APPENDIX 1: METHODS

Collection, Culture, and Identification of MSCs

Synovial fluid was obtained from the left knees of 12 rabbits with a meniscal injury of 4 weeks' duration (Injury group). Briefly, 2 mL of isotonic saline solution was injected into the knee joint, the knee was moved several times, and the synovial fluid was collected with saline solution. As a control, the synovial fluid was collected from contralateral knees that underwent sham operation (Sham group). Then, the synovial fluid was diluted with 5 volumes of phosphate-buffered saline (PBS) and filtered through a 70-μm filter to remove debris. After centrifugation, the collected cells were resuspended and plated in 6 culture dishes of 60 cm^2 in α-minimum essential medium (αMEM) complete medium with 10% fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin and incubated at 37°C in a 5% humidified CO_2 incubator. After 24 hours, the nonadherent cells were washed out with PBS, and the complete culture medium was changed every 3 days. The trilineage differentiation, culture, and immunophenotypic identification of MSCs were performed as described in a previous study.[3] Cells that adhered to the culture dish, formed cell colonies, expressed specific markers (positive for CD44, CD90, CD105, and CD166 and negative for CD14, CD34, CD45, and HLA-DR), and differentiated into chondrocytes, adipocytes, and osteoblasts in vitro were identified as MSCs.[1] The cells derived from synovial fluid were termed synovial fluid–derived MSCs (SMSCs). SMSCs at passage 2 and passage 3 were used in this study.

Colony-Forming Assay

Synovial fluid–derived cells from the Injury and Sham groups were plated in 60-cm^2 dishes at densities of 100 or 1000 cells per dish, cultured in complete medium for 2 weeks, and then stained with 0.5% crystal violet in methanol for 10 minutes. The number of colonies was counted. Colonies less than 2 mm in diameter and faintly stained colonies were disregarded.

Cell Viability

A CCK-8 (Cell Counting Kit-8) assay (Dojindo Laboratories) was performed to evaluate the viability of SMSCs cultured in the extracts (Mg extract group) according to the manufacturer's protocol. Briefly, at each time point, the cells were thoroughly washed with PBS and then submersed in 20 μL of CCK-8 working solution with 200 μL of fresh medium at 37°C for 2 hours. The optical density value was then measured at 450 nm by use of a plate reader. Cells cultured in complete medium with control extracts were used as a control (Control extract group).

Cell Adhesion Assay

SMSCs (1×10^6) suspended in 10 mL of PBS with 0, 1, 5, and 10 mM $MgSO_4$ and magnesium extracts were placed on 8-well culture slides with or without type I collagen coating (Becton Dickinson) for 10 minutes as previously described.[2] After the slides were washed with PBS, the nuclei were stained with Hoechst 33258 (1:800; Fanbo). Then, images of cells in 4 high-power fields (HPFs) were captured by use of confocal microscopy (Leica), and the numbers were counted with Image-Pro Plus 6.0 software (Media Cybernetics). Finally, the mean was determined. To neutralize against adhesion molecules, cells were preincubated in PBS with 20 mg/mL of integrin α2 (P1E6; Abcam), integrin α3 (P1B5; Santa Cruz Biotechnology), and integrin ß1 (P5D2; R&D Systems) antibodies for 1 hours.

Cell Chemotaxis Assay

A Transwell system (Costar 3422; Corning Inc) was used to determine whether magnesium extracts can regulate the migration of SMSCs through an in vitro chemotaxis assay as previously described.[20] In brief, 1×10^5 SMSCs suspended in 100 mL of serum-free αMEM plus 0.5% bovine serum albumin were added to the upper chamber. To induce chemotaxis, 60 mL of magnesium extract (serum-free αMEM) was added to the lower chamber (–/+).In addition, magnesium extracts were added to the upper and lower chambers to reveal chemokinesis (+/+). Because chemokine receptor 4 (CXCR4) is a specific cognate receptor expressed on the migratory cells, SMSCs were pretreated with AMD3100 (CXCR4-specific antagonist) to evaluate whether CXCR4 plays a role

in migration. SMSCs were pretreated with AMD3100 (10 μg/mL) for 2 hours at 37°C (AMD3100 –/+). Twenty-four hours later, the upper surface of the filters was scraped free of SMSCs and debris. The SMSCs that had migrated through the filter were fixed in 4% (vol/vol) paraformaldehyde and stained with Hoechst 33258. Then, the numbers were calculated as described above.

