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

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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

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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

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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

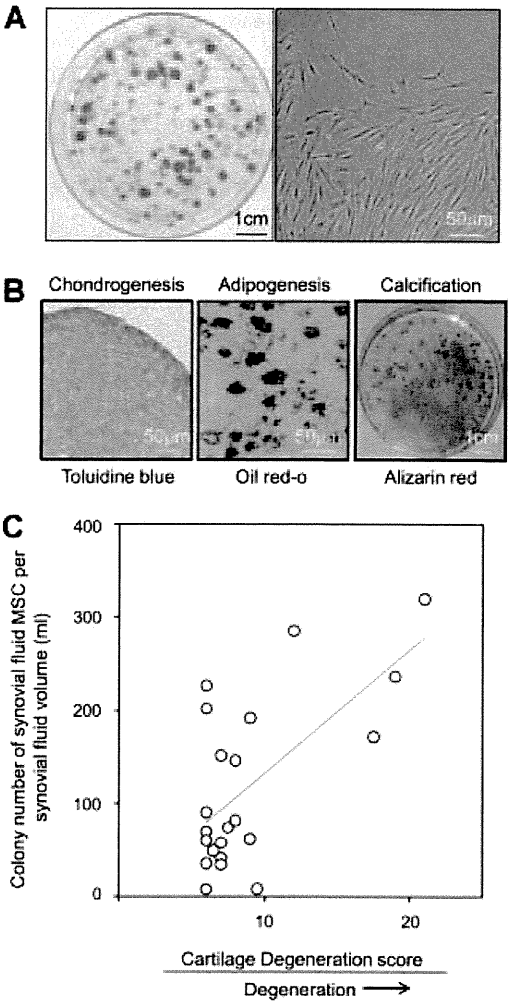

Figure 1. Colony-forming cells in synovial fluid derived from ACL injury patients. (A) Representative cell colonies stained with crystal violet and cell morphology. (B) Multidifferentiation potential. (C) Relationship between cartilage degeneration score and colony number of synovial fluid MSCs per synovial 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

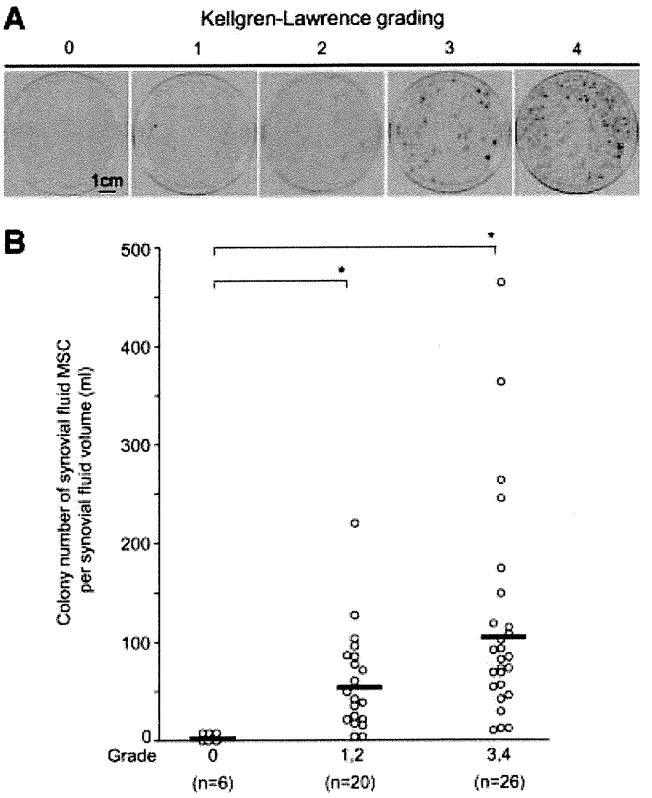

Figure 2. MSCs in synovial fluid derived from osteoarthritis patients. (A) Representative dishes for colonies of synovial fluid MSCs according to the Kellgren–Lawrence grading system. (B) Relationship between the osteoarthritis grading and the colony number of synovial fluid MSCs per synovial fluid volume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

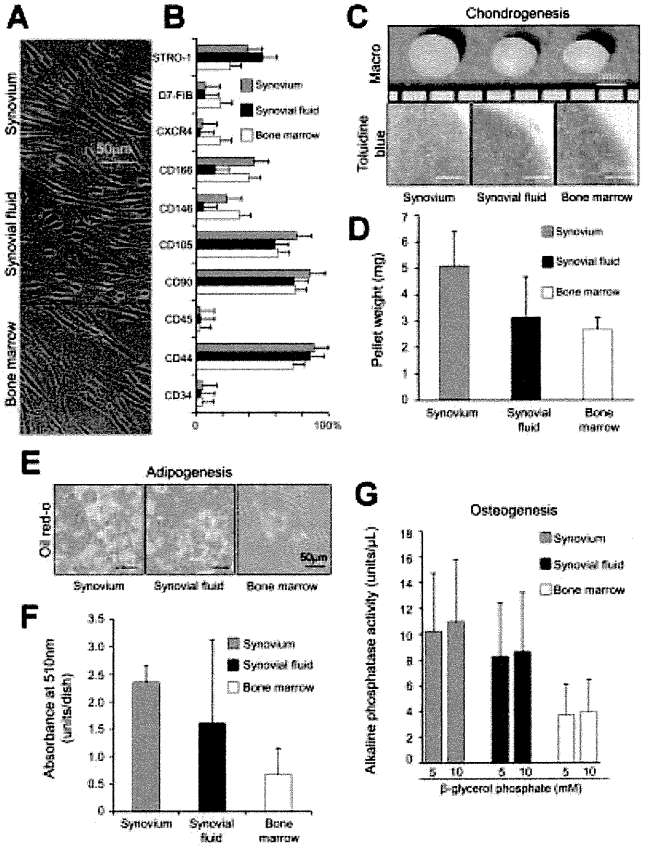

Figure 3. Comparison among synovium MSCs, synovial fluid MSCs, and bone marrow MSCs from osteoarthritis patients. (A) Representative morphologies. (B) Surface epitopes of MSCs. Positive expression rates are displayed as the average and standard deviation ($n = 5$). (C) Chondrogenesis. (D) Wet weight 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

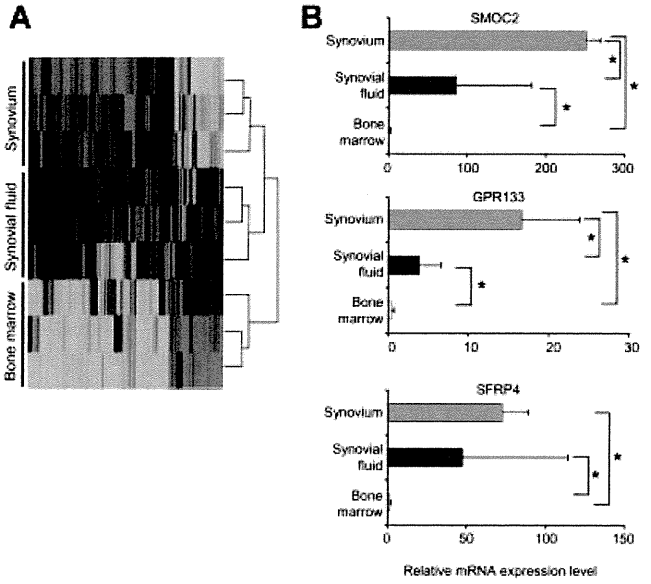

Figure 4. Comparison of the gene expression profiles among synovium MSCs, synovial fluid MSCs, and bone marrow MSCs from osteoarthritis patients. (A) Hierarchical clustering analysis ($n = 3$). (B) Quantitative RT-PCR analysis for the top three genes shown in Table 1. Relative amount of PCR products 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.

