Synovial fluid-derived synovial fragments represent an improved source of synovial mesenchymal stem cells in the temporomandibular joint

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Abstract. Surgery-obtained synovium specimens (SSSs) can provide a source of synovial mesenchymal stem cells (SMSCs) for experimental studies. However, these specimens contain diverse tissues, including the intima and subintima; therefore, these SMSCs are not entirely derived from the intima and their cell source is heterogeneous. The present study isolated synovial fragments (SFs) from synovial fluid dilutions extracted from patients with temporomandibular joint (TMJ) osteoarthrosis. Unlike SSSs, SFs, which are membranous and translucent, consist of only several cell layers, indicating the presence of only the intima. In the present study, SF cells (SFCs) and SSS cells (SSSCs) exhibited a homogeneous, fibroblast-like, spindle-shaped morphology after passaging *in vitro*. Furthermore, both cell types exhibited similar proliferative and differentiation potentials *in vitro*. However, SFCs exhibited more uniform surface markers compared with SSSCs when analysed by flow cytometry. Taken together, these results indicated that SFs contained a greater amount of unmixed intima than SSSs, and that SFCs exhibited more homogeneous characteristics than SSSCs, thereby offering an improved source of SMSCs in the TMJ.

Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic, self-renewing cells that are capable of clone-forming and multilineage differentiation (1). MSCs have been detected in numerous adult tissues, including bone marrow, skeletal muscles, adipose tissues, synovial fluid and synovium (2-5). The presence and characteristics of MSCs in synovium specimens were first reported by De Bari *et al* (6) and have been studied extensively in recent years (7-13). Synovium specimen-derived MSCs (SSMSCs) have a higher proliferative capacity and chondrogenic potential than MSCs derived from other sources; therefore, these cells are regarded as a promising cell source for MSC-based therapeutic strategies used to treat cartilage damage (5,11,14-17).

Generally, synovium specimens are obtained through surgery, including open surgery or arthroscopic surgical procedures (18-21). Previous studies have demonstrated that MSCs can be isolated from surgery-obtained synovial specimens (SSSs) using the same protocol as that employed for synovial fibroblast cultivation. These cells exhibit ultrastructural and morphological features similar to those of type B synoviocytes (6,22). However, SSS cells (SSSCs) exhibit heterogeneity. For example, Harvanova *et al* (19) reported that 40-50% of SSSCs are cluster of differentiation (CD)105+ subpopulation cells prior to immunomagnetic separation. These data suggest that SSMSCs correspond to a subset of adherent cells in SSSs.

SSSs generally consist of at least two anatomically distinct layers: The synovium (intima) and the underlying layer (subintima). However, since there is currently no effective method for the separation of these two tissue layers, SSMSCs reported in previous studies were not entirely derived from the intima (5,6,9). Furthermore, no specific marker of synovial MSCs (SMSCs), which are derived from the intima only,

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has been identified to date. Therefore, the characteristics of SMSCs remain poorly understood.

The present study isolated and characterized synovial fragments (SFs) present in synovial fluid dilutions extracted from patients with temporomandibular joint (TMJ) osteoarthrosis. These synovial fluid-derived SFs consisted of several cell layers, indicating that they originated from the intima. Subsequently, the histological characteristics of SFs were compared with those of SSSs. Following isolation and expansion *in vitro*, the characteristics of both cell types were investigated, including *in vitro* proliferation and morphology, surface marker expression, and multilineage differentiation capabilities.

Materials and methods

Ethics statement. The present study was approved by the Institutional Ethics Board of the Hospital of Stomatology, Sun Yat-sen University (Guangzhou, China). Written informed consent was obtained from all subjects.

Collection of SFs and SSSs. SFs were collected, between October 2014 and April 2016, during TMJ arthrocentesis from patients with TMJ osteoarthrosis that showed no response to conservative treatment. Briefly, a no. 8 needle was punctured into the upper joint compartment. A total of 2.0 ml lidocaine was infused and then withdrawn. Diluted synovial fluid samples were collected from ~800 patients (age, 16-68 years), and SFs were obtained from 17 of these samples. These 17 patients (age, 18-61 years) had no other systemic diseases; among these patients, 3 were male and 14 were female. In addition, 8 SSSs (~0.3x0.5 cm) were obtained aseptically from patients with TMJ osteoarthrosis at the time of surgical debridement treatment for osteoarthrosis or joint disk perforation. The 8 donors (age, 25-50 years) had no other systemic diseases; among these patients, 1 was male and 7 were female.

Culture of human SFCs and SSSCs. SFs from the synovial fluid were washed three times and were then digested with 4 mg/ml type I collagenase for 2.5 h at 37˚C. The specimens were dispersed by pipetting and then filtered through a 200-mesh screen. Cells were centrifuged at 300 x g at room temperature for 5 min and cultured with complete culture medium [α-minimum essential medium (α-MEM)] supplemented with 10% fetal bovine serum (FBS) and 1X GlutaMAX (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in 5% CO_2. The SSSCs were isolated and cultured in the same manner as the SFCs.

Surface antigen expression profile. A total of 3 SFs and 6 SSSs samples were employed for surface antigen expression analysis. For surface marker detection, ~300,000 dissociated cells were collected. Following incubation with primary antibodies or isotype control antibodies for 30 min, the cells were centrifuged at 300 x g at room temperature for 8 min. The supernatant was discarded prior to resuspension of the cells. Flow cytometric analysis was performed using an FC 500 flow cytometer (Beckman Coulter, Miami, FL, USA), and the results were analysed using MXP Software version 2.0 (Beckman Coulter). The antibodies used are listed in Table I.

Cells obtained from 3 SFs and 3 SSSs (samples 4-6) were used for subsequent experiments.

Cell proliferation assay. SFCs and SSSCs derived from 3 samples were mixed and seeded in 96-well plates at a density of 800 cells/well at passage 4.

Cell proliferation was evaluated using Cell Counting kit-8 (Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China). The reagent was employed with the concentration of 10% per well. Culture medium with the reagent served as a blank control. The optical density (OD) of the supernatant was measured using a microplate reader (Infinite 200; Tecan Group, Ltd., Männedorf, Switzerland) after a 2-h incubation at 37˚C. Repeated measurements (n=3) were conducted at each time point. The population doubling (PD) and doubling time (DT) were evaluated using the following formulas: PD = (lnN - lnN0)/ln2 and DT = T/PD. N, OD_{cells} - $OD_{blank\ control}$ at the end point; N0, OD_{cells} - $OD_{blank\ control}$ at the initial time point; T, time interval; lnN=log(e,N).

