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Original Article

Membrane-free stem cell components suppress osteoclast differentiation: Implications for oral regenerative treatment

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Abstract *Background/purpose:* Membrane-free stem cell components (MFSCCs) have been developed by removing cell membranes with antigens to overcome the limitations associated with cell-based therapies and isolate effective peptides. MFSCCs have been reported to have effects on oral infection sites. Chronic inflammatory diseases cause excessive bone resorption. This study investigated the effects of MFSCCs on osteoclast differentiation in the context of the high prevalence of inflammatory bone resorption.
Materials and methods: Bone marrow macrophages (BMMs) were treated with macrophage colony-stimulating factor and receptor activator of nuclear factor kappa-B ligand. Osteoclast differentiation was assessed based on the MFSCC concentrations. Tartrate-resistant acid phosphatase (TRAP)-stained mature osteoclasts and multinucleated cells derived from BMMs were analyzed using light microscopy. The messenger RNA (mRNA) expression levels of genes related to osteoclast differentiation were measured using real-time polymerase chain reaction (RT-PCR). The relative expression levels of the key transcription factors c-fos and nuclear factor of activated T cells (NFATc1) were determined using quantitative RT-PCR and western blotting.
Results: After treatment with MFSCCs, the cell viability was similar, depending on the level of BMMs. As the MFSCC concentration increased, the number of TRAP-positive cells decreased. The mRNA and protein expression of cathepsin K, TRAP, dendritic cell-specific transmembrane protein, c-fos, and NFATc1 decreased as the MFSCC concentration increased.

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Conclusion: Our findings demonstrate that MFSCCs suppress osteoclast differentiation by downregulating transcription factors, particularly, c-fos and NFATc1. Therefore, MFSCCs may serve as a conservative treatment option for chronic inflammatory bone resorption diseases of the oral cavity by suppressing excessive bone resorption.

Introduction

Infectious bone-destructive diseases of the maxilla and mandible are closely associated with excessive osteoclast activity.[1,2] Chronic inflammatory conditions lead to increased inflammatory cytokine levels, inducing pathological osteoclast differentiation and promoting excessive bone resorption.[3–5] Osteomyelitis of the jaw is defined as progressive bone and bone marrow inflammation that can mostly occur in the jaw after chronic odontogenic infection.[6] Osteomyelitis has various classifications, of which acute, secondary chronic, and primary chronic osteomyelitis represent clinicopathological infections.[7] The number of activated osteoclasts in osteomyelitis was reported to be significantly higher than in the normal healthy group.[8,9] The representative periodontitis-causing pathogen, *Porphyromonas gingivalis* acts on periodontal tissues and leads to osteoclast activation through receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) expression as part of an inflammatory response, resulting in severe bone losses.[10]

Various regenerative approaches have been used to reconstruct defective bones, and the effects of materials have been explored to mimic the natural biological properties of the native bone.[2,11,12] Many studies have revealed the use of mesenchymal stem cells for bone defects in the oral and maxillofacial areas.[10] For example, when adipose-derived stem cells (ADSCs), a type of mesenchymal stem cell, were combined with bone morphogenetic protein-2, bone healing was observed with enhanced osteogenic potential.[13] However, cell-based therapies have several limitations because the cells must be grown, preserved, and transported.[14] Before autologous cell-based therapies can be used in clinical trials, side effects such as tumor formation and unwanted immune reactivity must be considered.[15] Previous articles reported that the limitations associated with cell-based therapies could be overcome by applying non-cell-based stem cell components extracted from stem cell-conditioned media.[14–17]

Membrane-free stem cell components (MFSCCs) are intracellular parts of human adipose tissue that remove cell membranes, possibly offering an advantage of the ADSCs to overcome the disadvantages of cellular immune reactions.[15] According to He et al.,[18] MFSCCs exhibit an anti-inflammatory effect on the cellular system. MFSCCs suppress RANKL and the mitogen-activated protein kinase signaling pathways in interleukin-1α-stimulated rat chondrocytes;[19] such signaling is critical for inflammatory responses and normal osteoclast differentiation and activation.[20] If MFSCCs could inhibit osteoclast-induced bone destruction, they could be used as adjuvants for severe inflammatory bone destruction disease owing to its synergistic effects. This study is the first to demonstrate the significant therapeutic effect of MFSCCs through the regulation of osteoclastogenesis, aiming to elucidate the mechanisms by which MFSCCs regulate osteoclast differentiation for potential applications in bone defect sites.

