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Paracrine effect of the stromal vascular fraction containing M2 macrophages on human chondrocytes through the Smad2/3 signaling pathway

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Abstract

The adipose-derived stromal vascular fraction (SVF) is composed of a heterogeneous mix of adipose-derived stem cells (ADSCs), macrophages, pericytes, fibroblasts, blood, and other cells. Previous studies have found that the paracrine effects of SVF cells may be therapeutic, but their role in osteoarthritis treatment remains unclear. This study aimed to investigate the therapeutic effect of SVF cells on chondrocytes. Chondrocytes were seeded on culture plates alone (control) or co-cultured with SVF or ADSCs on cell culture inserts. After 48 h of co-culture, chondrocyte collagen II, TIMP-3, and MMP-13 mRNA expression levels were evaluated using RT-PCR, and the TGF- β levels in the supernatant were measured using ELISA. Immunohistochemical staining and flow cytometry were used to evaluate the macrophages in the SVF. These macrophages were characterized according to phenotype using the F4/80, CD86, and CD163 markers. To determine whether the Smad2/3 signaling pathways were involved, the chondrocytes were pre-treated with a Smad2/3 phosphorylation inhibitor and stimulated with the SVF, and then Smad2/3 phosphorylation levels were analyzed using western blot. The mRNA expression levels of various paracrine factors and chondrocyte pellet size were also assessed. Collagen II and TIMP-3 expression was higher in the SVF group than in the ADSC group and controls, while MMP-13 expression was the highest in the ADSC group and the lowest in the controls. TGF- β levels in the SVF group were also elevated. Immunohistochemical staining and flow cytometry revealed that the macrophages in the SVF were of the anti-inflammatory phenotype. Western blotting showed that the SVF increased Smad2/3 phosphorylation, while Smad2/3 inhibitors decreased phosphorylation. Smad2/3 inhibitors also reduced the expression of various other paracrine factors and decreased chondrocyte pellet size. These findings suggested that

the paracrine effect of heterogeneous cells, such as anti-inflammatory macrophages, in the SVF partly supports chondrocyte regeneration through TGF- β -induced Smad2/3 phosphorylation.

Running Title: Paracrine effect of SVF via Smad2/3 pathway

Keywords: Chondrocytes, Smad2 Protein, Smad3 Protein, Macrophage, Stromal Vascular Fraction.

Introduction

Regenerative medicine is a relatively new and constantly advancing field that involves the direct mending of damaged tissues using stem cells. Adipose tissues are a rich and accessible source of autologous stem cells used in regenerative medicine (De Ugarte et al., 2003; Zhao, Kaye, & Abd-Elseyed, 2018). In 2001, mesenchymal stem cells from adipose tissue, which were subsequently named adipose-derived stem cells (ADSCs), were first identified and characterized by Zuk et al. (2001). ADSCs are easily obtained in large quantities from liposuction waste. When stem cells are harvested in this manner, there is minimal donor site morbidity compared to that from the traditional methods used to harvest bone marrow-derived stem cells (BMSCs). In addition to the ease of harvesting, ADSCs are equivalent to BMSCs in terms of their therapeutic effects on nerve injury and diabetic ulcer in animal models as well as cell differentiation in adipogenic, osteogenic, chondrogenic, and myogenic lineages (Zuk et al., 2001; Feng et al., 2010; Li et al., 2018; Pomatto et al., 2021). Therefore, regenerative medicine using ADSCs is becoming an increasingly common adjuvant therapy that has shown promising results; for example, a previous study reported that integrating tissue-engineering techniques with ADSCs could facilitate spinal interbody fusion (Helder et al., 2007). However, the preparation of ADSCs involves cell isolation from the stromal vascular fraction (SVF) and culture of the isolated cells, a process that takes a few weeks to complete before the cells can be used for therapeutic applications (Bora & Majumdar, 2017). The SVF is composed of heterogeneous cells, including ADSCs, macrophages, pericytes, fibroblasts, blood cells, and vessel-forming cells such as endothelial, smooth muscle cells, and their progenitors, which are obtained via mechanical or enzymatic digestion of adipose tissue without culture or multiple

passages (McIntosh et al., 2006; Han et al., 2010). The SVF can be prepared within 3–4 h after tissue collection, and the harvesting process only involves cell separation and seeding of scaffold cells. Since the method of SVF collection is simpler compared to that of other stem cell sources, its use allows for a one-step surgical procedure for regenerative therapy (Fraser et al., 2004).

Osteoarthritis (OA) is one of the key targets for SVF use. The therapeutic properties of the SVF are currently being investigated in multiple clinical trials, and SVF application to knee OA has become widespread with promising results (Hong et al., 2019; Biazzo et al., 2020). However, there is insufficient data on the mechanism underlying the effects of the SVF on chondrocytes. Recent studies have reported that the paracrine effects of both the SVF and ADSCs on chondrocytes are key contributors to their suppressive effects on OA development (Shi et al., 2017; Kuroda et al., 2019). The ADSC proportion in the SVF varies substantially, and values ranging from less than 1% to over 16% have been reported (Aronowitz, Lockhart, & Hakakian, 2015). Thus, the therapeutic effect of the SVF appears to be due to the function of various cells, and the mechanism underlying the paracrine effect of these cells in the SVF on chondrocytes needs to be investigated (van Dijk et al., 2011; Semon et al., 2013).

Macrophages can polarize to a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype. The latter is associated with wound-healing through the production of pro-chondrogenic cytokines, such as transforming growth factor (TGF)- β and interleukin (IL)-10 (Fernandes et al., 2020). Recent studies have revealed that TGF- β plays an important role in both cartilage homeostasis and repair by stimulating the expression of tissue inhibitors of metalloproteinases (TIMPs), which are natural inhibitors of matrix metalloproteinases (MMP) (Qureshi, Ricci, & Zafarullah, 2008; Qureshi et al., 2005).

