in dilution, ab1671, Abcam, Cambridge, UK) was applied to sections incubated for 1 h. After extensive washes with PBS, the sections were incubated in the secondary antibody of biotinylated horse anti-mouse IgG (1:200 in dilution; Vector Laboratories) or anti-goat IgG (1:200 in dilution; Vector Laboratories) for 30 min. Immunostaining was detected with the Vectastain ABC reagent (Vector Laboratories) followed by diaminobenzidine staining. The sections were counterstained with hematoxylin.

2.8. LacZ staining

For LacZ staining, a LacZ tissue staining kit (Invivo gen, San Diego, USA) was used. The cryosection was fixed with a fixative solution (0.2% glutaraldehyde, 2 mM $MgCl_2$ and 5 mM EDTA) in PBS for 30 min and treated with an X-gal staining solution under incubation at 37 °C for 3 h and rinsed in PBS.

2.9. TUNEL staining

For TUNEL staining, an in situ apoptosis detection kit (Takara Bio Inc., Shiga, Japan) was used. Paraffin-embedded sections were deparaffinized in xylene and pretreated with 0.4 mg/ml proteinase K (DAKO, Carpinteria, CA) for 15 min. TdT enzyme (5 μl) and labeling safe buffer (45 μl) was dropped on the sections and incubated at 37 °C for 90 min.

For nuclear staining, Hoechst 33342 (1:1000 indilution, Dojindo Laboratories, Kumamoto, Japan) was applied to sections incubated at room temperature for 1 min. The sections were washed.

2.10. In vivo bioluminescent imaging

A noninvasive bioimaging system IVIS (Xenogen, Alameda, CA) was used for analysis using IGOR (WaveMetrics, Lake Oswego, OR) and IVIS Living Image (Xenogen) software packages [9]. Also, for transplanted cell tracking in vivo, D-luciferin (30 μl 10 μg/μl) was injected into the knee joint of rats under anesthesia with isoflurane. The signal intensity was quantified as the difference between injected knee joint and the opposite side photon flux in units of photons per seconds in the region of interest.

2.11. Fluorescent microscopic examination

The dissected meniscus tissue was immediately fixed in a 4% paraformaldehyde solution. The fixed specimens were incubated for 3 h in 5%, 10%, 15% and 20% sucrose solution, respectively. A 30% OCT (Sakura Finetek, Tokyo, Japan) in sucrose solution was added into a holder. Then, the fixed specimens were mounted gently on the holder. The holder was frozen in hexan chilled by dry ice and stored at −80 °C. Cryosections (5 μm) were prepared with an ultracut S microtome (Reichert, Wien, Austria) and a Microm HM560 cryostat.

2.12. Statistical analysis

The StatView 5.0 program (SAS Institute, Cary, NC) was used for statistical analyses. Comparisons between two groups were analyzed using the Mann–Whitney *U* test. Comparisons between multi groups were analyzed using the Kruskal–Wallis test and the Scheffe test. A *P*-value of <0.05 was considered statistically significant. Comparison between each control and group was analyzed using the Steel test. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Appearance of aggregates of rat synovial MSCs

Rat synovial MSCs were aggregated using the hanging drop technique (Fig. 1A). The size of the aggregate was dependent on the number of MSCs (Fig. 1B). In aggregates consisting of 500 MSCs, the whole region was comprised of round cells. In aggregates consisting of 5000 and 25,000 MSCs, the superficial layer was comprised of spindle cells parallel to the surface, whereas the deep layer was comprised of round cells (Fig. 1C). TUNEL positive cells were observed in each aggregate but were small in number (Fig. 1D).

3.2. Meniscal regeneration by transplantation of aggregates of synovial MSCs

After meniscectomy (Fig. 1E), 50 aggregates consisting of 500 MSCs, 5 aggregates consisting of 5000 MSCs or 1 aggregate consisting of 25,000 MSCs were placed on the sites of meniscal defects (Fig. 1F). At 4 weeks, the regenerated meniscus was observed in cases in which the meniscectomy had been performed in all groups, and the size appeared to be dependent on the groups (Fig. 2A). The areas of the regenerated menisci in the "50 aggregates consisting of 500 MSCs" and "5 aggregates consisting of 5000 MSCs" groups were significantly larger than those in the "untreated" and "suspension of 25,000 MSCs" groups (Fig. 2B). There were no significant differences between each aggregation group containing 25,000 MSCs totally and suspension group containing 5 million MSCs. At 12 weeks, the regenerated meniscus was further enlarged in all the "untreated", "50 aggregates consisting of 500 MSCs" and "5 aggregates consisting of 5000 MSCs" groups. The area of the regenerated menisci was significantly larger in the "50 aggregates consisting of 500 MSCs" and "5 aggregates consisting of 5000 MSCs" groups than in the "untreated" group.

Histologically, the contours of the regenerated menisci was sharper, and the stainability of type II collagen in the matrices was higher in each "aggregate(s)" group than in the untreated and suspension groups at 4 weeks (Fig. 3A). Histological scores for the regenerated menisci were significantly lower in each "aggregate(s)" group than in the untreated and suspension groups (Fig. 3B), indicating the meniscal regeneration was promoted in each "aggregate(s)" group. There was no significant difference of the histological scores for the regenerated menisci between each "aggregate(s)" group and the suspension group containing 5 million MSCs. At 12 weeks, the contours of the regenerated menisci were sharpened in all the "untreated", "50 aggregates consisting of 500 MSCs" and "5 aggregates consisting of 5000 MSCs" groups, but the stainability of safranin-o and type II collagen in the matrices was still higher in the "50 aggregates consisting of 500 MSCs" and "5 aggregates consisting of 5000 MSCs" groups than in the "untreated" group. Histological scores for the regenerated menisci were still significantly lower in the "50 aggregates consisting of 500 MSCs" and "5 aggregates consisting of 5000 MSCs" group than in the "untreated" group.

3.3. Prevention of cartilage degeneration by transplantation of aggregates of synovial MSCs

In the untreated group, a non-smooth area of the articular cartilage stained with India ink was extensively observed at the medial tibial plateau. In contrast, a non-smooth area was hardly observed in the "50 aggregates consisting of 500 MSCs" group at 12 weeks (Fig. 3C). Quantification analysis demonstrated that areas of the degenerated cartilages were significantly smaller in the "50