Materials and methods

Chemicals and reagents

MFSCCs were obtained from T-Stem Co. (Changwon, Gyeongsagnam-do, Korea).[15] The recombinant receptor activator of RANKL was purchased from Prospec (Rehovot, Central District of Israel, Israel), and macrophage colony-stimulating factor (M-CSF) was obtained from PeproTech (Rocky Hill, NJ, USA). The leukocyte acid phosphatase (TRAP) staining kit and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Alpha-modified Eagle's medium (α-MEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco (Grand Island, NY, USA). Anti-NFATc1 and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the anti-c-fos antibody was obtained from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies against rabbit and mouse immunoglobulin G were purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Cell culture

Bone marrow-derived macrophages (BMMs) were cultured in α-MEM supplemented with 10% FBS and 100 U/mL penicillin-streptomycin and were maintained in an incubator with 5% CO_2 at 37 °C. Subculturing was conducted using 0.05% trypsin-EDTA (Gibco) when the cells were confluent.

Osteoclast differentiation and TRAP staining

For osteoclast differentiation, BMMs isolated from 5-week-old ICR mice were seeded at a density of 3×10^4 cells/48-well and cultured in α-MEM supplemented with 10% FBS, 100 U/mL penicillin-streptomycin, 30 ng/mL M-CSF, and 100 ng/mL recombinant RANKL for 2–4 days. Different concentrations of MFSCCs were added to the cultures at the indicated times, and the medium was changed every 2 days. After culture, mature osteoclasts were stained with

TRAP using a TRAP staining kit (Sigma-Aldrich) following the manufacturer's instructions. TRAP-positive cells with three or more nuclei were counted under a light microscope.

Cell counting kit-8 assay

Cell proliferation and viability were determined using the cell counting kit-8 (CCK-8) assay kit (Enzo Life Sciences Inc.). BMMs were seeded in 48-well culture plates at a density of 3×10^4 cells/well and treated with MFSCCs for 24, 48, and 72 h. The cells were treated with the CCK-8 reagent in the culture medium for 2 h; the absorbance of the samples was measured at a wavelength of 450 nm using a microplate reader. All experiments were repeated at least three times to ensure reproducibility.

Western blotting

Total cell lysates for western blotting were prepared using cold passive lysis buffer (Promega, Madison, WI, USA), gently sonicated, and incubated on ice for 20 min. The total protein concentration in the cell lysates was determined using a Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated using 8—10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were subsequently blocked with 5% milk in phosphate-buffered saline containing Tween-20 (PBST) for 30 min at room temperature and incubated with primary antibodies at 4 °C overnight. Primary antibodies against NFATc1 (1:1000 dilution), c-fos (1:1000 dilution), and actin (1:2000 dilution) were used. The membranes were washed three times with PBST buffer at 10-min intervals and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After the membrane was washed with the PBST buffer, the protein bands were detected using an enhanced chemiluminescence solution (Immobilon Classico reagent, Millipore) and monitored using a LAS4000 (GE Healthcare Life Sciences, Marlborough, MA, USA). Band images were quantified using ImageJ software (NIH, Bethesda, MD, USA) and normalized against suitable loading controls.

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA from BMMs was isolated using the RiboEx reagent (GeneAll Biotech, Seoul, Korea), following the manufacturer's instructions, and quantified using Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). Thereafter, 1 μg of total RNA was reverse-transcribed into cDNA using a HiSenScript RH (-) RT PreMix Kit (Intronbio, Seongnam, Gyeonggi, Korea). The messenger RNA (mRNA) expression levels of osteoclast-related genes were evaluated using a QuantStudio 1 real-time polymerase chain reaction (PCR) system (Thermo Fisher Scientific) and SYBR Green Q-PCR Master Mix with a Low Rox kit (Smart Gene, Seoul, Korea). The following PCR cycling conditions were employed: 40 cycles for 15 s at 95 °C, denaturation, and 1 min at 60 °C, amplification. The data represent the results of three independent experiments, and relative mRNA expression was determined using the comparative Ct method, with β-actin as the control gene. Primer sequences are listed in Table 1.

Statistical analysis

All experimental data are expressed as the mean ± standard deviation (SD) of triplicate independent samples. Student's *t*-test was performed, and $P < 0.05$ was considered to indicate significance ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

No significant cytotoxicity in BMMs treated with MFSCCs

Different concentrations of MFSCCs (1—20 μg/mL) were added to BMMs to determine potential toxicity and osteoclastogenesis effects using the CCK-8 assay. The viability of BMMs was not compromised by 1—20 μg/mL of MFSCC (Fig. 1A). MFSCC proliferation was evaluated at 24, 48, and 72 h using the CCK-8 assay (Fig. 1B). Based on the results, 1—20 μg/mL of MFSCC did not induce significant cytotoxicity in BMMs. Therefore, MFSCC concentrations of 1—20 μg/mL were employed to assess its effects on osteoclast differentiation.