Colony-forming assay for SFCs and SSSCs. SFCs and SSSCs (passage 4) were plated at a density of 5 cells/cm². After culturing in complete medium for 2 weeks, the cells were fixed in 4% paraformaldehyde for 10 min at room temperature and were then stained with 0.5% crystal violet for counting.

Differentiation of isolated SFCs and SSSCs.
Osteogenic differentiation. Cells were seeded at a density of 5,000 cells/cm² in 6-well plates and were induced to differentiate the next day. The differentiation medium consisted of high-glucose Dulbecco's modified Eagle's medium (H-DMEM) containing 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.), 10 mM sodium β-glycerophosphate (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), 100 nM dexamethasone (MP Biomedicals, LLC, Santa Ana, CA, USA) and 50 μg/l ascorbic acid-2-phosphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Adipogenic differentiation. Cells were seeded at a density of 5,000 cells/cm² in 6-well plates and were induced to differentiate the next day. The medium consisted of H-DMEM containing 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.), 200 mM indomethacin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 0.5 mM isobutyl methylxanthine, 1 mM dexamethasone and 10 mg/ml insulin (both MP Biomedicals, LLC). The medium was replaced every 3 days.

Chondrogenic differentiation. Approximately 300,000 cells were collected in a 15-ml centrifuge tube and were centrifuged at 300 x g at room temperature for 5 min. Subsequently, cells were resuspended in 450 μl medium consisting of H-DMEM, 1X insulin-transferrin-selenium-sodium pyruvate (both Gibco; Thermo Fisher Scientific, Inc.), 100 nM dexamethasone (MP Biomedicals, LLC), 50 mM ascorbic acid, 40 mg/ml proline (both Sigma-Aldrich; Merck KGaA) and 10 ng/ml transforming growth factor-β1 (PeproTech, Inc., Rocky Hill, NJ, USA) . After a 10 min centrifugation at 450 x g at room temperature, the cells were incubated at 37˚C in an atmosphere containing 5% CO_2.

Evaluation of osteogenic, adipogenic, and chondrogenic differentiation. After a 4-week osteogenic induction, the

Table I. Antibodies used in flow cytometry.

Antibody	Dilution	Supplier
Peridinin chlorophyll protein-Cy5.5-conjugated anti-human CD105	1:20	BD Biosciences[a]
Allophycocyanin-conjugated anti-human CD73	1:20	BD Biosciences
Fluorescein isothiocyanate-conjugated anti-human CD90	1:20	BD Biosciences
Phycoerythrin-conjugated anti-human CD44	1:20	BD Biosciences
Phycoerythrin-conjugated anti-human CD45/CD34	1:20	BD Biosciences
Phycoerythrin-conjugated anti-human CD45/CD34 CD11b/CD19/HLA-DR isotype control	1:20	BD Biosciences

[a]BD Biosciences, Franklin Lakes, NJ, USA. CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR; antibodies' kit cat. no. 562245.

cells were washed with PBS and were fixed with 4% paraformaldehyde at room temperature for 10 min. Subsequently, cells were stained with fresh 0.1% Alizarin Red S solution for 30 min at 37˚C and examined under an inverted phase contrast microscope (Axiovert 40; Carl Zeiss AG, Oberkochen, Germany).

Adipogenesis was assessed by Oil Red O staining after 4 weeks of induction. The cells were washed and fixed as aforementioned. Subsequently, 0.3% Oil Red O solution was used to stain the cells for 150-180 sec. The cells were then examined under an inverted phase contrast microscope (Axiovert 40; Carl Zeiss AG).

Histological staining was employed to assess chondrogenic differentiation after a 3-week induction. Cartilage nodules formed by the cells were fixed with 4% paraformaldehyde at 4˚C overnight and were then embedded in paraffin. The blocks were cut into 5 μm sections. The expression levels of collagen type II were then detected. Sections were incubated with rabbit anti-human collagen type II antibodies (Sigma-Aldrich; dilution, 1:80; cat. no. SAB4500366) in blocking buffer (10% goat serum sealant, cat. no. SL038; Solarbio, Beijing, China) for 16 h at 4˚C. Biotinylated goat anti-rabbit immunoglobulin G (cat. no. SA1022; Wuhan Boster Biological Technology, Ltd., Wuhan, China) was used as a secondary antibody incubating at 37˚C for 30 min and was detected using streptavidin-biotin complex reagent (cat. no. SA1022; Wuhan Boster Biological Technology, Ltd.). The staining was visualized with 3,3'-diaminobenzidine (cat. no. AR1022; Wuhan Boster Biological Technology, Ltd.) and was observed under a light microscope (Axioskop 40; Carl Zeiss AG). Sections incubated without the primary antibody served as a control.

Sulfated glycosaminoglycan (GAG) assays were performed to determine the levels of GAG. Chondrogenic nodules were digested overnight at 56˚C in 50 μg/ml proteinase K solution Santa Cruz Biotechnology, Inc.) diluted in 100 mM Na$_2$HPO$_4$ (pH 8.0), followed by inactivation for 10 min at 90˚C. After centrifugation, 500 μl working 1,9-dimethylmethylene blue (DMMB) solution (Santa Cruz Biotechnology, Inc.; 100 ml 1M GuHCl, 1 g sodium formate, 1 ml 98% formic acid, 25 ml 0.64‰ DMMB ethanol solution completed to 500 ml with distilled water was defined as solution A; 100 ml 1M GuHCl, 1 g sodium formate, 1 ml 98% formic acid, 25 ml 100% ethanol completed to 500 ml with distilled water was defined as solution B; working DMMB solution was made by rapidly mixing solution A with solution B) was added to 50 μl treated sample or standard sample. Subsequently, the samples were mixed vigorously for 30 min and centrifuged for 10 min at 12,000 x g at 4˚C. Once the supernatant was discarded, 1 ml DMMB decomplexation solution [50 mM sodium acetate (pH 6.8) added with 10% methyl alcohol, 4M GuHCl] was added. After a further 30 min of agitation, absorbance was examined at 656 nm using a microplate reader (Infinite 200; Tecan Group, Ltd.). Double-stranded DNA, which was obtained from the chondrogenic nodules digestion fluid, as detected by Quant-iT PicoGreen dsDNA reagent (Invitrogen; Thermo Fisher Scientific, Inc.), was used as an endogenous control.