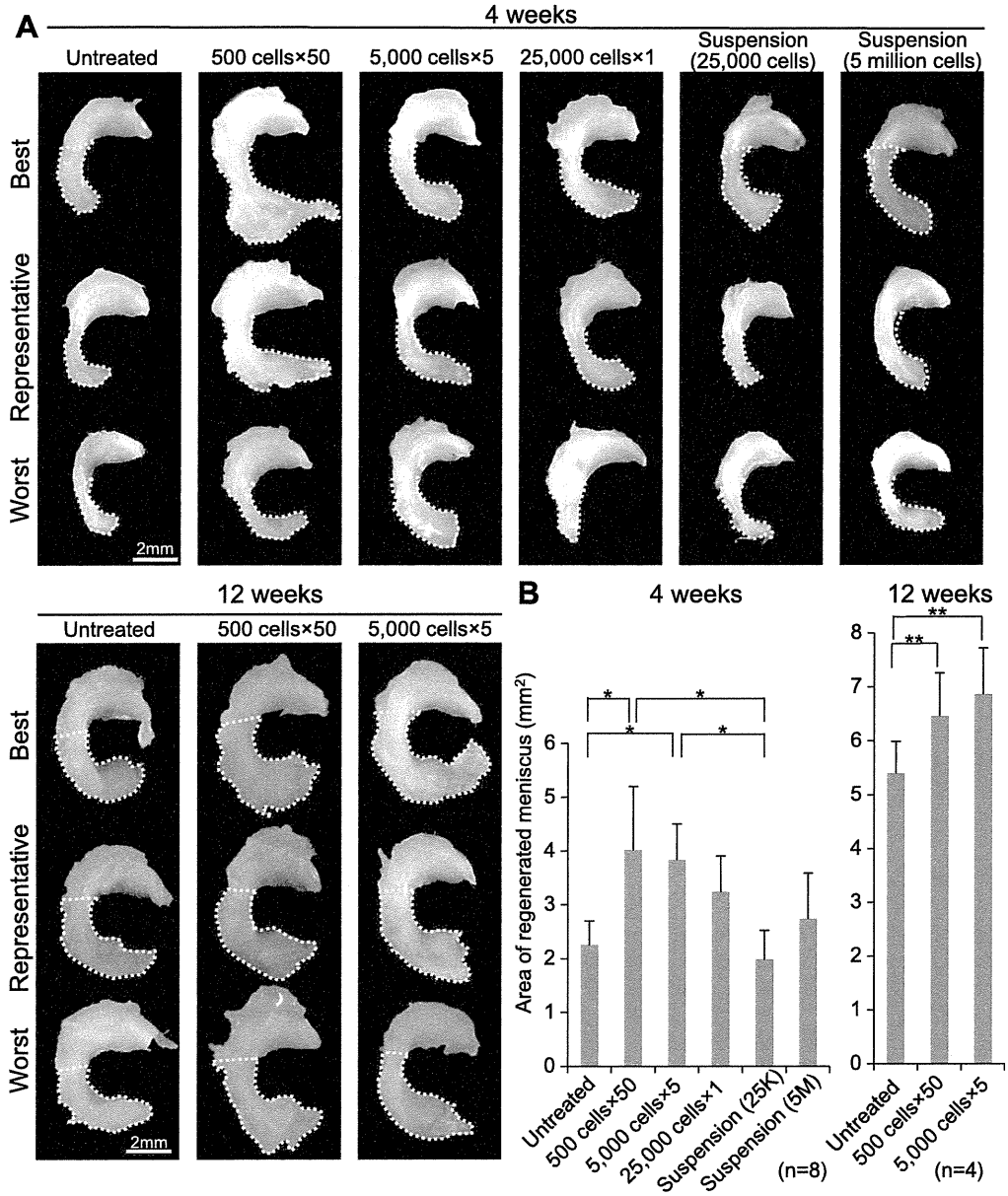

Fig. 2. Macroscopic analysis of regenerated menisci after transplantation of aggregates. (A) Macroscopic findings of the regenerated menisci at 4 and 12 weeks. Regenerated areas are surrounded with yellow dotted lines. (B) Quantification for areas of the regenerated menisci. Values are averages with standard deviations ($n = 8$ at 4 weeks and $n = 4$ at 12 weeks). *$p < 0.05$ by the Kruskal–Wallis test and the Scheffe test. **$p < 0.05$ by the Steel test. (For interpretation of color in this figure legend, the reader is referred to the web version of this article).

aggregates consisting of 500 MSCs" group than in the untreated group (Fig. 3D).

3.4. Transplanted aggregates remain in the knee joint

In vitro imaging of luciferase activity showed that as few as one thousand MSCs were detected over the background in the linear dose-dependent output of luminescence (Fig. 4A and B).

When the suspension of 25,000 MSCs expressing luciferase gene was injected into the knee after meniscectomy, the photons from these MSCs could not be detected at 6 weeks and thereafter (Fig. 4C). On the other hand, when 50 aggregates consisting of 500 MSCs were placed on the site of the meniscal defect, the photons from MSCs could be detected even at 12 weeks. Substantial luminescence light could not be detected in any other organs of either group. Sequential quantification demonstrated that luminescence intensity was highest at 2 weeks in both groups (Fig. 4D). In comparison to each group, luminescence intensity in the "50 aggregates consisting of 500 MSCs" group was lower at 1 and 2 weeks, but became higher at 3 weeks and remained higher thereafter in the suspension group.

When the "50 aggregates consisting of 500 MSCs" labeled with DiI was transplanted into the meniscal defect, the DiI positive area was confirmed around the meniscal defect at day 1 (Fig. 4E). Interestingly, the DiI positive area was enlarged at 4 weeks. Histologically, regenerated meniscus consisted of both DiI positive cells and DiI negative cells.

When 50 aggregates consisting of 500 MSCs expressing GFP gene were transplanted into the meniscal defect, GFP positive cells were still detected in the regenerated meniscus macroscopically and histologically at 12 weeks (Fig. 4F). Regenerated meniscus also consisted of GFP positive cells and GFP negative cells.