MFSCCs suppress RANKL-induced osteoclast formation of mature TRAP-positive multinucleated cells in BMMs

To determine the effects of MFSCCs on osteoclast differentiation and maturation in BMMs, TRAP-stained mature osteoclasts derived from BMMs treated with different concentrations of MFSCCs (Fig. 2A) and multinucleated cells (MCCs) (Fig. 2B) were analyzed using light microscopy. Treatment of BMMs with RANKL and M-CSF for 3—4 days induced osteoclast differentiation and maturation (Fig. 2A).

Table 1 Primer sequences used for real-time polymerase chain reaction.

Genes	Primers	Sequences (5′—3′)
CTSK	Forward	ATATGTGGGCCAGGATGAAAGTT
	Reverse	TCGTTCCCCACAGGAATCTCT
DC-STAMP	Forward	GGGTGCTGTTTGCCGCTG
	Reverse	CGACTCCTTGGGTTCCTTGCT
TRAP	Forward	CGACCATTGTTAGCCACATACG
	Reverse	TCGTCCTGAAGATACTGCAGGTT
c-fos	Forward	ACTTCTTGTTTCCGGC
	Reverse	AGCTTCAGGGTAGGTG
NFATc1	Forward	CCAGTATACCAGCTCTGCCA
	Reverse	GTGGGAAGTCAGAAGTGGGT
β-actin	Forward	GATATCGCTGCGCTGGTCG
	Reverse	GCCCACGATGGAGGGGAATA

CTSK: cathepsin K, DC-STAMP: dendritic cell-specific transmembrane protein, NFATc1: nuclear factor of activated T-cells 1, TRAP: tartrate-resistant acid phosphatase.

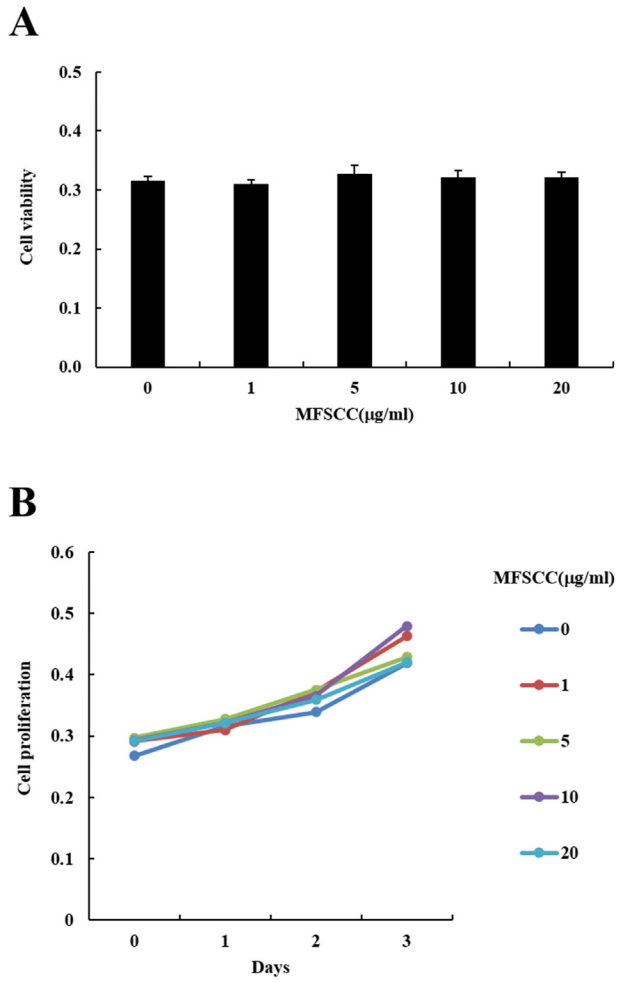

Figure 1 MFSCCs do not exhibit cytotoxic effects on the cell viability and proliferation of BMMs. BMMs were treated with the indicated MFSCCs concentrations. (A) Cell viability and (B) cell proliferation were determined using a cell counting kit-8 assay. All data are expressed as the mean ± standard deviation. BMM: bone marrow-derived macrophage, MFSCC: membrane-free stem cell component.

As the MFSCC concentration increased, the number of TRAP-positive MCCs decreased significantly ($P < 0.001$) (Fig. 2).