IL-10 exerts chondroprotective effects in cartilage by downregulating the expression of inflammatory cytokines and chondrocyte apoptosis (Wojdasiewicz, Poniatowski, & Szukiewicz, 2014; Wang & Lou, 2001). Additionally, TGF- β and IL-10 are secreted by articular chondrocytes, which also revealed the expression of their respective receptor chains (Iannone et al., 2001; Parker et al., 2007). A previous study reported that macrophages constitute approximately 20% of the SVF (Morris et al., 2015), and the majority of macrophages in the SVF were the M2 phenotype (Lumeng et al., 2008; Potente, Gerhardt, & Carmeliet, 2011). Therefore, M2 macrophages are expected to play important roles in the therapeutic effect of the SVF on chondrocytes. However, there remains insufficient data on the paracrine effects of the SVF on chondrocytes, the effect of macrophages present in the SVF on chondrocytes, and the mechanisms underlying these phenomena.

Therefore, the primary aim of this study was to evaluate the therapeutic effects of heterogeneous cells, including macrophages, present in the human SVF on human chondrocytes. The secondary aim was to investigate the mechanism underlying the paracrine effects of the SVF on chondrocytes.

Materials and Methods

Chondrocyte Extraction and Culture

Human chondrocytes were extracted from healthy-looking cartilage tissue from the femoral condyles and tibial plateaus of 24 patients, including 2 males and 22 females (mean age, 65.2 ± 5.5 years; body mass index, 25.5 ± 3.0 kg/m²), undergoing total knee arthroplasty in our institution. Cartilage tissues were washed with Dulbecco's modified Eagle's medium (DMEM), and the cartilage surfaces were gently scraped to remove

blood, synoviocytes, and the synovial fluid. Cartilage tissues were stripped, diced, and digested in DMEM with 0.2% collagenase D for 2 h and 30 min, and the undigested cartilage was removed using a 70 mm nylon filter. The filtrate was centrifuged at 1200 rpm for 5 min at 25 °C, and the supernatant was removed. The chondrocyte pellet was resuspended in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO, USA), 100 mg/mL streptomycin solution (Sigma-Aldrich), and 0.1 mM dexamethasone (Sigma-Aldrich). Chondrocytes were cultured and passaged when confluence was reached. The second passage of chondrocytes was used for the present study.

Preparation of the SVF and ADSCs

The SVF was extracted from 24 patients, including 6 males and 18 females (mean age, 64.7 ± 4.9 years; body mass index, 25.8 ± 3.2 kg/m²), undergoing intraarticular SVF injection into the knee joint for treatment of osteoarthritis. The Celution® 800/CRS system (Cytori Therapeutics Inc., San Diego, CA, USA) was used to extract the SVF from the abdominal or breech subcutaneous fat of the patients. All subjects underwent a liposuction procedure under general anesthesia to obtain 100–360 mL of adipose tissue. The extracted tissue was then processed using the Celution® 800/CRS System according to the method used by Tsubosaka et al. (2020). Briefly, the subcutaneous adipose tissue was removed, minced, and incubated with Celase® GMP, which is a mixture of highly purified collagenase, to digest the aspirated adipose tissue. After digestion, the SVF was concentrated through centrifugation at 1500 rpm for 5 min and washed to remove the Celase® reagent. Thereafter, “fresh” SVF was prepared by extracting the SVF from the system and suspending it in 100 µL of phosphate-buffered

saline (PBS). Additionally, adherent stem cells in the SVF were cultured in DMEM containing 10% FBS and passaged when confluence was reached. The fourth passage was used as the ADSCs in this study.

Colony-Forming Unit (CFU-F) Assay

The SVF was seeded at a density of 2×10^3 cells/cm² onto tissue culture plates containing DMEM with 10% FBS, 100 IU penicillin, 100 mg/mL streptomycin, and 5 ng/mL fibroblast growth factor-2 (FGF-2; R&D Systems, Minneapolis, MN, USA). The medium was changed to remove the non-adherent cells every 2–3 days for 10–14 days (n = 5). Adhered cells were obtained from the culture designated as ADSCs. After colony formation, the medium was removed, and the plates were stained with 0.5% crystal violet (Kanto Chemical Co. Inc., Tokyo, Japan) in methanol for 10 min. The colonies consisting of at least 40 cells were counted, and the number of colonies was normalized to the number of plated cells (Güven et al., 2012).

Monolayer Co-Culture Assay

To confirm the paracrine effect of the SVF and ADSCs on the chondrocytes, we used a co-culture system. Chondrocytes (1×10^5 cells) were seeded in 12-well plates (BD Falcon® Cell Culture Insert Companion Plates, BD Biosciences, Franklin Lakes, NJ, USA), and co-cultured with the SVF (1×10^5 cells) or ADSCs (1×10^4 cells) in cell culture inserts (0.4 mm pores; BD Falcon®, BD Biosciences) in DMEM with 10% FBS. In the preliminary CFU-F assay, the ADSC ratio in the SVF was found to be about 10%, which defined the doses of 1×10^5 SVF and 1×10^4 ADSCs. The control group

was established with only chondrocytes in 12-well plates (Figure 1-A). The cells were incubated at 37 °C in 5% CO₂ for 48 h.

Water-Soluble Tetrazolium (WST)-8 Assay

Control, ADSC, and SVF group viabilities were measured using the WST-8 cell proliferation assay kit (Cayman Chemical, Ann Arbor, MI, USA) (n = 5/group). Following monolayer co-culture, the cell culture inserts were removed, and the WST-8 reagents were added to the 12-well plates. Absorbances at 450 and 655 nm were measured using a microplate reader (Bio-Rad, Hercules, CA, USA) after 2 h of incubation. Absorbances (A₄₅₀–A₆₅₅) are assumed to be proportional to the number of living cells.

Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The mRNA expression levels of chondrocytes in the control group, ADSC group, and SVF group (n = 5/group) were assessed relative to glyceraldehyde 3-phosphate dehydrogenase levels using RT-PCR. Following monolayer co-culture, total RNA was extracted from chondrocytes using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) and reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RT-PCR amplification of the cDNA was performed in duplicate using the Applied Biosystems 7500 real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences were purchased from Thermo Fisher Scientific Inc. PCR was performed with primers: *GAPDH* (forward 5'-CTGGGCTACACTGAGCACC-3', reverse 5'-AAGTGGTCGTGAGGGCAATG-3'), collagen II (forward 5'-

CCCAGAGGTGACAAAGGAGA-3', reverse 5'-CACCTTGGTCTCCAGAAGGA-3'), *TIMP-3* (forward 5'-CTTCCCAGATGATGACAATGAA-3', reverse 5'-ACCCCATATGACAGCATAGACC-3'), and *MMP-13* (forward 5'-TGCTGCATTCTCCTTCAGGA-3', reverse 5'-ATGCATCCAGGGGTCCTGGC-3').

