Human Mesenchymal Stem Cells in Synovial Fluid Increase in the Knee with Degenerated Cartilage and Osteoarthritis

Ichiro Sekiya,[1] Miyoko Ojima,[2] Shiro Suzuki,[2] Mika Yamaga,[2] Masafumi Horie,[2] Hideyuki Koga,[1] Kunikazu Tsuji,[3] Ken Miyaguchi,[4] Soichi Ogishima,[4] Hiroshi Tanaka,[4] Takeshi Muneta[2]

[1]Section of Cartilage Regeneration, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan, [2]Section of Orthopedic Surgery, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan, [3]Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases, Tokyo Medical and Dental University, Tokyo, Japan, [4]Department of Bioinformatics, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan

Received 11 January 2011; accepted 17 November 2011
Published online 6 December 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.22029

ABSTRACT: We investigated whether mesenchymal stem cells (MSCs) in synovial fluid (SF) increased in the knee with degenerated cartilage and osteoarthritis. SF was obtained from the knee joints of 22 patients with anterior cruciate ligament (ACL) injury during ACL reconstruction, and cartilage degeneration was evaluated arthroscopically. SF was also obtained from the knee joints of 6 healthy volunteers, 20 patients with mild osteoarthritis, and 26 patients with severe osteoarthritis, in which the grading was evaluated radiographically. The cell component in the SF was cultured for analyses. Synovium (SYN) and bone marrow (BM) were also harvested during total knee arthroplasties. The MSC number in SF was correlated with the cartilage degeneration score evaluated by arthroscopy. The MSC number in the SF was hardly noticed in normal volunteers, but it increased in accordance with the grading of osteoarthritis. Though no significant differences were observed regarding surface epitopes, or differentiation potentials, the morphology and gene profiles in SF MSCs were more similar to those in SYN MSCs than in BM MSCs. We listed 20 genes which were expressed higher in both SYN MSCs and SF MSCs than in BM MSCs, and 3 genes were confirmed by quantitative RT-PCR. MSCs in SF increased along with degenerated cartilage and osteoarthritis. © 2011 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 30:943–949, 2012

Keywords: mesenchymal stem cells; synovial fluid; cartilage degeneration; osteoarthritis; synovium

Mesenchymal stem cells (MSCs) can be defined as being derived from mesenchymal tissue and having the functional capacity for self-renewal, commonly identified by colony-forming unit fibroblast assay[1] and generation of a number of differentiated progeny.[2] We previously reported that MSCs in synovial fluid (SF) from anterior cruciate ligament (ACL) injury patients were 100 times more in number than those from healthy volunteers, and that the MSC number was positively correlated with post-injury period.[3] During ACL reconstruction, cartilage degeneration was also observed at high rates,[4] and ACL transection is one of the most widely used animal models for cartilage degeneration.[5] Therefore, there seems to be three intercorrelations among three conditions; ACL injury, cartilage degeneration, and MSCs in SF. The first purpose of this study was to investigate a direct relation between the number of MSCs in SF and cartilage degeneration in the ACL injured knees.

The existence of MSCs in SF of osteoarthritis knees was first reported by Jones et al.[6] They further reported in 2008 that MSCs in SF numerically increased in early osteoarthritis.[7] This review is of value because it shed light on the roles of MSCs in SF of osteoarthritis knees. However, the design of their study seemed to be inappropriate. All SF examined was obtained from patients with unexplained knee pain and from those who underwent arthroscopy. Furthermore, the nonosteoarthritis group they studied consisted of individuals with meniscal tears in addition to individuals without damage to the articular cartilage. Our second purpose was to investigate more strictly whether MSCs in SF increased in the knee with osteoarthritis.

MSCs can be obtained from various tissues, and they contain common features. However, an increasing number of reports have described variant properties dependent on cell sources.[8–11] In ACL injured knees, the gene profile of SF MSCs was more similar to that of synovium (SYN) MSCs than to that of bone marrow (BM) MSCs,[3] however, different pathological conditions may have led to different properties of synovial MSCs. Our third purpose was to perform patient-matched quantitative comparisons of the properties of SF MSCs, synovial MSCs, and BM MSCs. The properties examined included cell morphology, surface epitopes, chondrogenic, adipogenic, and osteogenic differentiation potentials, and gene profiles.

MATERIALS AND METHODS

Synovial Fluid Derived from ACL Injury Patients

The study was approved by an institutional review board, and informed consents were obtained from all study subjects. SF was obtained from the knee joints of 22 patients with ACL injury of the knees during ACL reconstruction under spinal anesthesia. The average age was 26 years, and the range was 12–45 years. During the operation, 6 areas consisting of medial femoral condyle, medial tibial plateau, lateral femoral condyle, lateral tibial plateau, patella, and femoral groove of cartilage were evaluated arthroscopically as 1 (normal), 2 (softening), 3 (fissure), 4 (more than half

Ichiro Sekiya and Miyoko Ojima contributed equally to this study.

Correspondence to: Ichiro Sekiya (T: +81-3-5803-4020; F: +81-3-5803-4020; E-mail: sekiya.orj@tmd.ac.jp)

© 2011 Orthopaedic Research Society. Published by Wiley Periodicals, Inc.

cartilage thickness), 5 (less than half cartilage thickness), 6 (full thickness cartilage defect), or 7 (bone defect), respectively, and the total number[6–28] was calculated for the cartilage degeneration score.

Synovial Fluid Derived from Osteoarthritis Patients

Synovial fluid was obtained from the knee joints of 6 healthy volunteers, 35-year old on average, ranging from 30 to 45, and from 20 patients with osteoarthritis of the knee at Grades 1 and 2 of Kellgren–Lawrence at our outpatient clinic. The patients were 50-year old on average, ranging from 40 to 60. SF was also obtained from the knee joints of 26 patients with osteoarthritis of the knee at Grades 3 and 4 of Kellgren–Lawrence during total knee arthroplasty under spinal anesthesia. The patients were 75-year old on average, ranging from 65 to 85. Kellgren–Lawrence grading was evaluated by radiographs of weight bearing posterior–anterior view at 45° of flexion of the knee.[12]

Cultures of Colony-Forming Cells in Synovial Fluid

Synovial fluid was diluted with phosphate-buffered saline (PBS), filtered through a 70 μm nylon filter (Becton Dickinson, Franklin Lakes, NJ) to remove debris, and plated in six culture dishes of 60 cm^2 (Nalge Nunc International, Rochester, NY) in complete culture medium: α-modified essential medium (α-MEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Invitrogen). The dishes were incubated at 37°C with 5% humidified CO_2. After 24 h, the adherent cells were washed with PBS. Fourteen days after initial plating, three dishes were stained with 0.5% crystal violet (Wako, Osaka, Japan) in 4% paraformaldehyde for 5 min, and the number of colonies was counted. Colonies less than 2 mm in diameter and faintly stained colonies were ignored. The other three dishes were harvested with 0.25% trypsin and 1 mM EDTA (Invitrogen; Passage 0), replated at 500 cells/cm^2 in a 145-cm^2 culture dish (Nalge Nunc International) and cultured for 14 days for further analyses.

In Vitro Differentiation Assay

For chondrogenesis, 250,000 cells were placed in a 15 ml polypropylene tube (Becton-Dickinson, Franklin Lakes, NJ), centrifuged at 450*g* for 10 min, and cultured in chondrogenesis medium containing 1,000 ng/ml BMP-7 (Stryker Biotech, Hopkinton, MA), 10 ng/ml transforming growth factor-β3 (R&D Systems, Minneapolis, MN), and 100 nM dexamethasone (Sigma–Aldrich Corp., St. Louis, MO) for 14 days. For microscopy, the pellets were embedded in paraffin, cut into 5 μm sections, and stained with toluidine blue.[13]

For adipogenesis, cells were cultured in adipogenic medium which consisted of complete medium supplemented with 100 nM dexamethasone (Sigma–Aldrich Corp.), 0.5 mM isobutyl-methylxanthine (Sigma–Aldrich Corp.), and 50 μM indomethacin (Wako) for 21 days. The adipogenic cultures were fixed in 4% paraformaldehyde and then stained with fresh oil red-O solution.[14] For quantification, isopropyl alcohol was added to the stained culture dish. After 5 min, the absorbance of the extract was assayed by a spectrophotometer at 510 nm after dilution to a linear range.[15]

For osteogenesis, cells were cultured in osteogenesis medium that comprised complete medium consisting of 1 nM dexamethasone, 10 mM β-glycerol phosphate (Wako), and 50 μg/ml ascorbate-2-phosphate (Sigma–Aldrich Corp.) for 21 days. The dishes were stained with 0.5% alizarin red solution.[16] For alkaline phosphatase activity (ALP), the cells were harvested with lysis buffer (0.1 M Tris–HCl, 5 mM $MgCl_2$, 2% Triton-X 100, and 1 mM phenylmethylsulfonyl fluoride) and sonicated. An aliquot (10 μl) of supernatant was added into 100 μl 50 mM *p*-nitrophenylphosphatase hexahydrate containing 1 mM $MgCl_2$, and the mixture was incubated at 37°C for 30 min. The absorption at 405 nm was measured with a spectrophotometer. ALP activity represented millimoles of *p*-nitrophenol release after 30 min of incubation at 37°C.[16]

Isolation and Culture of Synovium and Bone Marrow MSCs

During total knee arthroplasty, SYN and BM were also collected. SYN was harvested from the bony side of the suprapatellar pouch, digested in a 3 mg/ml collagenase D solution (Roche Diagnostics, Mannheim, Germany) for 3 h. BM was aspirated from the tibia, and the nucleated cells were separated with a Ficoll density gradient (Ficoll-Paque; Pharmacia Biosystems, Uppsala, Sweden). Nucleated cells from SYN and BM were plated in a 60-cm^2 dish and cultured in a similar method as described earlier.

Epitope Profile

One million cells were resuspended in 200 μl PBS containing 20 μg/ml antibody. After incubation for 30 min at 4°C, the cells were washed with PBS and re-suspended in 1 ml PBS for flowcytometric analysis. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-coupled antibodies against CD34, CD45, CD90, and CD146 (BD), CD44 (eBioscience, San Diego, CA), CD105, CD166 (Ancell, Bayport, MN), and CXCR4 (R&D Systems) were used. For STRO-1 staining, the cells were incubated for 30 min with an antibody against STRO-1 (mouse IgM; Genzyme-Techne Minneapolis, MN). The cells were then incubated with a secondary antibody (fluorescein-conjugated goat anti-mouse IgM; Vector Laboratories, Burlingame, CA) for 30 min. For D7-FIB staining, the cells were incubated for 30 min with an antibody against D7-FIB (mouse IgG; Serotec, Kidlington, UK). The cells were then incubated with a secondary antibody (fluorescein-conjugated rabbit anti-mouse IgG; Serotec) for 30 min. The cells were also incubated with a secondary antibody (fluorescein-conjugated rabbit anti-goat IgG; Southern Biotech, Birmingham, AL) for 30 min. For isotype control, FITC- or PE-coupled nonspecific mouse immunoglobulin G (IgG; BD) was substituted for the primary antibody. Cell fluorescence was evaluated by flow cytometry using a FACSCalibur instrument (BD). The data were analyzed using CellQuest software (BD).

Microarray Analysis

Three samples in each group were analyzed. Human genome-wide gene expression was examined on a total 28,000 genes with the Human Gene 1.1 ST Array (GeneChip; Affymetrix, Santa Clara, CA). Total RNA was prepared from colony-forming cells (Passage 1) derived from SYV, SF, and BM using TRIzol solution (Invitrogen). Sense-strand cDNA probes for hybridization were synthesized using the WT Expression Kit for Affymetrix GeneChip Whole Transcript Expression Arrays (Ambion, Foster City, CA). The microarray data were analyzed using the Robust Multichip Average method[17] (Bioconductor, http://www.bioconductor.org). A heatmap was constructed by hierarchical clustering on highly differentially expressed genes which show >5.0- or

<0.2-fold changes in any pairs of samples (SYV/SF, SYV/BM, and SF/BM). To identify the genes showing (1) high expressions in both SYV and SF, and (2) low expressions in BM, expression values in triplicates were averaged and filtered by the following threshold for fold changes; SYV/BM > 5 and SF/BM > 5; and listed in descending order of SYV/BM.

Quantitative Real-Time PCR

First-strand cDNAs were synthesized using a Transcriptor First-strand cDNA synthesis kit (Roche Applied Sciences, Indianapolis, IN), and Q-PCR analyses were performed using a LightCycler 480 Probe Master system (Roche). β-Actin was used as an internal control. Relative mRNA expression levels were calculated as described by Niikura et al.[18] PCR primers were as follows: SMOC2 5′-AAGGAAGTATACCCAGG AGCAA-3′ (forward), 5′-GTGTAGCTGTGACACTGGACCT-3′ (reverse); GPR133 5′-TATACGCGGGACAATTCCAT-3′ (forward), 5′-AAATAGGACATGAGTCCAA TAGGG-3′ (reverse); SFRP4 5′-GCCTGAAGCCATCGTCAC-3′ (forward), 5′-CCAT-CATGTCTGGTGTGATGT-3′ (reverse); β-actin 5′-ATTGGC-AATGAGC GGTTC-3′ (forward), 5′-TGAAGGTAGTTTCGT-GGATGC-3′ (reverse).