MFSCCs inhibit osteoclastogenesis by interfering with the regulators of differentiation, fusion, and maturation

To evaluate the effects of MFSCC on osteoclastogenesis, BMMs were treated with M-CSF and RANKL, and the mRNA expression levels of the regulatory factors of differentiation and activation were examined using different concentrations of MFSCCs. As the concentration of MFSCCs increased, the mRNA expression level of c-fos and NFATc1, osteoclastic transcription factors that act as key regulators of osteoclast multinucleation, decreased. The cells were more sensitive to a lower concentration of MFSCCs on day 2 than on day 3, and multi-nucleation was significantly affected by higher concentrations of MFSCCs (Fig. 3A and B).

The mRNA expression levels of dendritic cell-specific transmembrane protein (DC-STAMP), a cell surface receptor involved in cell-to-cell fusion, and cathepsin K (CTSK) and TRAP, enzymes involved in degradation, decreased in MFSCCs, with greater statistical significance observed on day 3 than on day 2 (Fig. 3C–E), suggesting that the anti-osteoclastic effect of MFSCCs is mediated by the inhibition of osteoclast-related marker genes.

MFSCCs suppress the expression of the RANKL-induced osteoclast-specific transcription factors c-fos and NFATc1

Upon treatment with MFSCCs on day 2, the protein expression levels of c-fos and NFATc1 significantly decreased (Fig. 4A and B). The expression of the transcriptional regulators was increasingly suppressed at higher concentrations of MFSCCs (Fig. 4C and D). These results indicate that suppressing the levels of transcription factors involved in osteoclast differentiation may be related to the regulatory effects of MFSCCs.

Discussion

Excessive bone resorption due to inflammatory diseases of the oral cavity significantly impairs a patient's quality of life. Complete restoration of the original condition is challenging, even with procedures such as bone grafting. In oral and maxillofacial surgery, various conservative treatments have been explored to address infectious bone resorption. Previous studies focused on the mechanisms of inflammation, the treatment of inflammatory bacteria, and inflammation-related effects by regulating a pivotal mediator in RANKL signaling.[18–20] RANKL promotes osteoclast differentiation and activates NFATc1 through c-fos stimulation and bone resorption.[21–23] Bone research, particularly, research on changes in osteoclast differentiation due to inflammation and subsequent alveolar bone resorption which, in turn, affect future prosthetic rehabilitation, has been insufficient. This study pioneers the exploration of MFSCCs in bone research, emphasizing their role in regulating osteoclast differentiation and potentially suppressing bone resorption. In our study, MFSCCs were identified as an essential regulator of osteoclast differentiation at the mRNA and protein expression levels.

Inflammatory sites within the oral cavity exhibit excessive osteoclast activity, leading to bone resorption and the formation of bone defects.[8,9] Osteoclast differentiation can occur through RANKL-dependent and RANKL-independent processes.[24] The osteoclast differentiation pathway is a complex process, and specific proteins play key roles (TRAP, CTSK, c-fos, and NFATc1) in osteoclast differentiation.[25] Because various intermediate processes of the RANKL signaling pathways are involved in the molecular regulation of osteoclast differentiation,[26] the level of relative mRNA expression according to the RANKL pathway was investigated. RANKL-induced osteoclast differentiation is critical because it regulates TRAP (one of the proteins expressed due to osteoclast marker genes) and CTSK (one of the most potent proteases).[23] To determine the effect of MFSCCs on osteoclast differentiation and bone resorption in the