Enzyme-Linked Immunosorbent Assay (ELISA)

After 48 h of monolayer co-culture, the supernatants were collected, and the concentration of released cytokines in the control group, ADSC group, and SVF group (n = 5/group) was determined using a sandwich ELISA kit (TGF- β : DB100B, IL-10: HS100C, IL-1 β : DLB50, and IL-6: D6050, R&D Systems) according to the manufacturer's protocol. The results were normalized to the quantity of DNA in the control group.

Immunohistochemical Staining

Fresh SVF and ADSCs were washed in PBS for 5 min and processed in 0.3% H₂O₂ and methanol for 20 min. The cells were incubated in a blocking buffer containing 5% normal goat serum and 0.3% Triton X-100 in PBS at 37 °C for 1 h.

Immunohistochemical staining was performed by double staining with an anti-F4/80 antibody (1:200; DF7762, Affinity Biosciences, Cincinnati, OH, USA) and an anti-CD86 antibody (1: 200; GTX74650, GeneTex Inc., Irvine, CA, USA). Additionally, double staining with anti-F4/80 and anti-CD163 antibodies (1:200; NB110-40686, Novus Biologicals, Centennial, CO, USA) was performed for the other SVF and ADSCs (n = 5/group). F4/80 was used as a unique macrophage marker (Yue et al., 2017). CD86 was used as an M1 phenotype marker, and CD163 served as an M2

phenotype marker (Simkin et al., 2017). After incubation with the primary antibodies at room temperature for 1 h, the samples were incubated with Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (1:1000; ab150073, Abcam, Tokyo, Japan) or Alexa Fluor® 594-conjugated goat anti-mouse (1:1000; A90-131D4, Bethyl Laboratories, Montgomery, AL, USA) at room temperature for 2 h. The cells were finally counterstained with DAPI (P36931, Thermo Fisher Scientific) and fluorescent signals were captured using a fluorescence microscope. Additionally, among the F4/80-positive cells, the ratio of CD163-positive cells compared with CD86-positive cells was evaluated. The number of cells was examined in five fields by two blinded observers (YT and MF). The average percentages of CD86- and CD163-positive cells relative to F4/80-positive cells were calculated.

Flow Cytometry

The phenotypes of the macrophages in the SVF were determined by detecting cell markers using flow cytometry. For cellular staining, FITC rat anti-human F4/80 antibodies (ab60343, Abcam, Tokyo, Japan), PE mouse anti-human CD86 antibodies (ab77226, Abcam, Tokyo, Japan), and APC mouse anti-human CD163 antibodies (ab134416, Abcam, Tokyo, Japan) were used. The fresh SVF cells were washed with PBS two times and incubated with antibodies in the dark for 20 min at room temperature. Fluorescence intensity was measured using an Attune acoustic-focusing flow cytometer (Thermo Fisher Scientific) (n = 4).

Western Blotting

To evaluate whether the Smad2/3 signaling pathways were involved in the paracrine effect of the SVF on the chondrocytes, chondrocyte Smad2 and Smad3 phosphorylation were examined via western blotting. The chondrocytes were maintained in serum-deficient DMEM for 24 h and subjected to a variety of treatments. The chondrocytes were pre-treated with or without the specific Smad2 inhibitor SB203580 (10 μ M; AG-CR1-0030, Adipogen Life Science, Liestal, Switzerland) and the specific Smad3 inhibitor SIS3 (10 μ M; 15945, Cayman Chemical Company, Ann Arbor, MI, USA) for 1 h, and then stimulated with the SVF using the monolayer co-culture assay for 48 h (n = 6/group). Whole lysates of the chondrocytes were collected to assess the protein content using a protein assay reagent (Bio-Rad) through the Bradford method. These lysates were also diluted to equal concentrations with a hypotonic buffer. Whole samples were separated using sodium dodecyl sulfate (SDS) polyacrylamide gradient gel (Biocraft, Tokyo, Japan) electrophoresis under reducing conditions, and transblotted electrically onto a blotting membrane (Amersham Biosciences Corp., Arlington Heights, IL, USA). The membranes were blocked for 1 h and incubated with primary polyclonal rabbit anti-human Smad2 (1:1000; #5339; Cell Signaling Technology, Danvers, MA, USA), Smad3 (1:1000; #9513; Cell Signaling Technology), phosphorylation of Smad2 (1:1000; #3108; Cell Signaling Technology) and phosphorylation of Smad3 (1:1000; #9520; Cell Signaling Technology) overnight at 4 °C. The anti-rabbit IgG HRP-linked whole antibody (1:10000; GE Healthcare Bio-Sciences, Piscataway, NJ, USA) was used as the secondary antibody. Images of the blot showing the protein expression were obtained using the Chemilumino analyzer LAS-3000 mini. Relative band intensities were quantified using the ImageJ software (<http://imagej.nih.gov/ij/>).

Gene Expression Assessment After Smad2 and Smad3 Inhibition

For the inhibition studies, the chondrocytes were pre-treated in medium with or without 10 μ M of SB203580 and 10 μ M of SIS3 for 1 h and then stimulated with or without the SVF and ADSCs via a monolayer co-culture for 48 h (n = 8/group). After culturing, the chondrocyte collagen II, *TIMP-3*, and *MMP-13* mRNA expression levels were measured using RT-PCR.