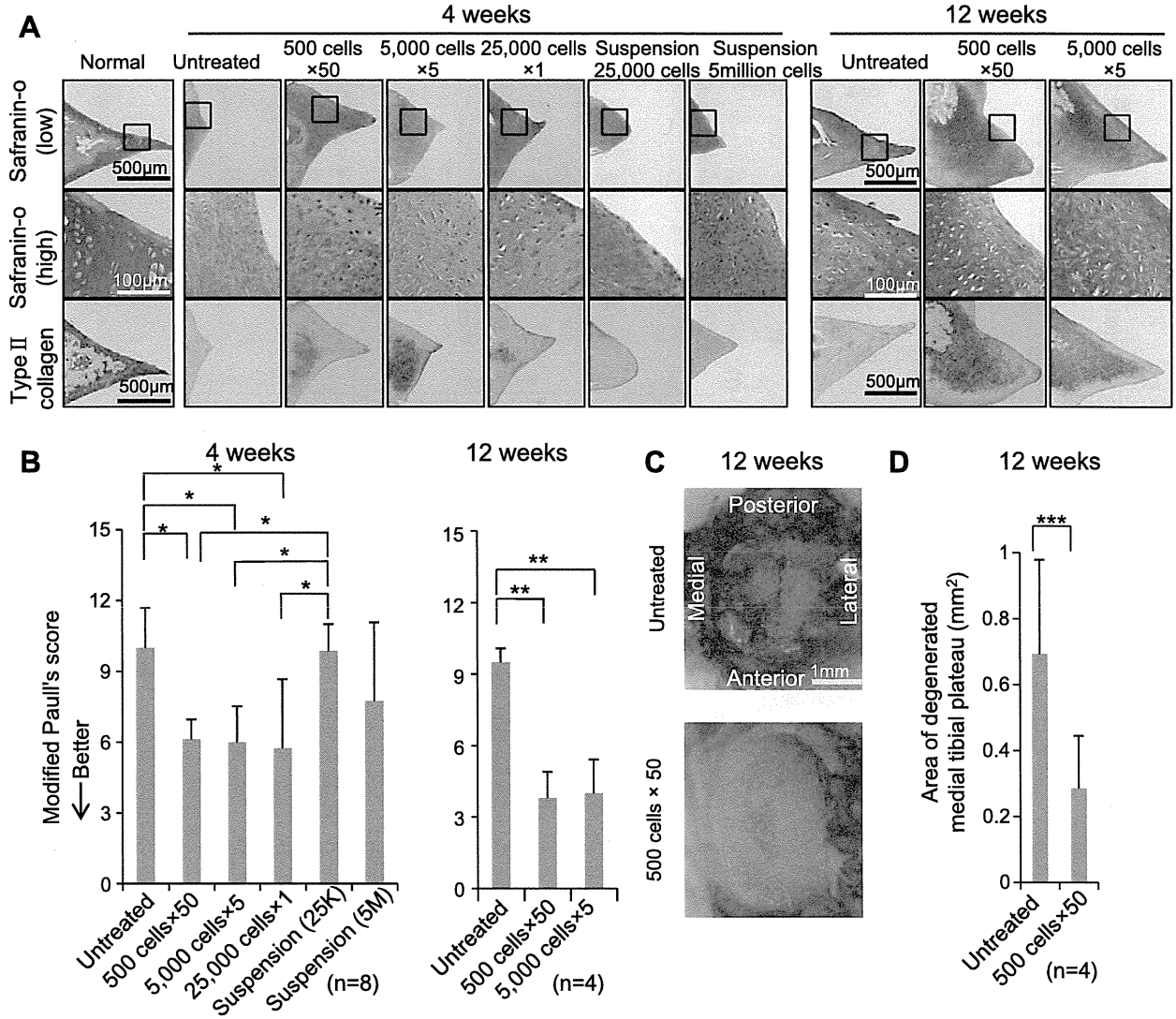

Fig. 3. Histological observation of regenerated menisci after transplantation of aggregates. (A) Representative sections of normal meniscus and regenerated menisci at 4 and 12 weeks. (B) Modified Pauli's score for histology. Values are averages with standard deviations ($n = 8$ at 4 weeks and $n = 4$ at 12 weeks; $^{*}p < 0.05$ by the Kruskal–Wallis test and the Scheffe test, $^{**}p < 0.05$ by the Steel test). Analysis for articular cartilage at the medial tibial plateau. (C) Macroscopic findings of the joint surface of the tibia at 12 weeks. Both of the cartilages were stained with India ink. (D) Quantification for areas of the degenerated cartilages at the medial tibial plateaus. Values are averages with standard deviations ($n = 4$ at 12 weeks; $^{***}p < 0.05$ by the Mann–Whitney's U test).

When 50 aggregates consisting of 500 MSCs expressing luciferase gene were transplanted into the meniscal defect, Lac Z positive cells were surrounded with matrix stained with type II collagen at 4 weeks (Fig. 4G). This showed that MSCs differentiated into meniscal cells directly.

3.5. Aggregates of synovial MSCs disperse shortly

One hour after 50 aggregates consisting of 500 MSCs were placed on the sites of meniscal defects, aggregates of synovial MSCs were surrounded by a large population of erythrocytic and leukocytic cells (Supplemental Fig. 1). At 3 days, aggregates consisting of 500 MSCs were already dispersed into single cells, and each cell was seen in the coarse connective tissue. At 7 days, each DiI positive cell looked like a spindle and was assembled with other DiI positive cells and DiI negative cells with similar orientation. During this phase, most DiI positive cells and some cells around the DiI positive cells were positive for CXCR4.

4. Discussion

In this study, we demonstrated that transplantation of aggregates of synovial MSCs regenerates meniscus more effectively than intraarticular injection of synovial MSCs if the number used was the same. We discuss advantages of transplantation of aggregates from the following three viewpoints.

Firstly, aggregation of synovial MSCs may increase chondrogenic potentials in rats similar to that in humans which we previously reported [6]. Also, aggregates of human synovial MSCs at 3 days increased expressions of SOX9, a master gene for chondrogenesis and expressions of BMP-2, a growth factor for chondrogenesis of MSCs [10]. Further experiments are required to investigate whether aggregates of synovial MSCs increase chondrogenic potential in rats as well as in humans.

Secondly, the number of synovial MSCs attached to the meniscal defect would have been higher using aggregates of synovial MSCs than when using suspension of synovial MSCs. According to another study of ours, one day after GFP synovial MSCs were in-