RESULTS

Synovial Fluid MSCs and Cartilage Degeneration

A large number of colonies consisting of spindle shaped cells were observed after 14 days culturing of cell components in SF of the knee with ACL injury (Fig. 1A). The colony-forming cells differentiated into chondrocytes, adipocytes, and calcified when cultured in the differentiation medium (Fig. 1B). These findings indicate that colony-forming cells, which originally existed in syonvial fluid, had characteristics of MSCs. The MSC number in the SF was correlated to the cartilage degeneration score evaluated with arthroscopy (Fig. 1C).

Synovial Fluid MSCs and Osteoarthritis

Representative culture dishes indicated that the colony number of SF MSC increased according to the radiological osteoarthritis grading by Kellgren–Lawrence (Fig. 2A). Comparing the normal (Grade 0), mild (Grades 1 and 2), and severe (Grades 3 and 4) osteoarthritis groups, the MSC number in the SF increased along with the grading of the osteoarthritis (Fig. 2B).

Comparison among Synovium MSCs, Synovial Fluid MSCs, and Bone Marrow MSCs from Osteoarthritis Patients

Morphologically, SF MSCs appeared to be closer to SYN MSCs than to BM MSCs, in that SYN MSCs and SF MSCs were narrower, and their nuclei were more obvious (Fig. 3A). For surface epitopes, 3 populations of MSCs were positive for CD44, CD90, CD105, and negative for CD34, CD45. These results were identical to those of distinctive MSCs, and there were no significant differences of surface epitopes among the 3 populations (Fig. 3B). After MSC pellets were cultured in chondrogenic medium, they became cartilage pellets whose matrix appeared as purple from toluidine blue staining in the 3 populations (Fig. 3C). There were no significant differences of pellet weights, an indicator of

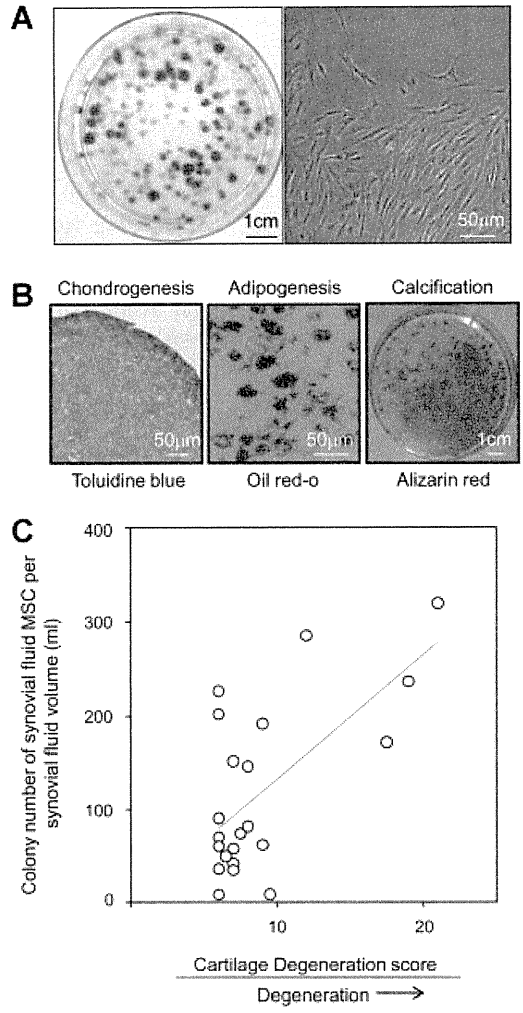

Figure 1. Colony-forming cells in synovial fluid derived from ACL injury patients. (A) Representative cell colonies stained with crystal violet and cell morphology. (B) Multidifferentiation potential. (C) Relationship between cartilage degeneration score and colony number of synovial fluid MSCs per synovial fluid volume (ml; $r^2 = 0.64$, $P = 0.002$ by correlation analysis).

cartilage matrix synthesis, among the 3 populations (Fig. 3D). After culturing in adipogenic medium, lipids showing red by oil red-O staining were observed in the 3 populations (Fig. 3E). There were no significant differences of absorbance at 510 nm for extraction of oil red-O among the 3 populations (Fig. 3F). In addition, there were no significant differences of ALP activity among the 3 populations of MSCs both when MSCs were cultured in osteogenesis medium containing 10 mM β-glycerol phosphate and in osteogenesis medium containing 20 mM β-glycerol phosphate (Fig. 3G).

Comparison of the Gene Expression Profiles among Synovium MSCs, Synovial Fluid MSCs, and Bone Marrow MSCs from Osteoarthritis Patients

Hierarchical clustering analysis demonstrated that gene profiles in SF MSCs were more similar to those in SYN MSCs than to those in BM MSCs (Fig. 4A). To demonstrate specific genes which were expressed higher in both SYN MSCs and SF MSCs than in BM

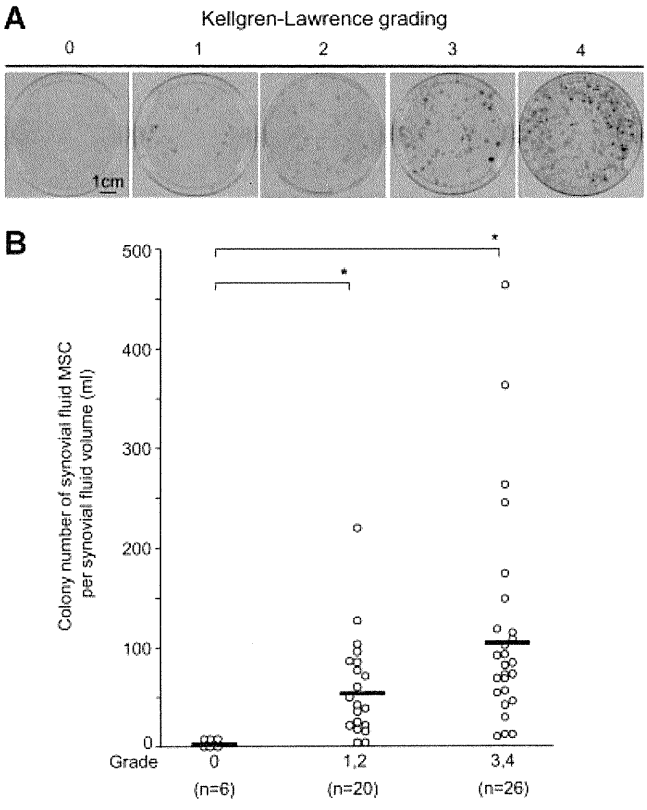

Figure 2. MSCs in synovial fluid derived from osteoarthritis patients. (A) Representative dishes for colonies of synovial fluid MSCs according to the Kellgren–Lawrence grading system. (B) Relationship between the osteoarthritis grading and the colony number of synovial fluid MSCs per synovial fluid volume (ml). Average values are shown as bars ($P = 0.002$ by Kruskal–Wallis test; $^*P < 0.05$ by Steel–Dwass test).

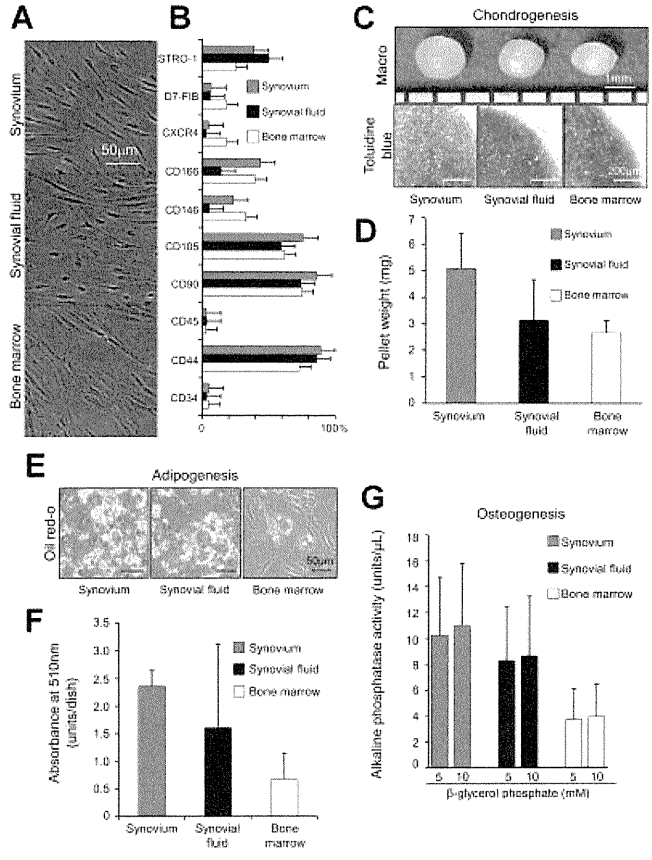

Figure 3. Comparison among synovium MSCs, synovial fluid MSCs, and bone marrow MSCs from osteoarthritis patients. (A) Representative morphologies. (B) Surface epitopes of MSCs. Positive expression rates are displayed as the average and standard deviation ($n = 5$). (C) Chondrogenesis. (D) Wet weight of the cartilage pellets. (E) Adipogenesis. (F) Quantification of adipogenesis by absorbance at 510 nm for extraction of oil red-O. (G) Alkaline phosphatase activity. MSCs were cultured in osteogenesis medium containing 5 and 10 mM β-glycerol phosphate.

MSCs, 20 genes are listed in Table 1. For SMOC2, GPR 133, and SFRP4 genes, which are listed as the top 3, quantitative RT-PCR analyses were performed in five other donors with osteoarthritis, and expressions of these genes were much higher in SYN MSCs and SF MSCs than in BM MSCs (Fig. 4B).

DISCUSSION

In this study, we directly demonstrated a correlation between number of MSCs in SF and cartilage degeneration in the ACL injured knees. However, variability still seems to exist in a population with a minimum cartilage degeneration score which is shown in Figure 1C. The number of SF MSCs per volume was 119 ± 92 in ACL injured knees, while it was 60 ± 52 in Grade 1, 2 osteoarthritis knees, and 115 ± 109 in Grade 3, 4 osteoarthritis knees. Though the degree of cartilage degeneration was much higher in Grade 3, 4 osteoarthritis knees, the number of SF MSCs per volume was similar between ACL injured knees and Grade 3, 4 osteoarthritis knees. This will be due to that ACL injury itself affected the number of SF MSCs in addition to the cartilage degeneration.[3] More detailed analysis will be of value in the same population from the standpoints of post-injury period, stability of the knee, appearance of ACL remnants, and so on.

Though the existence of MSCs in SF from the knee with osteoarthritis patients was previously reported,[6,7] we first demonstrated that the MSC number in SF increased along with radiological osteoarthritis grading. Our previous study showed that autologous SF enhanced expansion of MSCs in tissue culture of SYN from osteoarthritis patients.[19] These results suggest one mechanism by which SF derived from osteoarthritis promotes mobilization of SYN MSCs into SF. Similar to ACL injured knees, variability also still seems to exist in the mild and severe osteoarthritis groups, respectively. A future study to analyze osteoarthritis of the same grade from the standpoints of spur formation, SYN conditions, subjective scores, and so on would be important.

Here, SF MSCs were more similar to SYN MSCs than to BM MSCs from the morphological and gene profile views. However, similar characteristics between SF MSCs and SYN MSCs may be due to the environmental cues; both kinds of MSCs are constantly exposed to the same cytokines and growth factors in the knee joint, the environment of both kinds of MSCs is

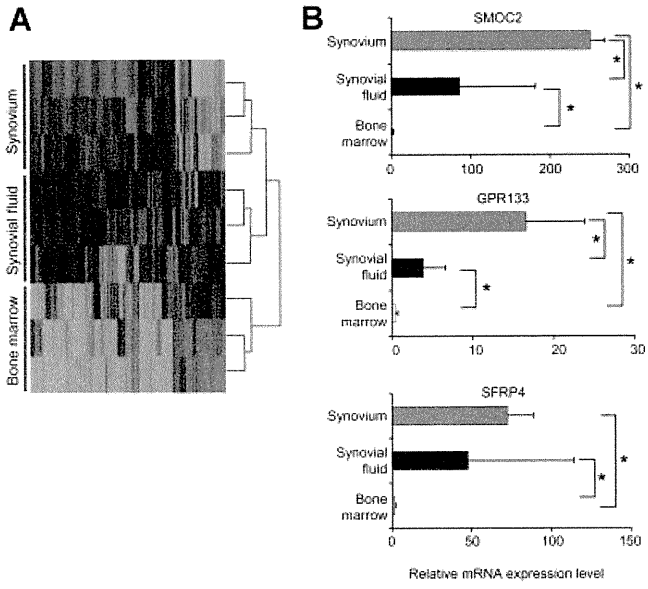

Figure 4. Comparison of the gene expression profiles among synovium MSCs, synovial fluid MSCs, and bone marrow MSCs from osteoarthritis patients. (A) Hierarchical clustering analysis ($n = 3$). (B) Quantitative RT-PCR analysis for the top three genes shown in Table 1. Relative amount of PCR products to that in bone marrow MSCs are displayed as the average and standard deviation ($n = 5$; $^*P < 0.05$ by Steel–Dwass test). The tissues were harvested from five donors in addition to the donor analyzed in the microarray.

quite different from that of BM, and, thus resulting in a similar gene expression pattern. The gene expression pattern of MSCs that are recruited from BM through systemic circulation may change after they are sent to SF. It would be quite interesting to examine whether BM MSCs which incubate in SF resemble the phenotypes as they do in SYN MSCs.