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南江堂

滑膜間葉幹細胞の役割と低侵襲な軟骨再生への応用

関節液中の細胞成分を培養皿に培養すると，ある割合の細胞がコロニー（細胞集団）を形成する．条件をかえてこの細胞を培養すると軟骨・骨・脂肪に分化する．関節液中にはコロニーを形成し，多分化能を有する間葉幹細胞が存在する．正常膝関節液中の間葉幹細胞はわずかにしか存在しないが，前十字靱帯損傷や変形性関節症の膝の関節液中には100倍以上多くの間葉幹細胞が存在する[1]．

関節液中間葉幹細胞の遺伝子発現を網羅的に解析すると，滑膜由来間葉幹細胞の遺伝子発現に類似することが示される[2]．動物モデルで前十字靱帯・軟骨・半月板をそれぞれ欠損させ，滑膜間葉幹細胞を関節内注射すると損傷部位に接着し，組織修復が促進する[3]．滑膜は間葉幹細胞のリザーバであり，関節内組織損傷時には滑膜から関節液中に幹細胞が動員され，損傷部位に接着し，修復に寄与する機序が存在すると考えられる．関節内組織損傷の自然治癒に限界があるのは動員される幹細胞の絶対数が少ないためであり，体外で滑膜由来の幹細胞を増殖して移植すれば自然治癒力を増強する可能性がある．

間葉幹細胞の軟骨分化能を *in vitro* および *in vivo* で比較すると，滑膜や骨髄由来のものは皮下脂肪や骨格筋由来のものよりも軟骨分化能が高い[4,5]．自己血清による培養で，滑膜間葉幹細胞は骨髄液由来のものよりも初代細胞を多く確保できるという利点がある[6]．滑膜間葉幹細胞の浮遊液を軟骨欠損部に10分間静置すると，約60%の細胞が接着する[7]．

これらの基礎研究をもとに，筆者らは軟骨再生の臨床研究を開始している．採取した滑膜を酵素処理後，自己血清を使用して14日間，本学の細胞治療センターで培養し，関節鏡視下で軟骨欠損部に細胞浮遊液を10分間静置して移植する（図1）．翌日より可動域訓練，2週後より部分荷重，6週後より全荷重を開始する．これまで20例以上に行い，多くの症例で自覚症状が改善し，MRIで軟骨欠損部が修復されている結果を得ている．

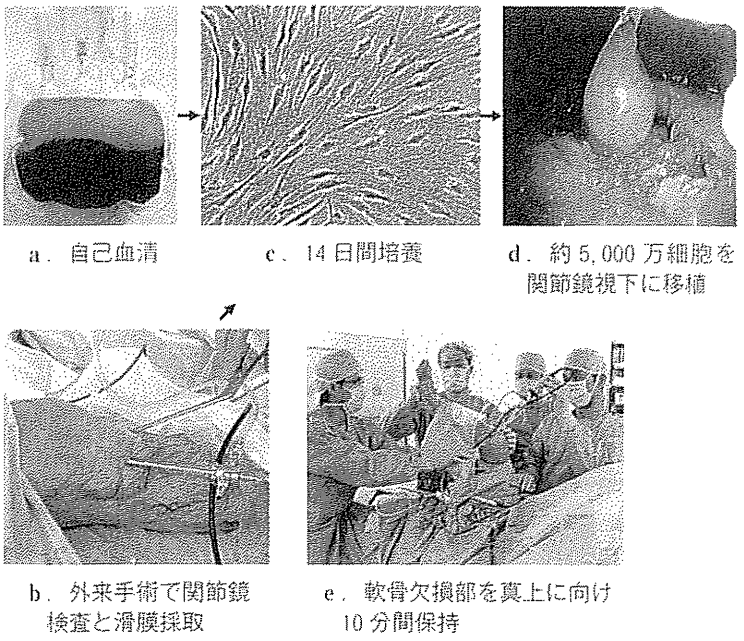

図1．滑膜間葉幹細胞による軟骨再生医療のスキーム．はじめに自己血清を準備する．外来手術で関節鏡検査と滑膜採取を行う．約0.5 gの滑膜を酵素処理後，14日間自己血清を使用して細胞治療センターで培養する．細胞浮遊液を関節鏡視下で軟骨欠損部に静置し，10分間肢位を保持する．

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滑膜由来の幹細胞による再生医療

Regenerative medicine for osteoarthritis using mesenchymal stem cells from synovium

関矢 一郎* 宗田 大**
Sekiya Ichiro Muneta Takeshi

抄録▶変形性膝関節症において，滑膜から間葉系幹細胞が関節液中に動員され，軟骨変性部に接着し，軟骨基質の産生を促す機序の存在が予測される．滑膜由来の間葉系幹細胞は軟骨分化能が高く，確実に細胞数を確保できるため，軟骨再生医療の細胞源として有用である．滑膜間葉系幹細胞の浮遊液を軟骨欠損部に10分間静置すると，効率よく細胞が接着し，軟骨の再生が認められる．この方法は自然修復を促進するものと考えられ，また低侵襲な軟骨再生を可能にする．さらに変形性膝関節症への応用も期待できる．

Key Words 間葉系幹細胞，滑膜，関節液，骨髄，軟骨再生

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変形性膝関節症の再生医療

変形性膝関節症は，膝関節軟骨の磨耗・消失と，骨棘形成を特徴とする，進行性の関節疾患である．軟骨は代謝の低い組織であるが，正常膝では軟骨基質の合成と分解のバランスが調和し，基質の量が維持される．変形性膝関節症の進行過程では，軟骨基質の合成よりも分解が上回るため，軟骨基質の全体量は減少する．変形性膝関節症の再生を考える場合，軟骨基質の合成を司る自然機序を促進させることが戦略のひとつとなる．

間葉系幹細胞について

骨髄液を直接培養用ディッシュに播種し，2週間培養すると1つの細胞由来と考えられる細胞集団，いわゆるコロニーを形成する．このコロニー形成細胞をまとめて回収し，条件を変えて培養すると，骨，軟骨，脂肪に分化し，多分化能が示される．このコロニー形成細胞は特有の表面抗原パターンを示し，間葉系幹細胞と呼ばれる．間葉系幹細胞は生体の恒常性を維持し，組織損傷時の修復に寄与する．