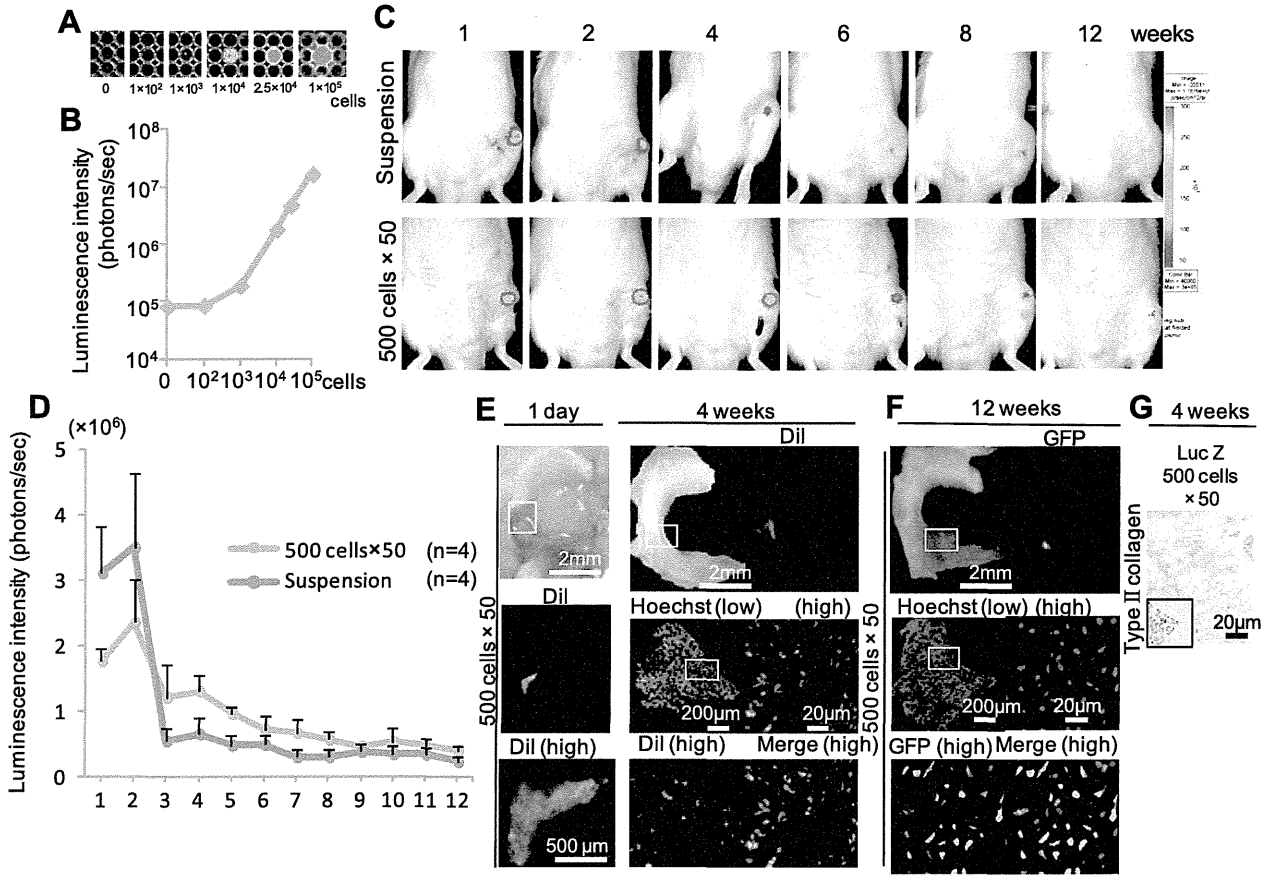

Fig. 4. Tracking the transplanted MSCs. (A) In vitro bioluminescent imaging of varying numbers of synovial MSCs from transgenic rats expressing luciferase. (B) Quantification for bioluminescent imaging. (C) Imaging of photons from MSCs expressing luciferase. 25,000 MSCs suspended in PBS were injected into the knee after meniscectomy or 50 aggregates consisting of 500 MSCs were placed on the site of the meniscal defect. Luciferin was injected into the knee to monitor luminescence driven by MSCs. (D) Sequential quantification of luminescence intensity. Values are shown as averages with standard deviations ($n = 4$). (E) Observation after transplantation of 50 aggregates consisting of 500 MSCs labeled with DiI. Nuclei are shown in blue. "High" indicates high magnification and "low" indicates low magnification. (F) Observation after transplantation of 50 aggregates consisting of 500 MSCs expressing GFP. (G) Type II collagen expression after transplantation of 50 aggregates consisting of 500 MSCs expressing Lac Z (blue). (For interpretation of color in this figure legend, the reader is referred to the web version of this article).

jected into the knee joint in a rat cylindrical meniscus defect model, only very faint fluorescence was visible when 1 million cells were injected [5]. Aggregates of MSCs are visible and can be placed around the meniscal defect directly. The use of aggregates of MSCs will help avoid loss of MSCs from targeted defects.

Thirdly, synovial MSCs may have survived longer in the knee joint when aggregates of synovial MSCs were transplanted than when suspension of synovial MSCs were transplanted. The luminescence intensity was related to the number of MSCs from transgenic rats expressing luciferase (Fig. 4A). Also, the luminescence intensity was correlated with viability of the cells [7]. Using our in vivo imaging analysis, the luminescence intensity in the aggregates group remained higher at 3 weeks and thereafter than in the suspension group. This indicates that synovial MSCs in the aggregates maintained the living cell number and its viability longer than those in suspension.

We confirmed that synovial MSCs transplanted as aggregates existed in the regenerated meniscus stained with type II collagen at 4 weeks. This indicates that synovial MSCs were directly differentiated into chondrocytes. However, DiI or GFP positive cells in the regenerated meniscus were limited and observed only focally and partially. Since King's report over 70 years ago [11], it has been known that when meniscus is injured, adjacent synovial tissue is induced and contributes to the meniscal repair during the natural course of meniscal healing. These findings suggest that transplanted synovial MSCs also expressed trophic factors and stimulated adjacent synovial tissue, then progenitors of meniscus were induced thus contributing to the meniscal regeneration.

In this histological observation, the distribution of matrix positive for type II collagen appeared to be different between the normal and regenerated menisci at 12 weeks (Fig. 3A). In the normal menisci, the peripheral area was positive for type II collagen and the center was ossified, while in the regenerated menisci, the peripheral area was mostly negative for type II collagen. During the process of the meniscus regeneration in this model, the matrix positive for type II collagen initially emerged at the center area, the positive area enlarged toward the periphery, and then the center area ossified. These results suggest that regenerated meniscus did not yet fully mature at 12 weeks. It would be interesting to investigate whether the peripheral matrix becomes positive for type II collagen and whether the center further ossifies after 12 weeks. This seems to be similar to the process during meniscus formation in skeletogenesis [12,13] though the details of meniscal development still remain unclear.

When the number of synovial MSCs was adjusted to 25,000, there were significant differences of regenerated meniscus between the "50 aggregates consisting of 500 MSCs" group, the "5 aggregates consisting of 5000 MSCs" group and the "untreated" group at 4 weeks. Therefore, we selected these groups for further analyses.

Immunohistological analyses showed that aggregates consisting of synovial MSCs were positive for the chemokine receptor 4

(CXCR4). CXCR4 mediated trafficking and migrating of MSCs to the sites of injury [14]. Only aggregation of human synovial MSCs increased expressions of CXCR4 [6]. These findings suggest that aggregation of synovial MSCs has an advantage from the standpoint of CXCR4.

Some papers have reported transplantation of aggregates of chondrocytes and MSCs for cartilage regeneration. Schedel et al. transplanted aggregates of chondrocytes, referred to as chondrospheres, into cartilage defect in SCID mice and showed that aggregates of chondrocytes promoted cartilage repair [15]. We previously reported the usefulness of aggregates of synovial MSCs as a source for cartilage regeneration [6]. Our current study is the first report demonstrating meniscal regeneration with aggregates of cells including MSCs and meniscal cells.

From a clinical relevance perspective, aggregates of MSCs have some advantages. They are easily formed, visible and solid enough to aspirate with a syringe. Aggregates of MSCs can be collected in a shorter time than MSCs attached to dishes, indicating that it is easy to adjust the time to harvest MSCs during the transplant time for a patient. Aggregates sink faster in the suspension medium then disperse MSCs, and they avoid loss of MSCs from the targeted defect. The use of aggregates is practically convenient for meniscal regeneration with MSCs.

In conclusion Transplantation of aggregates of synovial MSCs regenerated meniscus more effectively than intraarticular injection of synovial MSCs when the same number of cells was used in a rat massive meniscectomized model.

Acknowledgments

This study was supported by grants from the Project for Realization of Regenerative Medicine and the Global Center of Excellence (GCOE) Program by the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.026.