Hematoxylin and eosin (HE) staining of SF and SSS sections and histological immunostaining of SSS sections. Sections were stained with Mayer's hematoxylin for 15 min and with eosin for 1 min. After gradient dehydration and clearing, the sections were mounted and observed under a light microscope (Axioskop 40; Carl Zeiss AG). Histological immunostaining of SSS sections was performed in the same manner as mentioned above. Mouse anti-human CD105 antibody (cat. no. ab11414; dilution, 1:200; Abcam, Cambridge, UK) was used as the primary antibody and biotinylated goat anti-mouae immunoglobulin G (cat. no. A1001; Wuhan Boster Biological Technology, Ltd.) was used as the secondary antibody.

Evaluation of gene expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol reagent (Invitrogen: Thermo Fisher Scientific, Inc.) and cDNA was synthesized using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturers' protocols. qPCR analyses were performed using a LightCycler® 480 Probes Master system (Roche Diagnostics) with an initial denaturation at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. A melting curve analysis was then performed. GAPDH was used as an internal control. Relative mRNA expression levels were evaluated using the following formula: $(Cq_{target\ gene} - Cq_{gapdh})$
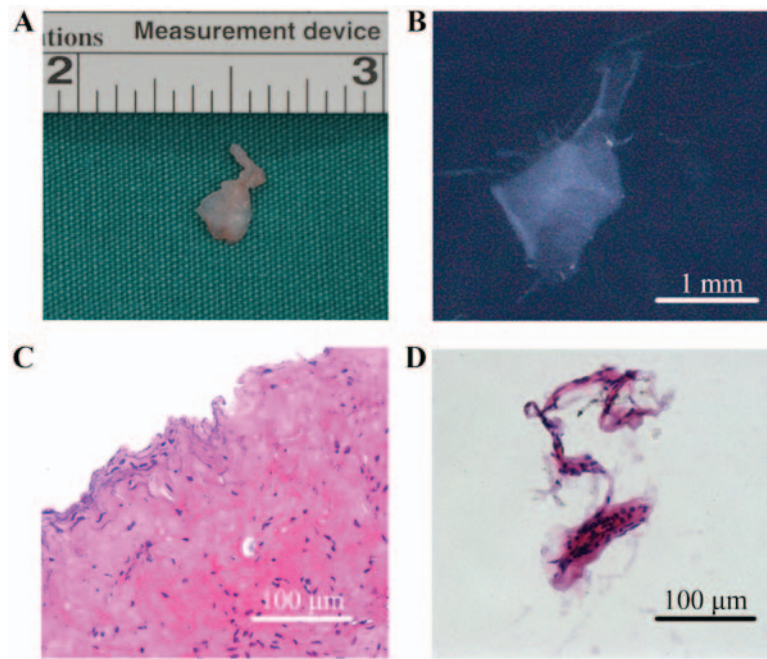
Figure 1. Characteristics of SFs and SSSs collected from patients with temporomandibular joint osteoarthrosis. (A) Gross morphology of SSSs; (B) gross morphology of SFs; (C) HE-stained histological section of SSSs; (D) HE-stained histological section of SFs. HE, hematoxylin and eosin; SFs, synovial fragments; SSSs, surgery-obtained synovium specimens.

Table II. Oligonucleotide primers used in quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')
GAPDH	F: GACAGTCAGCCGCATCTTCT
	R: TTAAAAGCAGCCCTGGTGAC
RUNX-2	F: TCAACGATCTGAGATTTGTGGG
	R: GGGGAGGATTTGTGAAGACGG
OCN	F: CCACCGAGACACCATGAGAG
	R: TCAGCCAACTCGTCACAGTC
ALP	F: ACCATTCCCACGTCTTCACATTTG
	R: AGACATTCTCTCGTTCACCGCC
PPARG2	F: GCAAACCCCTATTCCATGCTG
	R: CACGGAGCTGATCCCAAAGT
LPL	F: CAAGAGTGAGTGAACAAC
	R: AATTATGCTGAAGGACAAC
SOX-9	F: ACACACAGCTCACTCGACCTTG
	R: AGGGAATTCTGGTTGGTCCTCT
COL-2	F: GGCAATAGCAGGTTCACGTACA
	R: CGATAACAGTCTTGCCCCACTT

ALP, alkaline phosphatase; *COL-2*, type II collagen; F, forward; *LPL*, lipoprotein lipase; *OCN*, osteocalcin; ; *PPARG2*, peroxisome proliferator-activated receptor γ, transcript variant 2; R, reverse; *RUNX-2*, runt-related transcription factor 2; *SOX-9*, sex-determining region Y-box 9.

$_{sample}$ - $(Cq_{target\ gene}$ - $Cq_{gapdh})_{control}$ (23).PCR primer sequences are listed in Table II.

Statistical analysis. Data were statistically analysed using SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA). Experiments were repeated at least 3 times and numerical data are presented as the mean ± standard deviation. Student's t-test was used to analyze results for 2 independent groups. Comparisons between multiple groups were conducted using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization of SFs, SSSs, SFCs and SSSCs expanded in vitro. The SFs collected from patients with TMJ osteoarthrosis were membranous and translucent, whereas SSSs were masses of tissue (Fig. 1A and B). HE staining revealed that SSSs exhibited a more complex histological structure, containing intima and subintima (Fig. 1C), whereas the SFs were formed of several layers of cells only, indicating that they were obtained from the intima (Fig. 1D).

Adherent cells were obtained from SFs and SSSs. The SFCs and SSSCs both exhibited a typical fibroblastic spindle shape (Fig. 2A and B). In addition, both cell types exhibited clone-forming potential (Fig. 2C and D). The clone-forming rate of SFCs was slightly lower than that of SSSCs (Fig. 2E; P=0.014).

Cell proliferation curves demonstrated that SFCs exhibited a growth pattern similar to that of SSSCs (Fig. 2F). The PD and DT of SFCs were 2.58±1.01 and 30.73±5.90 h, respectively, whereas the PD and DT of SSSCs were 3.18±1.38 and 24.88±5.07 h, respectively, at passage 4.