Pellet Co-Culture Assay

The chondrocyte pellets (1×10^5 cells) were converted into a chondrogenic medium by adding DMEM with 15% FBS (Sigma–Aldrich), 100 U/mL penicillin (Sigma–Aldrich), 100 mg/mL streptomycin solution (Sigma–Aldrich), 0.1 mM dexamethasone (Sigma–Aldrich), 50 mM ascorbate-2-phosphate (Sigma–Aldrich), 0.4 mM proline (Sigma–Aldrich), 1% insulin-transferrin-sodium selenite (Sigma–Aldrich), and 500 ng/mL bone morphogenetic protein-6 (Sigma–Aldrich). The pellets were collected at the bottom of a 15 mL tube by centrifuging aliquots of chondrocytes cells at 1200 rpm for 5 min. The pellets and SVF (1×10^5 cells) or ADSC (1×10^4 cells) were separated with sterilized culture plate inserts (Millicell; Millipore, Burlington, MA, USA). Additionally, the pellets stimulated with the paracrine effect of SVF were cultured with or without the Smad2 and Smad3 phosphorylation inhibitors to evaluate whether the Smad2/3 signaling pathway was involved in pellet growth (Figure 5-A). The pellets were incubated at 37 °C in 5% CO₂, and the medium was changed every 2–3 days (n = 5/group). After 3 weeks, the pellet size was measured, and stained with toluidine blue.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software (version 16.0, IBM Corp., Armonk, NY, USA). The results are shown as the mean $\pm$ standard deviation (SD). Comparisons among groups were performed using the chi-squared test or one-way analysis of variance followed by a post hoc Tukey's test. A probability (p) value of <0.05 was considered statistically significant.

Ethics and Consent

The protocols of the present study were approved by the local Ethics Committee (Permission No; B190138), and the patients provided informed consent for participation.

Results

Cell Viability

The cell viability of the chondrocytes co-cultured with the SVF and ADSCs was significantly higher compared to that of chondrocytes cultured alone. The viability of the chondrocytes co-cultured with the SVF was significantly higher than that of the chondrocytes co-cultured with ADSCs (Figure 1-B).

Chondrocyte Gene Expression After ADSC and SVF Stimulation

RT-PCR analysis revealed that the collagen II and *TIMP-3* mRNA expression levels were significantly upregulated in the ADSC and SVF groups compared to those in the control group. Additionally, the collagen II and *TIMP-3* expression in the SVF group was significantly greater than that in the ADSC group. The *MMP-13* mRNA expression

levels were significantly downregulated in the ADSC and SVF groups compared with those in the control group. However, significantly decreased *MMP-13* expression was observed in the SVF group compared to that in the ADSC group (Figure 1-C).

Paracrine Factors in the Co-culture Medium

TGF- β and IL-10 levels in the co-culture medium were significantly increased in the SVF and ADSC groups than in the control group. Additionally, TGF- β and IL-10 levels were significantly higher in the SVF group than in the ADSC group. On the contrary, IL-1 β and IL-6 levels in the co-culture medium of SVF and ADSC groups were significantly decreased compared with the control group, and IL-1 β and IL-6 levels in the SVF group were significantly lower than those in the ADSC group (Figure 1-D).

Immunohistochemical Staining of Macrophages in the SVF

Macrophages were labeled with F4/80 in the fresh SVF. Most macrophages in the SVF were double-stained with F4/80 and CD163 rather than double-stained with F4/80 and CD86. No cell was labeled with F4/80, CD86, or CD163 in the ADSCs (Figure 2-A, B). The ratio of CD163-positive cells in the F4/80-positive cells was significantly higher than CD86-positive cells (Figure 2-C).

Fluorescence Staining of Macrophages in the SVF

Flow cytometry was performed to detect the proportion of F4/80-, CD86-, and CD163-positive cells. The proportion of F4/80-positive cells was approximately $20.7\% \pm 8.4\%$, and the proportion of cells double-stained with F4/80 and CD163 was significantly higher than that of cells double-stained with F4/80 and CD86 (Figure 2-D, E).

Chondrocyte Smad2/3 Signaling Pathways Phosphorylation is Stimulated by the Paracrine Effect of the SVF

Our results showed high levels of TGF- β and IL-10 in the SVF group, however, the level of TGF- β was more prominent than IL-10. Thus, we investigated the phosphorylation of the Smad2/3 signaling pathway. Smad2 and Smad3 were phosphorylated by the paracrine effect of the SVF for 48 h. We tested the specificity of the inhibitory effects of SB203580 and SIS3. SB203580 treatment significantly inhibited SVF-induced Smad2 phosphorylation without affecting Smad3 phosphorylation (Figure 3-A, B). SIS3 treatment significantly inhibited SVF-induced Smad3 phosphorylation without affecting Smad2 phosphorylation (Figure 3-A, C).

Suppression of Chondrocyte Gene Expression by Specific Inhibitors of Smad2 and Smad3

The SVF and ADSC-induced collagen II and *TIMP-3* mRNA expression levels were significantly suppressed by SB203580 and SIS3 treatments, while the SVF and ADSC-induced decrease in the *MMP-13* mRNA expression levels were significantly increased by SB203580 and SIS3 treatment (Figure 4).

Pellet Co-Culture

To assess chondrocyte proliferation when cultured in the presence of the SVF and ADSC paracrine factors, we performed pellet co-culture. The pellets from all groups were positively stained with toluidine blue (Figure 5-B). Pellet size analysis revealed that chondrocytes co-cultured with ADSCs and the SVF formed larger pellets than those

formed in the control group, and the pellet size of the SVF group was significantly larger than that of the ADSC group. Additionally, supplementation with the SVF and SB203580 or SIS3 exhibited significantly smaller pellet sizes compared with only that of the SVF group (Figure 5-C).

Discussion

The most important finding of the present study was that the heterogeneous cells, including the M2 macrophages, in the SVF could secrete TGF- β and partly contribute to the therapeutic effect of the SVF on chondrocytes through the Smad2/3 signaling pathway. To the best of our knowledge, this is the first study to attribute therapeutic potential to heterogeneous cells in the SVF for regenerative therapy of chondrocytes. Based on this finding, transplantation of the SVF may be an effective treatment option for the regenerative therapy of damaged cartilage tissues.