References

[1] P.S. Walker, M.J. Erkman, The role of the menisci in force transmission across the knee, Clin. Orthop. Relat. Res. (1975) 184–192.
[2] B. Tucker, W. Khan, M. Al-Rashid, H. Al-Khateeb, Tissue engineering for the meniscus: a review of the literature, Open Orthop. J. 6 (2012) 348–351.
[3] Y. Sakaguchi, I. Sekiya, K. Yagishita, T. Muneta, Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source, Arthritis Rheum. 52 (2005) 2521–2529.
[4] H. Koga, M. Shimaya, T. Muneta, A. Nimura, T. Morito, M. Hayashi, S. Suzuki, Y.J. Ju, T. Mochizuki, I. Sekiya, Local adherent technique for transplanting mesenchymal stem cells as a potential treatment of cartilage defect, Arthritis Res. Ther. 10 (2008) R84.
[5] K. Mizuno, T. Muneta, T. Morito, S. Ichinose, H. Koga, A. Nimura, T. Mochizuki, I. Sekiya, Exogenous synovial stem cells adhere to defect of meniscus and differentiate into cartilage cells, J. Med. Dent. Sci. 55 (2008) 101–111.
[6] S. Suzuki, T. Muneta, K. Tsuji, S. Ichinose, H. Makino, A. Umezawa, I. Sekiya, Properties and usefulness of aggregates of synovial mesenchymal stem cells as a source for cartilage regeneration, Arthritis Res. Ther. 14 (2012) R136.
[7] Y. Hakamata, T. Murakami, E. Kobayashi, "Firefly rats" as an organ/cellular source for long-term in vivo bioluminescent imaging, Transplantation 81 (2006) 1179–1184.
[8] C. Pauli, S.P. Grogan, S. Patil, S. Otsuki, A. Hasegawa, J. Koziol, M.K. Lotz, D.D. D'Lima, Macroscopic and histopathologic analysis of human knee menisci in aging and osteoarthritis, Osteoarthritis Cartilage 19 (2011) 1132–1141.
[9] P.R. Contag, I.N. Olomu, D.K. Stevenson, C.H. Contag, Bioluminescent indicators in living mammals, Nat. Med. 4 (1998) 245–247.
[10] I. Sekiya, B.L. Larson, J.T. Vuoristo, R.L. Reger, D.J. Prockop, Comparison of effect of BMP-2, -4 and -6 on in vitro cartilage formation of human adult stem cells from bone marrow stroma, Cell Tissue Res. 320 (2005) 269–276.
[11] K. Don, The healing of semilunar cartilages, J. Bone Joint Surg. Am. (1936) 333–342.
[12] G. Hyde, R.P. Boot-Handford, G.A. Wallis, Col2a1 lineage tracing reveals that the meniscus of the knee joint has a complex cellular origin, J. Anat. 213 (2008) 531–538.
[13] S.P. Henry, C.W. Jang, J.M. Deng, Z. Zhang, R.R. Behringer, B. de Crombrugghe, Generation of aggrecan-CreERT2 knockin mice for inducible Cre activity in adult cartilage, Genesis 47 (2009) 805–814.
[14] T. Kitaori, H. Ito, E.M. Schwarz, R. Tsutsumi, H. Yoshitomi, S. Oishi, M. Nakano, N. Fujii, T. Nagasawa, T. Nakamura, Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model, Arthritis Rheum. 60 (2009) 813–823.
[15] T. Schubert, S. Anders, E. Neumann, J. Schölmerich, F. Hofstädter, J. Grifka, U. Müller-Ladner, J. Libera, J. Schedel, Long-term effects of chondrospheres on cartilage lesions in an autologous chondrocyte implantation model as investigated in the SCID mouse model, Int. J. Mol. Med. 23 (2009) 455–460.

Intraarticular Injection of Synovial Stem Cells Promotes Meniscal Regeneration in a Rabbit Massive Meniscal Defect Model

Daisuke Hatsushika,[1] Takeshi Muneta,[1,2] Masafumi Horie,[1] Hideyuki Koga,[1] Kunikazu Tsuji,[2] Ichiro Sekiya[3]

[1]Department of Joint Surgery and Sports Medicine, Tokyo Medical and Dental University, Tokyo, Bunkyo-ku, Japan, [2]Global COE Program, Tokyo Medical and Dental University, Tokyo, Bunkyo-ku, Japan, [3]Department of Cartilage Regeneration, Tokyo Medical and Dental University, 1-5-45 YushimaBunkyo-ku, Tokyo, 113-8519, Japan

Received 14 December 2012; accepted 13 March 2013
Published online 17 April 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.22370

ABSTRACT: We investigated whether single intraarticular injection of synovial MSCs enhanced meniscal regeneration in a rabbit massive meniscal defect model. Synovium were harvested from the knee joint of rabbits, and the colony-forming cells were collected. Two weeks after the anterior half of the medial menisci were excised in both knees, 1×10^7 MSCs in 100 μl PBS were injected into the right knee. The MSC and control groups were compared macroscopically and histologically at 1, 3, 4, and 6 months ($n = 4$). Articular cartilage of the medial femoral condyle was also evaluated histologically at 6 months. Multipotentiality of the colony-forming cells was confirmed. Injected MSCs labeled with DiI were detected and remained in the meniscal defect at 14 days. The size of meniscus in the MSC group was larger than that in the control group at 1 and 3 months. The difference of the size between the two groups was indistinct at 4 and 6 months. However, histological score was better in the MSC group than in the control group at 1, 3, 4, and 6 months. Macroscopically, the surface of the medial femoral condyle in the control group was fibrillated at 6 months, while looked close to intact in the MSC group. Histologically, defect or thinning of the articular cartilage with sclerosis of the subchondral bone was observed in the control group, contrarily articular cartilage and subchondral bone were better preserved in the MSC group. Synovial MSCs injected into the knee adhered around the meniscal defect, and promoted meniscal regeneration in rabbits. © 2013 Orthopaedic Research Society Published by Wiley Periodicals, Inc. J Orthop Res 31:1354–1359, 2013.

Keywords: meniscus; regeneration; synovium; mesenchymal stem cells; rabbit

The meniscus is a wedge-shaped fibrocartilage and plays important roles in load distribution and knee joint stability,[1] but it has a poor regenerative potential.[2–4] For a massive meniscal defect in a clinical situation,[5,6] meniscal allograft is indicated but its invasiveness and selection difficulty of proper allograft are not low. A new strategy to regenerate a meniscus with low invasiveness is required for a massive meniscal defect.

Mesenchymal stem cells (MSCs) are an attractive cell source for meniscal regeneration.[7–10] It was reported that intraarticular injection of synovial MSCs promoted meniscal regeneration in a rat massive meniscal defect model.[11] However, its effect remained unknown for other larger animals because of different meniscal properties dependent on animal species.[12,13]

In this study, we removed half of the medial meniscus in rabbits, and we examined the effect of intraarticular injection of synovial MSCs on regeneration of the meniscus. Injected MSCs were chased to investigate the role of the cells, and the articular cartilage was also evaluated to examine the function of the regenerated meniscus. We hypothesized that synovial stem cells injected into the knee would adhere around the meniscal defect, promote meniscal regeneration, and prevent cartilage degeneration in rabbits.