2000年以降になると骨髄以外の皮下脂肪や骨格筋などの種々の間葉組織から，間葉系幹細胞が採取できることが多数報告されるようになった[1]．間葉系幹細胞は，元の組織によらない共通した特性を有する一方，元の組織に依存する特性も報告されるようになっている[2,3]．

私たちは軟骨再生に対して間葉系幹細胞を用いる際に，どの組織由来のものが最適か検討を重ねてきた．膝関節を構成する組織で，手術中に採取が容易な骨髄液，滑膜，骨膜，骨格筋，皮下脂肪から同等な手法で間葉系幹細胞を採取し，増殖させ，その特性を検討した．すると骨髄液と滑膜由来のものが，軟骨に分化する能力の高いことが明らかになった[4-6]．軟骨組織は骨髄と滑膜に隣接することが，その理由になると考えられる[7]．獲得できる細胞数を比較すると，骨髄よりも滑膜由来の間葉系幹細胞のほうが，

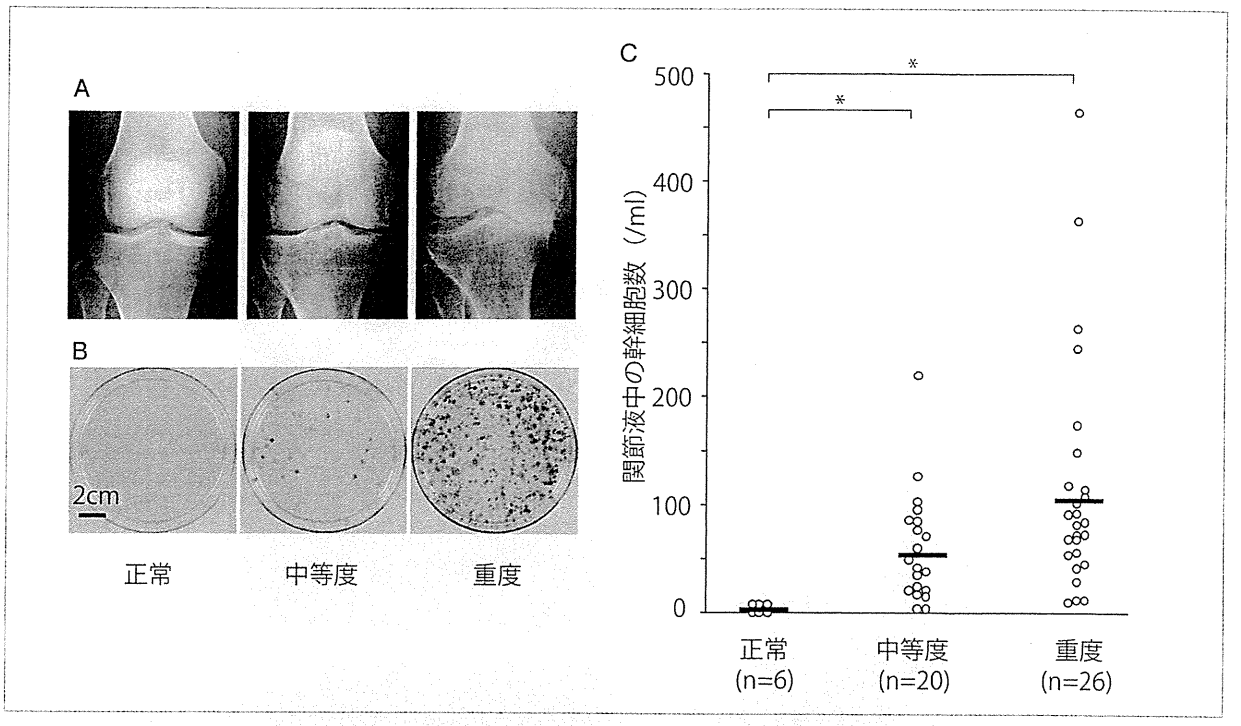

図1　変形性膝関節症の関節液中に含まれる間葉系幹細胞

(A) 立位伸展位正面のレントゲン像．Kellgren Lawrence分類でグレード1と2を中等度，グレード3と4を重度の変形性膝関節症とした．(B) 穿刺した関節液をフィルターに通しdebrisを除去後，全細胞成分の1/6をディッシュに播種し14日間培養後，クリスタルバイオレッドで染色したもの．間葉系幹細胞のコロニーが観察される．(C) 変形性膝関節症のグレード毎にプロットした関節液1mlあたりの間葉系幹細胞の数．平均値をバーで示す（p=0.002 by Kruskal-Wallis test; *=p<0.05 by Steel-Dwass test）．（文献10より）

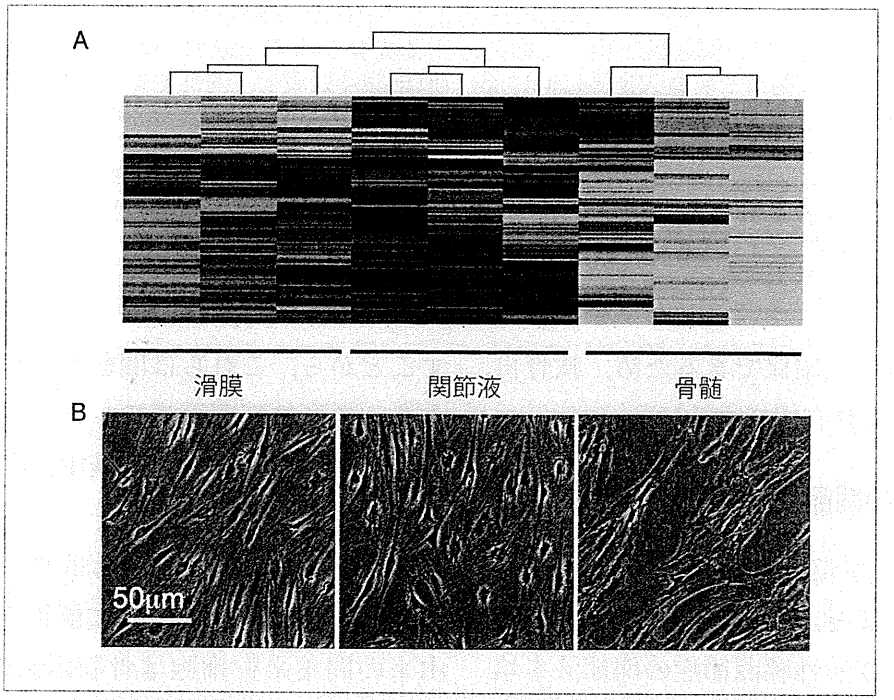

図2　滑膜，関節液，骨髄由来の間葉系幹細胞に関する特性の比較

(A) 3名の変形性膝関節症の方から手術時に各組織を採取し，同一条件で間葉系幹細胞を分離後，total RNAを抽出し，マイクロアレイによる遺伝子プロファイル解析を行った．発現が強い遺伝子が緑に，弱い遺伝子が赤く示されている．階層的クラスター分析の結果，関節液由来の間葉系幹細胞は，骨髄由来よりも滑膜由来のものに遺伝子プロファイルが類似する．(B) 滑膜，関節液，骨髄由来の間葉系幹細胞の形態．（文献10より）