Surface marker assays for SFCs and SSSCs. The results of a flow cytometric analysis indicated that >95% of SFCs derived from all 3 SFs expressed positive markers of MSCs, including CD90, CD44, CD73 and CD105. Negative markers of MSCs: CD11b, CD19, CD34, CD45 and human leukocyte antigen (HLA)-DR, were positive in <2% of cells (Fig. 3). In addition,
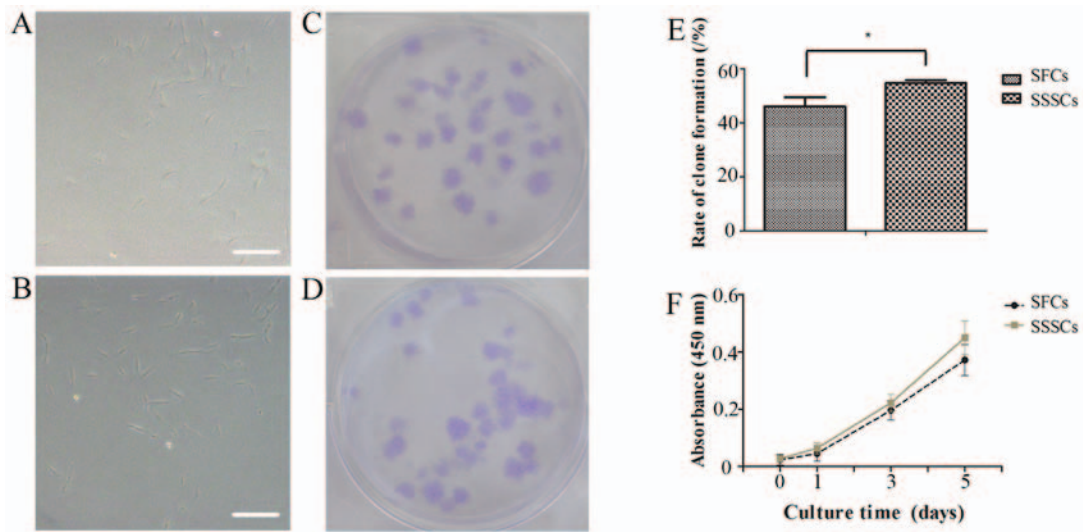
Figure 2. Clone-forming and proliferation assays for SFCs and SSSCs. Typical morphology of (A) SFCs and (B) SSSCs expanded *in vitro*. Scale bars, 100 μm. Clone formation of (C) SFCs and (D) SSSCs after 14 days of culture. (E) Clone-forming rates of SFCs and SSSCs after culturing *in vitro* for 2 weeks. (F) Cell growth curves for SFCs and SSSCs. *P<0.05. SFCs, synovial fragment cells; SSSCs, surgery-obtained synovium specimen cells.
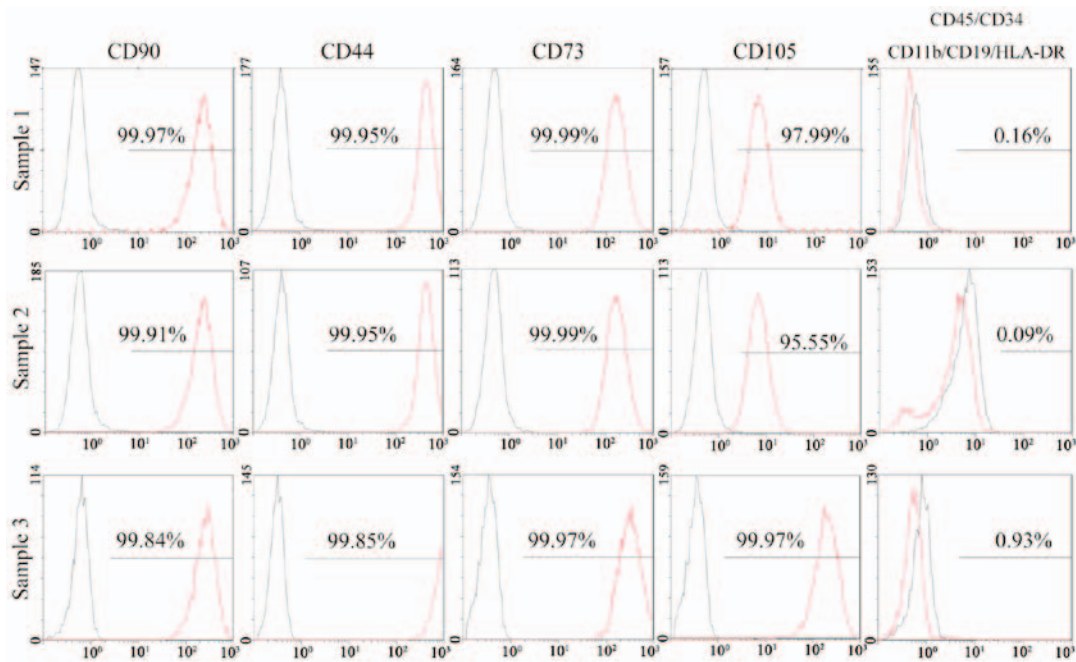

Figure 3. Flow cytometric analysis of SF cells derived from 3 SFs. The black lines represent negative controls and the red lines represent experimental groups. CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR; SF, synovial fragment.

95% of SSSCs derived from all 6 SSSs expressed CD90, CD44 and CD73. However, the percentage of CD105+ cells in two of the SSSs was much lower (32.36 and 74.08%), whereas that in the other four samples was >95%. Negative markers of MSCs were expressed in >2% of cells for 3 SSSs (Fig. 4). The results of immunohistochemical staining demonstrated that CD105+ cells were located in the intima and subintima, indicating that CD105 was not a specific marker for SMSCs (Fig. 5).

Differentiation potential of SFCs and SSSCs.
Osteogenic differentiation. Calcium deposits were detected in both groups after a 4-week osteogenic induction, as confirmed by Alizarin Red S staining (Fig. 6A-L). The expression levels of osteogenesis-associated genes, including runt-related transcription factor 2 (Fig. 6M), osteocalcin (Fig. 6N) and alkaline phosphatase (Fig. 6O), were significantly upregulated in induced SFCs and SSSCs compared with those in the corresponding control groups. However, there were no differences between the two induced groups. Results are presented in Table III.