Real-Time Polymerase Chain Reaction Analysis

Real-time polymerase chain reaction (RT-PCR) was performed to determine gene expression. The expression of fibrochondrogenesis (*COL1Al, COL2A1, ACAN, TNC,* and *SOX9*), adhesion (integrin ß$_1$ [ITGB1], integrin α$_2$ [*ITGA2*], and integrin α$_3$ [ITGA3]), and chemotaxis gene markers (CXCR4) was assessed. For in vitro fibrochondrogenic differentiation, SMSCs were cultured in chemically defined medium (αMEM supplemented with 0.1 μM dexamethasone, 50.0 μg/mL ascorbate 2 phosphate, 40.0 μg/mL *L*-proline, and 100.0 μg/mL sodium pyruvate) and its supplements (6.25 μg/mL insulin, 6.25 μg/mL transferrin, and 6.25 ng/mL selenous acid) with 10 ng/mL transforming growth factor ß$_3$ (PeproTech) and 100 ng/mL connective tissue growth factor (PeproTech). After 2 weeks, total RNA of SMSCs cultured in the Mg extract group (fibrochondrogenic differentiation culture medium with magnesium extract medium) and the Control extract group (fibrochondrogenic differentiation culture medium with control extract medium) was isolated using TRIzol reagent (TaKaRa). Isolated RNA was reverse-transcribed into cDNA by use of a TaKaRa PrimeScript RT Reagent Kit. RT-PCR analysis was performed with the Bio-Rad CFX96 Real-Time PCR System with SYBR Premix Ex Taq (TaKaRa). The PCR primers are shown in Table 1. Cells were cultured in αMEM without any differentiation culture medium as a blank control. The expression levels of target genes were normalized against those of GAPDH (housekeeping gene), and relative levels were calculated by the $2^{-\Delta\Delta CT}$ method.

TABLE A1

Primer Sequences Used for Real-Time Polymerase Chain Reaction

Function	Gene	Forward Primers (5′-3′)	Reverse Primers (5′-3′)
Fibrochondrogenesis	COL1A1	TGGCAAGAACGGAGATGACG	GCACCATCCAAACCACTGAA
	COL2A1	CCACGCTCAAGTCCCTCAAC	AGTCACCGCTCTTCCACTCG
	ACAN	CGTGGTCTGGACAGGTGCTA	GGTTGGGGTAGAGGTAGACG
	TNC	TCTCTGCACATAGTGAAAAACAATACC	TCAAGGCAGTGGTGTCTGTGA
	SOX9	AGTACCCGCACCTGCACAAC	TACTTGTAGTCCGGGTGGTCTTTC
Adhesion	ITGB1	ACCAACCGTAGCAAAGGCAC	TGGGGTAGTCTTCAGCACGC
	ITGA2	CACTGAATCCCACTTGCGACA	AGAAAAGGCTGGGACGTTAGG
	ITGA3	CCCCGACTACAGGCGGAACAT	AGAAGGAGCCGTGGAGGACAG
Chemotaxis	CXCR4	GATGATGGAGTAGATGGTGGG	ACACTTCAGACAACTACACGGAG
Control	GAPDH	CCATCACCATCTTCCAGGAG	GATGATGACCCTTTTGGCTC

REFERENCES

1. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8(4):315-317.

2. Shimaya M, Muneta T, Ichinose S, Tsuji K, Sekiya I. Magnesium enhances adherence and cartilage formation of synovial mesenchymal stem cells through integrins. *Osteoarthritis Cartilage*. 2010;18(10):1300-1309.

3. Zhang Z, Ding Y, Li W, Song B, Yang R. Interleukin-17A- or tumor necrosis factor alpha-mediated increase in proliferation of T cells cocultured with synovium-derived mesenchymal stem cells in rheumatoid arthritis. *Arthritis Res Ther*. 2013;15(5):R169.