# Adipose Tissue Derived Mesenchymal Stem Cell Inhibits Osteoclast Differentiation Through Tumor Necrosis Factor Stimulated Gene-6

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# Adipose tissue derived mesenchymal stem cell inhibits osteoclast differentiation through tumor necrosis factor stimulated gene-6

Directed by Professor Yong-Beom Park

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This certifies that the Doctoral Dissertation of Kwang-Hoon Lee is approved.

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ABSTRACT

# Adipose tissue derived mesenchymal stem cell inhibits osteoclast differentiation through tumor necrosis factor stimulated gene-6

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Systemic and local bone loss in rheumatic diseases is common and significantly increases the risk of fracture in patients with rheumatic diseases. Mesenchymal stem cells (MSCs) inhibit osteoclast differentiation and activation and seem to be promising in treating these conditions, but the underlying mechanisms remain to be clarified. Tumor necrosis factor activated gene-6 (TSG-6) is a potent anti-inflammatory, tissue-protective molecule that inhibits osteoclast activation. This study aimed to investigate whether TSG-6 mediates the MSC's inhibition of osteoclast differentiation. Human adipose tissue-derived MSCs (ADMSCs) and bone marrow-derived monocyte/macrophage (BMMs) from DBA/1J or B6 mouse were co-cultured in the presence of M-CSF 10 ng/mL and RANKL 10 ng/mL. In

some groups, ADMSCs were transfected with siRNA targeting TSG-6 or osteoprotegerin (OPG). Tartrate-resistant acid phosphatase (TRAP) activity in culture supernatant and mRNA expression of osteoclast markers were investigated. TRAP+ multinucleated cells and F-actin ring formation were counted. ADMSCs significantly inhibited osteoclast differentiation under osteoclastogenic conditions, but pretreatment with TNF-α did not enhance the anti-osteoclastogenic effect of ADMSCs. Suppression of TSG-6 significantly reversed the inhibition of osteoclast differentiation in a degree similar to that of OPG based on TRAP activity, mRNA expression of osteoclast markers, and numbers of TRAP+ multinucleated cell and F-actin ring formation. This study demonstrated that ADMSCs inhibit osteoclast differentiation through TSG-6 under osteoclastogenic conditions, which did not necessitate pretreatment of ADMSCs with TNF-α.

Key words : TSG-6, mesenchymal stem cell, osteoclast

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## I. INTRODUCTION

Systemic and local bone loss is common in rheumatic diseases such as rheumatoid arthritis (RA)[1] and systemic lupus erythematosus (SLE).[2] Osteoporosis associated with inflammatory rheumatic diseases increases the risk of fragility fracture, which poses a substantial clinical burden in these patients.[3, 4] In fact, the risk of osteoporotic fracture in patients with RA doubles that of the general population.[5]

Inflammation and immobility are among the principal pathways leading to bone loss in rheumatic disease, but other mechanisms, which were mainly studied in postmenopausal osteoporosis, can also be considered in bone loss in rheumatic diseases.[6] The receptor

activator of the nuclear factor kappa-B ligand (RANKL)/osteoprotegerin (OPG) and Wnt/β catenin pathways are the major regulators of bone remodeling.[7] RANKL, a cytokine of the tumor necrosis factor (TNF) family, and with its decoy molecule OPG regulates the osteoclast activity. RANKL is known to be essential in osteoclast development.[8] On the contrary, Wnt/β catenin pathway activates the transcription of osteoblast-specific genes and regulates osteoblast maturation and osteogenesis. Wnt inhibitors, Dickkopf-related protein 1 (Dkk-1), and sclerostin counteract the Wnt system activity.[7] In rheumatic diseases, Wnt inhibitor and RANKL production is upregulated, resulting in increased osteoclastogenesis and decreased osteogenesis. In addition, inflammatory mediators in rheumatic diseases, such as TNF-α, IL-1 and IL-6, lead to the activation of osteoclast (OC).[6]

The mainstream treatment options for treating bone loss in rheumatic disease are drug-based agents. Bisphosphonates decrease bone resorption by inducing osteoclast apoptosis.[9] Denosumab and calcitonin are alternative anti-resorptive agents.[10, 11] These anti-resorptive agents significantly reduced the risk of fragility fractures, but their long-term uses are associated with serious side effects such as osteonecrosis of the jaw and atypical fracture of the femur.[12] For this reason, efforts have been directed toward developing therapies that target anabolic pathways in bone and restore a population of bone-forming cells and osteoblasts, which led to the development of romosozumab and abaloparatide.[13, 14]

Mesenchymal stem cells (MSCs) are pluripotent cells that are emerging as potent tools in allogeneic cell therapy. They have been shown to have immunomodulatory activities in proinflammatory microenvironment and showed therapeutic potential in various inflammatory disorders.[15] MSCs are known to exert immunomodulation by interacting with various cells of innate and adaptive immune cells either through direct cell-to-cell contact [16] or by secreting soluble mediators including IL-10, TGF-β, PGE2, CCL-2, etc.[17]

MSCs were also shown to inhibit the differentiation and activation of OC [18], which is implicated in systemic bone loss and bone erosion in inflammatory rheumatic diseases, probably by secreting osteoprotegerin (OPG)[18] and IL-10[19], which highlights their potential in treating these conditions.

Tumor necrosis factor activated gene-6 (TSG-6) is an inflammation related, secreted protein that has anti-inflammatory and tissue protective effect in diverse tissues. TSG-6 was shown to mediate the immunomodulatory effect of MSC in various disorders including myocardial infarction, psoriasis, inflammatory bowel disease and neuroinflammation, etc.[20-24] Notably, TSG-6 was also shown to inhibit OC activity in synergism with OPG.[25] To the best of our knowledge, it has not been addressed whether TSG-6 mediates the inhibition of OC differentiation and activation by MSCs.

In this study, we aimed to determine whether adipose tissue derived MSC (ADMSC) inhibits the differentiation from OC precursors to mature OCs and whether the inhibition is mediated by TSG-6.

## II. MATERIALS AND METHODS

### 1. Cell isolation and culture conditions

ADMSCs were used because they are relatively easy to acquire compared to other tissues. ADMSCs (PromoCell, Heidelberg, Germany) were expanded in low-glucose Dulbecco's modified Eagle's medium (L-DMEM; Corning Inc., Corning, NY, USA) supplemented with 10% FBS (Corning Inc.), 1% nonessential amino acids (Gibco, Grand Island, NY, USA), and 1% penicillin/streptomycin (Gibco) for five to six passages (P5 or P6). Cells were harvested using 0.25% trypsin-EDTA solution (Gibco) for detachment of ADMSCs after Dulbecco's phosphate-buffered saline (DPBS; Corning Inc.) washing.