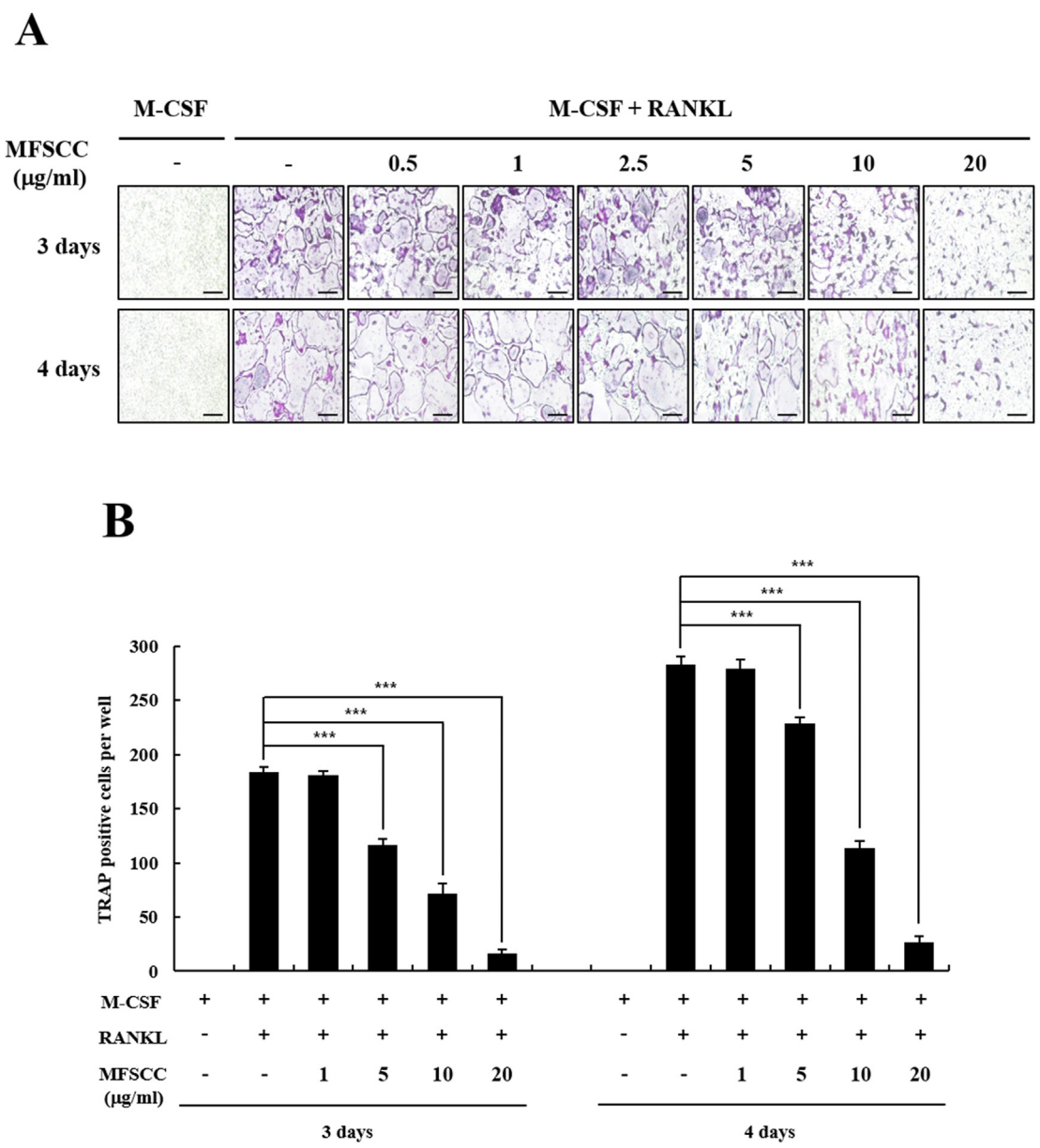

Figure 2 MFSCCs inhibit the maturation of TRAP activity in osteoclasts derived from BMMs. BMMs were treated with M-CSF and RANKL at the indicated MFSCCs concentrations on days 3 and 4. (A) Mature osteoclasts derived from BMMs were stained using a TRAP staining kit; microphotographs were analyzed using a light microscope (scale bars: 200 μm). (B) TRAP-positive cells containing three or more nuclei per wall were counted on days 3 and 4. All data are expressed as the mean ± standard deviation (***$P < 0.001$). BMM: bone marrow-derived macrophage, M-CSF: macrophage colony-stimulating factor, MFSCC: membrane-free stem cell components, RANKL: receptor activator of nuclear factor kappa B ligand, TRAP: tartrate-resistant acid phosphatase.

presence of RANKL, we observed that the expression levels of TRAP and CTSK, which are osteoclast-specific markers, decreased, suggesting that MFSCCs inhibit the RANKL-dependent signaling pathway for osteoclast bone resorption. Suppression of CTSK activity can prevent bone resorption.[27] Thus, TRAP and CTSK can be used to evaluate specific biochemical markers of osteoclastic functional activity. The relative mRNA expression of TRAP and CTSK was significantly inhibited by MFSCCs.

c-fos is a critical transcription factor involved in osteoclastogenesis. In a previous study, the role of c-fos was further established through experiments in which c-fos knockout mice exhibited an osteopetrotic phenotype.[28] In this experiment, treatment with MFSCCs led to a decrease in the mRNA expression level of c-fos. The activation of fos/activator protein-1, NF-κB, and NFATc1 constitutes an essential central part of the signals leading to osteoclastic bone loss.[29] This suggests that MFSCCs can downregulate c-fos expression by affecting the expression of signaling molecules associated with osteoclast differentiation. c-fos initiates the transcription of osteoclast-specific genes and induces differentiation and maturation of osteoclast precursors after binding to NFATc1.[29] The differentiation of osteoclast precursor cells into osteoclasts occurs even in