Conflicts of interest: none.
Grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology, Japan (Project for Realization of Regenerative Medicine and the Global Center of Excellence (GCOE) Program).
Correspondence to: Ichiro Sekiya (T: +81-3-5803-4020; F: +81-3-5803-4020; E-mail: sekiya.orj@tmd.ac.jp)

METHODS

Cell Isolation and Culture

This study was approved by the Animal Experimentation Committee of our institution. Skeletally mature female Japanese white rabbits weighing an average of 3.0 kg were used. Under anesthesia, synovium was harvested from the medial, lateral, and suprapatellar regions of the knee joints. The harvested synovium was digested in a 3 mg/ml collagenase type V (Sigma, St. Louis, MO) in α-minimal essential medium (αMEM; Invitrogen, Carlsbad, CA) for 3 h at 37°C. The digested tissues were filtered through a 70 μm cell strainer (Becton-Dickinson and Company, Franklin Lakes, NJ). The obtained cells were seeded at 5×10^4 cells/cm^2 in 145-cm^2 culture dishes (Nalge Nunc International, Rochester, NY) and cultured with complete medium, αMEM containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. The media was replaced to remove nonadherent cells 2 days later. After being cultured for 7 days, the cells were harvested with 0.25% trypsin–EDTA (Invitrogen) and cryopreserved at 1×10^6 cells/ml in αMEM with 5% dimethylsulfoxide (Wako, Osaka, Japan) and 10% FBS. The frozen cells from synovium were thawed, plated at 3×10^3 cells/cm^2 in 145-cm^2 culture dishes, and incubated for 5 days. Harvested cells were resuspended at 1×10^6 cells/ml in αMEM, and a DiI (Molecular Probes, Eugene, OR) fluorescent lipophilic tracer was added at 5 μl/ml in αMEM. After incubation for 20 min at 37°C with 5% humidified CO_2, the cells were centrifuged at 450g for 5 min and washed twice with PBS.[12] The obtained cells were used for further analyses.

Colony-Formation

One hundred synovial MSCs at passage 1 were plated in 60-cm^2 dishes, cultured in complete medium for 14 days, and stained with 0.5% crystal violet in methanol for 5 min.

In Vitro Differentiation Assay

For chondrogenesis, 250 thousand synovial MSCs were placed in a 15 ml polypropylene tube (Becton-Dickinson),

centrifuged at 450*g* for 10 min, and cultured for 21 days in chondrogenic medium which contained 1,000 ng/ml bone morphogenetic protein 7 (Stryker Biotech, Boston, MA), 10 ng/ml transforming growth factor-β3 (R&D Systems, Inc., Minneapolis, MN), and 100 nM dexamethasone. The pellets were embedded in paraffin, cut into 5 μm sections, and stained with 1% toluidine blue.[14]

For adipogenesis, synovial MSCs were cultured in adipogenic medium which consisted of complete medium supplemented with 100 nM dexamethasone (Sigma), 0.5 mM isobutyl-methylxanthine (Sigma), and 50 μM indomethacin (Wako) for 21 days. The adipogenic culture was fixed in 4% paraformaldehyde and then stained with fresh oil red-O solution.[15]

Synovial MSCs were cultured in calcification medium that comprised complete medium consisting of 1 nM dexamethasone, 10 mM β-glycerol phosphate (Wako), and 50 μg/ml ascorbate-2-phosphate (Sigma) for 21 days. The dishe was stained with 0.5% alizarin red solution.[16]

Meniscectomy and Synovial MSC Injection

A straight incision was made on the anterior side of the knee, the anteromedial side of the joint capsule was cut, and the anterior horn of the medial meniscus was exposed. Then the meniscus was dislocated anteriorly with a forceps, cut vertically at the level of the medial collateral ligament, and the anterior half of medial meniscus was excised in both knees. Capsule and skin were closed in layers with absorbable suture.

Two weeks after the skin incision was closed, 1×10^7 synovial MSCs in 100 μl PBS were injected into the meniscal defect in each of the right knees using a 23 gauge needle. In left knees, the same volume of plain PBS was injected as control. Immediately after the injection, the medial side of the injected knee was kept down for 10 min so that injected synovial MSCs could be attached around the defect of the meniscus.[17] The rabbits were allowed to walk freely in the cage. Medial menisci and femoral condyle from both knees were harvested at 4-, 12-, 16-, and 24-week endpoints. A procedure was performed on the 16 rabbits.

DiI Labeling

On the day of implantation, MSCs were labeled for cell tracking by the fluorescent lipophilic tracer 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes). For labeling, the cells were resuspended at 1×10^6 cells/ml in αMEM, and DiI was added at 5 μl/ml in αMEM. After incubation for 20 min at 37°C with 5% humidified CO_2, the cells were centrifuged at 450*g* for 5 min and washed twice with PBS, and then they were resuspended in PBS for the transplantation.[18,19]

Quantification of Size of Regenerated Menisci

The tibial plateaus were photographed, and the sizes of medial menisci and lateral menisci were quantified with Image J software (version 1.43, National Institutes of Health, Bethesda, MD). We calculated the ratio of the medial meniscus area to the lateral meniscus area.

Histological Analysis

Specimens of medial menisci and medial femoral condyles were fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin. The specimens were then sectioned into slices 5 μm thick radially for the meniscus and sagittally for the condyles. For quantification of histology for regenerated meniscus, Pauli's scoring system which was modified was used (Table 1).[20] For quantification of histology for articular cartilage, the OARSI Osteoarthritis Cartilage Histopathology Assessment scoring system was used.[21] The score was evaluated by two investigators blinded to treatment category.

Statistical Methods

The paired t test was used for comparison between the MSC group and the control group at each period. p Values <0.05 were considered significant.

RESULTS

Fourteen days after 100 synovial cells were cultured, approximately 50 colonies were observed. Colony-forming cells derived from rabbit synovium differentiated into chondrocytes, adipocytes, and were calcified, when cultured in their respective differentiation media (Fig. 1).

Two weeks after anterior half of medial menisci were excised in both knees, 1×10^7 MSCs in 100 μl PBS were injected into the right knee (Fig. 2A). Two weeks after the injection, the meniscal defect was already filled with synovial tissue (Fig. 2B), whereas the DiI positive area was partially detected macroscopically (Fig. 2C). In the radial histological sections, DiI positive cells were located mostly in the center of the synovial tissue (Fig. 2D).

Macroscopically, the meniscectomized anterior half of the medial meniscus consisted of swollen soft tissue 2 weeks after the injection of MSCs (Fig. 3A). Thereafter, medial meniscus became similar to the intact one in the MSC group along with the time course. Contrarily, the meniscal defect in the control group was still observed at 4 and 12 weeks. Quantification analysis demonstrated that the medial meniscus area was larger in the MSC group than in the control group at 4 and 12 weeks (Fig. 3B).

Histologically, at 4 weeks, the anterior half of the medial meniscus in the MSC group was still poorly organized and consisted of crowded cells without lacunae (Fig. 4A). At 12 weeks, the contour of the meniscus became closer to the normal one, and the cell density in the center decreased. At 16 weeks, the cells in the center contained lacunae, while the anterior half of the medial meniscus in the control group was still poorly organized. At 24 weeks, the matrix staining increased in the MSC group, while the surface was still not smooth in the control group. The histological score in the MSC group was better than that in the control group at each point we examined (Fig. 4B).

For articular cartilage of the medial femoral condyle, thinning of the cartilage was observed in one specimen among four in the MSC group at 24 weeks (Fig. 5A). In the control group, osteochondral lesions were observed in two specimens and sclerosis of subchondral bone in the other two specimens, one of which had thinning of the articular cartilage. The OARSI score was better in the MSC group than in the control group (Fig. 5B).