There were no significant differences of surface epitopes among SYN MSCs, SF MSCs, and BM MSCs. One of the surface epitopes we examined was D7-FIB, whose antigen is a fibroblast-specific molecule of unknown function. According to Jones et al.,[20] D7-FIB is a marker of BM MSCs, and $CD45^{low}D7\text{-}FIB^{+}LNGFR^{+}$ BM cells express classic markers of cultured MSCs CD73/SH3, CD105/SH2, and CD106/VCAM.[21] In our results, the positive rate for D7-FIB was less than 20% in the 3 populations of MSCs.

In SF MSCs, the differentiation potentials were not correlated to the cartilage degeneration score and to the MSC number in SF. In 10 donors we examined, SF MSCs differentiated into all three lineages, though the quantitative values varied. SF MSCs had common properties in all donors, however, each property varied individually.

The volume of SF taken from the patients was 9.9 ± 6.9 ml in Grade 1, 2 osteoarthritis knees, and 11.1 ± 9.5 ml in Grade 3, 4 osteoarthritis knees. There

Table 1. Specific Genes Which Were Expressed Higher in Both Synovium MSCs (SYB) and Synovial Fluid MSCs (SF) Than in Bone Marrow MSCs (BM)

				Fold Ratio of Expression		
No.	GenBank No.	Gene Name	Symbol	SYV/BM	SF/BM	SYV/SF
1	NM_022138	SPARC-related modular calcium binding 2	**SMOC2**	13.4	13.8	1.0
2	NM_198827	Secreted frizzled-related protein 4	**GPR133**	13.1	10.5	1.2
3	NM_003014	Peptidase inhibitor 16	**SFRP4**	11.9	8.5	1.4
4	NM_153370	Transmembrane and tetratricopeptide repeat containing 1	PI16	9.8	8.2	1.2
5	NM_175861	Calcium channel, voltage-dependent, beta 4 subunit	TMTC1	9.0	6.1	1.5
6	NR_030672	KIAA1324-like	KIAA1324L	8.9	8.1	1.1
7	NM_000726	Calcium channel, voltage-dependent, beta 4 subunit	CACNB4	8.5	6.2	1.4
8	NR_002196	H19, imprinted maternally expressed transcript	H19	8.4	6.6	1.3
9	BC006141	Erythrocyte membrane protein band 4.1-like 3	EPB41L3	7.8	6.1	1.3
10	BC101809	Prostaglandin I2 (prostacyclin) synthase	PTGIS	7.8	8.8	0.9
11	NR_001284	Tenascin XA	TNXA	7.7	10.3	0.7
12	NM_004753	Dehydrogenase/reductase (SDR family) member 3	DHRS3	7.6	6.2	1.2
13	NM_020858	Sema domain, transmembrane domain (TM), and cytoplasmic domain (semaphorin), 6D	SEMA6D	6.5	5.5	1.2
14	NM_198148	Carboxypeptidase X (M14 family), member 2	CPXM2	6.4	4.8	1.3
15	NM_001276	Chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1	6.3	5.5	1.1
16	NM_002214	Integrin, beta 8	ITGB8	6.2	5.0	1.2
17	NM_015419	Matrix-remodeling associated 5	MXRA5	6.2	5.7	1.1
18	NM_000062	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	SERPING1	6.0	4.4	1.4
19	NM_005807	Proteoglycan 4	PRG4	5.6	8.4	0.7
20	NM_001354	Aldo-keto reductase family 1, member C2	AKR1C2	5.3	5.2	1.0

The genes are listed in descending order of SYV/BM. Genes in bold were confirmed by real-time PCR.

was a weak tendency between SF volume and synovial MSC number per SF volume in the osteoarthritis knees (data not shown). In this study, only SF with translucency viewed with naked eyes were analyzed, and the blood test for CRP was negative in most cases. However, the volume of SF is one of the indexes for inflammation in the osteoarthritis knee. Mild inflammation undetectable with our examinations might have affected the number of SF MSCs in the osteoarthritis knees.

The volume of SF taken from the patients was 3.8 ± 3.1 ml in ACL injured knee, and there was no tendency between SF volume and synovial MSC number per SF volume. In ACL injury knees, several other factors in addition to cartilage degeneration, as mentioned above, might have affected SF MSCs and negated the factor of SF volume.

We demonstrated that the MSC number in SF increased along with the radiological grading of osteoarthritis as shown in Figure 2B. The age distribution of the three groups was different; the average age was 35 in the normal group, 50 in the mild group, and 75 in the severe osteoarthritis group. This may have influenced the data because several studies have reported that aging affects properties of MSCs derived from several kinds of mesenchymal tissues, though the subjects diagnosed were elderly people with osteoarthritis or osteoporosis in most of the previous studies.[22,23] To remove the factor of aging, normal control groups at the same age are required, however, it is practically difficult to collect SF from elderly people with normal knees.

We showed 20 specific genes whose expression was higher in both SYN MSCs and SF MSCs in comparison to that in BM MSCs. We further confirmed expressions of the top three genes by RT-PCR. SMOC2 stands for SPARC-related modular calcium binding 2. SPARC (secreted protein acidic and rich in cysteine) is highly expressed during embryogenesis, and its expression becomes more restricted in adult tissues.[24] SMOC-2 is expressed highly in the heart, muscle tissue, spleen, and ovary.[25] Only the function of SMOC2 reported previously involves stimulation of endothelial cell proliferation.[26] In comparison with normal cartilage, SMOC-2 mRNA expression was higher in osteoarthritis cartilage.[27] GPR133 encodes a G-protein-coupled receptor (GPCR). GPCRs are involved in osteoclast function and regulation of bone mineral density and cell growth.[28] A genome-wide association study recently demonstrated that genetic variation in GPR133 was associated with body height.[29] SFRP4 stands for secreted frizzled-related protein 4. Secreted frizzled-related protein is one of the regulators for the Wnt signaling pathway, which regulates various normal and pathological developmental processes of many organs. Some studies reported that SFRP4 is a negative regulator of peak bone mineral density.[30] In most genes listed in Table 1, including the three genes mentioned above, their relationships to SYN or SF remains unknown, however, these genes would be interesting as signatures in that they distinguish the SYN and SF MSCs from BM MSCs. This knowledge would be greatly helpful to clarify the mechanisms of treatment for osteoarthritis. For example, we would be able to answer the question about the tissue origin of the MSCs, which appear in the degenerated site of articular cartilage induced by abrasion arthroplasty.

What is the role of MSCs in SF? MSCs are postulated to participate in tissue homoeostasis, remodeling, and repair by ensuring the replacement of mature cells lost to physiological turnover, senescence, injury, or disease. Stem cell populations are found in most adult tissues, and in general, their differentiation potential may reflect the local cell population.[8,9] Developmentally, intraarticular tissues are differentiated from common progenitors, referred to as common interzone cells.[31] SYN MSCs have a high chondrogenic potential[8–10]; intraarticular injection of SYN MSCs promoted cartilage regeneration in rabbit cartilage defect model[32]; and intraarticular injection of SYN MSCs contributed to meniscal regeneration in a rat meniscus defect model.[33] We speculate that synovial tissue may serve as a reservoir of stem cells that mobilize following intraarticular tissue diseases and migrate to the site to participate in the repair response.

Why is the number of SF MSCs increased in accordance with the degree of cartilage degeneration? In the adult cartilage, because of enzymatic activities and the mechanical stress imposed onto the joints, cartilage damage always occurs. In normal circumstances, this is compensated by the turnover of the matrix components synthesized by chondrocytes. Thus, in normal adult articular cartilage, there is an equal balance between anabolism and catabolism. In osteoarthritis, catabolism becomes stronger than the anabolic capacities of chondrocytes, the cartilage matrix degenerates, and the joint cartilage gets damaged.[34] We speculate that MSCs were mobilized from SYN through SF toward degenerative cartilage. Seemingly, the number of mobilized MSCs is limited, therefore osteoarthritis progresses in its natural course. Our next step is to investigate whether intraarticular injection of SYN MSCs can inhibit the progression of osteoarthritis.

In conclusion, the MSC number in SF was correlated to cartilage degeneration evaluated with arthroscopy in the ACL injured knees. The MSC number in SF increased along with the radiological grading of osteoarthritis. SF MSCs were more similar to SYN MSCs than to BM MSCs from the morphological and gene profile views.

ACKNOWLEDGMENTS

The present study was supported by grants from the Japanese Ministry of Education Global Center of Excellence Program, International Research Center for Molecular Science in Tooth and Bone Diseases to T.M., from the Japan Society for the Promotion of Science (16591478), and from Health and Labor Sciences Research Grant, Research on

Regenerative Medicine for Clinical Application to I.S. Recombinant human BMP-7 was distributed by Stryker Biotech. We thank Izumi Nakagawa for management of our laboratory.

REFERENCES

1. Friedenstein AJ. 1976. Precursor cells of mechanocytes. Int Rev Cytol 47:327–359.
2. Horwitz EM, Le Blanc K, Dominici M, et al. 2005. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy 7:393–395.
3. Morito T, Muneta T, Hara K, et al. 2008. Synovial fluid-derived mesenchymal stem cells increase after intra-articular ligament injury in humans. Rheumatology (Oxford) 47: 1137–1143.
4. Asano H, Muneta T, Ikeda H, et al. 2004. Arthroscopic evaluation of the articular cartilage after anterior cruciate ligament reconstruction: a short-term prospective study of 105 patients. Arthroscopy 20:474–481.
5. Hayashi M, Muneta T, Ju YJ, et al. 2008. Weekly intra-articular injections of bone morphogenetic protein-7 inhibits osteoarthritis progression. Arthritis Res Ther 10:R118.
6. Jones EA, English A, Henshaw K, et al. 2004. Enumeration and phenotypic characterization of synovial fluid multipotential mesenchymal progenitor cells in inflammatory and degenerative arthritis. Arthritis Rheum 50:817–827.
7. Jones EA, Crawford A, English A, et al. 2008. Synovial fluid mesenchymal stem cells in health and early osteoarthritis: detection and functional evaluation at the single-cell level. Arthritis Rheum 58:1731–1740.
8. Sakaguchi Y, Sekiya I, Yagishita K, et al. 2005. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. Arthritis Rheum 52:2521–2529.
9. Yoshimura H, Muneta T, Nimura A, et al. 2007. Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. Cell Tissue Res 327:449–462.
10. Koga H, Muneta T, Nagase T, et al. 2008. Comparison of mesenchymal tissues-derived stem cells for in vivo chondrogenesis: suitable conditions for cell therapy of cartilage defects in rabbit. Cell Tissue Res 333:207–215.
11. Segawa Y, Muneta T, Makino H, et al. 2009. Mesenchymal stem cells derived from synovium, meniscus, anterior cruciate ligament, and articular chondrocytes share similar gene expression profiles. J Orthop Res 27:435–441.
12. Rosenberg TD, Paulos LE, Parker RD, et al. 1988. The forty-five-degree posteroanterior flexion weight-bearing radiograph of the knee. J Bone Joint Surg Am 70:1479–1483.
13. Shimaya M, Muneta T, Ichinose S, et al. 2010. Magnesium enhances adherence and cartilage formation of synovial mesenchymal stem cells through integrins. Osteoarthritis Cartilage 18:1300–1309.
14. Sekiya I, Larson BL, Vuoristo JT, et al. 2004. Adipogenic differentiation of human adult stem cells from bone marrow stroma (MSCs). J Bone Miner Res 19:256–264.
15. Sekiya I, Larson BL, Smith JR, et al. 2002. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells 20:530–541.
16. Sakaguchi Y, Sekiya I, Yagishita K, et al. 2004. Suspended cells from trabecular bone by collagenase digestion become virtually identical to mesenchymal stem cells obtained from marrow aspirates. Blood 104:2728–2735.
17. Irizarry RA, Bolstad BM, Collin F, et al. 2003. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 31:e15.
18. Niikura T, Hak DJ, Reddi AH. 2006. Global gene profiling reveals a downregulation of BMP gene expression in experimental atrophic nonunions compared to standard healing fractures. J Orthop Res 24:1463–1471.
19. Zhang S, Muneta T, Morito T, et al. 2008. Autologous synovial fluid enhances migration of mesenchymal stem cells from synovium of osteoarthritis patients in tissue culture system. J Orthop Res 26:1413–1418.
20. Jones EA, Kinsey SE, English A, et al. 2002. Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. Arthritis Rheum 46:3349–3360.
21. Jones EA, English A, Kinsey SE, et al. 2006. Optimization of a flow cytometry-based protocol for detection and phenotypic characterization of multipotent mesenchymal stromal cells from human bone marrow. Cytometry B Clin Cytom 70:391–399.
22. Mochizuki T, Muneta T, Sakaguchi Y, et al. 2006. Higher chondrogenic potential of fibrous synovium- and adipose synovium-derived cells compared with subcutaneous fat-derived cells: distinguishing properties of mesenchymal stem cells in humans. Arthritis Rheum 54:843–853.
23. Murphy JM, Dixon K, Beck S, et al. 2002. Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. Arthritis Rheum 46:704–713.
24. Porter PL, Sage EH, Lane TF, et al. 1995. Distribution of SPARC in normal and neoplastic human tissue. J Histochem Cytochem 43:791–800.
25. Vannahme C, Gosling S, Paulsson M, et al. 2003. Characterization of SMOC-2, a modular extracellular calcium-binding protein. Biochem J 373:805–814.
26. Rocnik EF, Liu P, Sato K, et al. 2006. The novel SPARC family member SMOC-2 potentiates angiogenic growth factor activity. J Biol Chem 281:22855–22864.
27. Wu J, Liu W, Bemis A, et al. 2007. Comparative proteomic characterization of articular cartilage tissue from normal donors and patients with osteoarthritis. Arthritis Rheum 56:3675–3684.
28. Hsiao EC, Boudignon BM, Chang WC, et al. 2008. Osteoblast expression of an engineered Gs-coupled receptor dramatically increases bone mass. Proc Natl Acad Sci USA 105:1209–1214.
29. Tonjes A, Koriath M, Schleinitz D, et al. 2009. Genetic variation in GPR133 is associated with height: genome wide association study in the self-contained population of Sorbs. Hum Mol Genet 18:4662–4668.
30. Nakanishi R, Akiyama H, Kimura H, et al. 2008. Osteoblast-targeted expression of Sfrp4 in mice results in low bone mass. J Bone Miner Res 23:271–277.
31. Archer CW, Dowthwaite GP, Francis-West P. 2003. Development of synovial joints. Birth Defects Res C Embryol Today 69:144–155.
32. Koga H, Shimaya M, Muneta T, et al. 2008. Local adherent technique for transplanting mesenchymal stem cells as a potential treatment of cartilage defect. Arthritis Res Ther 10:R84.
33. Horie M, Sekiya I, Muneta T, et al. 2009. Intra-articular injected synovial stem cells differentiate into meniscal cells directly and promote meniscal regeneration without mobilization to distant organs in rat massive meniscal defect. Stem Cells 27:878–887.
34. Aigner T, Soeder S, Haag J. 2006. IL-1beta and BMPs—interactive players of cartilage matrix degradation and regeneration. Eur Cell Mater 12:49–56, discussion 56.