Our results showed that the paracrine effect of ADSCs and the SVF increased chondrocyte cell viability and collagen II and *TIMP-3* mRNA expression levels. ADSCs and the SVF also downregulated chondrocyte *MMP-13* mRNA expression levels. The paracrine effect of ADSCs and SVF on cell viability, promotion of anabolic factors, and inhibition of catabolic factors of chondrocytes has been demonstrated previously (Kuroda et al., 2019; Kamenaga et al., 2021). Several authors compared the therapeutic effect of ADSCs with SVFs for nerve regeneration, hypertrophic scar remodeling, treatment of acute myocardial infarction, and cartilage regeneration in animal models, where an almost equal effect was usually observed (van Dijk et al., 2011; You et al., 2015; Domergue et al., 2016; Shimizu et al., 2018). Biazzo et al. reported that both intra-articular injections of ADSCs and the SVF exert clinical and radiological

improvements for knee OA (Biazzo et al., 2020). However, there is no study on the therapeutic effect of the cells that compose the SVF other than ADSCs. In this study, the observed paracrine effect was greater in the SVF (1×10^5 cells) than in ADSCs (1×10^4 cells). We also determined the ADSC ratio in the SVF to be 10% using the CFU-F assay. These results suggest that the therapeutic effect of the heterogeneous cells in the SVF is greater than that of ADSCs alone. ELISA results revealed high levels of TGF- β and IL-10 and low levels of IL-1 β and IL-6 during co-culture of the SVF and ADSCs. Previous studies have suggested that the paracrine factors of adipose stem cells include TGF- β and IL-10 (Nagasaki et al., 2011; Dykstra et al., 2020; Laidding et al., 2020), but there has been no study focusing on heterogeneous cells. Our results showed higher levels of TGF- β and IL-10 in the SVF group than in the ADSC group, indicating that the paracrine factors of the SVF could be secreted from not only ADSCs but also other heterogeneous cells. Wang et al. (2018) reported that the levels of inflammatory cytokines, including IL-1 β and IL-6, in the supernatant were decreased after co-culture with chondrocytes and umbilical cord stem cells compared with chondrocytes only. Consistent with this previous finding, our results demonstrated the anti-inflammatory effects mediated by the paracrine factors of SVF on chondrocytes.

Immunohistochemical staining showed that the majority of macrophages in the SVF were of anti-inflammatory phenotypes, which were identified through double-staining with F4/80 and CD163. However, no cell was stained with F4/80 in the ADSCs as ADSCs are pure stem cells. Previous studies reported that macrophages compose approximately 20% of the SVF (Morris et al., 2015). Approximately 80% of these cells were also positive for F4/80, which is a common cell surface marker of macrophages, and approximately 70% were positive for M2 anti-inflammatory markers (Potente,

Gerhardt, & Carmeliet, 2011; Lumeng et al., 2008), supporting our results. In addition, we performed flow cytometry to detect the proportion of F4/80-, CD86-, and CD163-positive cells. We found that the proportion of CD163-positive macrophages was significantly higher than that of CD86-positive macrophages. These results also suggested that M2 polarization was higher than M1 polarization, similar to the results of immunohistochemical staining. Previous studies suggested that M2 macrophages played an important role in tissue repair, including cartilage regeneration (Tateiwa, Yoshikawa, & Kaito, 2019; Benoit, Desnues, & Mege, 2008). M2 macrophages were reported to secrete TGF- β and IL-10, which induce the expression of collagen II and decrease MMP-13 expression in chondrocytes (Dai et al., 2018). Additionally, M2 macrophages were also reported to suppress T-helper 1 immune responses and enhance the T-helper 2 responses, which support tissue remodeling (Martinez et al., 2008). Previous authors have also reported that macrophages acquire the M2 phenotype and upregulate the expression of anti-inflammatory cytokines, including IL-10 and TGF- β , when co-cultured with MSCs and ADSCs (Shohara et al., 2012; Jiang et al., 2013). These changes potentially provide a protective environment in the joints of patients with OA. Furthermore, previous studies have suggested that the osteoarthritic synovium contains a higher number of macrophages than the pre-OA model; M1 macrophages in the synovium could exacerbate experimental OA, and M2 macrophages could alleviate OA progression in vivo (O'Brien et al., 2017; Zhang et al., 2018). The joint space environment has the capability of influencing the synovial microenvironment, shifting the phenotype of macrophages from M1 to M2 (Castrogiovanni et al., 2019). In clinical cases, intra-articular injections of the SVF or ADSCs may increase the ratio of M2

macrophages in the joint space and support a protective joint environment for patients with OA. Future studies investigating these mechanisms in vivo are required.

Previous studies have reported that TGF- β plays an important role in the control of both cartilage homeostasis and repair (Verdier et al., 2005). It has also been reported that Smad2/3 signaling is a critical mediator of TIMP-3 and collagen II induced by TGF- β (Qureshi, Ricci, & Zafarullah, 2008; Re'em et al., 2012). Therefore, we investigated the mechanism underlying the SVF-induced TGF- β secretion in chondrocytes. The results of western blotting showed phosphorylation of the Smad2/3 pathway in chondrocytes following co-culture with the SVF and ADSCs. Wu et al. reported the paracrine effect of ADSCs, where Smad2/3 phosphorylation was observed in breast cancer cells through stimulation of TGF- β released by the ADSCs (Wu et al., 2019). Our results are in line with that report. Additionally, specific inhibitors of Smad2 and Smad3 phosphorylation, SB203580 and SIS3, inhibited SVF-induced Smad2/3 phosphorylation. RT-PCR showed that collagen II and *TIMP-3* levels, which were upregulated by the SVF and ADSCs, were suppressed by the specific inhibitors, while the *MMP-13* levels were downregulated by the SVF and increased by the ADSCs. These results suggest that the paracrine effects of the SVF and ADSCs promote the phosphorylation of the Smad2/3 pathway to influence the chondrocytes. Several studies reported that SB203580 and SIS3 inhibit the phosphorylation of Smad2/3 stimulated by TGF- β and suppress TIMP-3 and collagen II expression in chondrocytes (Qureshi, Ricci, & Zafarullah, 2008; Zhu et al., 2017). However, there have been no studies investigating the paracrine effect of the SVF on the phosphorylation of the Smad2/3 pathway. In the pellet co-culture, all the pellets were positively stained with toluidine blue, but the supplementation with SB203580 and SIS3 with the SVF caused

significantly smaller pellet sizes compared with adding the SVF only. These results suggest that paracrine factors, including TGF- β , partly contribute to the promotion of anabolism and the inhibition of catabolism in chondrocytes through the Smad2/3 signaling pathway. In contrast, some studies suggest that ERK and MAPK pathways are essential for the induction of TIMP-3 expression by TGF- β and that the complex relationship between Smad2/3, ERK1/2, and MAPK pathways could play a pivotal role in mediating the signals triggered by TGF- β (Qureshi et al., 2005; Leivonen et al., 2013). Wang et al. (2014) demonstrated the correlation between phosphorylation of ERK1/2 and the Smad2/3 signaling pathway and suggested that ERK1/2 could activate the Smad2/3 signaling pathway. The results of RT-PCR and pellet co-culture showed that Smad2/3 inhibitors diminished the effect of SVF stimulation on chondrocytes; however, these inhibitors could not completely suppress the effect of SVF stimulation compared with the control groups. This result suggests the existence of pathways other than the Smad2/3 signaling pathways in our study. Further studies on the relationship between Smad2/3, ERK1/2, and MAPK pathways in the treatment of chondrocytes with SVF are required in the future.