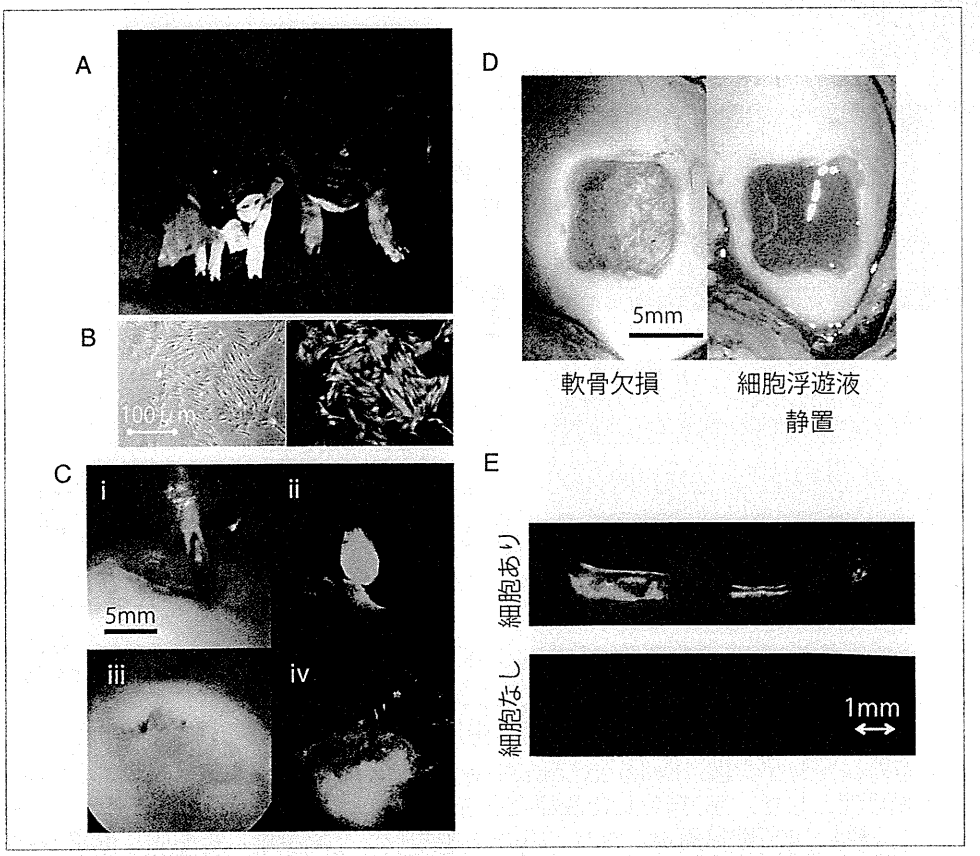

図3　軟骨欠損部に細胞浮遊液を10分間静置する方法による，細胞接着効果のブタを用いた検討

(A)蛍光を照射すると全身が緑に発色する遺伝子改変ブタ．特に目および白い鼻や四肢が強く緑に発色している．(B)遺伝子改変ブタ由来の滑膜間葉系幹細胞の形態．(C)蛍光を検出する関節鏡を用いた観察．(i)野生型ブタの大腿骨内顆に軟骨欠損を作成し，注射針を軟骨欠損部に向ける．(ii)細胞浮遊液を注射器で軟骨欠損部に静置する．(iii)10分後に膝関節内を還流液で満たす．(iv)関節鏡の尖端から還流液が勢いよく流れているにもかかわらず，細胞が軟骨欠損部に接着している．(D)ブタの膝関節大腿骨内顆の荷重面に軟骨欠損を作成し，赤く標識した滑膜間葉系幹細胞の浮遊液を10分間静置した．(E)1週間後に組織学的に観察し，移植細胞が軟骨欠損部に接着していることが確認される．(文献15より)

確実に多くの細胞数を確保できるため，軟骨再生の細胞源としてより有用である[8,9]．

変形性膝関節症の関節液中に含まれる幹細胞

正常膝の関節液を培養用ディッシュに播種し，培養しても，ほとんど細胞のコロニーを認めない．中等度の変形性膝関節症の関節液を培養すると少数の，高度の変形性膝関節症の関節液では多数のコロニー形成細胞を認める(図1)．これらのコロニー形成細胞は培養条件を変えることにより，骨，軟骨，脂肪に分化し，多分化能を有する．また特有の表面抗原パターンを示すことから，これらは間葉系幹細胞の特徴を有する．変形性膝関節症のレントゲン分類による重症度が増すほど，関節液中に含まれる間葉系幹細胞の数が増す[10]．

骨髄，滑膜，関節液から間葉系幹細胞を採取し，遺伝子発現を網羅的に解析すると，関節液由来の間葉系幹細胞は骨髄由来のものよりも，滑膜由来のものに類似する(図2)．また細胞形態も，より細長く，核が明瞭である点で，関節液由来の間葉系幹細胞は骨髄由来のものよりも，滑膜由来のものに類似する．