Knee Surg Sports Traumatol Arthrosc (2012) 20:88–94
DOI 10.1007/s00167-011-1541-x

KNEE

Surgical management of grade 3 medial knee injuries combined with cruciate ligament injuries

Hideyuki Koga · Takeshi Muneta · Kazuyoshi Yagishita · Young-Jin Ju · Ichiro Sekiya

Received: 20 December 2010 / Accepted: 2 May 2011 / Published online: 10 May 2011
© Springer-Verlag 2011

Abstract
Purpose Although various surgical procedures have attempted to restore valgus stability in medial knee injuries, so far none has achieved satisfactory results. The purpose of this study was to assess clinical outcome for patients with grade 3 valgus instability who were treated according to our surgical management strategy.
Methods Eighteen patients with both acute and chronic grade 3 medial knee injuries, all of which had combined cruciate ligament injuries, were treated with a proximal advancement of both the superficial medial collateral ligament (MCL) and posterior oblique ligament together with underlying deep MCL and joint capsule, in conjunction with cruciate ligament reconstructions in chronic phase. Augmentation with doubled semitendinosus tendon was added in 7 patients whose medial knee stability had been considered to be insufficient with only the proximal advancement procedure. They were evaluated preoperatively and at final follow-up.
Results Manual valgus laxities at 0° and 30°, as well as side-to-side difference in medial joint opening in stress radiograph, were significantly improved at final follow-up. The Lysholm knee scale was also significantly improved. Median values of the subjective evaluations of the patients' satisfaction, stability and sports performance level measured with visual analogue scale at final follow-up were 82 (60–100), 94 (71–100) and 88 (60–100), respectively.
Conclusions Clinical outcomes of our surgical management strategy were reasonable in terms of restoring medial knee stability. This treatment protocol can help determine the surgical management of grade 3 medial knee injuries combined with cruciate ligament injuries.
Level of evidence Retrospective case series, Level IV.

Keywords Medial collateral ligament (MCL) · Medial knee injury · Operative procedure · Posterior oblique ligament · Multiple ligament injury

Introduction

Even though medial knee injury is one of the most common injuries of the knee joint, its treatment strategy is still a matter of controversy. Although some reports have recommended simultaneous surgical treatment for both anterior cruciate ligament (ACL) and medial collateral ligament (MCL) injuries if ACL injury is accompanied by grade 2 MCL injury with valgus laxity [2, 8, 26], it is generally accepted that the majority of patients who sustain incomplete or isolated complete MCL tears can be treated nonoperatively [3, 6, 12, 14, 28]. Both animal and human clinical studies have shown that MCL has excellent healing capacity [1, 18, 25, 31]. On the other hand, in cases of complete medial knee injuries combined with cruciate ligament injuries, complex and problematic instability could be pronounced, because the functional deficiency of one ligament might affect the healing of the others [19]. However, for lack of evidence [20], the treatment of complete medial knee injuries associated with combined ligamentous injuries still continues to be controversial as to whether the medial knee structures should be treated nonoperatively or operatively [7, 10, 11, 15, 29]. In addition, in cases of surgical treatment, whether the medial knee structures should be repaired in acute phase [4, 16, 27] or

H. Koga (✉) · T. Muneta · K. Yagishita · Y.-J. Ju · I. Sekiya
Department of Orthopaedic Surgery, Tokyo Medical and Dental University Hospital of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
e-mail: koga.orj@tmd.ac.jp

should be reconstructed in chronic phase [7, 17, 33] is also controversial.

In this case series, acute complete medial knee injuries associated with combined cruciate ligament injuries have been treated conservatively until knee range of motion is recovered, and the following surgical management strategy has been applied in chronic phase; (1) a proximal advancement of both the superficial MCL and posterior oblique ligament (POL) together with underlying deep MCL and joint capsule is performed for residual grade 3 valgus instability in conjunction with cruciate ligament reconstructions. (2) Augmentation with doubled semitendinosus tendon is added in cases that are considered to be insufficient with only the proximal advancement procedure. The purpose of this study was to assess the clinical outcome for patients with grade 3 valgus instability who were treated according to our surgical management strategy. The hypothesis underling this study was that the surgical management strategy applied in this case series could restore medial knee stability in patients with grade 3 medial knee injuries with concomitant cruciate ligament injuries.

Materials and methods

Between 1997 and 2007, 24 patients with grade 3 valgus laxity combined with cruciate ligament injuries were treated according to our surgical management strategy. The grade of valgus laxity was defined with manual valgus test in both 0° and 30° of flexion as grade 1–3 according to the grading of Fetto and Marshall [8]. Among those patients, 18 patients with no history of ligamentous injuries in contralateral knee, no history of injuries in ipsilateral knee and with a minimum follow-up of 2 years were included in this study. They comprised 9 male and 9 female patients with a median age at the time of surgery of 24 years (range 17–44 years). The median time from the surgery to the latest follow-up was 26 months (range 24–75 months). Informed consent was obtained from each patient enrolled in this study.

Of these 18 patients, 10 patients had been treated at our institution as acute injuries, while the remaining 8 patients were diagnosed with chronic grade 3 medial knee injuries. Combined cruciate ligament injuries were observed in all patients, with anterior cruciate ligament (ACL), posterior cruciate ligament (PCL) and both ACL and PCL injuries in 14, 1 and 3 patients, respectively. For chronic combined ligament injuries, we essentially attempted to surgically correct all the instabilities. For acute cases combined with cruciate ligament injuries, they were initially treated conservatively, followed by subsequent operative treatment for residual grade 3 valgus instability combined with cruciate ligament reconstructions after swelling had subsided and range of motion was recovered, thereby avoiding limited recovery of range of motion after the surgery due to arthrofibrosis. The combined cruciate ligament injuries were reconstructed using semitendinosus tendon, bone-patellar tendon-bone or quadriceps tendon graft. Medial knee injuries were treated using only the proximal advancement procedure even in chronic cases if the underlying joint capsule was strong enough to obtain sufficient valgus stability. Meanwhile, augmentation with doubled semitendinosus tendon was added in 7 patients in whom only the proximal advancement procedure was considered to be insufficient, such as in patients in whom valgus instability would be expected to remain with only the proximal advancement procedure due to insufficient strength of remaining MCL and joint capsule, in contact sports athletes, in patients with PCL injury and in patients with general laxity and valgus alignment. Concomitant meniscal surgeries (either meniscectomy or meniscal repair) were performed in 5 patients. The median length of time from injury to surgery was 5 months (range 2–156 months).

Surgical technique

A 3–4 cm longitudinal incision was made on the femoral attachment of the superficial MCL[22]. After an incision was made on the femoral fascia overlying superficial MCL and POL, torn or slack superficial MCL was identified, and proximal insertion of the superficial MCL was released transversely from the attachment site with a knife. The POL, the medial joint capsule and the deep MCL were released from the femoral fascia and from the femoral bone anteriorly and posteriorly. The attachment sites of the superficial and deep MCL and the medial joint capsule were decorticated with a small curette or a small rasp. Four Krackow sutures [21] using No. 5 braided polyester sutures were placed at the anterior, middle and posterior part of the superficial MCL and the POL together with underlying deep MCL and joint capsule. Two anchor staples were placed 10 mm proximal to the proximal insertion of the superficial MCL parallel to the insertion site. Sutures placed at the anterior and middle part of the superficial MCL were tied to the anteriorly placed anchor staple, and sutures placed at the posterior part of the superficial MCL and the POL were tied to the posteriorly placed anchor staple. Each suture was tied with manual maximum pull at 30° of flexion (Fig. 1a).

Augmentation with doubled semitendinosus tendon was added in patients in whom only the proximal advancement procedure was considered to be insufficient. Semitendinosus tendon was harvested, and Krackow sutures using No. 5 braided polyester sutures were placed at both

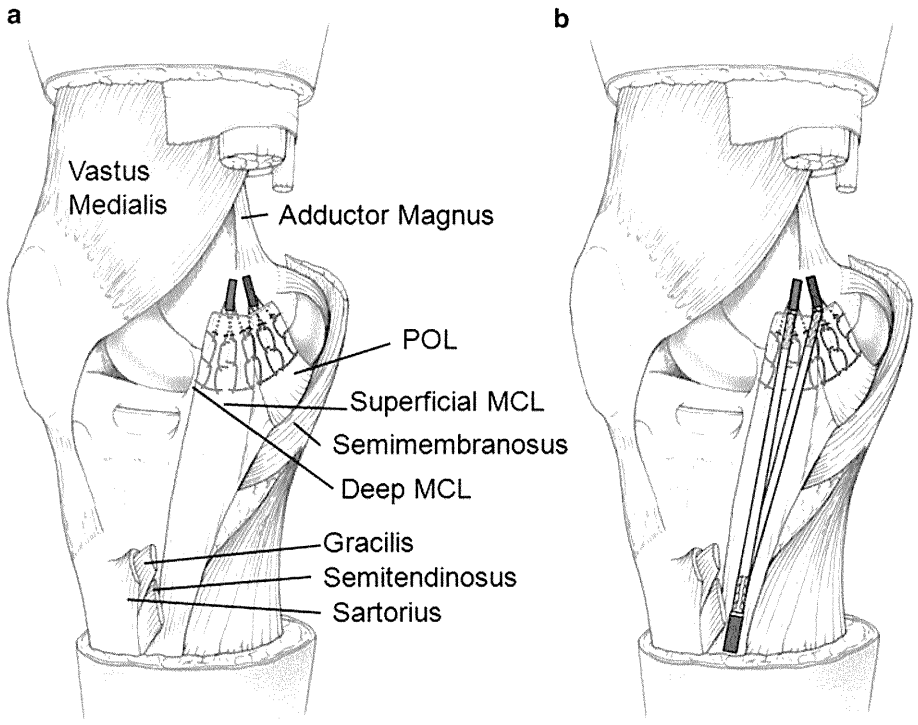

Fig. 1 Surgical technique. **a** Proximal advancement procedure. Four Krackow sutures were placed at the anterior, middle and posterior part of the superficial MCL and the POL together with underlying deep MCL and joint capsule. Sutures placed at the anterior and middle part of the superficial MCL (*red*) were tied to the anteriorly placed anchor staple (*red*), and sutures placed at the posterior part of the superficial MCL and the POL (*blue*) were tied to the posteriorly placed anchor staple (*blue*) with manual maximum pull at 30° of flexion. **b** Augmentation with doubled semitendinosus tendon (*yellow*). Krackow sutures were placed at both ends of the semitendinosus tendon. An anchor staple was placed at the distal insertion site of the superficial MCL (*black*). A Krackow suture was also placed at the stranded side of the doubled tendon and tied to the proximal leg of the anchor staple. The sutures placed at the both ends were tied to the anchor staples placed at the proximal site with manual maximum pull at 30° of flexion in conjunction with the fixation of the proximal advancement procedure

ends of the tendon. An anchor staple was placed at the distal insertion site of the superficial MCL. A Krackow suture using a No. 5 braided polyester suture was also placed at the stranded side of the doubled tendon and tied to the proximal leg of the anchor staple. The sutures placed at both ends were tied to the anchor staples placed at the proximal site with manual maximum pull at 30° of flexion in conjunction with the fixation of the proximal advancement procedure. The augmented tendon was sutured to the surrounding superficial MCL and the medial capsule with multi-stitches of 3–0 absorbable sutures (Fig. 1b).