The present study has some limitations. First, the adipose tissue and chondrocytes were not collected from the same donors in this study, although autologous transplantation is more common in clinical practice. Thus, the replication status and confluence of the culture conditions may be variables that can influence the outcomes of this study. In the future, more global and unbiased comparisons should be performed between the adipose tissues and chondrocyte populations. Second, to clarify the exact mechanisms underlying the role of the SVF and ADSCs in chondrocytes, the present study compared the SVF (1×10^5 cells) with ADSC (1×10^4 cells), which were used at

different cell concentrations. The outcomes of this study could not compare the effects of the SVF and ADSCs in a clinical setting, thus further research is required to compare the therapeutic properties of the SVF and ADSCs in clinical applications of regenerative therapy of chondrocytes using the same dose. Third, the present study only evaluated the paracrine effect between adipose stem cells and chondrocytes. Intra-articular injections of the SVF and ADSCs influence not only chondrocytes but also synovial tissue metabolism, thus a more complex model is required to evaluate more exact therapeutic effects. Fourth, the chondrocytes were not cultured in the pro-inflammatory environment in this study; hence, IL-1 β pre-treatment is desirable in an additional study to simulate the environment of the osteoarthritis joint. Finally, the assessments presented in this study were mainly semi-quantitative analyses. More objective and precise quantitative methods are needed for more accurate evaluations.

Conclusion

The present study confirms that the SVF and ADSCs have therapeutic effects on chondrocytes by promoting anabolic factors and inhibiting catabolic factors. The heterogeneous cells, including M2 macrophages, in the SVF, can contribute to the therapeutic effects on chondrocytes by promoting the secretion of growth factors and chondrocyte-protecting cytokines. TGF- β secreted from the cells of the SVF, including ADSCs and M2 macrophages, may partly contribute to the regeneration of chondrocytes via the Smad2/3 signaling pathway.

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Conflict of Interest Statement

There are no conflicts of interest to declare.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Figure legends

Figure 1. (A) Monolayer co-culture system. The ADSC and SVF groups were established using ADSCs and the SVF in cell culture inserts and chondrocytes in 12-well plates. The control group was established using only chondrocytes in 12-well plates. (B) The viability of the chondrocytes was assessed using the cell counting kit-8 (n = 5/group). (C) Expression of collagen II, TIMP-3, and MMP-13 in the total RNA extracts of the chondrocytes co-cultured with ADSCs, the SVF, and the chondrocyte monoculture (n = 5/group). (D) TGF- β , IL-10, IL-1 β and IL-6 levels in the supernatant following co-culture with ADSCs and SVF and the chondrocyte monoculture (n = 5/group). ADSCs, adipose-derived stem cells; SVF, stromal vascular fraction; TIMP-3, tissue inhibitor of metalloproteinases; MMP-13, matrix metalloproteinase-13; TGF- β , transforming growth factor- β ; IL-10, interleukin-10; IL-1 β , interleukin-1 β ; IL-6, interleukin-6.

Figure 2. (A) Representative double immunostaining for human-F4/80 (green) and human-CD86 (red) in the ADSC and SVF groups. Scale bar: 20 μ m for the panels on the right and 50 μ m for the other panels. (B) Representative double immunostaining for human-F4/80 (green) and human-CD163 (red) in two groups. Scale bar: 20 μ m for the panels on the right and 50 μ m for the other panels. (C) Comparison of the ratio of CD86-positive cells and CD163-positive cells in the F4/80-positive cells via immunohistochemical staining (n = 5). (D) The proportion of F4/80-positive cells in SVF and M1/M2 phenotype balance in F4/80-positive cells determined via flow cytometry (n = 4). (E) Comparison between the proportion of CD86-positive cells and

CD163-positive cells in F4/80-positive cells via flow cytometry (n = 4). ADSCs, adipose-derived stem cells; SVF, stromal vascular fraction.

Figure 3. (A) P-Smad2, Smad2, P-Smad3, Smad3, and β -actin levels were assessed via western blotting. The blots were stripped and re-probed with regular antibodies to detect the total amount of the respective proteins. (B) P-Smad2 expression induced by the paracrine effect of the SVF and blocking by the inhibition of the Smad2 signaling pathway (n = 6/group). (C) P-Smad3 expression induced by the paracrine effect of the SVF and blocking by the inhibition of the Smad3 signaling pathway (n = 6/group). ADSCs, adipose-derived stem cells; SVF, stromal vascular fraction; P-Smad2, phosphorylation of Smad2; P-Smad3, phosphorylation of Smad3.

Figure 4. Expression of collagen II, TIMP-3, and MMP-13 in the total RNA extracts from chondrocytes induced by the paracrine effect of ADSCs and the SVF, with or without SB203580 and SIS3 treatment (n = 8/group). ADSCs, adipose-derived stem cells; SVF, stromal vascular fraction; TIMP-3, tissue inhibitor of metalloproteinase-3; MMP-13, matrix metalloproteinase-13.

Figure 5. (A) Separated pellet co-culture system. The ADSC and SVF groups were established using ADSCs and the SVF in the membrane plate and chondrocytes in a 15-mL tube. In SB203580 and SIS3 group, the chondrocytes were treated with the SVF following pre-treatment with specific inhibitors (n = 5/group). The control group was established using no cells in the membrane plate and only chondrocytes in a 15-mL tube. (B) Chondrocytes pellets and toluidine blue staining of the pellets in five groups.

The bottom panels indicate the magnification of the middle panels. Scale bar: 200 μm for middle panels and 50 μm for lower panels. (C) Comparison of pellet size between five groups. ADSCs, adipose-derived stem cells; SVF, stromal vascular fraction.

Figure 1.