Bone marrow-derived monocyte/macrophages (BMMs) medium was prepared using alpha-minimum essential medium (α-MEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Corning Inc.) and 1% penicillin/streptomycin (Gibco). For osteoclast differentiation analysis, bone marrow (BM) cells were isolated from the tibias and femurs of 10-week-old C57BL/6 or DBA/1J mice (Orient Bio Inc., Sungnam, Republic of Korea) by flushing the bone marrow cavity with BMM medium using a sterile 1-mL syringe equipped with a 27-gauge needle. The flushed BM cells were filtered through 70 μm cell strainers (SPL Life Sciences, Pocheon, Republic of Korea), and the red blood cells were removed using RBC lysis buffer (BioLegend, San Diego, CA, USA). BM cells were dispensed in α-MEM-based BMM medium in T-75 culture flasks (Thermo Fisher Scientific, Waltham, MA, USA) at $1 \times 10^8$ cells/T-75 and a final volume of 30 mL and were incubated for approximately 2 days to separate non-adherent and adherent cells. During the morning of the second day, non-adherent cells were collected and cultured in 10-cm dishes (Corning Inc.) at a density of $5 \times 10^7$ cells/plate in BMM medium with 10 ng/mL recombinant mouse macrophage colony stimulating factor (rmM-CSF; R&D Systems, Minneapolis, MN, USA) for the selection of BMMs and adequate cell expansion. After incubation for 3 days, the adherent BMMs were detached using cell detachment solution (Amsbio, Cambridge, MA, USA) and a sterile cell scraper (SPL Life Sciences) and

collected for further culture to induce osteoclast differentiation. The cells were maintained in a cell incubator at 37 °C and 5% $CO_2$. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Health System (approval number: 2020-0291).

**2. Small interfering RNA (siRNA) transfection**

Human TSG-6 (cat. no. sc-39819) and human OPG (cat. no. sc-40152) siRNA were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Negative control siRNA (cat. no. 12935-400) was purchased from Thermo Fisher Scientific. Briefly, $3.2 \times 10^4$ or $7 \times 10^3$ ADMSCs (P5 or P6) were resuspended in L-DMEM-based growth medium and then cultured in a 6-well or 24-well plate using a SPLInsert™ Hanging (0.4 μm pore size; SPL Life Sciences) overnight to reach over 40% confluence. The growth medium was changed to fresh L-DMEM (serum- and antibiotic-free) for transfection. Each siRNA (final concentration per well; 12.5 nM or 5 nM) and Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA, USA) were separately diluted in fresh L-DMEM and incubate for 5 min at room temperature. The two solutions were mixed and incubated for 20 min at room temperature. The siRNA-RNAiMAX Reagent mixture was then added to the cells, and these cells were incubated for 8h at 37 °C.

**3. ADMSC/BMM co-culture system for osteoclast differentiation**

The SPLInsert™ Hanging 6-well or 24-well cell culture inserts (0.4 μm pore size; SPL Life Sciences) were used for co-culture. Briefly, BMMs were seeded in the lower wells at a density of $2.5 \times 10^5$ cells/well or $8.3 \times 10^4$ cells/well and incubated with 10 ng/mL of rmM-CSF (R&D Systems) for 2 days to reach over 60% confluence. Before co-culture for osteoclast differentiation, the fresh L-DMEM of the transfected ADMSCs on the SPLInsert™ Hanging (upper wells) was replaced with L-DMEM-based growth medium containing 10 ng/mL of recombinant human (rh) TNFα (R&D Systems) overnight to induce TSG-6 expression. The next morning, the transwell inserts containing the TNFα-

stimulated ADMSCs were washed with PBS, picked, and transferred onto the BMMs cultured in the lower wells. For induction of osteoclast differentiation, BMMs and ADMSCs were cultured in α-MEM-based medium supplemented with rmM-CSF (10 ng/ml; R&D Systems) and receptor activator of nuclear factors κB ligand (rmRANKL; 10 ng/ml or 20 ng/ml; R&D Systems) at a final volume of 4 mL/6-well or 1 mL/24-well. The medium was refreshed every second day until mature osteoclasts had formed. 200 μL of the culture supernatant per well was harvested every single day for TRAP activity assay.

**4. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)**

Cells were washed with DPBS to remove serum, trypsin-EDTA, and phenol red, and total RNA from the BMMs and ADMSCs was extracted using a RNeasy Micro kit (QIAGEN, Hilden, Germany) and converted to complementary DNA (cDNA) using a RT PreMix cDNA synthesis kit (iNtRON-Biotechnology, Seongnam, Republic of Korea), following the manufacturers' protocols. qRT-PCR was performed using a Viia7 system (Applied Biosystems, Waltham, MA, USA) with qPCRBIO SyGreen Mix (PCR Biosystems, London, UK) according to the manufacturer's instructions. The mRNA abundance of mouse tartrate-resistant acid phosphatase (TRAP), mouse cathepsin-K (CTS-K), mouse matrix metalloproteinase (MMP)-9, mouse nuclear factor of activated T cells 1 (NFATc1), mouse cFos-1, human TSG-6, and human OPG was measured with qRT-PCR. Relative RNA expression was normalized to mouse *β-actin* or human *Gapdh* messenger RNA (mRNA), a housekeeping gene, using the $2^{-\triangle\triangle Ct}$ calculation method. The primers used are summarized in table 1.

**Table 1. Primers used for RT-PCR analysis.**

| Genes | Forward | Reverse |
|---|---|---|
| TRAP | 5′-CGA GAA CGG TGT GGG CTA TG-3′ | 5′-AGT GAA ACC GCA AGT AGC CG-3′ |
| *CTS-K* | 5′-GTG GGC CAG GAT GAA AGT TGT-3′ | 5′-GGC TTT CTC GTT CCC CAC AG-3′ |
| *MMP-9* | 5′-AAA CCT CCA ACC TCA CGG ACA-3′ | 5′-TCT GAA GCA TCA GCA AAG CC-G-3′ |
| *NFATc1* | 5′-CGA GTT CAC ATC CCA CAG CC-3′ | 5′-CTG TGC TCT GCT TCT CCA CG-3′ |
| *cFos-1* | 5′-CCA CTC TGG TCT CCT CCG TG-3′ | 5′-CAT TCC CGC TCT GGC GTA AG-3′ |
| *β-actin* | 5′-TGT TTG AGA CCT TCA ACA CCC-C-3′ | 5′-TGT GGT ACG ACC AGA GGC AT-3′ |
| *TSG-6* | 5′-AAG CAC GGT CTG GCA AAT ACA AGC-3′ | 5′-ATC CAT CCA GCA GCA CAG ACA TGA-3′ |
| *OPG* | 5′-GCG TGT GCG AAT GCA AGG AA-3′ | 5′-TAT TTC GCT CTG GGG TTC CAG C-3′ |