Postoperative treatment

A postoperative rehabilitation programme for isolated ACL reconstruction was applied to patients with ACL reconstruction, whereas one for isolated PCL reconstruction was applied to patients with PCL reconstruction and to patients with both ACL and PCL reconstruction. Namely, range of motion exercise from full extension to 120° of flexion was started at 2 days after the surgery. Twenty kilogram partial weight bearing was allowed at 2 days and was gradually increased. Crutches were removed at 4 weeks. Patients with PCL reconstruction were allowed restricted walking in extended position with a knee brace for 4 weeks, and open kinetic chain active knee flexion exercise was prohibited for 3 months. Instead, passive protective knee flexion exercise and closed kinetic chain knee flexion exercise were encouraged. Running was allowed at 3–6 months with progressive squatting exercise, and patients progressed to full activity at 6–10 months.

Evaluation

The knee joint condition at the preoperative and the latest follow-up period was evaluated based on side-to-side differences between the injured and uninjured leg. A full evaluation was performed by 2 examiners blindly and independently on the same day. If differences were found between the 2 examiners, a re-evaluation was performed together until consensus was obtained. The difference in

the range of motion between the two knees was recorded, with knee extension and flexion angle described in 1° and 5° increments, respectively.

Manual knee laxity tests for ACL (Lachman test and pivot shift test) and PCL (posterior drawer test) were graded according to the International Knee Documentation Committee (IKDC) form [13]. The anterior knee laxity measured with the KT-1000 arthrometer (MEDmetric, San Diego, CA) at manual maximum pull was expressed as the difference between the injured and uninjured legs in 0.5-mm increments.

Valgus instability was evaluated with manual valgus test in both 0° and 30° of flexion, and the differences between the injured and uninjured legs were categorized as negative, 1+, 2+ or 3+ in the same manner. Valgus instability was also assessed with valgus stress radiographic examination according to the IKDC form (knee in 20° of flexion, applied stress force 134 N), and the differences in length of the medial joint opening between the injured and uninjured legs were measured in 1-mm increments.

The Lysholm knee scale was used as a general knee evaluation method [30]. The subjective evaluations of the patients' satisfaction, stability and sports performance level compared to pre-injury level were measured on a 100-mm visual analogue scale (VAS) in 1-mm increments at final follow-up.

Statistical analysis

Statistical analyses were performed by use of a paired t-test for KT measurement and valgus stress radiographic examination. Wilson signed-rank test was used for Lysholm score, Lachman test, pivot shift test, posterior drawer test and manual valgus test. Significance level was set at $P < 0.05$. The results are shown as the median with range.

Results

There was no patient with a greater than 5° extension or 10° flexion deficit at final follow-up, while two patients with PCL reconstruction required postoperative manipulation at the time of anchor staple removal because of 15° of flexion deficit. There were no other significant intraoperative or postoperative complications during the study period.

Valgus knee stability

Manual valgus tests in 0° and 30° were both significantly improved at the final follow-up (Table 1). Valgus laxities in 0° were 2+ in 9 patients and 1+ in 9 patients preoperatively and became negative in all patients except one ($P < 0.001$). Valgus laxities in 30° were 3+ in 11 patients

Table 1 Pre- and postoperative valgus knee stability in 18 patients

Patient	Combined ligamentous surgery	Augmentation with ST[a]	Comments	0°		30°	
				Pre-op	Post-op	Pre-op	Post-op
1	ACL	None		2+	–	3+	1+
2	ACL	None		1+	–	3+	–
3	ACL	Yes	Judo player	2+	–	3+	1+
4	ACL	None		1+	–	3+	1+
5	ACL	Yes	Insufficient strength of remaining MCL and joint capsule	2+	–	3+	–
6	ACL	None		2+	–	3+	–
7	ACL	None		1+	–	2+	1+
8	ACL	None		1+	–	2+	–
9	ACL/PCL	None	Both ACL and PCL were injured	2+	–	2+	2+
10	PCL	Yes	PCL was injured	2+	–	3+	–
11	ACL	None		1+	–	3+	–
12	ACL	Yes	Judo player	2+	–	3+	–
13	ACL/PCL	Yes	Both ACL and PCL were injured	1+	–	3+	1+
14	ACL	None		1+	–	2+	1+
15	ACL	Yes	Valgus knee, general laxity	2+	–	3+	–
16	ACL	None		1+	–	2+	–
17	ACL	None	Soccer player, hyperextended knee and general laxity	2+	1+	2+	2+
18	ACL/PCL	Yes	Both ACL and PCL were injured	1+	–	2+	–

[a] *ST* semitendinosus tendon

Table 2 Clinical results

	Pre-op[a]	Post-op[a]	*P*
Valgus stress radiograph (mm)[b]	6 (3–13)	1 (0–2)	0.004
Lysholm score	81 (41–94)	91 (70–100)	0.001
KT measurements (mm)[c]	7.0 (4.5–16.0)	2.0 (0.0–6.0)	<0.001

[a] Results are shown as the median (range)

[b] Results from 7 patients in whom preoperative valgus stress radiograph was taken

[c] Results from 17 patients with ACL reconstruction

and 2+ in 7 patients and became negative in 10 patients, 1+ in 6 patients and 2+ in 2 patients ($P < 0.001$). Preoperative valgus stress radiograph was taken only in 7 patients in chronic phase, as grade 3 valgus instability was obvious with manual stress test in the rest of the patients. In addition, we wanted to avoid the possible risk of further injury of an intact or partially torn cruciate ligament by applying a large clinical valgus load to a knee with a severe medial knee structure injury especially in acute phase [23]. Side-to-side difference in medial joint opening in valgus stress radiograph was significantly improved in those 7 patients (Table 2). Postoperative valgus stress radiograph was taken in all patients, and the overall result was 1 mm (0–3) at final follow-up. None of the patients showed side-to-side differences in medial joint opening greater than 2 mm, except one patient with 3 mm side-to-side difference that also showed 1+ valgus laxity at 0° of flexion (patient 17).

Lysholm knee scale and subjective assessments

The total score on the Lysholm knee scale was significantly improved (Table 2). The subjective evaluations of the patients' satisfaction, stability and sports performance level compared to pre-injury level measured with VAS were 82 (60–100), 94 (71–100) and 88 (60–100) out of 100, respectively.

Anteroposterior stability

In patients with ACL reconstruction, reasonable ACL-relevant stability was obtained. Lachman test was negative in 13 out of 17 patients, and pivot shift was negative in 12 patients at final follow-up. KT measurement was also improved significantly (Table 2). However, 4 out of 5 patients with pivot shift positive also showed at least 1+ valgus laxity in 30°. In patients with PCL reconstruction, posterior drawer test became negative in 3 out of 4 patients; however, 2+ instability remained in one patient in whom valgus instability also remained (patient 9).

Discussion

The most important finding of the present study was that the clinical outcomes of our surgical management strategy were found to be reasonable in terms of restoring medial knee stability in patients with grade 3 medial knee injuries with concomitant cruciate ligament injuries. Seventeen out of 19 patients could regain satisfactory valgus stability, whereas valgus laxity with concurrent residual laxity of reconstructed cruciate ligaments progressed in two patients postoperatively.

In the treatment of complete medial knee injuries combined with cruciate ligament injuries, some surgeons have recommended surgical primary repair and have reported relatively good results [16, 27, 32]. However, the most problematic complication could be postoperative stiffness that requires manipulation [27]. On the other hand, several surgeons reported that MCL reconstruction using hamstring tendons [33] or an artificial ligament [17] showed good results without the incidence of arthrofibrosis. Their limitations could be relatively complicated surgical methods and that only the anterior part of the superficial MCL was reconstructed. On the other hand, importance of POL has been reported and stressed [5, 9]. The advancement procedure was first reported by Mauck [24] as the advancement and reattachment of its distal insertion. This procedure is useful in a way that the method is relatively simple, while the problem of this method is that the quality of the remaining ligamentous tissue is unreliable. Also, only the superficial portion of the MCL is restored with this procedure.

Results from previous studies regarding repair or reconstruction of the medial knee structures in the setting of the multi-ligament-injured knee have not been reported to be successful. Ibrahim et al. [16] reported 18 patients with complete MCL ruptures combined with ACL and PCL injuries that underwent MCL repair in conjunction with ACL and PCL reconstruction. While 89% of patients were found to have clinically stable knees, mean postoperative Lysholm score was 79.2 and mean Tegner score decreased from 7.6 preoperatively to 4.7. Owens et al. [27] reported 11 knee dislocation patients with complete MCL ruptures that underwent primary repair of all torn ligaments. All patients obtained clinically stable knees; however, 27% of patients had stiff knees that required manipulation. In a recent randomized clinical trial, Halinen et al. [10, 11] randomized 47 patients with combined ACL and grade 3 MCL injuries into 2 groups. The MCL injury was treated with primary repair in group 1 ($n = 23$) and nonoperatively in group 2 ($n = 24$). In both groups, the ACL injury was treated with early reconstruction. In this study, they could not show any differences with respect to subjective function of the knee, postoperative stability, return to

activities, Lysholm score and overall IKDC evaluation. Rather, range of motion and muscle power were better in the nonoperatively treated group. Bin et al. [4] reported a two-stage protocol for knee dislocations. In this study, 10 patients sustained MCL injuries and were treated with repair. The collateral ligaments were repaired within 2 weeks after injury, and ACL/PCL reconstruction was performed after swelling had subsided. Even though incidence of arthrofibrosis was lowered with their protocol, only 70% of patients could restore valgus stability. Yoshiya et al. [33] reported 22 patients with combined ligament knee injuries that underwent operative management of the associated cruciate ligament injuries and MCL reconstruction using autograft semitendinosus/gracilis tendons. Four out of 22 patients showed grade 1 medial instability in this case series. More recently, Ibrahim et al. [17] reported successful results of 15 patients with multi-ligament reconstructions including MCL reconstruction using an artificial ligament. In this case series, 14 out of 15 patients had stable knees in manual valgus stress testing at 30° flexion. However, the authors failed to show knee functional scores, such as Lysholm score and IKDC score, in those patients.

Compared to these previous studies, the present study seemed to show better clinical results. Seventeen out of 18 patients recovered sufficient valgus stability in valgus stress radiograph, and the median Lysholm score was 91. This is probably because 1) the surgical technique used in this study (not only superficial MCL and POL are advanced, but also underlying deep MCL and joint capsule are pulled up together) might be better than normal primary repair and 2) we followed our surgical management protocol that defined indication for augmentation with doubled semitendinosus tendon.

There might be some potential problems on the proximal advancement procedure performed in the present study. First, this method is a nonanatomical procedure as the proximal attachment sites are advanced proximally. However, we tried to retain the functions of each repaired ligament by tying the superficial MCL and the POL to the anterior and posterior leg of the anchor staple, respectively. Second, the POL might have been overtightened in this case series. The POL was tied with manual maximum pull at 30° of flexion in our procedure, while the POL became tightest and its valgus load response became highest near full extension [9]. This could have overconstrained the knee, resulting in the entire surgical repair stretching out in the cases with residual laxity. Another problem might be that it is sometimes difficult to judge whether augmentation with doubled semitendinosus tendon is necessary. In this case series, valgus laxity became gradually manifested in two patients in whom immediate postoperative stability seemed good enough with only the proximal advancement procedure. One was a soccer player with hyperextended knee and general joint laxity (patient 17), where we should have added the augmentation. Another was the first patient with PCL injury (patient 9), where both 2+ valgus instability in 30° of flexion and 2+ posterior instability remained. After this patient, we added the augmentation in all patients with PCL injury, in whom both valgus and anteroposterior stability were improved at final follow-up. This might be because the functional deficiency of one ligament might affect the healing of the others more significantly in patients with PCL injury, and because more robust medial stabilization might be needed. The results from patients with ACL injury were not satisfactory either. Five patients showed pivot shift test positive, and 4 out of the 5 patients also showed at least 1+ valgus laxity in 30°. This might also be because residual valgus instability might affect the healing of reconstructed ACL, resulting in concurrent residual laxity of ACL. These results indicate that there is still room for improvement in the treatment strategy.