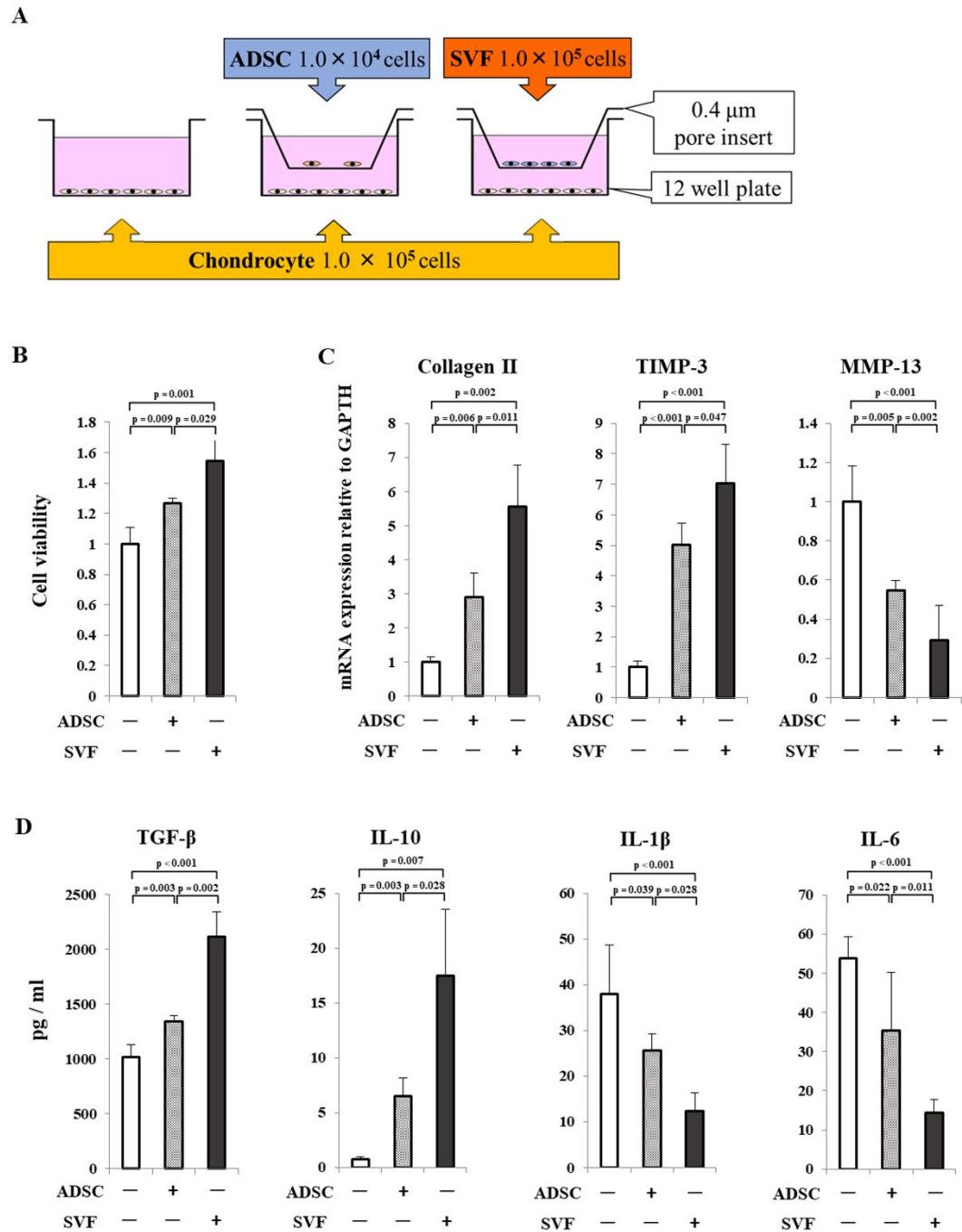


Figure 2.