私たちは過去に，前十字靱帯損傷後に得られ

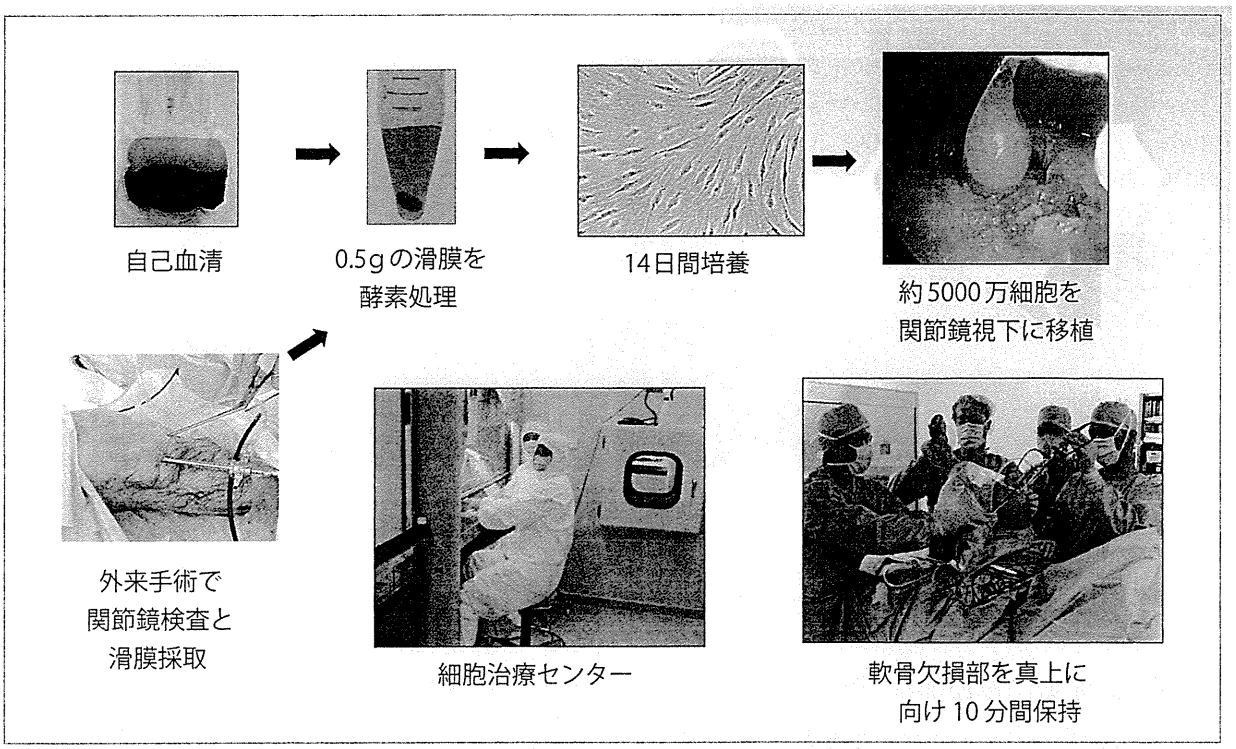

図5　自己滑膜間葉系幹細胞による軟骨再生医療のスキーム

外来手術で関節鏡検査と同時に滑膜を採取し，酵素処理後，自己血清を用いて14日間細胞治療センターで培養し，細胞浮遊液を関節鏡視下で軟骨欠損部に静置し，10分間肢位を保持して細胞を接着させる．

グから滑膜を採取し，間葉系幹細胞を採取した(図3)．注射器を用いて軟骨欠損部に滑膜間葉系幹細胞の浮遊液を軟骨欠損部に静置し10分間保持した後に，関節内を還流液で満たし，GFPを検出する関節鏡で観察すると，関節鏡の先端から勢いよく還流液が流れ出しているにもかかわらず，GFP陽性滑膜間葉系幹細胞は軟骨欠損部に接着していた．また蛍光を照射すると赤く発色する色素でラベルした滑膜間葉系幹細胞の浮遊液を10分間静置させ，1週後に観察すると軟骨欠損部にラベルされた細胞を検出できた．

さらにこの方法を用いて，ブタの軟骨欠損部に滑膜間葉系幹細胞を接着させ，再生過程を関節鏡で経時的に観察した．軟骨欠損を作成し細胞を投与しないコントロール群では，軟骨欠損部が時間経過とともに拡大した(図4)．一方，細胞を投与したものは，1カ月時に薄い膜で覆われ，2カ月時に膜が厚くなり，3カ月時には軟骨様の組織で覆われた．組織で評価すると，コントロールでは，1カ月時よりもさらに軟骨欠損部が拡大していることが確認される一方で，細胞投与群では1カ月時に膜様組織で欠損部が満たされ，3カ月時には軟骨基質が観察された[15]．このブタのモデルでは，軟骨再生が完了するまでに3カ月以上の期間を要するものと思われる．

滑膜間葉系幹細胞の鏡視下移植術の実際

私たちはこれまでの基礎研究の成果を基にして，膝関節軟骨欠損や局所に限定している変形性膝関節症に対して，自己滑膜間葉系幹細胞を関節鏡視下で移植する臨床研究を開始している(図5)．まず末梢血を採取し，自己血清を分離して用意する．外来手術で関節鏡検査と同時に滑膜を採取する．本学の手術室と同じフロアにある細胞治療センターで，滑膜を酵素処理後，10%自己血清を用いて滑膜間葉系幹細胞を14日間培養する．平均0.5gの滑膜と70 mlの自己血清から，14日間で平均5,000万細胞を採取できる．この細胞の浮遊液を関節鏡視下で軟骨欠損

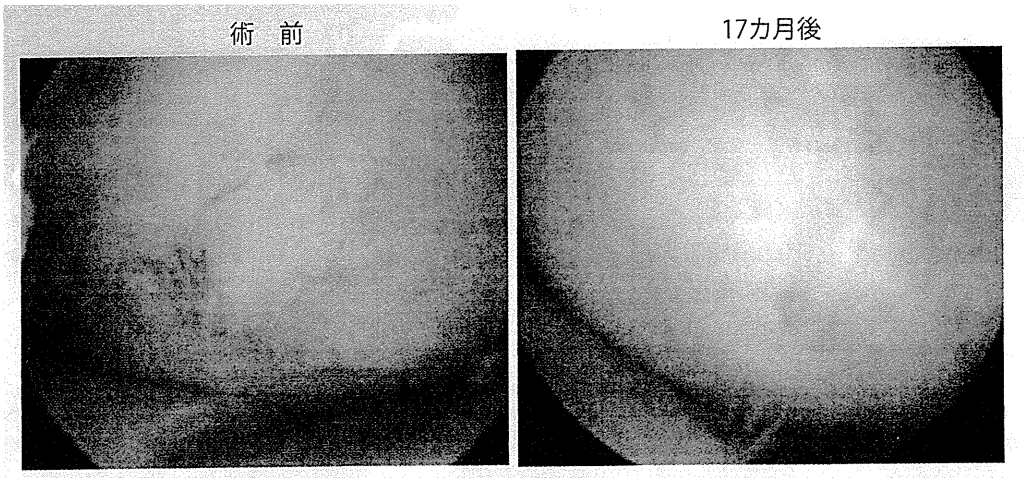

図6 臨床例

内側型変形性膝関節症に対して，高位脛骨骨切術後，自己滑膜間葉系幹細胞の浮遊液を軟骨変性部に静置し，10分間肢位を保持した．17カ月後の抜釘時に再鏡視を行った．細胞移植した大腿骨内顆の軟骨が厚くなっている．

部に10分間静置する．後療法は，外固定をせず，2週後から部分荷重，6週後から全荷重を開始する．この方法は動物血清や人工素材を必要とせず，低侵襲で実施可能である利点がある．これまで重篤な副作用を認めていない．多くの場合で自覚症状が改善し，MRIで軟骨が再生することを確認している．内反変形の強い変形性膝関節症の場合は高位脛骨骨切術を併用している．再鏡視で軟骨が厚くなる効果を確認しているが(図6)，骨切術のみの場合と比較する検討が必要と考えている．