Adipogenic differentiation. Oil red O-positive, lipid-laden fat cells were detected in SFCs and SSSCs after a 4-week adipogenic induction (Fig. 7A-F). In addition, the expression levels of peroxisome proliferator-activated receptor γ, transcript variant 2 (Fig. 7G) and lipoprotein lipase (Fig. 7H) were significantly upregulated in both induced cell types compared with in the control groups. No differences were detected
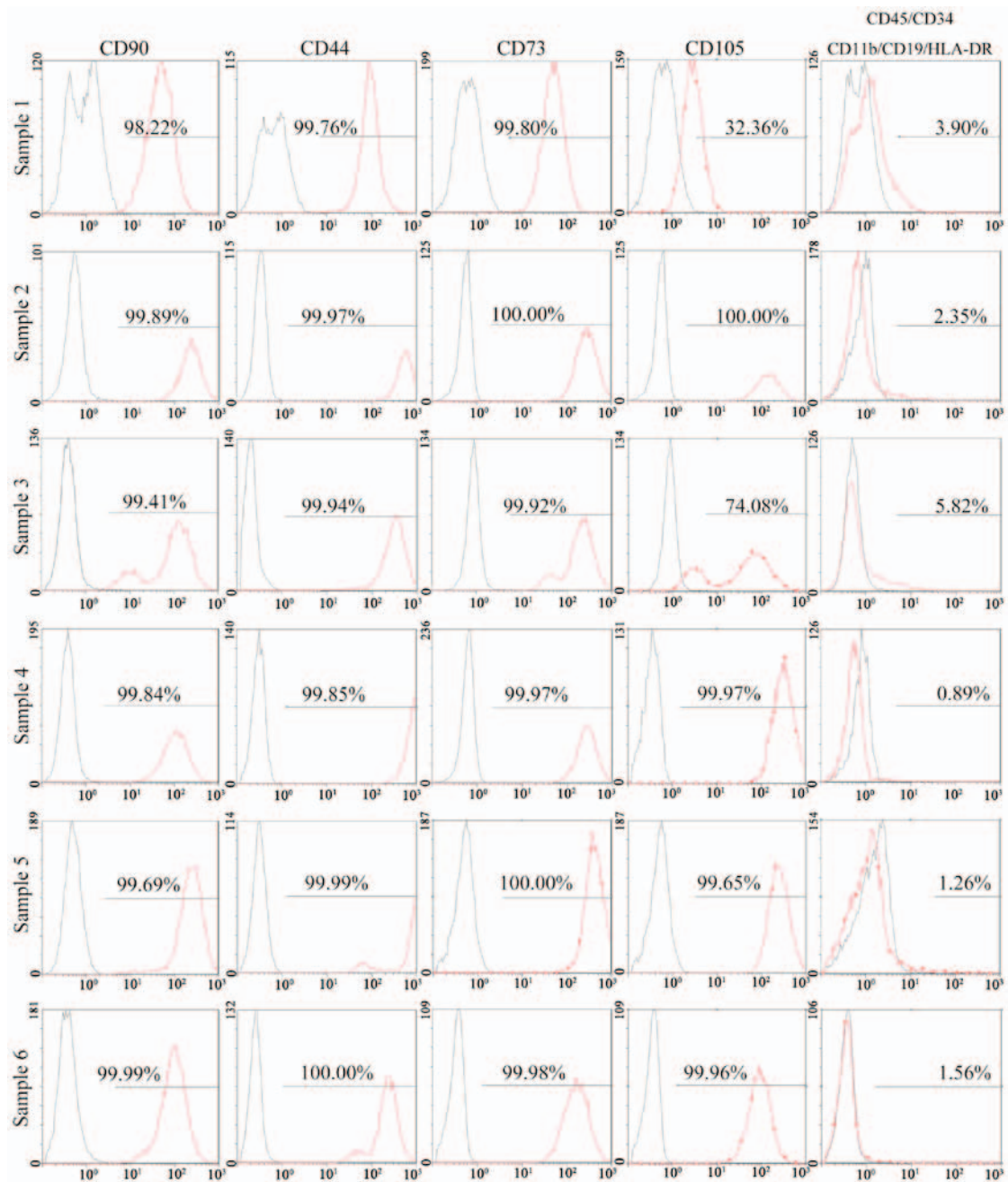
Figure 4. Flow cytometric analysis of SSS cells derived from 6 SSSs. The black lines represent negative controls and the red lines represent experimental groups. CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR; SSS, surgery-obtained synovium specimen.
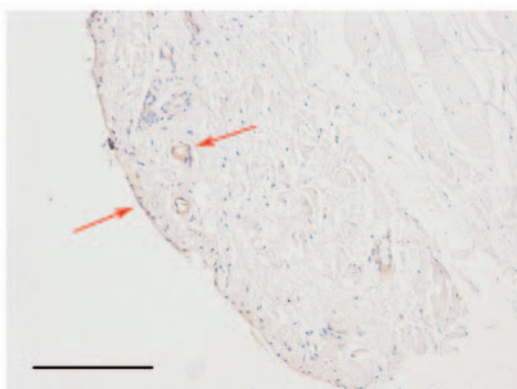

Figure 5. Immunohistochemical analysis of cluster of differentiation 105 in surgery-obtained synovium specimens. Scale bar, 100 μm.

between the two induced groups. Results are presented in Table III.