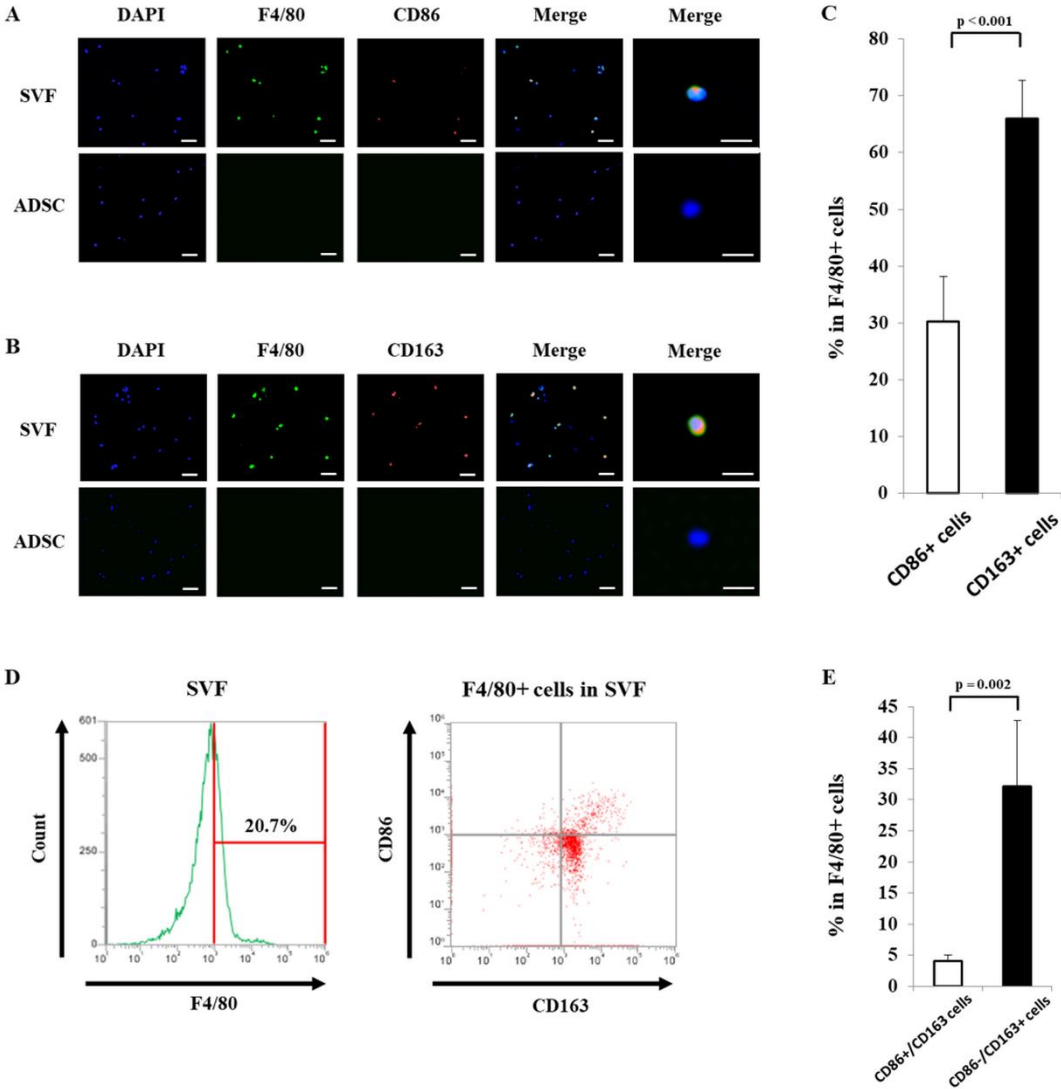


Figure 3.

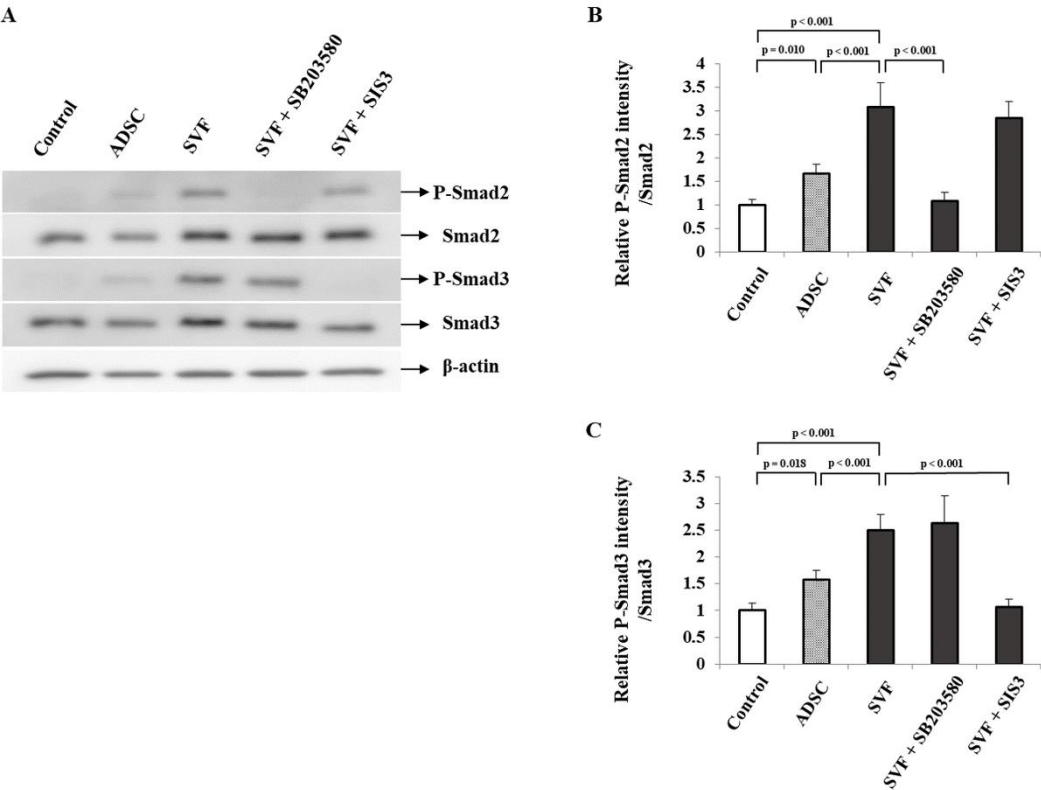


Figure 4.

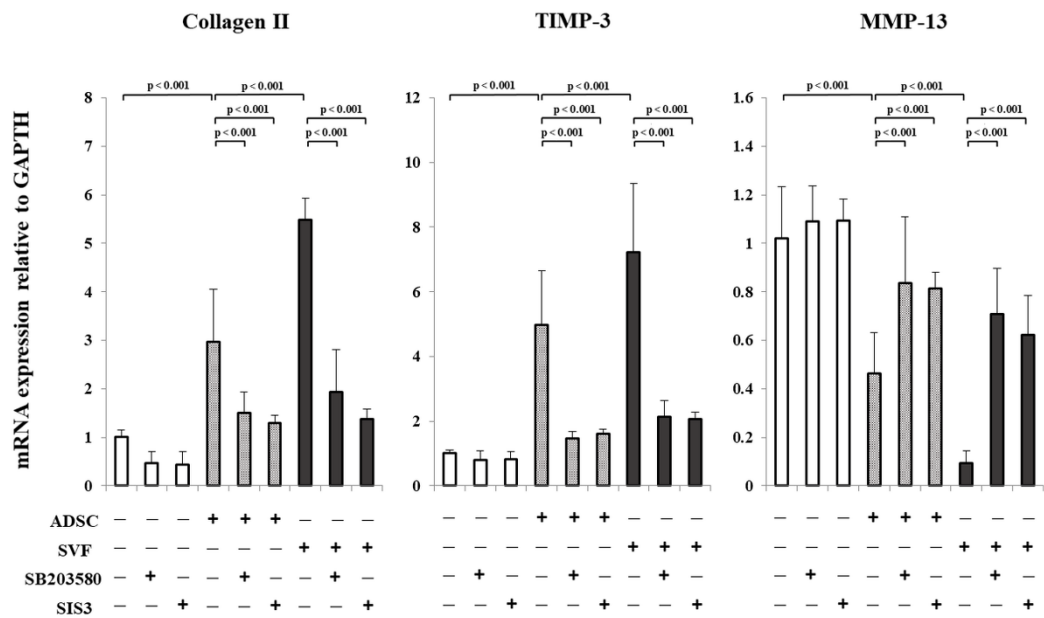


Figure 5.