There are also some potential limitations in this study. First, for patients with PCL reconstruction, we evaluated posterior instability with posterior drawer test, which is subjective for assessment of posterior tibial translation. PCL stress radiographic examination should have been performed to evaluate posterior instability more objectively. Second, a limited number of patients with preoperative radiograph could weaken the evidence that valgus instability was improved in this case series. However, we believe that significant improvement in manual valgus test in both 0° and 30° supports the effectiveness of our surgical management strategy. Third, this is a case series of mixed cohort of grade 3 valgus instability with combined cruciate ligament injuries. The patients with augmentation with semitendinosus tendon are also mixed in this study, due to the limited number of patients. Comparative study with larger number of cases is required to further evaluate the optimal treatment strategy for grade 3 valgus instability combined with cruciate ligament injuries.

Conclusion

Clinical outcomes of our surgical management strategy were found to be reasonable in terms of restoring medial knee stability in patients with grade 3 medial knee injuries with concomitant cruciate ligament injuries. Although valgus laxity with concurrent residual laxity of reconstructed cruciate ligaments progressed in some patients, which indicates that the treatment strategy should be improved further, this treatment protocol can help determine the surgical management of grade 3 medial knee injuries combined with cruciate ligament injuries.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Anderson DR, Weiss JA, Takai S et al (1992) Healing of the medial collateral ligament following a triad injury: a biomechanical and histological study of the knee in rabbits. J Orthop Res 10:485–495
2. Andersson C, Gillquist J (1992) Treatment of acute isolated and combined ruptures of the anterior cruciate ligament. A long-term follow-up study. Am J Sports Med 20:7–12
3. Ballmer PM, Jakob RP (1988) The non operative treatment of isolated complete tears of the medial collateral ligament of the knee. A prospective study. Arch Orthop Trauma Surg 107:273–276
4. Bin SI, Nam TS (2007) Surgical outcome of 2-stage management of multiple knee ligament injuries after knee dislocation. Arthroscopy 23:1066–1072
5. Coobs BR, Wijdicks CA, Armitage BM et al (2010) An in vitro analysis of an anatomical medial knee reconstruction. Am J Sports Med 38:339–347
6. Derscheid GL, Garrick JG (1981) Medial collateral ligament injuries in football. Nonoperative management of grade I and grade II sprains. Am J Sports Med 9:365–368
7. Fanelli GC, Edson CJ (2002) Arthroscopically assisted combined anterior and posterior cruciate ligament reconstruction in the multiple ligament injured knee: 2- to 10-year follow-up. Arthroscopy 18:703–714
8. Fetto JF, Marshall JL (1978) Medial collateral ligament injuries of the knee: a rationale for treatment. Clin Orthop Relat Res 132:206–218
9. Griffith CJ, Wijdicks CA, LaPrade RF et al (2009) Force measurements on the posterior oblique ligament and superficial medial collateral ligament proximal and distal divisions to applied loads. Am J Sports Med 37:140–148
10. Halinen J, Lindahl J, Hirvensalo E et al (2006) Operative and nonoperative treatments of medial collateral ligament rupture with early anterior cruciate ligament reconstruction: a prospective randomized study. Am J Sports Med 34:1134–1140
11. Halinen J, Lindahl J, Hirvensalo E (2009) Range of motion and quadriceps muscle power after early surgical treatment of acute combined anterior cruciate and grade-III medial collateral ligament injuries. A prospective randomized study. J Bone Joint Surg Am 91:1305–1312
12. Hara K, Niga S, Ikeda H et al (2008) Isolated anterior cruciate ligament reconstruction in patients with chronic anterior cruciate ligament insufficiency combined with grade II valgus laxity. Am J Sports Med 36:333–339
13. Hefti F, Muller W, Jakob RP et al (1993) Evaluation of knee ligament injuries with the IKDC form. Knee Surg Sports Traumatol Arthrosc 1:226–234
14. Holden DL, Eggert AW, Butler JE (1983) The nonoperative treatment of grade I and II medial collateral ligament injuries to the knee. Am J Sports Med 11:340–344
15. Hughston JC (1994) The importance of the posterior oblique ligament in repairs of acute tears of the medial ligaments in knees with and without an associated rupture of the anterior cruciate ligament. Results of long-term follow-up. J Bone Joint Surg Am 76:1328–1344
16. Ibrahim SA (1999) Primary repair of the cruciate and collateral ligaments after traumatic dislocation of the knee. J Bone Joint Surg Br 81:987–990
17. Ibrahim SA, Ahmad FH, Salah M et al (2008) Surgical management of traumatic knee dislocation. Arthroscopy 24:178–187
18. Indelicato PA (1983) Non-operative treatment of complete tears of the medial collateral ligament of the knee. J Bone Joint Surg Am 65:323–329
19. Inoue M, McGurk-Burleson E, Hollis JM et al (1987) Treatment of the medial collateral ligament injury. I: The importance of anterior cruciate ligament on the varus-valgus knee laxity. Am J Sports Med 15:15–21
20. Kovachevich R, Shah JP, Arens AM et al (2009) Operative management of the medial collateral ligament in the multi-ligament injured knee: an evidence-based systematic review. Knee Surg Sports Traumatol Arthrosc 17:823–829
21. Krackow KA, Thomas SC, Jones LC (1986) A new stitch for ligament-tendon fixation. Brief note. J Bone Joint Surg Am 68:764–766
22. LaPrade RF, Engebretsen AH, Ly TV et al (2007) The anatomy of the medial part of the knee. J Bone Joint Surg Am 89: 2000–2010
23. Laprade RF, Bernhardson AS, Griffith CJ et al (2010) Correlation of valgus stress radiographs with medial knee ligament injuries: an in vitro biomechanical study. Am J Sports Med 38:330–338
24. Mauck HP (1936) A new operative procedure for instability of the knee. J Bone Joint Surg 18:984–990
25. Nagineni CN, Amiel D, Green MH et al (1992) Characterization of the intrinsic properties of the anterior cruciate and medial collateral ligament cells: an in vitro cell culture study. J Orthop Res 10:465–475
26. Osti L, Papalia R, Del Buono A et al (2010) Simultaneous surgical management of chronic grade-2 valgus instability of the knee and anterior cruciate ligament deficiency in athletes. Knee Surg Sports Traumatol Arthrosc 18:312–316
27. Owens BD, Neault M, Benson E et al (2007) Primary repair of knee dislocations: results in 25 patients (28 knees) at a mean follow-up of four years. J Orthop Trauma 21:92–96
28. Reider B, Sathy MR, Talkington J et al (1994) Treatment of isolated medial collateral ligament injuries in athletes with early functional rehabilitation. A five-year follow-up study. Am J Sports Med 22:470–477
29. Shelbourne KD, Porter DA (1992) Anterior cruciate ligament-medial collateral ligament injury: nonoperative management of medial collateral ligament tears with anterior cruciate ligament reconstruction. A preliminary report. Am J Sports Med 20:283–286
30. Tegner Y, Lysholm J (1985) Rating systems in the evaluation of knee ligament injuries. Clin Orthop Relat Res 198:43–49
31. Woo SL, Inoue M, McGurk-Burleson E et al (1987) Treatment of the medial collateral ligament injury. II: Structure and function of canine knees in response to differing treatment regimens. Am J Sports Med 15:22–29
32. Yeh WL, Tu YK, Su JY et al (1999) Knee dislocation: treatment of high-velocity knee dislocation. J Trauma 46:693–701
33. Yoshiya S, Kuroda R, Mizuno K et al (2005) Medial collateral ligament reconstruction using autogenous hamstring tendons: technique and results in initial cases. Am J Sports Med 33:1380–1385

PLOS ONE

Isolation and Characterization of Multipotential Mesenchymal Cells from the Mouse Synovium

Ippei Futami[1,2], Muneaki Ishijima[1,2,3]*, Haruka Kaneko[1,2], Kunikazu Tsuji[4], Naoki Ichikawa-Tomikawa[5], Ryo Sadatsuki[1,2], Takeshi Muneta[4,6], Eri Arikawa-Hirasawa[5], Ichiro Sekiya[7], Kazuo Kaneko[1,2,3]

1 Department of Medicine for Motor Organ, Juntendo University Graduate School of Medicine, Tokyo, Japan, **2** Department of Orthopaedics, Juntendo University School of Medicine, Tokyo, Japan, **3** Sportology Center, Juntendo University Graduate School of Medicine, Tokyo, Japan, **4** International Research Center for Molecular Science in Tooth and Bone Diseases, Global Center of Excellence Program, Tokyo Medical and Dental University, Tokyo, Japan, **5** Research Institute for Diseases of Old Age, Juntendo University Graduate School of Medicine, Tokyo, Japan, **6** Section of Orthopaedic Surgery, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan, **7** Section of Cartilage Regeneration, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

Abstract

The human synovium contains mesenchymal stem cells (MSCs), which are multipotential non-hematopoietic progenitor cells that can differentiate into a variety of mesenchymal lineages and they may therefore be a candidate cell source for tissue repair. However, the molecular mechanisms by which this can occur are still largely unknown. Mouse primary cell culture enables us to investigate the molecular mechanisms underlying various phenomena because it allows for relatively easy gene manipulation, which is indispensable for the molecular analysis. However, mouse synovial mesenchymal cells (SMCs) have not been established, although rabbit, cow, and rat SMCs are available, in addition to human MSCs. The aim of this study was to establish methods to harvest the synovium and to isolate and culture primary SMCs from mice. As the mouse SMCs were not able to be harvested and isolated using the same protocol for human, rat and rabbit SMCs, the protocol for humans was modified for SMCs from the Balb/c mouse knee joint. The mouse SMCs obtained showed superior proliferative potential, growth kinetics and colony formation compared to cells derived from muscle and bone marrow. They expressed PDGFRá and Sca-1 detected by flow cytometry, and showed an osteogenic, adipogenic and chondrogenic potential similar or superior to the cells derived from muscle and bone marrow by demonstrating *in vitro* osteogenesis, adipogenesis and chondrogenesis. In conclusion, we established a primary mouse synovial cell culture method. The cells derived from the mouse synovium demonstrated both the ability to proliferate and multipotentiality similar or superior to the cells derived from muscle and bone marrow.

Citation: Futami I, Ishijima M, Kaneko H, Tsuji K, Ichikawa-Tomikawa N, et al. (2012) Isolation and Characterization of Multipotential Mesenchymal Cells from the Mouse Synovium. PLoS ONE 7(9): e45517. doi:10.1371/journal.pone.0045517

Editor: Dimas Tadeu Covas, University of Sao Paulo - USP, Brazil

Received April 6, 2012; **Accepted** August 20, 2012; **Published** September 18, 2012

Copyright: © 2012 Futami et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was funded in part by a High Technology Research Center Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (19791047 and 21791418 for M.I.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No additional external funding was received for this study.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ishijima@juntendo.ac.jp

Introduction

Mesenchymal stem cells (MSCs) are multipotential non-hematopoietic progenitor cells that can differentiate not only *in vitro*, but also *in vivo*, into a variety of mesenchymal lineages such as osteoblasts, chondrocytes and adipocytes. Although MSCs were initially isolated from bone marrow [1], they are now able to be isolated from various types of adult mesenchymal tissue, such as the synovium, skeletal muscle, and adipose tissue, in addition to bone marrow [2,3,4].

The synovium has a high regenerative capacity, as evidenced by its full healing after surgical and chemical synovectomy in rabbits [5,6,7]. The osteophytes observed at the synovium–cartilage junction in osteoarthritis are usually accompanied by excess cartilage formation [8]. When partial-thickness defects in the articular cartilage were formed in rabbits, the synovial membrane extension contributed to the repair of the cartilage [9]. Reconstructed ligaments are recovered by synovial tissue in the natural course of the healing processes [10]. All of these findings suggest that the synovium plays an important role in tissue repair in the joint.

Human synovial MSCs have a higher capacity for proliferation and greater chondrogenic potential than those from other cell sources, such as bone marrow [11]. The synovium can be collected relatively easily under the arthroscopy, while marrow aspiration is necessary for bone marrow collection. Thus, synovial MSCs are considered to be one of the appropriate candidate cell sources for tissue repair, especially for articular cartilage repair, and are now being investigated clinically as a treatment for cartilage defects [12]. Despite the impressive data reported from various investigations, there are still a lot of obstacles facing clinical research for a complete articular cartilage repair. Numerous basic research questions related to the developmental origin of these cells, their proposed pluripotency, and their molecular mechanisms of tissue repair, especially the regulation of cartilage differentiation, are also still largely unanswered [13].