**5. Western blot analysis**

After collecting BMMs and OCs, the cells were washed twice with PBS to remove the residual medium, after which they were lysed in RIPA buffer (Biosesang, Seongnam, Republic of Korea) containing a complete mini protease inhibitor cocktail (Roche Diagnostics, Pleasanton, CA, USA). The cell lysates were then centrifuged at 12,000 rpm for 15 min at 4 ℃ to remove cell debris. Proteins were quantified using a bicinchoninic acid (BCA) protein assay (GenDEPOT, Katy, TX, USA) according to the manufacturer's instructions. Equivalent amounts of the boiled cellular proteins were separated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then electrotransferred onto methanol-activated polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany) at 4 ℃ overnight. Non-specific binding to the membrane was blocked by block solution (TransLab, Elgin, IL, USA) for 2 h at room temperature, and the membranes were probed with primary antibodies for 2 h at room temperature. Afterward, the membranes were washed six times for 30 min in Tris-buffered saline with Tween 20 (TBS-T), followed by the species-appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Membranes were again washed eight times for 40 min, and proteins were visualized using West-Q Pico ECL solution (GenDEPOT) or EZ-Western Lumi Femto solution (DoGENBio, Seoul, Republic of Korea) and detected using an Amersham ImageQuant 800 biomolecular imager (Cytiva, Marlborough, MA, USA). The signal intensities of immunoblot bands were quantified using ImageJ software (NIH, Bethesda, MD, USA). Primary and secondary antibodies were used for western blotting. NFATc1 (D15F1; 1:1000 dilution), Cathepsin-K (E7U5N; 1:1000), and HRP-linked rabbit IgG (cat. no. 7074; 1:2000 or 1:4000) were purchased from Cell Signaling Technology (Danvers, MA, USA). MMP-9 (cat.

no. ab228402; 1:1000) and TRAP (cat. no. ab191406; 1:1000) were purchased from Abcam (Cambridge, UK). β-Actin (C4; 1:1000) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

**6. TRAP activity and staining**

The culture supernatant from differentiated BMMs into OCs was subjected to a TRAP activity assay using a TRACP & ALP assay kit (TAKARA Bio Inc., Kusatsu, Japan), following the manufacturer's instructions. This assay has been designed for the simple and quick detection of acid phosphatase (AP) activity in the presence of TRAP derived from OCs through the using pNPP (*p*-nitrophenyl phosphate) substrate. The absorbance was measured at 405 nm using a microplate reader (Molecular Devices, San Jose, CA, USA). Culture supernatant was collected every other day during the co-culture period before each replacement of the culture medium.

For TRAP staining, BMMs differentiated into OCs were fixed with fixation solution for 5 min at room temperature and then stained for TRAP expression using a TRACP & ALP double-stain kit (TAKARA Bio Inc.) according to the manufacturer's instructions. The cells were visualized using a Nikon Ti-U fluorescent inverted microscope (Tokyo, Japan) in the bright-field mode. Stained OCs (nuclei > 3) were considered TRAP-positive, indicating matured and differentiated multinucleated OCs. At least nine random fields per well were assessed, and the mean number of OCs was calculated using ImageJ software (NIH, Bethesda, MD, USA).

**7. F-actin ring staining**

For F-actin ring staining, the cells were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in DPBS for 5 min. Afterwards, the cells were washed with 5% FBS in DPBS and stained with FITC-conjugated phalloidin (1:1000 dilution; Abcam, UK) for at least 30 min to indicate the F-actin rings, including the cytoskeletons of OCs. OC nuclei were stained with DAPI solution (1:5000; Thermo Fisher Scientific) for 5 min, and the cells washed with DPBS several times and observed using a Nikon Ti-U fluorescent inverted microscope. The fluorescent images were analyzed to quantify the number of F-actin rings using the ImageJ software (NIH).

**8. Statistical analysis**

All experiments were independently repeated at least three times, and similar results were obtained. Data are represented as mean ± SEM. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). Statistical differences between two groups were determined by two-tailed Student's *t*-test for paired or unpaired data. Comparisons among three or more groups were performed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test. Statistical significance is denoted as follows: ns (not significant), *P < 0.05, **P < 0.01, and ***P < 0.001.

## III. RESULTS

### 1. Gene suppression by siRNA transfection

In order to determine whether a specific gene has a mediating role in the inhibition of osteoclast differentiation by ADMSC, gene suppression using siRNA transfection targeting TSG-6 and OPG was applied to ADMSCs. OPG is a well-known, potent inhibitory mediator of osteoclast differentiation [26] and was used as a comparator. Gene expressions for TSG-6 and OPG were markedly suppressed after siRNA transfection. (Fig 1.)

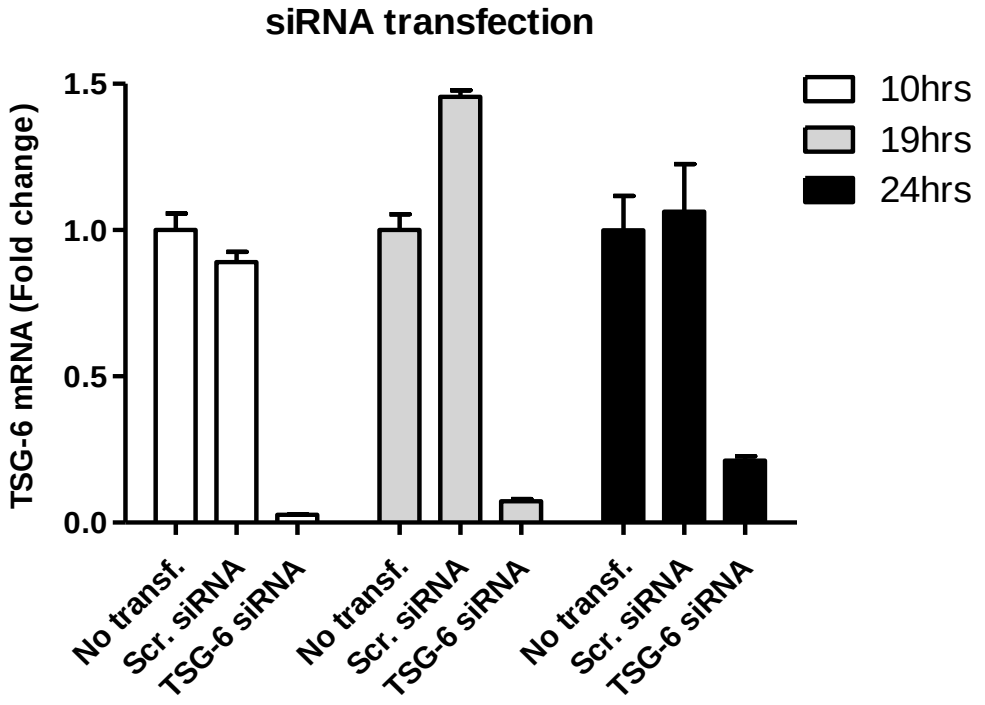


**Figure 1. Efficacy of siRNA transfection (TSG-6).** ADMSCs were transfected with siRNA targeting TSG-6 according to manufacturer's instructions. TSG-6 mRNA suppression was apparent as early as 10 hours after transfection and was maintained through 24 hours after.