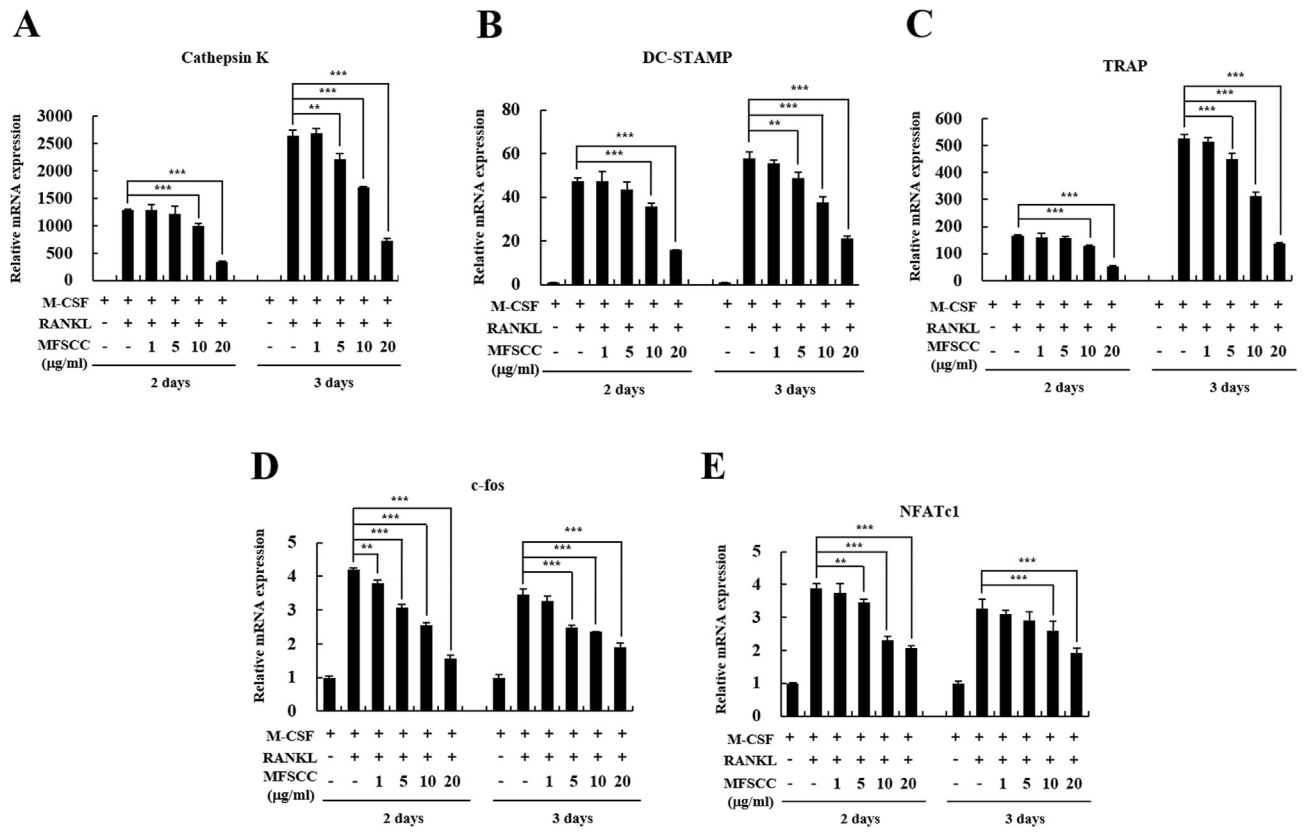

Figure 3 MFSCCs suppress the mRNA expression of genes associated with the differentiation of pre-osteoclasts derived from BMMs. BMMs were pretreated with M-CSF and RANKL at indicated MFSCC concentrations for indicated days. Total RNA from BMMs was isolated, and mRNA expression levels were evaluated using real-time PCR (RT-PCR). Relative mRNA expression levels of (A) c-fos, (B) NFATc1, (C) cathepsin K, (D) TRAP, and (E) DC-STAMP were evaluated. All data are expressed as the mean ± standard deviation (**$P < 0.01$ and ***$P < 0.001$). BMM: bone marrow-derived macrophage, DC-STAMP: dendritic cell-specific transmembrane protein, M-CSF: macrophage colony-stimulating factor, MFSCC: membrane-free stem cell components, NFATc1: nuclear factor of activated T-cells 1, RANKL: receptor activator of nuclear factor kappa B ligand, TRAP: tartrate-resistant acid phosphatase.