Mouse primary cell culture has enabled investigators to perform research to elucidate the molecular mechanisms of the phenomena because of the relatively easy gene manipulation in such cells,

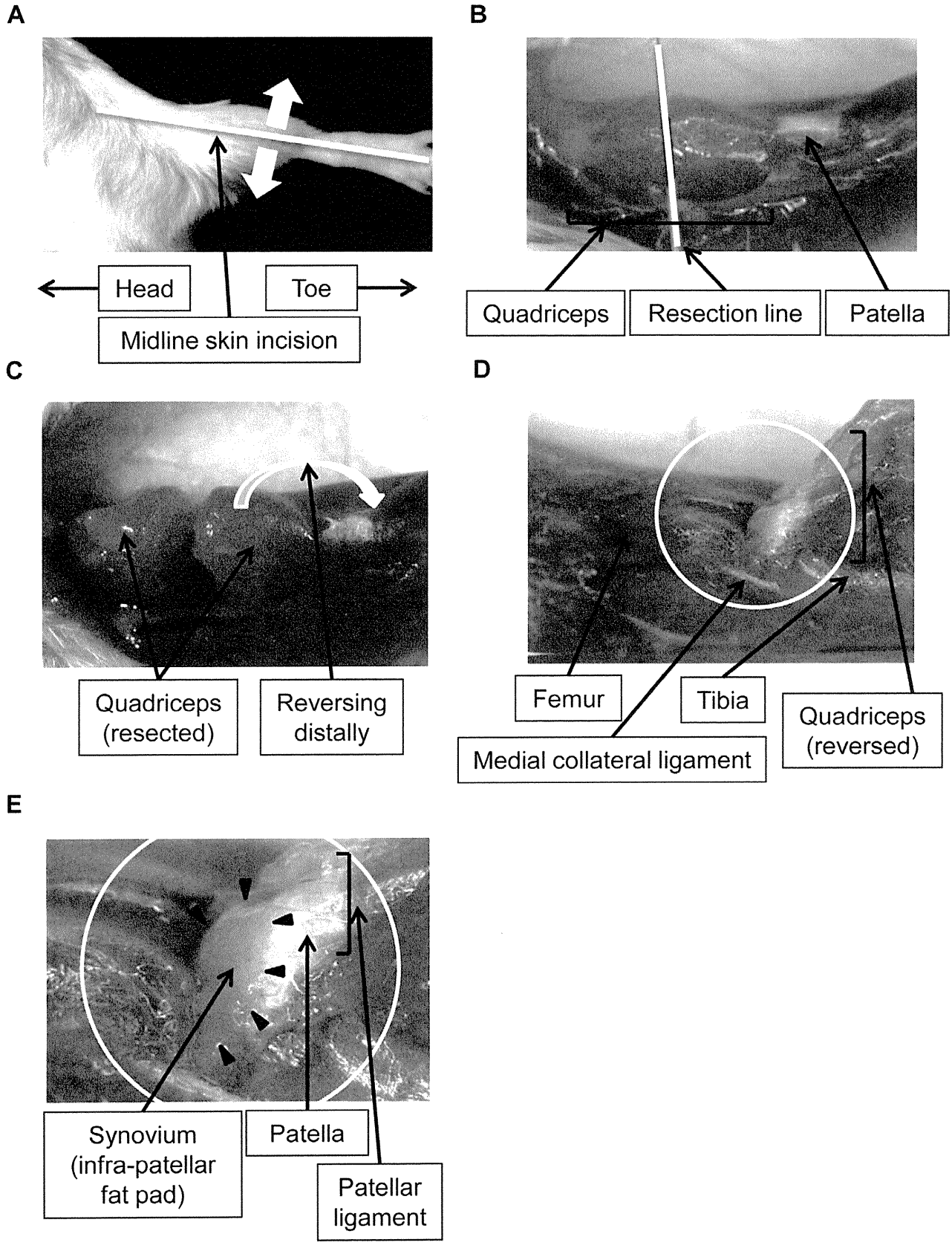

Figure 1. Microscopic photograph of a mouse knee to harvest the synovium. The knee joints were exposed by a midline skin incision (A). The quadriceps of the mice was microscopically resected at the middle (B) and reversed distally (C). As the femur, reversed patella and patellar ligament were exposed (D), the synovium on the infra-patellar fat pad could be easily visualized for resection purposes (E).
doi:10.1371/journal.pone.0045517.g001

which is indispensable for the molecular analysis. However, one of the obstacles we are currently confronting and have to overcome in this field is that mouse synovial MSCs have not been isolated and are not available for basic research, whereas rabbit [14], cow [15], and rat synovial MSCs [16] have been isolated, and are available for research, in addition to human synovial MSCs.

The aim of this study was to establish a primary synovial mesenchymal cell (SMC) culture method for cells isolated from the synovium of mouse knee joints, and to characterize these cells and determine whether they can function as MSCs.

Materials and Methods

Tissue Collection

Ten 10-week-old female Balb/c mice were prepared for the study. The synovium in the infra-patellar fat pad of these mice was harvested [details in the Results (**Figure 1**)]. Bone marrow was flushed from the femur and tibia of these mice. Muscle was obtained from their quadriceps. The protocol of this study was approved by the Institutional Animal Care and Use Committee of Juntendo University (Registration Number: 971, Permit Number: 220084, 230017). All experimental procedures were performed following the guidelines for the care and use of animals of Juntendo University.

Isolation and Culture of Mouse Cells

The mouse SMCs could not be isolated using the same protocol as that used for the isolation of human, rat and rabbit SMCs. Therefore, the protocol for human SMC culture had to be modified to obtain the appropriate conditions. The details of the methods used for the isolation and culture of mouse SMCs were also described in the Results (**Figure 1** and **Table 1**). The reagents used in this study were phosphate buffered saline (PBS), collagenase (Wako, Osaka, Japan), deoxyribonuclease I (DNase I: Sigma-Aldrich, St Louis, MO, USA) and Dulbecco's modified Eagle's medium (DMEM: Wako). Nucleated cells from the bone marrow were isolated with a density gradient (Ficoll-Paque; Amersham Biosciences, Uppsala, Sweden). Colony forming cells derived from muscle were used as muscle derived cells for this study. The muscle was digested in 0.1% collagenase 0.005% deoxyribonuclease I in DMEM at 37°C for 1 hour, and filtered through a 70-μm nylon filter (Becton Dickinson, Franklin Lakes, NJ, USA) The nucleated cells were plated in 6-well dishes for 24 hours in complete culture medium [DMEM containing 10% fetal bovine serum (FBS), 100 unit/mL penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA)] and incubated at 37°C with 5% humidified CO_2. The medium was changed every 3–4 days thereafter and then were cultured for 14 days as passage 0.

Table 1. The conditions for the isolation of mouse mesenchymal cells from the mouse knee joint.

	Human*/Rat**/ Rabbit***	Mouse
Minced size	2–3 mm	Less than 1 mm
Collagenase concentration	0.2–0.3%	0.1%
Collagenase reaction time	1–3 hr	15 min
Deoxyribonuclease (DNase) I	–	+

*[11], **[16], ***[14].
doi:10.1371/journal.pone.0045517.t001

Table 2. Monoclonal antibody list.

Antigen	Labeling	Clone	Isotype
CD29	PE	HMb1-1	Armenian Hamster IgG
CD34	FITC	RAM34	Rat IgG2a,k
CD44	APC	IM7	Rat IgG2b,k
CD45	APC	30-F11	Rat IgG2b,k
CD106(VCAM-1)	PE	429	Rat IgG2a,k
CD117(c-kit)	PE	2B8	Rat IgG2a,k
CD140á(PDGFRá)	APC	APA5	Rat IgG2a,k
Sca-1	FITC	D7	Rat IgG2a,k

APC: allophycocyaninFITC: Fluorescein isothiocyanate, PE: phycoerythrin.
VCAM-1: vascular cell adhesion molecule 1.
PDGFRα: platelet derived growth factor receptor alpha.
doi:10.1371/journal.pone.0045517.t002

Cell Expansion Assay

To compare the survival of the cells, cells derived from the synovium, muscle and bone marrow were replated every 14 days. The number of cells that were able to be replated ten times was counted. A Kaplan-Meier survival analysis was conducted to compare the survival rates of the cells.

Nucleated cells derived from the synovium, muscle and bone marrow were clonally expanded for 14 days, and cells at passage 2 were plated at 2,000 cells/cm^2 on 60-cm^2 dishes. Then, the cells were replated at 2,000 cells/cm^2 every 14 days until passage 10.

For the growth kinetics study, the cells derived from mouse synovium, muscle and bone marrow at passage 4 were plated at 1,000 cells/cm^2 and counted with a hemocytometer every two days until 14 days.

Colony-forming Efficacy

The cells at passage 4 were replaced at 1000 cells per 60-cm^2 dish, incubated for 14 days, and stained with 0.5% crystal violet in 4% paraformaldehyde for 5 minutes. The cells were then washed twice with distilled water, and the number of colonies per dish was determined. Colonies less than 2 mm in diameter and faintly stained colonies were ignored.

Flow Cytometry

One million cells at passage 3 were suspended in 500 μl PBS containing 20 μg/ml of antibody. After incubation for 30 minutes at 4°C, the cells were washed with PBS and suspended in 1 ml PBS for the analysis. Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or allophycocyanin (APC)- coupled antibodies against mouse CD29, CD34, CD117, and CD140á were obtained from eBioscience (San Diego, CA, USA). The CD44, CD45, CD106, and Sca-1 antibodies were from BioLegend (San Diego, CA, USA). For the isotype controls, FITC-, PE- or APC-coupled nonspecific rat and hamster IgG (eBioscience) was substituted for the primary antibody. The details of the antibodies used for flow cytometry are shown in **Table 2**. Cell fluorescence was evaluated by flow cytometry using a FACSAria instrument (Becton Dickinson, Franklin Lakes, NJ, USA), and the data were analyzed using the CellQuest software program (Becton Dickinson).

Osteogenesis in a Colony-forming Assay

One thousand cells were plated in 60-cm^2 dishes and cultured in complete medium for 7 days. The medium was switched to calcification medium that consisted of complete medium supple-

Table 3. Primers for specific polymerase chain reaction.

Primer	Forward	Reverse
β-actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT
C/EBPå	ACCGGGTTTCGGGACTTGA	CCCGCAGGAACATCTTTAAGTGA
Col1a1	GACATGTTCAGCTTTGTGGACCTC	GGGACCCTTAGGCCATTGTGTA
Col2a1	GGGCTCCAATGATGTAGAGATG	CCCACTTACCAGTGTGTTTCG
Col10a1	GCATCTCCCAGCACCAGA	CCATGAACCAGGGTCAAGAA
FABP4	TGGGAACCTGGAAGCTTGTCTC	GAATTCCACGCCCAGTTTGA
Lpl	AGAGGCTATAGCTGGGAGCAGAAAC	GCAAGGGCTAACATTCCAGCA
PPARã	GCCCAGGCTTGCTGAACGTGAAG	CACGTGCTCTGTGACGATCTGCC
Sox9	CAGCAAGACTCTGGGCAAG	TCCACGAAGGGTCTCTTCTC
Osteocalcin	GACCATCTTTCTGCTCACTCTG	GTGATACCATAGATGCGTTTGTAG
Osteopontin	CAGTGATTTGCTTTTGCCTGTTTG	GGTCTCATCAGACTCATCCGAATG
Runx2	GCACAAACATGGCCAGATCA	AAGCCATGGTGCCCGTTAG

C/EBPå: CCAAT/enhancer binding protein beta, FABP4: Fatty Acid Binding Protein 4, Lpl: Lipoprotein Lipase, PPARã: Peroxisome Proliferator-Activated Receptor gamma, RUNX2:Runt-related transcription factor 2.

doi:10.1371/journal.pone.0045517.t003

mented with 1 nM dexamethasone (Sigma-Aldrich), 20 mM glycerol phosphate (Sigma-Aldrich), and 50 μg/ml ascorbate-2-phosphate (Sigma-Aldrich) for an additional 21 days. The osteogenic medium was replaced every 3–4 days. The dishes were subsequently stained with fresh 0.5% alizarin red solution, and the number of alizarin red–positive colonies was determined. Colonies less than 2 mm in diameter and faintly stained colonies were ignored. The same calcification cultures were then stained with crystal violet, and the total number of cell colonies was determined [11,16].

Adipogenesis in a Colony-forming Assay

One thousand cells were plated in 60-cm^2 dishes and cultured in complete medium for 7 days. The medium was then switched to adipogenic medium that consisted of complete medium supplemented with 10 nM dexamethasone, 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), and 50 μM indomethacin (Sigma-Aldrich) for an additional 21 days. The adipogenic medium was replaced every 3–4 days. The adipogenic cultures were fixed in 4% paraformaldehyde, stained with fresh 0.5% oil red O solution, and the number of oil red O–positive colonies was determined. Colonies less than 2 mm in diameter and faintly stained colonies were ignored. The same adipogenic cultures were subsequently stained with crystal violet, and the total number of cell colonies was determined [11,16].