Table 1. Criteria and Scores for Histological Assessment of Regenerated Menisci

I. Surface Including Lamellar Layer:	
I–I. Femoral side:	
A Smooth	3
B Slight fibrillation or slightly undulating	2
C Moderate fibrillation or markedly undulating	1
D Severe fibrillation or disruption	0
I–II. Tibial side	
A Smooth	3
B Slight fibrillation or slightly undulating	2
C Moderate fibrillation or markedly undulating	1
D Severe fibrillation or disruption	0
II. Cellularity of chondrocyte	
A Normal cell distribution	3
B Moderately normal cell distribution	2
C Hypercellularity or hypocellularity	1
D No chondrocyte	0
III. Collagen fiber organization	
A Collagen fibers well organized, no separations or tears	3
B Collagen fibers moderately organized, slight separations or tears	2
C Collagen fiber unorganized, moderate separations or tears	1
D Collagen fiber unorganized, severe separations or tears	0
IV. Matrix staining (toluidine blue)	
A Well stained like normal meniscus	3
B Moderately stained	2
C Slightly Moderately stained	1
D Slightly stained	0

Note: If regenerated meniscus is not existed, total score is regarded as 0.

DISCUSSION

The cells from rabbit synovium formed colonies, and differentiated into chondrocytes, adipocytes, and were calcified when cultured in the appropriate differentiation medium. As MSCs are defined by adherence to plastic dish, colony formation, and trilineage differentiation,[22] our results indicate that the rabbit synovium-derived cells had characteristics of MSCs.

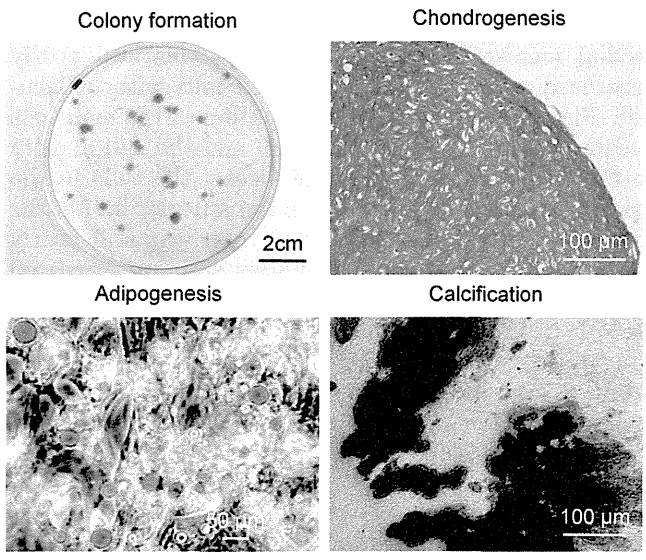

Figure 1. Colony formation and multipotentiality of synovial MSCs. Cell colonies were stained with crystal violet. Cartilage pellet was sectioned and stained with toluidine blue. Adipocytes were stained with oil red-O. Calcification was stained with alizarin red.

MSCs can be isolated from various adult mesenchymal tissues in addition to bone marrow. These MSCs have been assumed to be similar irrespective of their original tissue source since they all have colony formation and trilineage differentiation potential with common surface epitopes. However, there are increasing reports demonstrating the specificities dependent of their MSC source[8,23–25] When the chondrogenic potential of MSCs derived from bone marrow, synovium, adipose tissue, and skeletal muscle were compared in vitro and in vivo, the superiority of chondrogenesis in MSCs derived from bone marrow and synovium was demonstrated.[7] Synovial MSCs have an advantage in that they can proliferate more with autologous human serum than with bone marrow MSCs.[10] Therefore, we selected synovial MSCs among several kinds of MSCs.

To create a defect model of medial meniscus, we removed only the anterior half of the medial meniscus. Though total meniscus resection models may be more popular,[26] they seem to be highly invasive and complicated. In our current model, the medial collateral ligament could be preserved, and we could complete this model with low invasiveness, with relative ease, and with high reproducibility. Though our model mimics limited pathological conditions of meniscal

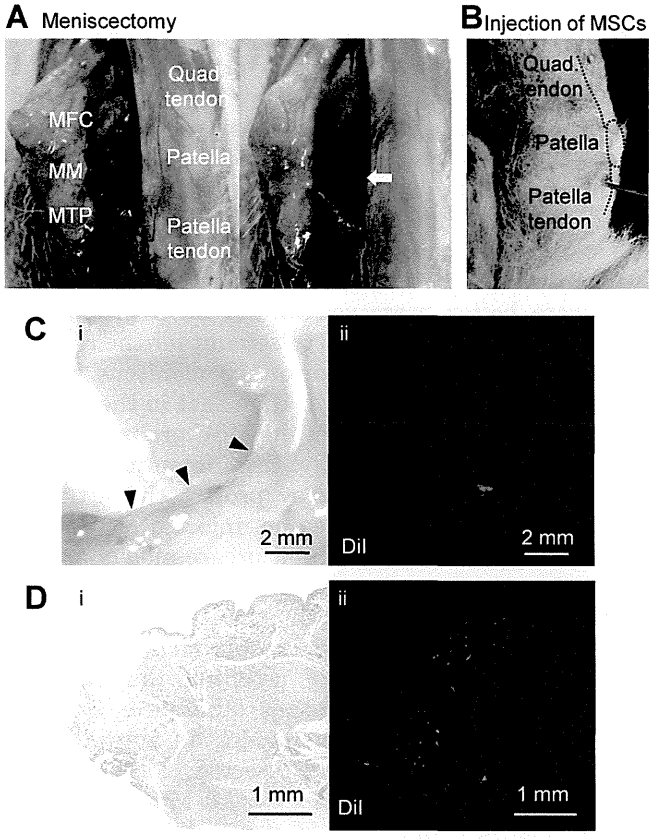

Figure 2. Procedure for removal of the medial meniscus and distribution of injected MSCs at 2 weeks. Medial capsule was incised longitudinally and the anterior half of the meniscus was removed. (A) Space where the meniscus was located is indicated with arrow. Two weeks after the removal of the meniscus, suspension of synovial MSCs was injected into the knee joint. (B) MM, medial meniscus; MFC, medial femoral condyle; MTP, medial tibial plateau. (C) Macroscopic images of tibial side of the knee joint in the bright field (i) and fluorescent field to detect injected MSCs labeled with DiI (ii). Area where meniscus was removed is shown with arrow heads. Histological images for regenerated meniscus sectioned radially. (D) Sections stained with toluidine blue (i) and for DiI (ii) are shown.