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治療

特集

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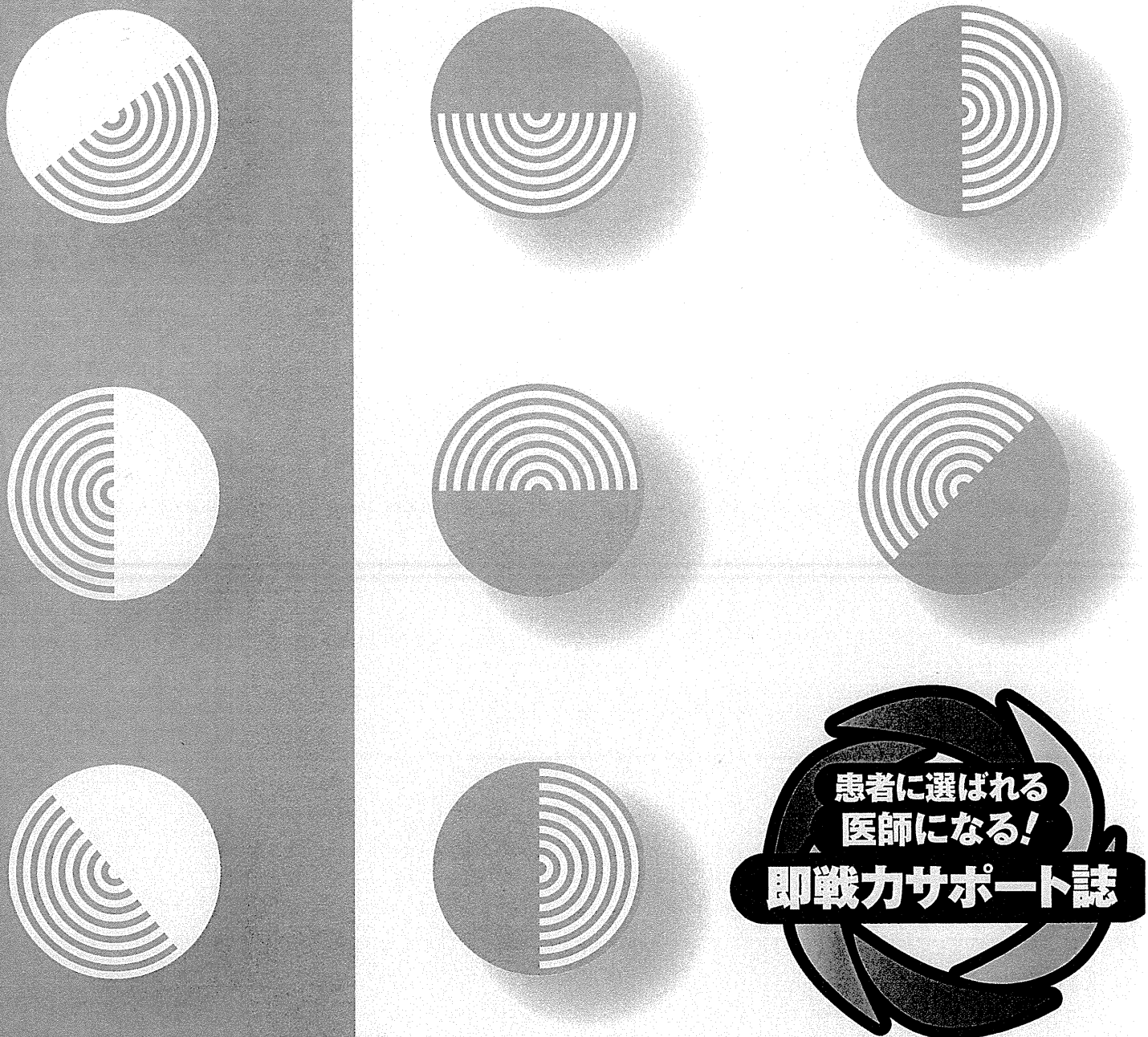

南山堂

滑膜幹細胞を用いた関節軟骨再生

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Summary

軟骨組織は細胞密度が低く，血行を欠くことから，再生能力が低い．そのため，軟骨欠損部に対して細胞成分を補うことが，再生させるための手段の１つになる．細胞源として間葉幹細胞は，軟骨組織を犠牲にせず，自分の細胞を使用でき，多数の細胞を確保できる点で有用である．骨髄液，滑膜，骨膜，皮下脂肪，筋肉から同一条件で間葉幹細胞を採取し比較すると，骨髄液と滑膜由来のものが軟骨分化能が高い．さらに滑膜由来のものは，自己血清を用いて優れた増殖を示すことから軟骨再生の細胞源として有用である．滑膜間葉幹細胞の浮遊液を軟骨欠損部に10分間静置すると約60%の細胞が接着し，軟骨修復を促進させることが実験的に示されている．これまでの基礎研究の成果を基にして，滑膜間葉幹細胞を関節鏡視下で移植する軟骨再生医療を開始している．重篤な副作用を認めず，多数の例で軟骨欠損部の再生，症状の改善を認めている．

はじめに

軟骨組織は細胞密度が低く，血行を欠くため，再生能力が低い．そのため，軟骨欠損に対して細胞成分を補うことが，軟骨再生を向上させるための手段の1つになる．細胞源として間葉幹細胞は，軟骨組織を犠牲にせず，自分の細胞を使用でき，多数の細胞を確保できる点で有用である．間葉幹細胞の定義はいまだ明確ではないが，本稿では間葉組織由来で，コロニー形成能を有し，*in vitro* で軟骨，骨，脂肪などに分化する能力を有する細胞集団とする．

Ⅰ 各種間葉幹細胞の増殖能の比較

間葉幹細胞の採取に関して，骨髄液であればフィコールを用いて単核球を分離後に，固形の組織であればコラゲナーゼ処理後に，ディッシュに播種し，培養する．培養過程で1細胞由来と考えられるコロニーを形成する．播種密度が低いと1ディッシュ当たりに得られる細胞数が少なくなりその後の解析が難しくなる．播種密度が高いとコロニー同士が接触し，コロニーのサイズが小さくなる(図1)．間葉幹細胞の形態，表面抗原，増殖能，分化能などの特性は，播種密度，培養期間，継代数などの影響を受けるので[1)]，細胞源が異なる間葉幹細胞の特性を比較する際には，同じ条件

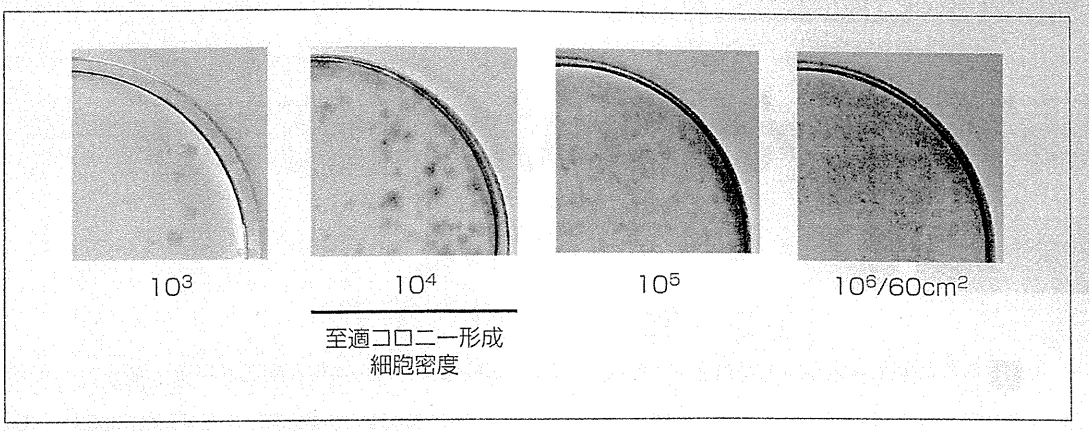

図1　細胞密度がコロニー形成に与える影響
滑膜を酵素処理後，有核細胞をディッシュ上に4種類の密度で播種し，14日間培養後，クリスタルバイオレッド染色した．この場合10^4で播種したものがコロニーが大きく，その数も多いため，至適コロニー形成密度となる．（文献2）より改変）