**2. Pretreatment of ADMSCs with tumor necrosis factor α (TNF-α)**

MSCs were shown to have biphasic functions depending on the local microenvironment they encounter. It has been shown that graft-versus host disease can be successfully treated by MSCs when intense inflammation is ongoing but less effectively treated when MSCs are infused before inflammation has begun [27, 28]. Accordingly, TNF-α treated MSCs were shown to inhibit osteoclastogensis while untreated MSCs promote osteoclastogenesis[29]. In addition, TSG-6 is known to be induced by inflammatory mediators including interleukin-1 (IL-1), lipopolysaccharide (LPS) and TNF-α [30]. In this study, ADMSCs were pretreated with TNF-α to guarantee their inhibitory action on osteoclastogenesis and to highlight the role of TSG-6. ADMSCs were treated with TNF-α (10ng/mL) for varying duration (0h, 6h and 24h) and the levels of mRNA expression for TSG-6 were determined. mRNA expression for TSG-6 markedly increased in a treatment time-dependent manner, while that of the ADMSCs with TSG-6 siRNA transfection remained markedly suppressed (Fig 2.)

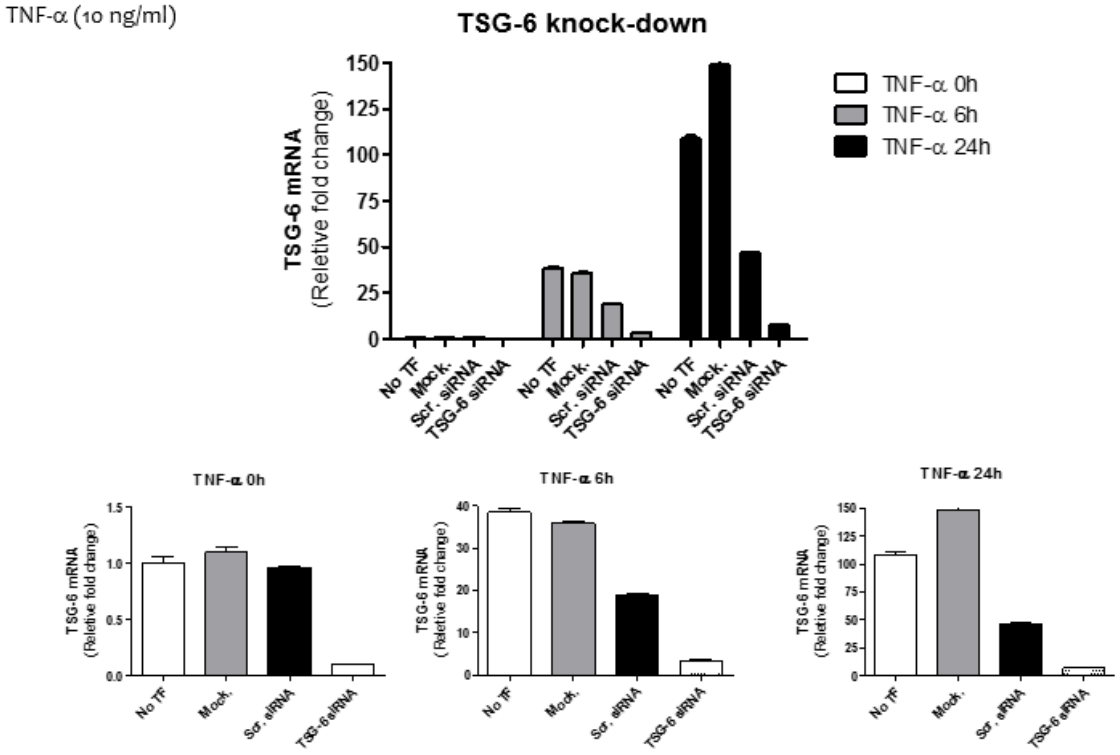


**Figure 2. TSG-6 mRNA expression levels of ADMSCs pretreated with rhTNF-α** ADMSCs were treated with rhTNF-α (10 ng/mL) for varying durations. TSG-6 levels dramatically increased after treatment with rhTNF-α in a time dependent manner. TSG-6 mRNA expression of ADMSCs with siRNA transfection for TSG-6 remained suppressed even after treatment with rhTNF-α.

### 3. Co-culture of ADMSC and OC precursors in osteoclastogenic conditions

ADMSCs with varying conditions were co-cultured with mouse BMMs in the presence of M-CSF 10ng/mL and RANKL 10ng/mL in a transwell co-culture system. Treatment groups comprised of 1) BMM only (BMM, used as negative control); 2) osteoclastogenesis (OC, without co-culture with ADMSC, used as positive control); 3) co-culture with untreated ADMSC (MSC); 4) coculture with TNF-α treated ADMSC (TNF MSC); 5) co-culture with TNF-α treated, TSG-6 suppressed ADMSC (TSG-6 KD); 6) co-culture with TNF-α treated, OPG suppressed ADMSC (OPG KD, used as comparator).

#### A. TRAP activity in co-culture supernatant

The supernatant of the co-culture was collected on co-culture day 2, 3, 4, 5 and was checked for TRAP activity. TRAP activity gradually increased as co-culture progressed in OC, TSG-6 KD and OPG KD group while those in BMM, TNF MSC and MSC group remained unchanged (Fig 3).