the absence of RANKL once NFATc1 is overexpressed.[30] NFATc1 affects osteoclast-specific genes and RANKL-dependent and independent differentiation signals. NFATc1 is an essential transcription factor for osteoclastogenesis. Treatment with MFSCCs reduced NFATc1 mRNA expression levels. Targeting the intermediate signaling factors of osteoclastogenesis can be performed in the clinic to suppress excessive osteoclast production in various bone destruction diseases.[31] Considering that MFSCCs suppress essential c-fos and NFATc1 transcription factors, the cells could be used in bone resorption diseases by targeting osteoclastogenesis. Moreover, cell-cell fusion is as critical as precursor cell differentiation for bone resorption; DC-STAMP is one of the key regulators of osteoclast cell-cell fusion.[32] The relative mRNA expression of DC-STAMP was significantly inhibited by MFSCCs; MFSCCs can affect cell-cell fusion processes, suggesting that MFSCCs suppress osteoclastogenesis by repressing osteoclast differentiation (via c-fos and NFATc1) and osteoclast maturation.

However, MFSCCs are relatively unfamiliar in dentistry, despite having been introduced in 2019 for their profiling and potential anti-inflammatory effects, as they are constructs derived from the internal contents of pluripotent adipose-derived stem cells.[15] While ADSCs are well-known for their pluripotency and regenerative benefits, their application in the oral cavity has been limited.[13] By removing the cell membrane and utilizing only the internal contents, MFSCCs can diminish unwanted immune responses. They have been developed to overcome limitations such as immune reactions and application sites to enhance their use. Numerous studies have demonstrated primarily their anti-inflammatory effects.[14,18,20] Research on the use of MFSCCs for regenerative tissue applications has gained popularity, but it has been limited to a few cell types, such as chondrocytes and keratocytes.[20,33] Therefore, this study is pioneering in examining the impact on the bone, as oral inflammation not only results in inflammatory responses but also leads to excessive bone resorption, which can worsen a patient's prognosis. Thus, it is crucial to look beyond inflammation and consider the effects on bone cells as well.[34]

In conclusion, this experimental study demonstrated that MFSCCs significantly inhibited osteoclast differentiation by regulating the levels of c-fos and NFATc1, which are key transcription factors and modulators of NF-κB activity (Fig. 5). If further experiments and evidence were to strengthen and validate our findings, our results suggest that MFSCCs can serve as therapeutic agents in oral