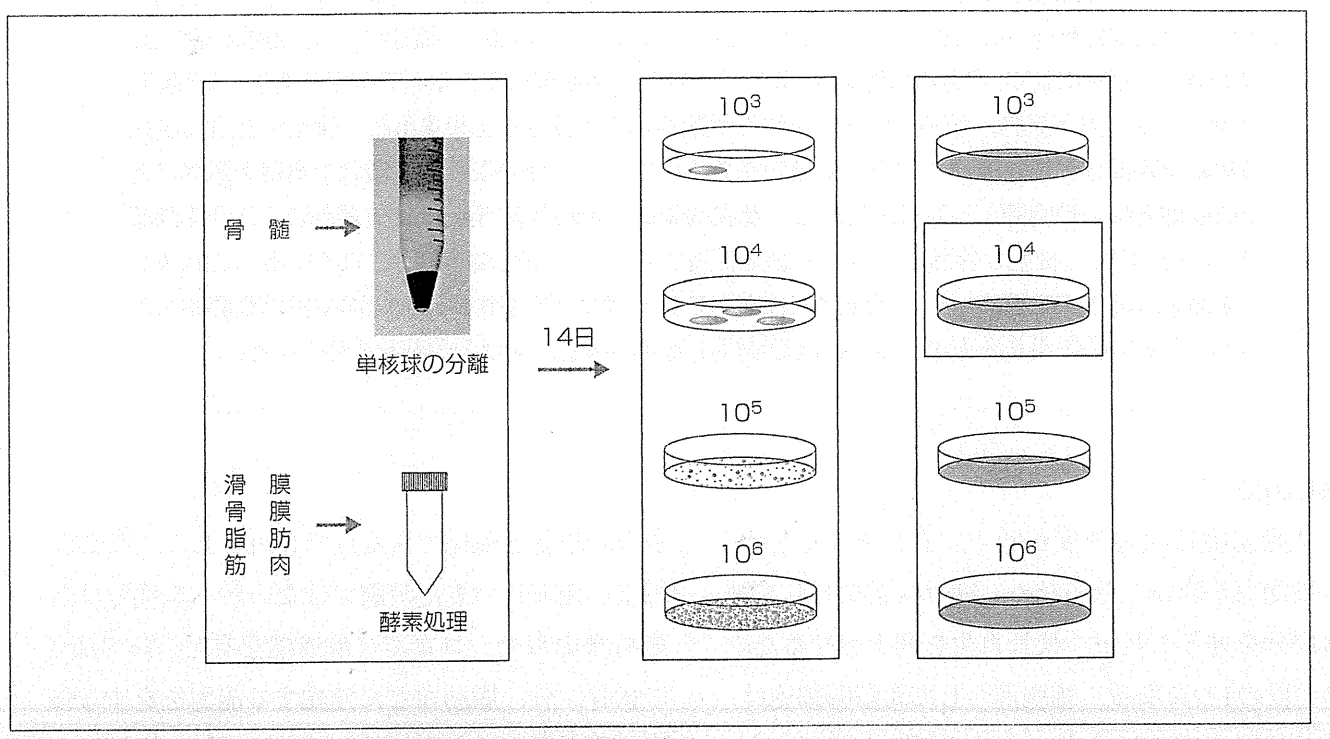

図2　5種の間葉組織から同じ条件で間葉幹細胞を培養する方法
骨髄はフィコールを用いて単核球を分離後に，滑膜，骨膜，脂肪，筋肉はコラゲナーゼ処理後に，それぞれ細胞密度を変えた条件で6枚ずつ播種し14日間培養する．半分のディッシュをクリスタルバイオレッドで染色し，至適コロニー形成密度を決定し，その密度で播種した残り3枚のディッシュから細胞を回収し解析を進める．この模式図では10^4で播種したものが至適コロニー形成密度となる．

で培養する必要がある．われわれは最も大きい細胞コロニーを形成し，かつ1ディッシュ当たりの細胞数が多くなる至適コロニー形成細胞密度を求め，この条件で得られた細胞を比較している（図2）[2]．

膝の前十字靱帯再建術時に得られた骨髄液，滑膜，骨膜，皮下脂肪，筋肉の有核細胞を至適コロニー形成細胞密度で14日間培養すると，いずれも紡錘形で小型の形態を呈する（図3a）．骨髄液由来のコロニー形成率はほかのものよりも1/100以下と低いが，1コロニー当たりの細胞数は多い（図3b）．