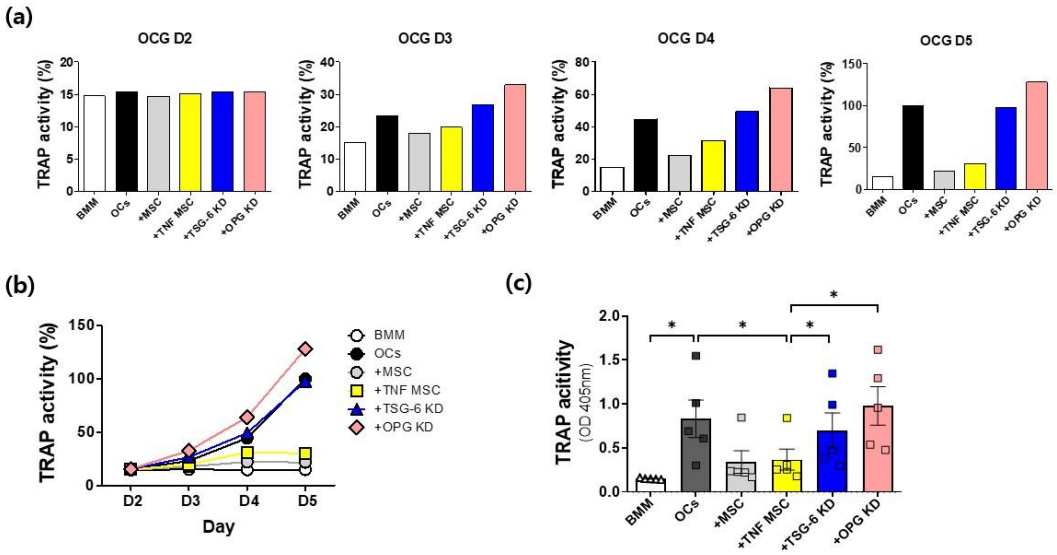


**Figure 3. Serially measured TRAP activity of the co-culture supernatant.** As the co-culture proceeded, TRAP activity gradually increased in TSG-6 KD and OPG KD group to a similar degree as those in OC group (positive control). TRAP activity of MSC and TNF MSC group did not increase during co-culture period. (a): comparisons of TRAP activity by co-culture day (b) merged graph of comparisons of TRAP activity by co-culture day (c) TRAP activity on co-culture day 5, *: $P<0.05$.

**B. Osteoclast marker expression by RT-PCR and western blot**

RT-PCR was performed to determine the mRNA expression levels of osteoclast markers including TRAP, CTS-K, MMP-9, NFATc1 and cFos-1 on the last day of co-culture. Though statistically not significant, cells co-cultured with untreated MSC and TNF-α treated MSC (MSC and TNF MSC group) showed decreased mRNA expression levels for osteoclast activity markers while TSG-6 KD and OPG KD group showed levels similar to that of OC group (positive control) (Fig 4).

Western blot was performed to determine if the findings above were still valid in the protein level. TRAP, CTS-K, MMP-9, and NFATc1 were decreased in TNF MSC group compared to positive control (OC group), which were reversed by gene silencing of TSG-6 (TSG-6 KD group) and OPG (OPG KD group). These differences were obvious for TRAP and CTS-K (Fig. 5).

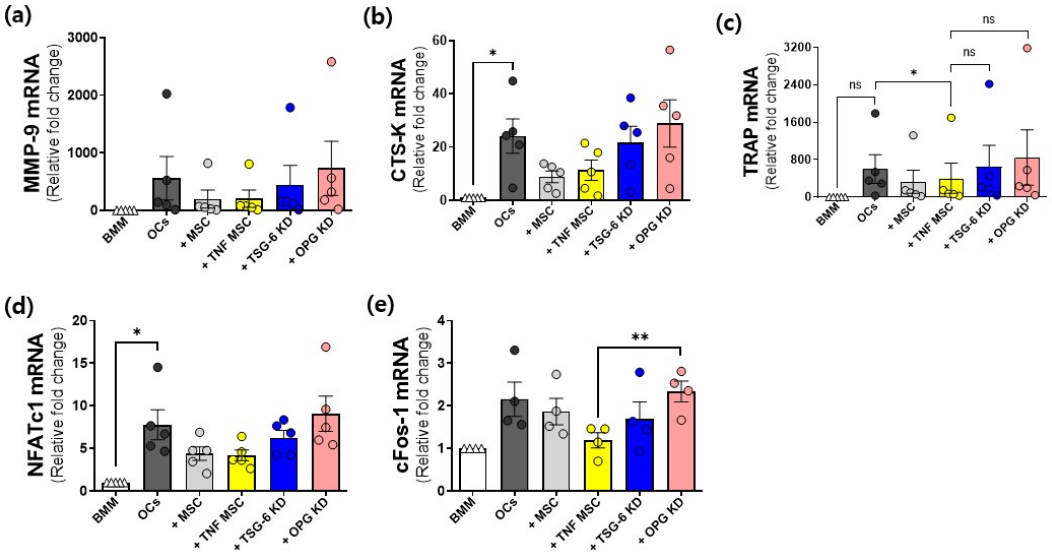


**Figure 4. mRNA expressions of osteoclast related markers at the end of co-culture.** RT-PCR was performed on the last day of co-culture. Though statistically not significant in all comparisons, all the measurements of markers [TRAP (a), CTS-K (b), MMP-9 (c), NFATc1 (d) and cFos-1 (e)] seem to be greater in TSG-6 KD and OPG KD group compared to MSC and TNF-MSC group.

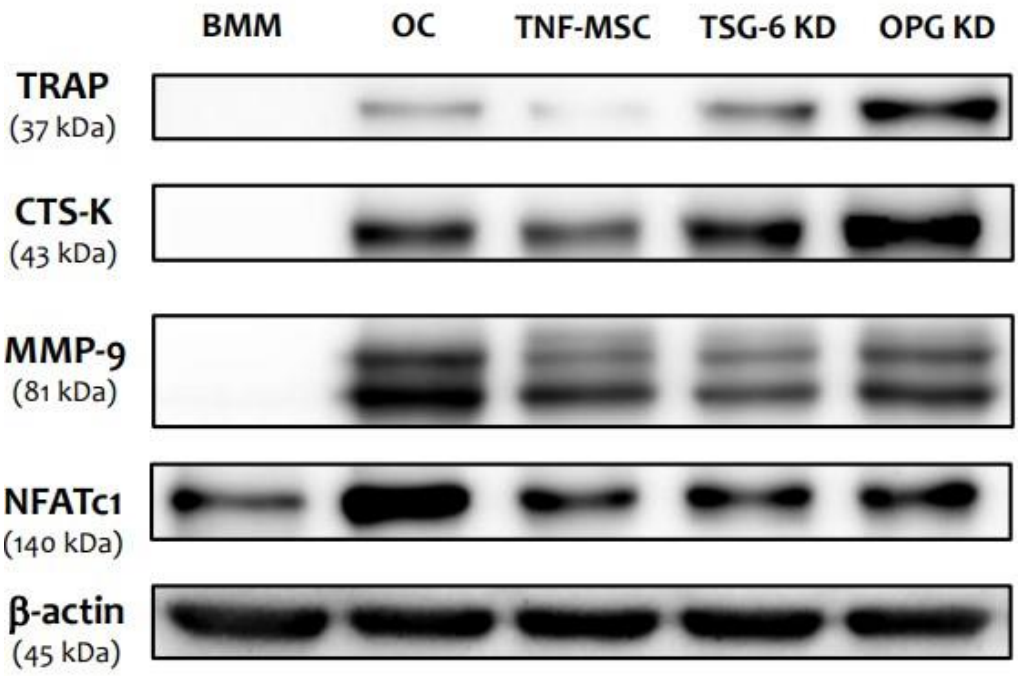


**Figure 5. Osteoclast activity markers by western blot.** TRAP, CTS-K, MMP-9, and NFATc1 were decreased in TNF-MSC group compared to OC group. These were reversed by gene silencing of TSG-6 (TSG-6 KD) and OPG (OPG KD), the difference of which was marked for TRAP and CTS-K.