Chondrogenic differentiation. Chondrogenic differentiation was assessed by detection of cartilage nodules and collagen type II expression (Fig. 8). Cartilage nodules were formed by SFCs (Fig. 8A, E and I) and SSSCs (Fig. 8C, G and K) after a 3-week chondrogenic induction. In addition, collagen type II expression was detected in cartilage nodule sections formed by SFCs (Fig. 8B, F and J) and SSSCs (Fig. 8D, H and L), as confirmed by immunohistochemical staining. Immunofluorescence staining was also conducted on cells without induction, which served as the control group; however, no immunostaining was detected (data not shown). In addition, the mRNA expression levels of sex-determining region
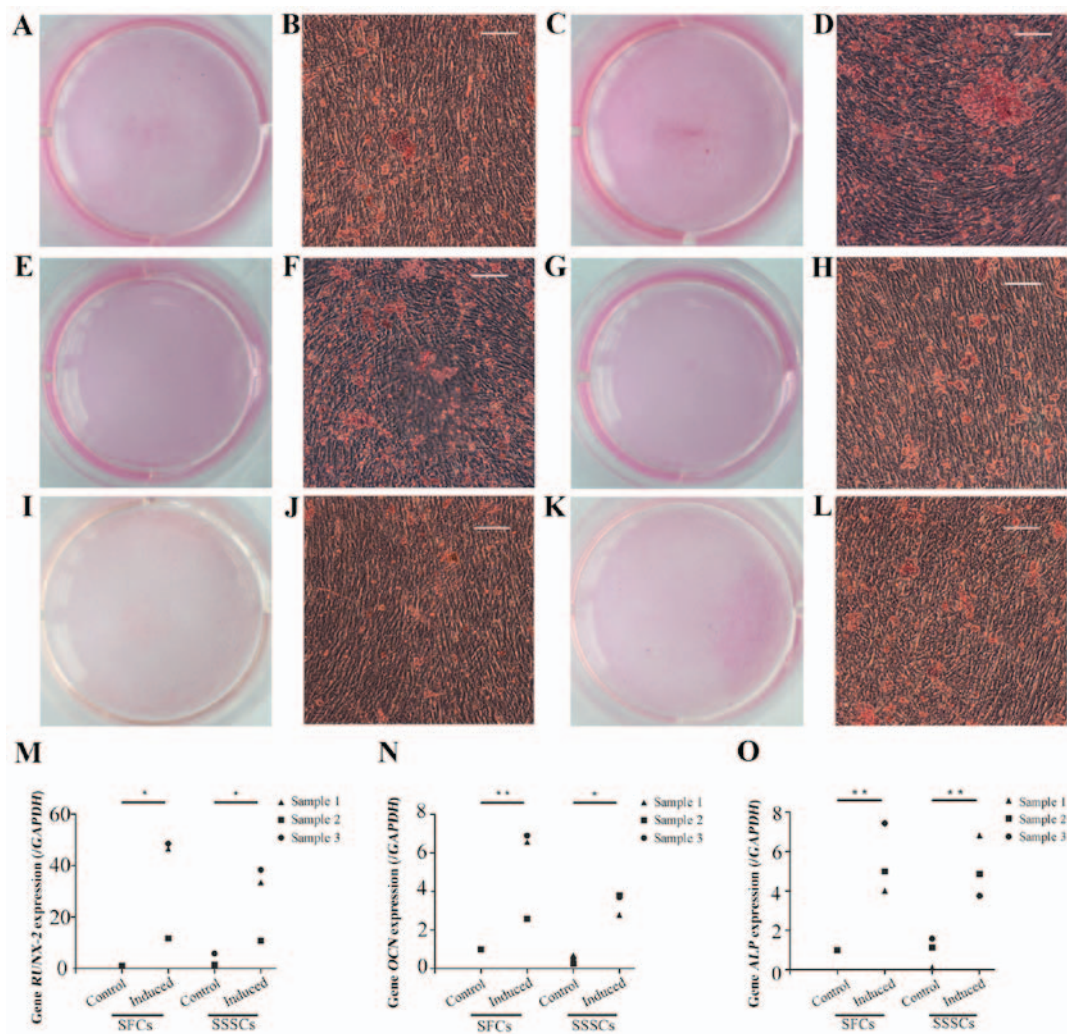
Figure 6. Osteogenic differentiation of SFCs and SSSCs. Calcium deposits were confirmed by Alizarin Red S staining in (A, B, E, F, I and J) SFCs and (C, D, G, H, K and L) SSSCs. Scale bars, 100 µm. qPCR results for (M) *RUNX-2*, (N) *OCN*, and (O) *ALP* expression in SFCs and SSSCs compared with those in the corresponding control groups. *P<0.05, **P<0.01. *ALP*, alkaline phosphatase; *OCN*, osteocalcin; *RUNX-2*, runt-related transcription factor 2; SFCs, synovial fragment cells; SSSCs, surgery-obtained synovium specimen cells.

Table III. Expression after multipotent differentiation.

| | | | | | P-values | | |
| | | | | | SFCs-control vs. | SSSCs-control vs. | SFCs-induced vs. |
Genes and proteins	SFCs-control	SFCs-induced	SSSCs-control	SSSCs-induced	SFCs-induced	SSSCs-induced	SSSCs-induced
RUNX-2	1.00±0.00	35.61±20.77	2.92±2.50	27.58±14.68	0.01	0.05	0.46
OCN	1.00±0.00	5.35±2.41	0.50±0.23	3.43±0.56	0.003	0.02	0.10
ALP	1.00±0.00	5.49±1.76	0.94±0.75	5.15±1.56	0.002	0.003	0.75
PPARG2	1.00±0.00	46.56±22.84	1.59±0.93	49.65±12.59	0.003	0.002	0.78
LPL	1.00±0.00	741.14±284.52	5.42±7.80	705.91±320.99	0.003	0.004	0.85
SOX-9	1.00±0.00	1.53±0.31	0.76±0.42	1.84±0.04	0.04	0.001	0.18
COL-2	1.00±0.00	6.00±0.82	0.88±0.15	6.58±0.31	<0.001	<0.001	0.15
GAG	1.36±0.06	7.61±0.55	1.36±0.06	8.07±1.81	<0.001	<0.001	0.57

ALP, alkaline phosphatase; *COL-2*, type II collagen; GAG, glycosaminoglycan; *LPL*, lipoprotein lipase; *OCN*, osteocalcin; *PPARG2*, peroxisome proliferator-activated receptor γ, transcript variant 2; *RUNX-2*, runt-related transcription factor 2; SFCs, synovial fragment cells; *SOX-9*, sex-determining region Y-box 9; SSSCs, surgery-obtained synovium specimen cells.

Table IV. Main differences between SFCs and SSSCs.

Properties	SFCs	SSSCs
Source of separated MSCs	Only from the intima (synovium)	Intima (synovium) and subintima, including MSCs from pericytes
Markers of MSC expression in primary cells	Higher uniformity	Less uniformity
Ethical controversy	Less	More