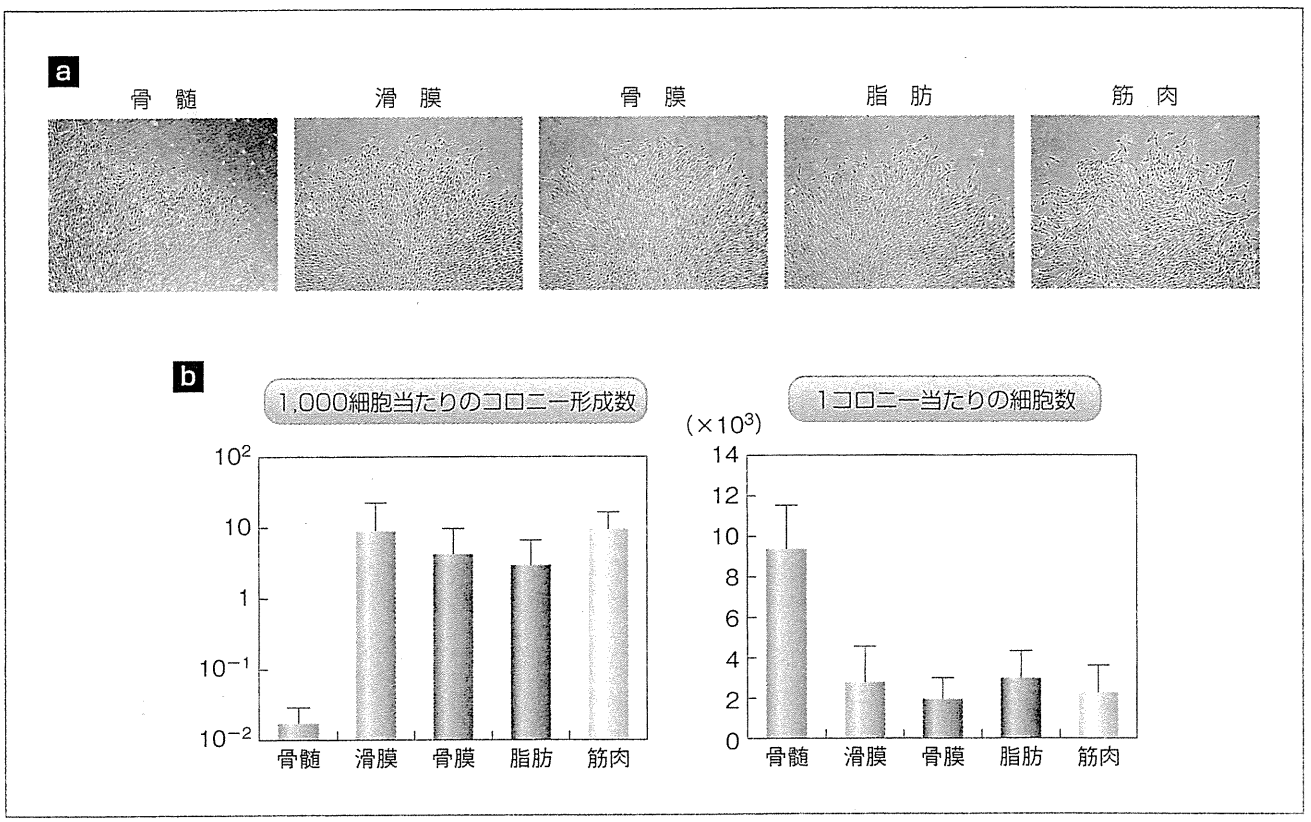

図3 骨髄，滑膜，骨膜，脂肪，筋肉由来間葉幹細胞の形態とコロニー形成能
(a)いずれも小型の紡錘形である．(b) 1,000細胞当たりのコロニー形成数は骨髄由来のものが低いが，1コロニー当たりの細胞数は多い．
(文献2)より改変)

Ⅱ 各種間葉幹細胞の脂肪分化・石灰化能の比較

脂肪への分化能を，脂肪分化したコロニーの割合で比較すると，滑膜と脂肪由来の間葉幹細胞はほかのものよりも高い(図4)．同様の方法を用いて石灰化する能力を検討すると，骨髄，滑膜，骨膜由来のものが脂肪，筋肉由来のものよりも高い傾向を示す(図5)．

Ⅲ 各種間葉幹細胞の*in vitro*軟骨分化の比較

25万の間葉幹細胞をチューブに入れ10分間遠心し細胞塊とした後に，形質転換成長因子(TGF-β)，デキサメタゾン，骨形成因子(BMP)を含む軟骨分化培地で培養すると，底に沈んでいた細胞塊が時間経過とともに丸く，大きくなり，軟骨塊を形成する(図6a)．このペレット培養の軟骨分化過程で軟骨塊が増大するのは，主に軟骨基質が産生されるためである[3]．細胞塊の大きさや重量は軟骨前駆細胞数と軟骨基質産生能を反映し，細胞集団の軟骨分化能の指標となる．骨髄液，滑膜，骨膜，脂肪，筋肉由来の間葉幹細胞を比較すると，滑膜や骨髄由来のものが大きい軟骨塊を形成し，トルイジンブルー陽性の軟骨基質を認める．骨膜由来のものも軟骨基質を産生するが，産生量は骨髄や滑膜のものと比較すると劣る．脂肪や筋肉由来のものはわれわれの検討では軟骨基質の産生が乏しかった(図6b)．

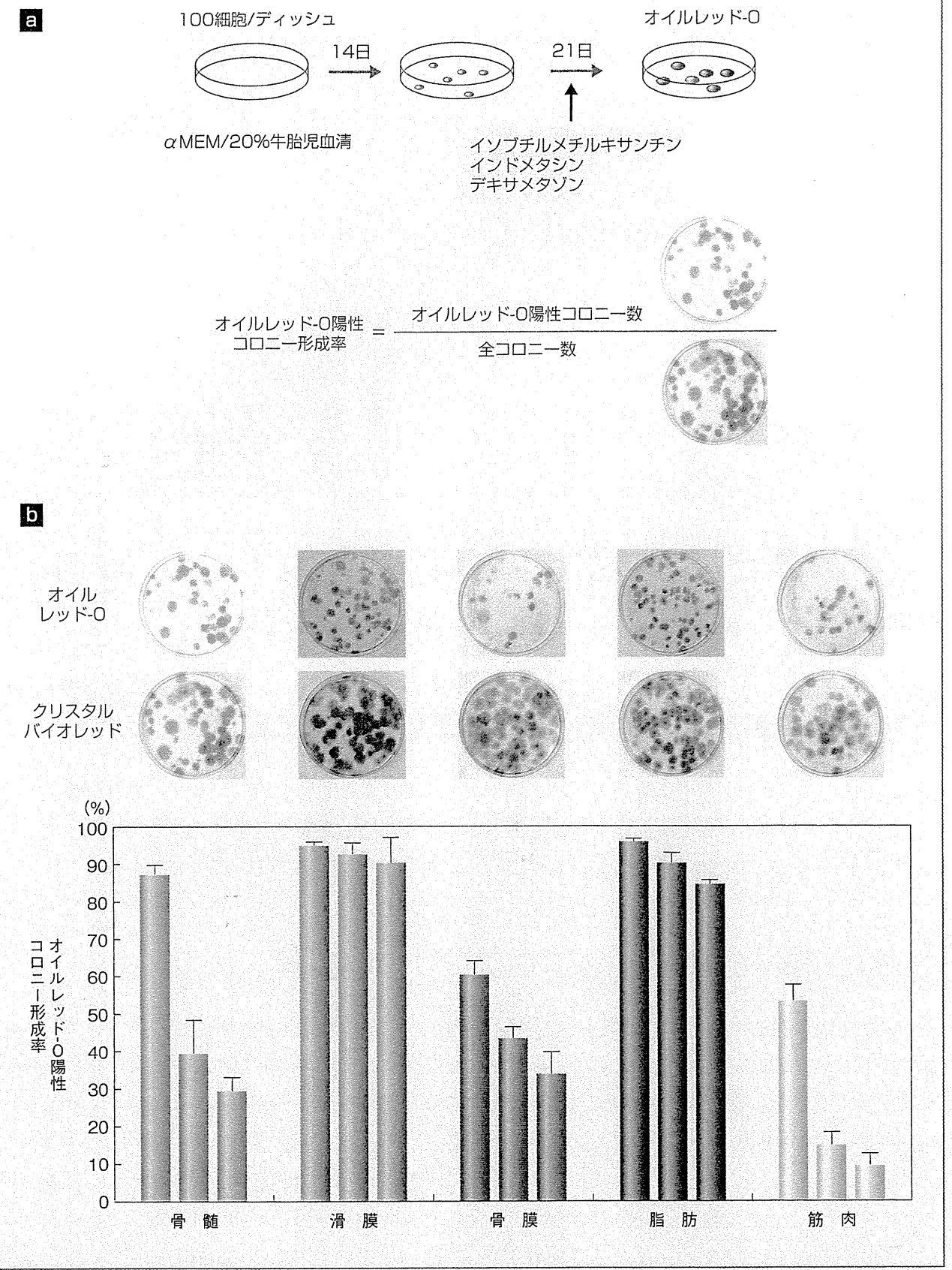

図4　脂肪分化能の比較
各種間葉幹細胞100個をディッシュに播種し14日間培養しコロニーを形成させる．その後，脂肪分化培地で21日間培養する．オイルレッド-Oで染色し，脂肪に分化したコロニー数をカウント後，同じディッシュをクリスタルバイオレッドで染色し全コロニー数をカウントし，オイルレッド-O陽性コロニー形成率を求める．3人のドナーの結果をそれぞれ示す．（文献2）より改変）