In vitro Chondrogenesis

2×10^5 cells were placed in a 15-ml polypropylene tube (BD Falcon, Franklin Lakes, NJ, USA) and centrifuged at 450×*g* for 10 minutes. The pellet was cultured at 37°C with CO_2 in 400 μl of chondrogenic medium that contained 500 μg/ml recombinant human bone morphogenetic protein 7 (rhBMP7: Stryker biotech,

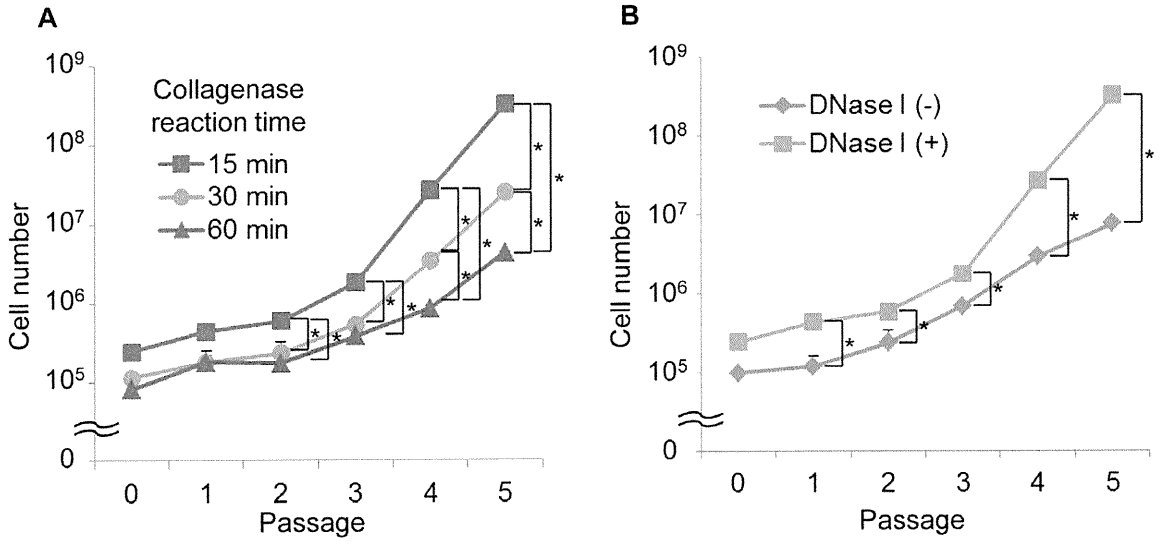

Figure 2. A comparison of the mouse SMC proliferation potentials following digestion by different collagenase reaction times. (A) and by the presence or absence of DNase I (B). DNase I; deoxyribonuclease I, SMC; synovial mesenchymal cell. * indicates $p<0.05$.

doi:10.1371/journal.pone.0045517.g002

Table 4. Date on the cell samples.

	Sample size	Nucleated cell no.
Bone marrow	0.15 (0.02) (ml)	1.72×10^7 (1.90×10^6) (cells/ml)
Synovium	1.29 (0.16) (mg)	4.35×10^4 (8.36×10^3) (cells/mg)
Muscle	13.84 (4.59) (mg)	3.73×10^4 (9.10×10^3) (cells/mg)

Data (SD) (unit).
doi:10.1371/journal.pone.0045517.t004

Cambridge, MA, USA) in high-glucose DMEM supplemented with 10 ng/ml transforming growth factor â3 (TGFâ3; R&D Systems, Minneapolis, MN, USA), 100 nM dexamethasone, 50 μg/ml ascorbate-2-phosphate, 40 μg/ml proline (Sigma-Aldrich), 100 μg/ml pyruvate (Sigma-Aldrich), and 1:100 diluted ITS+ Premix (Becton Dickinson). The medium was replaced every 3–4 days for 21 days. For the microscopy studies, the pellets were embedded in paraffin, cut into 5-μm sections, and stained with toluidine blue and type II collagen [17].

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA from cultured cells and cultured pellets was prepared by using the Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) with a random hexamer primer.

The qRT-PCR analyses of osteogenesis and adipogenesis were performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Green I PCR reagents (TOYOBO, Osaka, Japan) under the following condition; initial denaturation for 10 minutes at 94°C followed by 40 cycles consisting of 15 seconds at 94°C and 1 minute at 60°C. The copy number was expressed as the number of transcripts per nanogram total RNA. Experimental samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR protocol. The amounts of mRNA were shown as relative quantities in comparison to that of β-actin mRNA.

To evaluate chondrogenesis, a LightCycler 480 instrument (Roche Applied Science) was used with the FastStart Taqman Probe Master (Roche Applied Science). The qRT-PCR conditions were as follows: 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Normalization was performed using β-actin. Quantification was performed using the comparative Ct method [15].

For the qRT-PCR analysis, primers for the following target genes were used (**Table 3**): Runt-related transcription factor 2 (RUNX2), Osteocalcin, Osteopontin and Type I collagen for osteogenesis, Peroxisome proliferator-activated receptor ã (PPARã), CCAAT/enhancer binding protein â (C/EBPâ), Fatty acid binding protein 4 (FABP4) and Lipoproteinlipase (Lpl) for adipogenesis, Sex determining region Y-box 9 (Sox9), Type II and Type X collagen to evaluate chondrogenesis.

Statistical Analysis

To assess significance of difference, the log rank test was used for the Kaplan-Meier survival analysis and the Mann-Whitney U test was used for other analyses. *P* values less than 0.05 were considered to be significant.

Results

Harvesting the Synovium from the Mouse Knee Joint

Mice were fixed in the supine and legs extended position. The knee joints were exposed by a midline skin incision (**Figure 1A**). As the mouse synovium was too small and fragile to be isolated by a common lateral approach of the knee joint, the isolation of synovium was difficult. Thus, the quadriceps reversing approach was used, as explained below. The quadriceps was transversely resected at the middle (**Figure 1B**) and reversed distally (**Figure 1C**), enabling us to distinguish between the patella and patellar ligament (**Figure 1D**). As a result, the synovium of the infra-patellar fat pad attached to the patellar ligament could easily be seen and resected from the patellar ligament (**Figure 1E**).

Isolation and Culture of SMCs

The mouse SMCs could not be isolated using the same protocol used for the isolation of human, rat and rabbit SMCs, as shown in Table 2 [11,14,16,18]. When the mouse synovium was harvested and treated according to the conditions for human, rat, and rabbit cells, the cell proliferation was poor, and the cells became flat. In most cases, the cells harvested under the conditions used for human, rat and rabbit cells were not able to be cultured even twice (data not shown). Therefore, the protocol for human SMC culture had to be modified to obtain the appropriate conditions for isolation of mouse SMCs. We determined that the collagenase concentration for the mouse synovium had to be reduced to 0.1% for the mouse from 0.2–0.3% that used for human synovium (**Table 1**). In addition, a 15-minutes collagenase reaction time (**Table 1**), which was also reduced from 1–3 hours, which is the time used for human cells [11], was determined to be appropriate for the isolation and culture of SMCs based upon the results of comparison of the cell growth (**Figure 2A**). Although DNase was not used for human cells, the supplementation with DNase I in the isolation medium showed the superior isolation and culture results for the mouse SMCs in comparison to that without DNase I (**Figure 2B**).

Based on these results, the methods used for the isolation and culture of mouse SMCs were determined as follows: The harvested synovial tissue was minced into pieces (less than 1 mm) with a surgical knife, washed thoroughly with PBS to remove hematopoietic cells, and treated with 0.1% collagenase and 0.005% DNase I in DMEM at 37°C for 15 minutes. The digested cells were filtered through a 70-μm mesh nylon filter. The quantity of the harvested tissues was measured and the isolated cells were counted using a hemocytometer (**Table 4**). The nucleated cells from the tissues were placed in 6-well dishes for 3 hours in complete culture medium and incubated at 37°C with 5% humidified CO_2. Nonadherent cells were removed by changing the medium. The medium was changed every 3–4 days thereafter. The nucleated cells were cultured for 14 days at passage 0.

Expansion Capacity of Mouse SMCs

To examine the characteristics of the mouse SMCs obtained by the established conditions, described above, several experiments were conducted. First, the functional capacity for self-renewal was examined. All of the mouse SMCs we obtained were able to survive until passage 10, while the mouse primary cultured cells derived from muscle and bone marrow showed survival rates of 70% and 50%, respectively (**Figure 3A**). As statistical analysis revealed that the survival rate of mouse SMCs was significantly superior in comparison to that of cells derived from bone marrow (**Figure 3A**). Next, the growth kinetics of mouse SMCs was examined. The proliferation of mouse SMCs was superior or

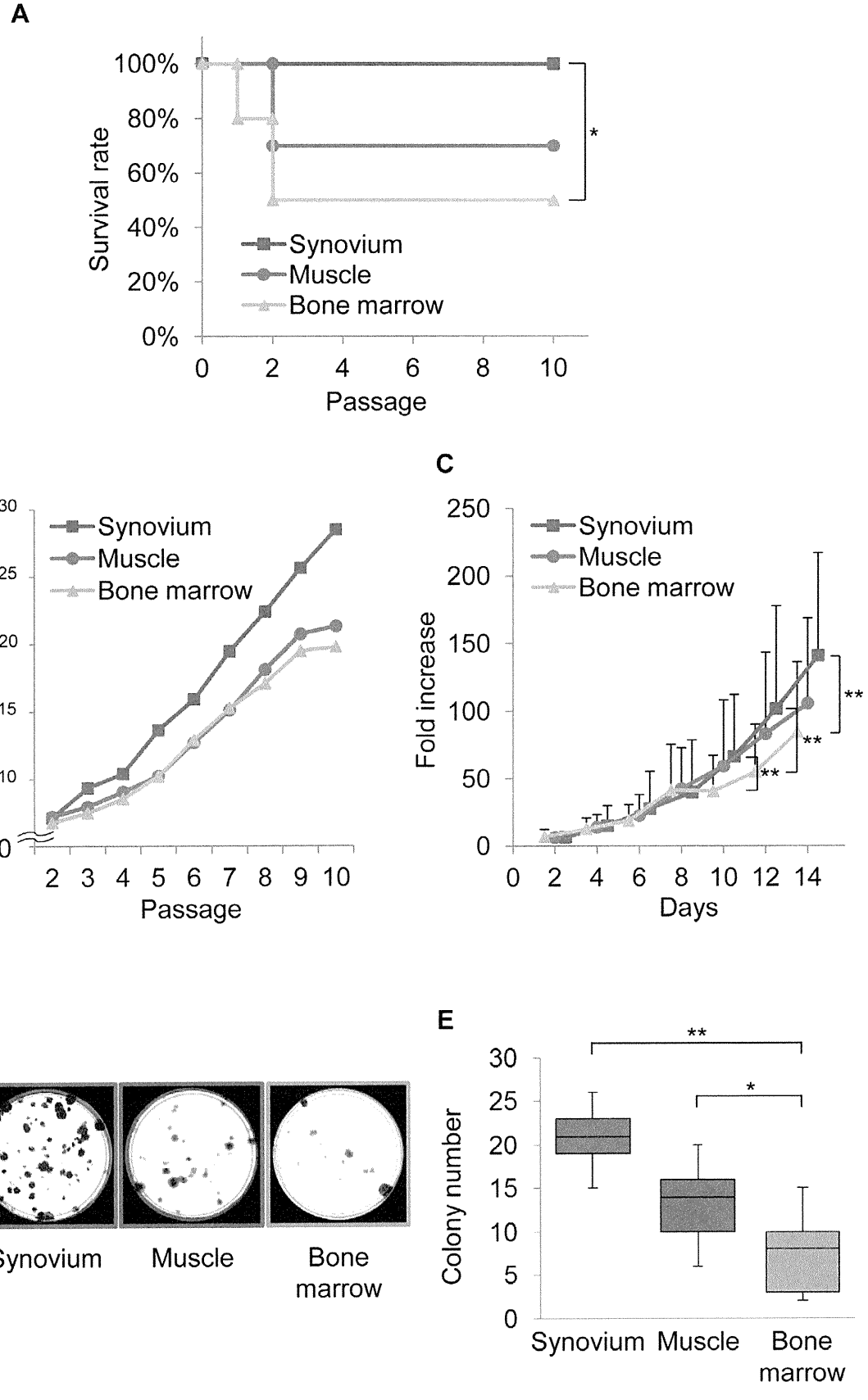

Figure 3. The expansion ability of mouse SMCs. (A): The survival rate of the cells. (B and C): The growth of the cells. (D): Crystal violet staining. (E): Quantification of the colony-forming ability. * and ** indicate $p<0.05$, and <0.01, respectively.
doi:10.1371/journal.pone.0045517.g003