**C. TRAP staining**

On the last day of co-culture, cells were fixed and stained for TRAP. TRAP positive, multinucleated cells were identified and counted as osteoclast. The total number of TRAP+ multinucleated cells were significantly greater in TSG-6 KD and OPG KD group compared to either MSC or TNF MSC group (Fig. 6).

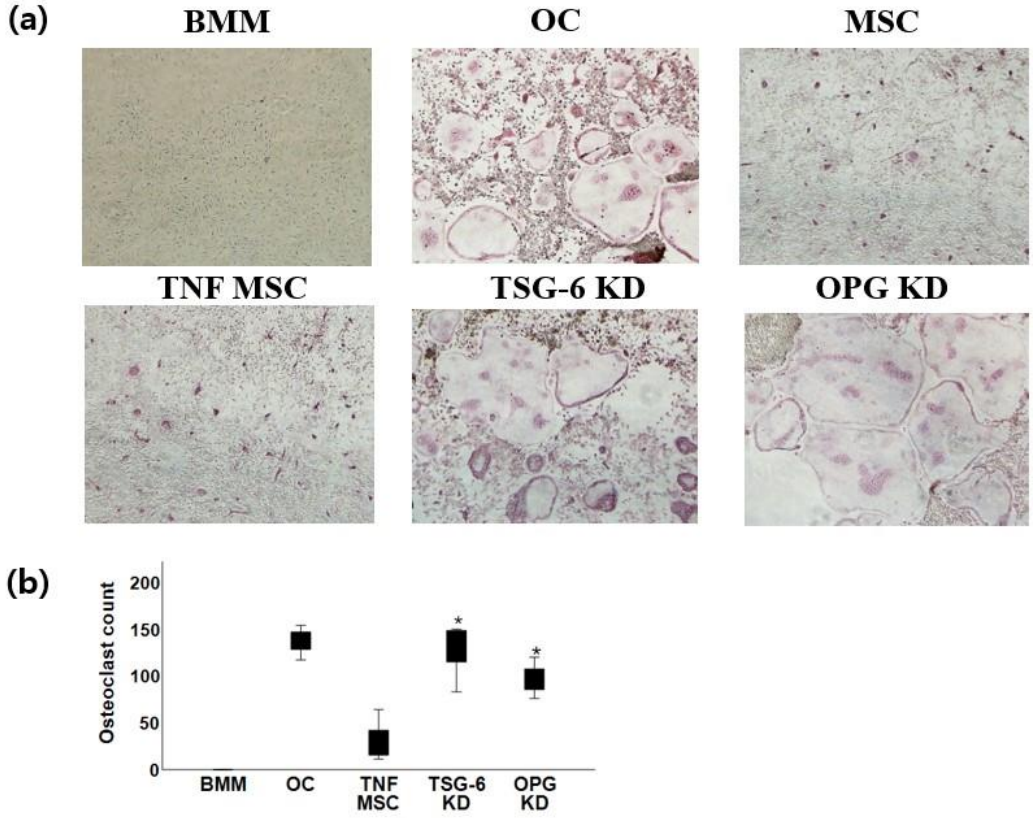


**Figure 6. TRAP staining.** (a) Representative TRAP staining images of each group are presented. TRAP+, multinucleated cells are rarely seen in MSC and TNF MSC group while suppression of TSG-6 (TSG-6 KD group) or OPG (OPG KD group) was associated with multiple, TRAP+ multinucleated cells. (b) The number of TRAP+, multinucleated cells (OC count) was significantly greater in TSG-6 KD and OPG KD group compared to MSC and TNF MSC group, *: $P<0.05$.

**D. F-actin ring formation**

On the last day of co-culture, cells were treated for F-actin ring identification in the method described above. The total number of F-actin rings was significantly greater in TSG-6 KD and OPG KD group compared to either TNF MSC or MSC group (Fig. 7). This supports that TSG-6 and OPG have roles in ADMSC's inhibition of osteoclastogenesis.

