다. 또한 뒤에서 응원의 말을 전해주신 박덕배 교수님께도 감사의 말을 전합니다. 아무것도 모르는 어수룩한 저에게 연구동향을 어떠한 방법으로 읽어나가야 할지를 조언해 주시고 많은 도전정신을 심어주신 박은정 교수님께도 감사의 말을 드립니다. 저는 3년의 실험실기간 동안 혼자 적응해야 했기에 외로움도 있었고 좌절의 순간도 많았습니다. 그때마다 격려해준 은진언니, 생화학 실험실 식구들, 김영미 박사님께도 감사 드립니다.

저는 이제 실험실 생활을 잠시 정리하고 임상의학과정을 공부하게 됩니다. 하지만 이런 것들이 별개의 과정으로 수학하는 것이 아니라 질병의 기전 발견을 위해 노력할 것이며, 기초과학과 임상과학이 어떻게 협력할 수 있는지 고민하고 그 연결점의 교두보 역할을 감당하기 위해 준비하는 시간이 될 것입니다.

마지막으로 사랑하는 가족들이 있었기에 제가 이 자리에 있을 수 있음을 고백합니다.