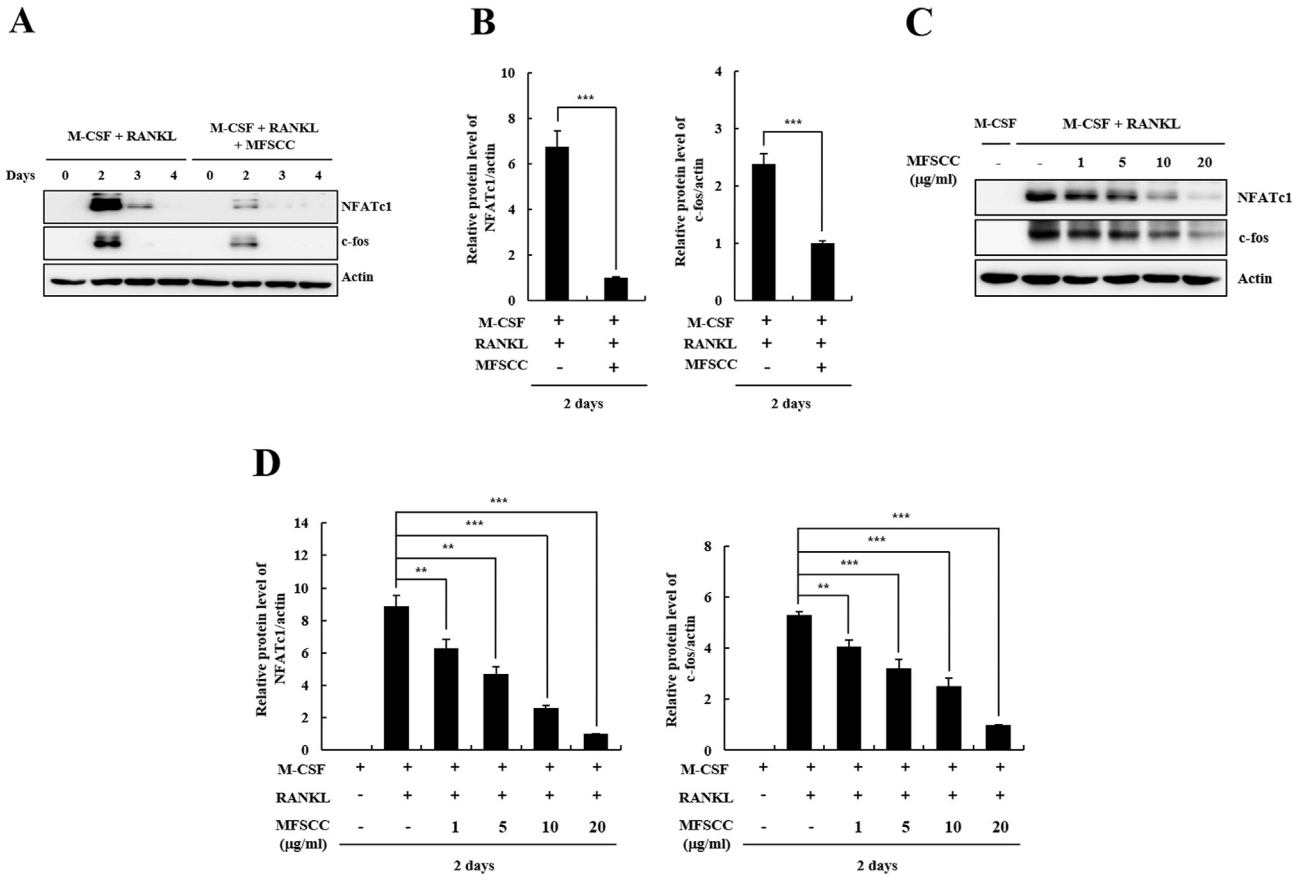

Figure 4 MFSCCs suppress the expression of proteins associated with the differentiation of pre-osteoclasts derived from BMMs. (A, B) BMMs were pretreated with M-CSF, RANKL, and 20 μg/mL MFSCCs for the indicated days. (C, D) BMMs were pretreated with M-CSF and RANKL at the indicated MFSCC concentrations. Protein expression of c-fos and NFATc1 was measured (A) over time and (C) in response to varying concentrations of MFSCCs using western blotting. Western blot analysis was used to determine the expression of proteins. Relative protein levels of differentiation-associated proteins/actin were evaluated (B) on day 2 (D) in response to varying concentrations of MFSCCs. All data are expressed as the mean ± standard deviation (**$P < 0.01$ and ***$P < 0.001$). BMM: bone marrow-derived macrophage, M-CSF: macrophage colony-stimulating factor, MFSCC: membrane-free stem cell components, NFATc1: nuclear factor of activated T-cells 1, RANKL: receptor activator of nuclear factor kappa B ligand.

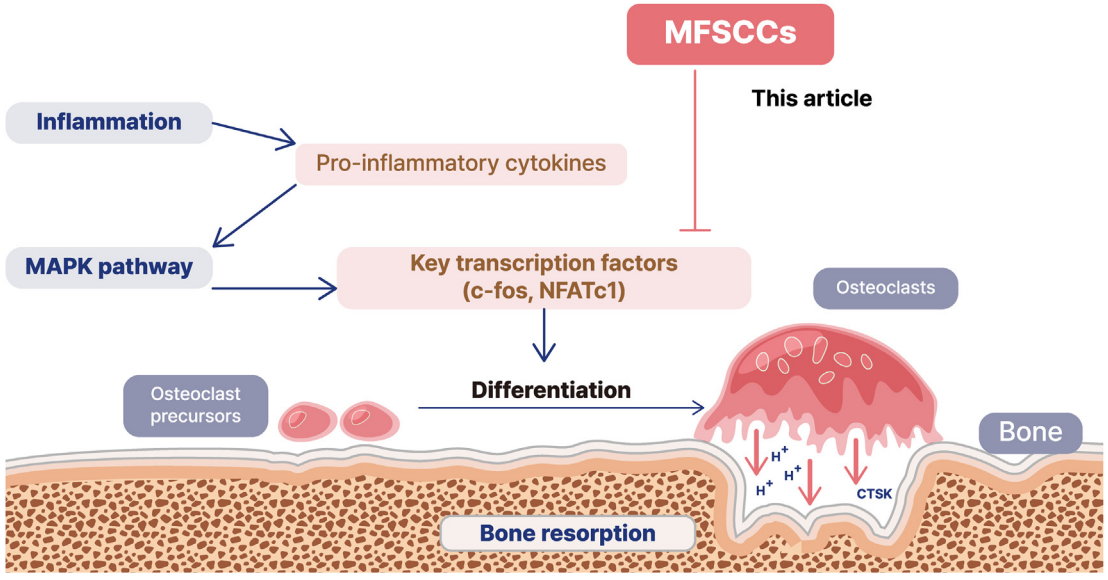

Figure 5 MFSCC-mediated regulatory network in osteoclast differentiation. CTSK: cathepsin K, MAPK: mitogen-activated protein kinase, MFSCC: membrane-free stem cell component, NFATc1: nuclear factor of activated T-cells 1.

regenerative medicine for inhibiting excessive inflammatory bone resorption.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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