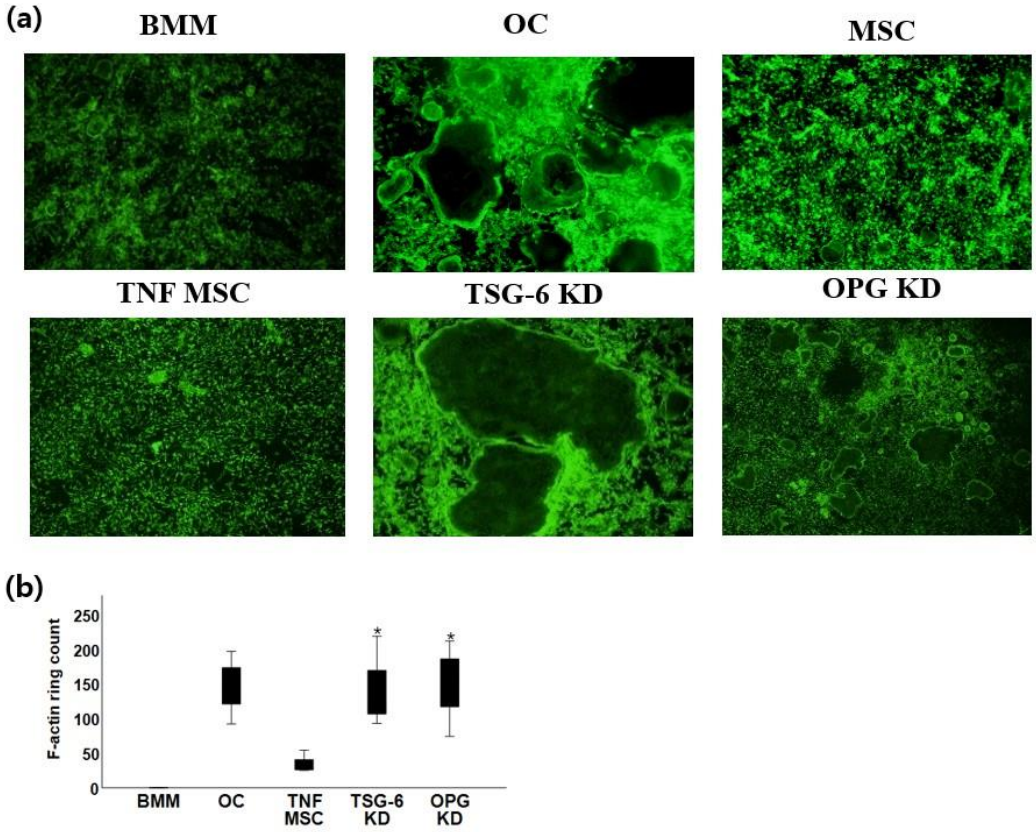


**Figure 7. F-actin ring staining.** (a) Representative F-actin ring images of each group are presented. While F-actin rings were rarely seen in MSC and TNF MSC group, F-actin rings were apparent in TSG-KD and OPG KD group. (b) The numbers of F-actin ring (F-actin ring count) in TSG-6 KD and OPG KD group were significantly greater than those in MSC and TNF-MSC group, **P*<0.05.

**E. Expression of TSG-6 and OPG mRNA at the end of co-culture**

mRNA expression levels of TSG-6 and OPG on day 5 of co-culture were obtained and compared to those of MSCs of varying conditions without co-culture. As expected, TSG-6 and OPG was effectively suppressed in TSG-6 KD and OPG KD group at the end with or without co-culture. Both TSG-6 and OPG mRNA expression increased after co-culture in MSC and TNF MSC group. Interestingly, TSG-6 mRNA expression level in OPG KD group was remarkably greater than other group, which was augmented by far after co-culture. On the contrary, OPG mRNA expression level of TSG-6 KD group with co-culture was lower than those of MSC and TNF MSCs group (Fig 8).

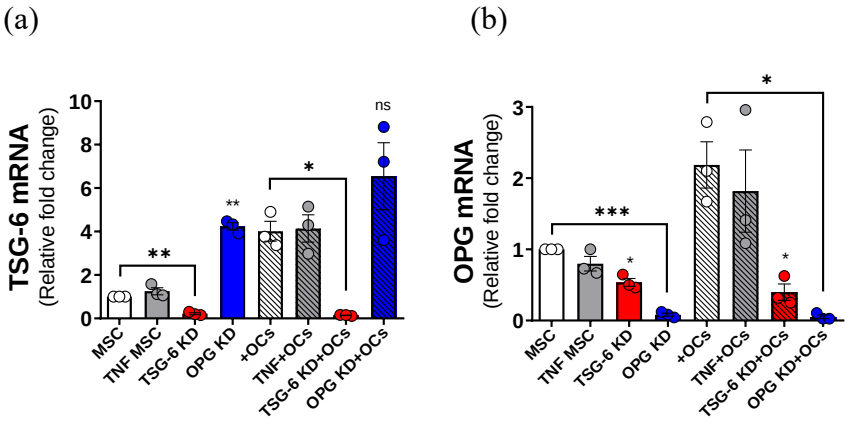


**Figure 8. mRNA expressions of TSG-6 and OPG on co-culture day 5** (a) TSG-6 mRNA expression levels were effectively suppressed in TSG-6 KD group (red bar) even after co-culture. TSG-6 mRNA expression levels of MSC (light grey bar) and TNF MSC group (dark grey bar) were similar. Note the increased level of TSG-6 mRNA expression in OPG KD group (blue bar) (b) OPG mRNA expression levels were effectively suppressed in OPG KD group (blue bar) after co-culture. Note the relatively low mRNA expression levels of OPG in TSG-6 KD group (red bar), **P*<0.05, ****P*<0.001

## IV. DISCUSSION

In this study, ADMSCs significantly inhibited OC precursors' differentiation into mature osteoclasts. Suppression of TSG-6 gene expression in ADMSCs significantly reversed this inhibition in a degree similar to suppression of OPG, which suggests the regulatory role of TSG-6 in this process.

One of the earlier studies reported that human bone marrow-derived MSCs promote osteoclastogenesis when co-cultured with OC precursors.[31] In contrast, later studies reported that MSCs inhibit osteoclastogenesis if the co-culture was stimulated with soluble RANKL and/or M-CSF.[32] Pretreatment of MSCs with TNF-α has also been highlighted to be necessary in polarizing MSCs toward the anti-osteoclastogenic side.[29] However, ADMSCs pretreated with TNF-α did not show superior inhibitory activity on osteoclastogenesis compared to those not pretreated. This may partly be explained by the differences in the source of MSCs. While previous studies used MSCs acquired from bone marrow[29], we used MSCs from adipose tissue. MSCs derived from different sources possess distinct characteristics, advantages and disadvantages, including their differentiation potential and proliferation capacity, which influence their applicability.[33]

Oshita et al [32] co-cultured bone marrow-derived MSCs with peripheral blood mononuclear cells (PBMCs) and stimulated them with relatively high levels of RANKL (50 ng/mL) and M-CSF (50 ng/mL), and MSCs exerted a suppressive effect on osteoclast differentiation. This suggests that relatively high levels of RANKL and M-CSF may have similar effect as TNF-α stimulating MSC's anti osteoclastic features.[34] However, low levels of RANKL (10 ng/mL) and M-CSF (10 ng/mL) seemed to be sufficient to drive the ADMSCs to be anti osteoclastogenic. This discrepancy may also be attributed to the different source of MSCs.[33]

TSG-6 is secreted product of the TNF-stimulated gene-6 that is upregulated in

response to inflammation though expressed constitutively in a few tissues.[35] TSG-6 is a small protein with a molecular mass of 35kDa and interacts with a large array of ligands, such as glycosaminoglycans, proteoglycan core proteins, chemokines, and bone morphogenic proteins (BMPs).[36] It has diverse biological functions including cell regulation, matrix organization as well as immune regulation. For bone related function, TSG-6 was shown to interact with BMPs, which is involved in osteoblastogenesis, inhibiting the BMP2-related differentiation of MSCs into osteoblasts.[37] TSG-6 has also been shown to inhibit RANKL-induced bone resorption by osteoclasts in synergism with OPG, but not the osteoclast differentiation.[25] In this regard, TSG-6 seems to have functions related with bone homeostasis.