SFCs, spinal fragment cells; SSSCs, surgery-obtained synovium specimen cells; MSCs, mesenchymal stem cells.
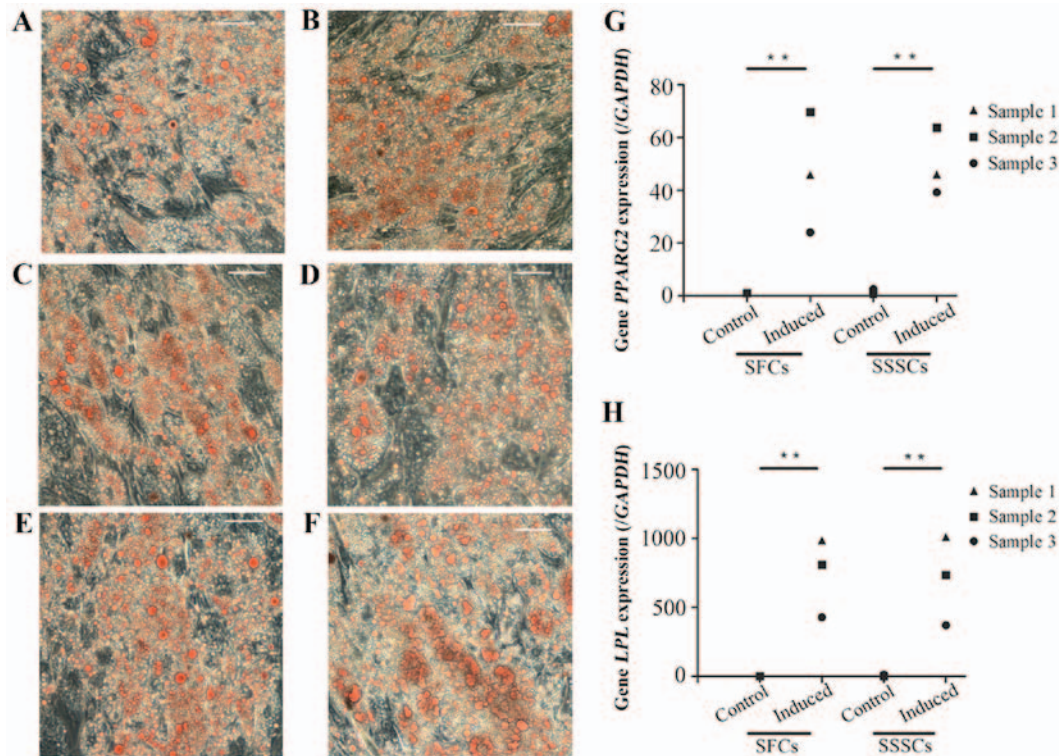

Figure 7. Adipogenic differentiation of SFCs and SSSCs. Oil red O staining of (A, C and E) SFCs and (B, D and F) SSSCs after induction for 4 weeks. Scale bars, 100 μm. qPCR results for (G) *PPARG2* and (H) *LPL* expression in SFCs and SSSCs compared with in the corresponding control groups. **P<0.01. *LPL*, lipoprotein lipase; *PPARG2*, peroxisome proliferator-activated receptor γ, transcript variant 2; SFCs, synovial fragment cells; SSSCs, surgery-obtained synovium specimen cells.

Y-box 9 (Fig. 8M) and type II collagen (Fig. 8N), and the production of GAG (Fig. 8O) were significantly upregulated in both cell types compared with in the control groups. No differences were detected between the two induced groups. Results are presented in Table III.

Discussion

The present study investigated the morphological characteristics of cells derived from SFs and SSSs. The main differences between SFCs and SSSCs are summarized in Table IV. The main similarities between the cell types were as follows: i) Positive expression of typical markers of MSCs, including CD44, CD73, CD90 and CD105, and negative expression of CD34, CD45, CD79α/CD19, CD14/CD11b and HLA-DR; ii) fibroblast-like and spindle-shaped morphology; iii) multi-lineage differentiation potential; and iv) clone-forming potential.

These cells exhibited similar morphological characteristics, and no significant differences in osteogenic, adipogenic or chondrogenic differentiation potential.

After the initial characterization of bone marrow stromal cells in the late 1960s, the concept of MSCs was established, and progenitor cells with similar properties have been isolated from various sources (24). In 2006, the International Society for Cellular Therapy (ISCT) set criteria for the definition of MSCs, defining MSCs as cells with the ability to adhere to plastic in standard culture conditions and to differentiate into osteogenic, adipogenic and chondrogenic lineages. Furthermore, according to the ISCT criteria, >95% of cells must express CD90, CD105 and CD73, whereas <2% of cells should express CD34, CD45, CD79α/CD19, CD14/CD11b and HLA-DR (14). In the present study, all 3 SFC samples met these criteria. Conversely, in 3 out of 6 SSS samples, the percentage of SSSCs that expressed negative markers was
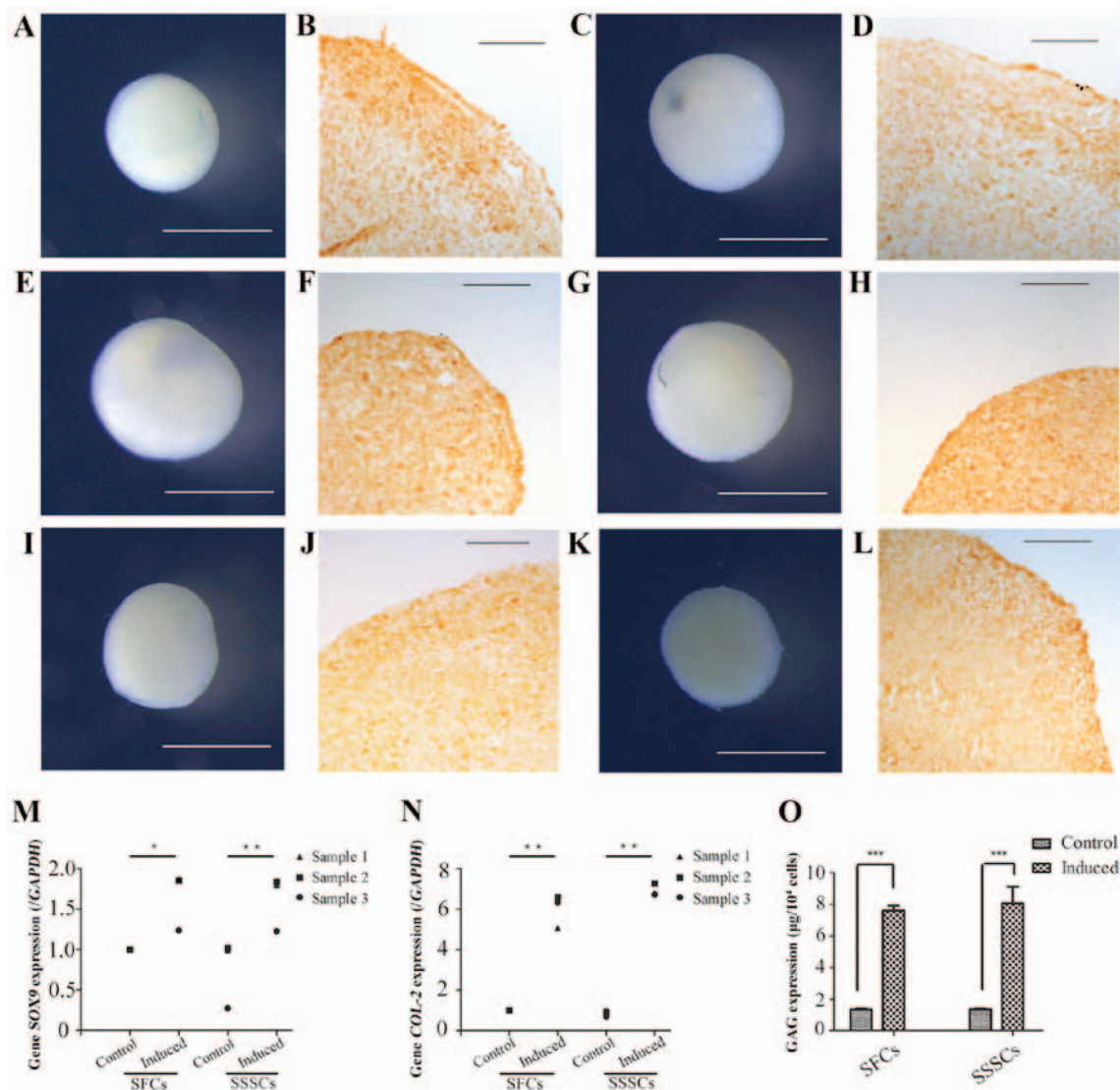
Figure 8. Chondrogenic differentiation of SFCs and SSSCs. Gross morphology of cartilage nodules formed by (A, E and I) SFCs and (C, G and K) SSSCs. Immunohistochemical staining of type II collagen in the cartilage nodules formed by (B, F and J) SFCs and (D, H and L) SSSCs. Scale bars, (B, D, F, H, J and L) 100 μm and (A, C, E, G, I and K) 1 mm. qPCR results for (M) *SOX-9* and (N) *COL-2* expression in SFCs and SSSCs compared with in the corresponding control groups. (O) GAG levels in cartilage nodules formed by SFCs and SSSCs. *P<0.05, **P<0.01 and ***P<0.001. *COL-2*, type II collagen; GAG, glycosaminoglycan; SFCs, synovial fragment cells; *SOX-9*, sex-determining region Y-box 9; SSSCs, surgery-obtained synovium specimen cells.