TSG-6 has also been shown to mediate many of the immunomodulatory actions of MSCs in various disease models. Yang et al reported that bone marrow MSCs attenuated neuropathic pain by secreting TSG-6 that inhibits TLR2/MyD88/NF-κB signaling pathway.[38] Ding et al showed that umbilical cord-derived MSCs alleviate psoriasis-like inflammation through TSG-6.[22] An et al reported that TSG-6 was the major factor in the relief of dextran sulfate sodium-induced colitis model.[39]

Our study demonstrated that TSG-6 plays a role in the ADMSC-mediated suppression of osteoclastogenesis for the first time. In addition to the previously known mediators like IL-10 [19], TGF-β [19], and OPG [32], TSG-6 seems to mediate MSC's inhibition of osteoclastogenesis. This contrasts with the previous studies that TSG-6 did not inhibit osteoclastogenesis though it did suppress osteoclast function. [25, 40] This could be attributed to the differences in the ways to demonstrate the function of TSG-6. While previous studies used a setting where rhTSG-6 was added to OC precursors in osteoclastogenic conditions, we used a co-culture system where some ADMSCs were transfected with siRNA for TSG-6. Though the latter setting

surely lacks TSG-6, it also allows many crosstalks other than TSG-6 between the ADMSCs and the OC precursors during co-culture. Based on the current findings of our study, it is speculated that ADMSCs suppress osteoclastogenesis through TSG-6. This finding adds to the current evidence that MSCs exert many of their immunomodulatory actions through TSG-6.

At the end of co-culture, the mRNA expression level of TSG-6 was augmented, while that of OPG was diminished in OPG KD group. ADMSCs in this group did not inhibit osteoclastogenesis. What can be learned from this finding is that ADMSCs may not be able to inhibit osteoclastogenesis without OPG even though they express high levels of TSG-6. In addition, ADMSCs in TSG-6 KD group were not able to inhibit osteoclastogenesis. Taken together, it is suspected that ADMSCs need both TSG-6 and OPG when inhibiting osteoclastogenesis. Similarly, Mahoney et al suggested a functional synergism between TSG-6 and OPG in suppressing osteoclast's activity.[25] However, the reason for low OPG mRNA expression levels in TSG-6 KD group is unclear for now, and further studies are needed to clarify the interactions between TSG-6 and OPG in osteoclastogenesis.

Though the mRNA expressions of genes related with osteoclast differentiation and activity at the end of co-culture were not significantly different between co-culture groups, co-culture with TNF-α treated ADMSCs suppressed the expressions of these genes, which was reversed by suppression of TSG-6 and OPG. The correlation between mRNA and protein expression in a cell is reported to be poor [41] and the discrepancy is generally attributed to the regulatory mechanism between transcript and protein product.[42]

Most of the systemically administered MSCs are reported to be trapped in the lung and do not get engrafted to the site of inflammation or injury, but they exert anti-inflammatory actions in a mouse model of myocardial infarction through

secretion of TSG-6.[20] Similarly, our results suggest that systemically administered ADMSCs may regulate osteoclastogenesis remotely through TSG-6 and OPG secretion.

Our study has some limitations. The experiments focused on osteoclastogenesis and did not address the aspects of osteoclast function. In addition, the molecular mechanism underlying TSG-6's action on osteoclastogenesis was not explored in this study. These issues should be addressed by further studies in the future.

## V. CONCLUSION

This is the first study that assessed the role of TSG-6 in ADMSC's inhibition of osteoclast differentiation. ADMSCs pretreated with TNF-α inhibited osteoclast differentiation through secretion of TSG-6 and OPG. Allogeneic stem cell therapy using ADMSCs might be promising in treating systemic and local bone loss in rheumatic diseases.

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ABSTRACT(IN KOREAN)

## 지방 세포 유래 중간엽 줄기세포의 파골세포 분화 억제에서 TSG-6의 역할

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류마티스 질환에 이환된 환자에게 국소 및 전신성 골소실은 흔히 발생하는 합병증으로 이들 환자들은 일반인보다 약 2배 이상 골절 위험이 증가되어 있다. 류마티스 질환과 연관된 골소실은 각종 신체 기관의 염증 반응과 receptor activator of the nuclear factor kappa-B ligand (RANKL)의 증가로 인해 파골세포는 활성화되고 조골세포는 억제되어 발생하는 것으로 알려져 있다. 중간엽 줄기세포는 염증성 미세환경에 노출될 경우 염증을 억제할 뿐만 아니라 파골세포의 분화와 활성화를 억제하는 것으로 알려져 있고 많은 전임상 실험에서 골다공증의 치료에 유용함이 보고된 바 있다. 그러나 중간엽 줄기세포의 골세포에 대한 작용의 정확한 기전에 대해서는 더 많은 연구가 필요하다. Tumor necrosis factor activated gene-6 (TSG-6)는 중간엽

줄기세포를 포함한 다양한 세포들에서 분비되는 물질로서 염증 환경에 노출될 경우 염증을 억제하고 조직을 보호하는 기능이 있다. 본 연구에서는 중간엽 줄기세포가 파골세포의 분화, 활성화를 억제함에 있어 TSG-6의 역할을 규명하고자 하였다. 인간 지방세포 유래 중간엽 줄기세포와 DBA/1J 및 B6 mouse에서 얻은 골수 유래 단핵구/대식세포 (bone marrow-derived monocyte/macrophage)를 파골세포 분화 유도 환경 (M-CSF 10 ng/mL 및 RANKL 10 ng/mL)에서 공배양하였고, 공배양 그룹 중 일부 그룹에서 인간 지방세포 유래 중간엽세포들은 TSG-6 및 osteoprotegerin (OPG) 유전자 발현 억제를 위해 siRNA transfection을 시행하였다. 공배양 중 공배양 2,3,4,5일째의 tartrate-resistant acid phosphatase (TRAP) 활성도를 측정하였고, 공배양 종료 후 파골세포 분화 연관 유전자들의 mRNA 활성도, 웨스턴 블롯, TRAP 염색 양성 다핵 세포 및 F-actin ring 형성을 평가 및 비교하였다. 실험 결과, TRAP activity, 파골세포 분화 연관 유전자들의 mRNA 활성도, TRAP 염색 양성 다핵 세포, F-actin ring number를 바탕으로 확인한 바, 인간 지방세포 유래 중간엽 줄기세포는 파골세포 분화 유도 환경에서 파골세포 분화를 억제하였고, siRNA transfection을 통해 인간 지방세포 유래 중간엽세포의 TSG-6 유전자를 억제할 경우 이러한 파골세포 분화 억제능이 소실되었다. 본 연구를 통해 인간 지방세포 유래 중간엽 줄기세포는 TSG-6를 분비함으로써 파골세포의 분화를 억제함을 확인할 수

있었고 류마티스 질환에 동반된 전신성 및 국소성 골소실의 치료에 있어 인간 지방세포 유래 중간엽 줄기세포가 유용할 수 있음을 추정할 수 있다.

핵심되는 말: 지방 유래 중간엽 줄기세포, 파골세포, TSG-6