>2%. Furthermore, in 2 out of 6 SSS samples, the percentage of SSSCs that expressed CD105 was <95%.

Synovium samples obtained through arthroscopic or surgical procedures consist of two layers: The intima (synovial membrane) and subintima. The intima consists of several cell layers, whereas the subintima consists of various cells lying over loose connective tissue, alongside matrix proteins, fibroblasts, macrophages, lymphocytes, blood vessels and MSCs (25-29). MSCs are present in various areas of the joint, including the intima and subintima (30-32). Since there is no effective method for the separation of the intima and subintima, the SSMSCs reported previously were not only from the synovium (intima), but also from the subintima. Therefore, MSCs specifically from the intima (SMSCs) remain poorly understood.

The present study demonstrated that SFs were present in the synovial fluid obtained from patients with TMJ osteoarthrosis. Shearing forces, natural remodelling and relative tissue weakening from lack of nutrients may contribute to the avulsion of the superficial lining (32,33). SFs consist of several layers of cells only, indicating that this tissue is shed from the intima and may serve as a better source of SMSCs. Furthermore, SFs can be obtained by arthrocentesis, which is a common more acceptable treatment strategy for patients with TMJ osteoarthrosis, as it is less invasive than open surgery. Accordingly, SFs are a patient-friendly source for SMSCs, which may be used in studies regarding TMJ osteoarthrosis.

MSCs can be isolated from synovium samples and are thought to be a promising cell type for cartilage repair due to anatomical position (34,35). Compared with other MSC sources, the synovium is the closest tissue to articular cartilage, and SSSCs exhibit a higher chondrogenic capacity, and can be harvested through routine arthroscopic or surgical procedures (9,18,36,37). The sources of these MSCs are complex and include the intima, subintima and peripheral circulation. Due to vascular recruitment, MSCs located around the blood vessels

in the subintima may have misled studies regarding the origin of synovial membrane-derived MSCs (SMMSCs) (20,38,39). Li and Makarov generated animal models of rheumatoid arthritis and demonstrated that arthritic fibroblast-like synoviocytes contain a substantial (>30%) fraction of bone marrow-derived precursors that can differentiate into various mesenchymal cell types *in vitro* (40). Furthermore, the higher chondrogenic capacity of SMMSCs remains poorly understood. The present study indicated that the differentiation potentials of SFCs, including chondrogenic differentiation potential, were not significantly different from those of SSSCs, thus suggesting that the higher chondrogenic capacity of SMMSCs was not determined by MSCs residing in the intima.

No specific markers for SMSCs have been identified to date. Harvanova *et al* reported that 40-50% of cells isolated from synovial samples are positive for CD105 (19). CD105 serves as an immunomagnetic separation marker for isolating SMMSC populations (19). The present study also confirmed that CD105⁻ cells were present in the cell population isolated from SSSs, but not that isolated from SFs. However, CD105⁺ cells were present in the intima and subintima, indicating that CD105 was not a specific marker for SMSCs. Since SFs consist of only intima, this tissue may allow for the exploration of markers specific to SMSCs.

In conclusion, the present study isolated MSCs from SFs and demonstrated that these SFCs were similar in morphology, growth and trilineage differentiation potential to SSSCs. However, SFCs exhibited more homogeneous characteristics than SSSCs. Not only were SFCs more uniform than SSSCs in terms of MSC surface marker expression in primary cells, but SFCs derived from SFs also consisted only of intima (synovium), thus exhibiting more homogeneity with regards to cell source. Conversely, SSSCs were derived from SSSs, which consisted of intima and subintima. In addition, the source of separated MSCs from SSSs exhibited heterogeneity, since SSSCs may contain MSCs from pericytes. Unlike other joints, including knee joints, the size of SSSs are limited; therefore, removing excessive synovial specimens from the TMJ raises ethical concerns. However, the process for obtaining SFs is simpler and less invasive, thereby making it more acceptable to patients and more in compliance with ethical and moral standards. Therefore, SFs may serve as an improved cell source for the study of SMSCs. Notably, obtaining MSCs from the intima is a key step in the exploration of specific SMSC markers. In the present study, although SFCs were confirmed as MSCs derived from the intima, specific markers for SMSCs remain to be identified; therefore, our future studies aim to investigate these markers.

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