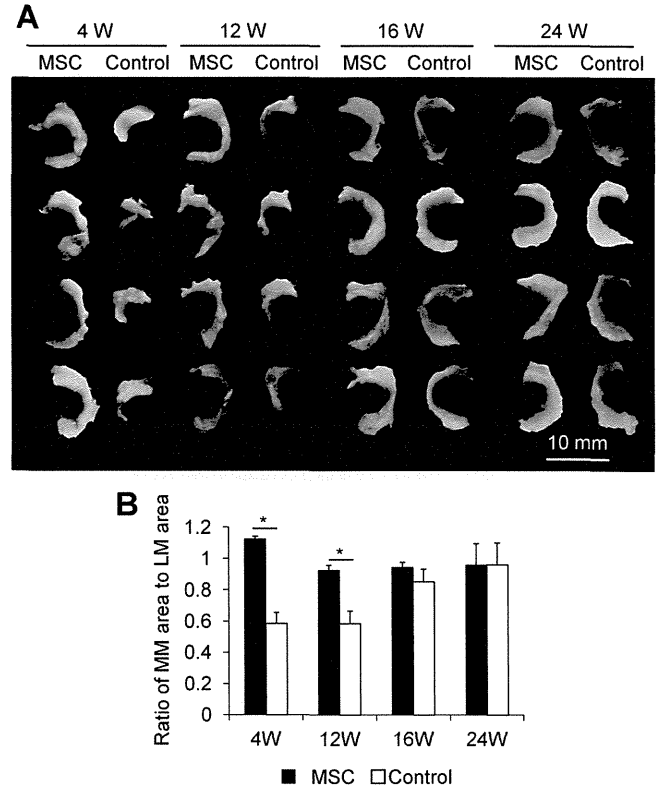

Figure 3. Macroscopic analyses. (A) Features of whole medial menisci are shown. Four pairs of medial menisci in the right knee (MSC group) and in the left knee (control group) are shown, respectively. Quantification of medial meniscus area. (B) Medial meniscus area and intact lateral meniscus area were measured in each knee. The ratio of the medial meniscus area to the lateral meniscus area was calculated. The average values with standard deviations are shown ($n = 4$, $p < 0.05$ by paired t-test).

lesions, it was useful to investigate effectiveness of an unestablished treatment for meniscal regeneration.

In our previous ex vivo study using human and rabbit samples, a suspension of synovial MSCs was placed on the fullthickness defect of the articular cartilage fragment, and approximately 60% of the cells were attached to the defect within 10 min.[17] A recent study reported that the addition of magnesium to the cell suspension increased the number of synovial MSCs attached to the cartilage defect.[27] In this study, the medial side of the injected knee was kept down for 10 min immediately after the injection so that injected synovial MSCs could be attached around the defect of the meniscus. We speculated that the injected MSCs adhered to the meniscal defect by gravity.

In this study, we quantified the meniscus regeneration based on the area rather than volume or weight. The measurement of the volume or the weight for the regenerated meniscus will provide more precise data, however, they seem to require more additional works than the measurement of area. The area of the regenerated meniscus will correlate to the volume and weight of the regenerated meniscus. We previously evaluated meniscal regeneration by the area in three other papers.[11,28,29] In our current study, we newly calculated the ratio of the regenerated medial meniscus area to the native lateral meniscus area to consider the differences between individuals.

We showed that the meniscus area in the MSC group was significantly larger than those in the control group at 4 and 12 weeks. Though there were no significant differences between the two groups at 16 and 24 weeks based on macroscopic analysis, the histological score in the MSC group was better than that in the control group at each point we examined. These results demonstrated that intraarticular injection of synovial MSCs promoted meniscal regeneration.

Two weeks after intraarticular injection of synovial MSCs, meniscal defect was already filled with synovial tissue, whereas the DiI positive area was partially detected macroscopically. In the radial histological section, DiI labeled cells were located mostly in the center of the synovial tissue. We examined the location of DiI labeled cells at 4 weeks and thereafter, but we

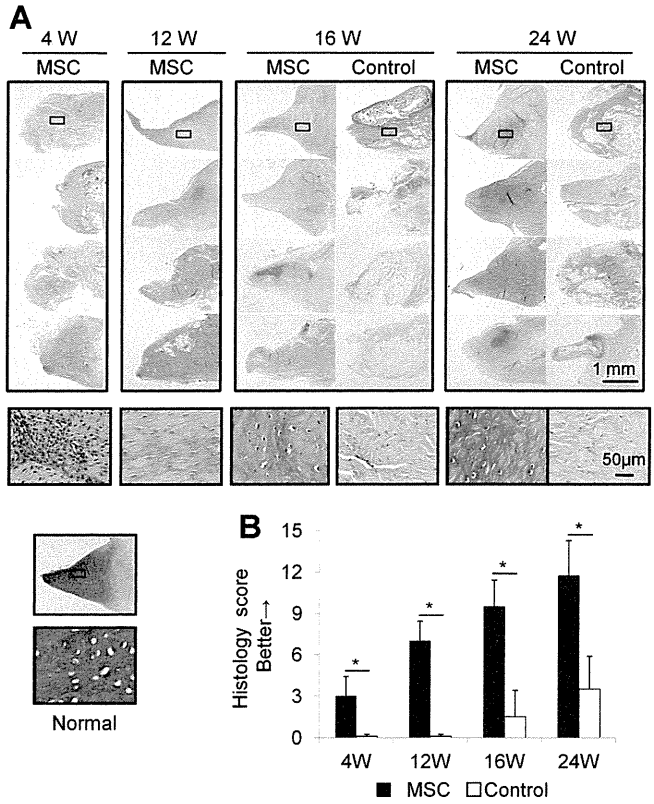

Figure 4. Histological analyses. (A) Radial histological sections of regenerated medial menisci stained with toluidine blue. At 4 and 12 weeks, four menisci in the right knee (MSC group) of each rabbit were shown, respectively. At 16 and 24 weeks, four pairs of menisci in the right knee (MSC group) and in the left knee (control group) were shown, respectively. Area indicated with square is magnified. Quantification of histology evaluated by our modified scoring system (Table 1) according to the Pauli's system.[20] (B) The average values with standard deviations are shown ($n = 4$, $p < 0.05$ by paired t-test).

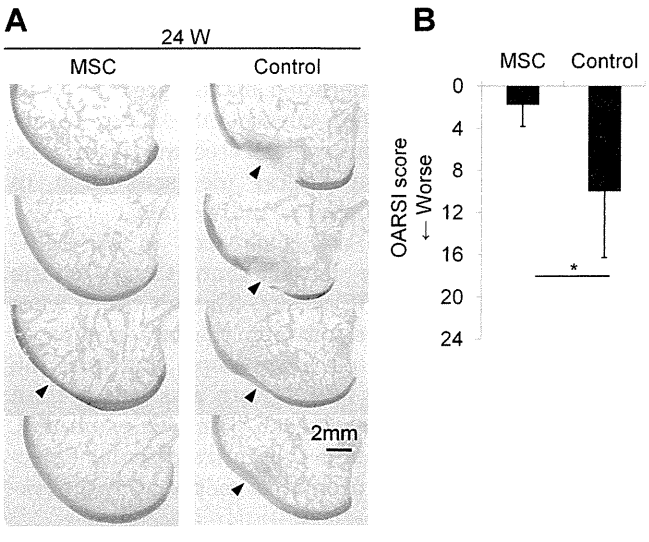

Figure 5. Histological analyses for articular cartilage adjacent to the medial meniscus at 24 weeks. (A) Sagittal histological sections of medial femoral condyles stained with toluidine blue. Four pairs of the medial femoral condyle in the right knee (MSC group) and in the left knee (control group) were shown, respectively. Cartilage lesion is indicated by arrow head. Quantification of histology evaluated by OARSI scoring system. (B) The average values with standard deviations are shown ($n = 4$, $p < 0.05$ by paired t-test).

could not find any. These findings indicate that injected MSCs adhered around the defect of meniscus at first, then they induced synovial tissue formation, after that the newly formed synovium differentiated into meniscal tissue. In this rabbit model, injected synovial MSCs did not differentiate into meniscal cells directly, which is different from our results in a rat model in which injected synovial MSCs differentiate into meniscal cells directly.[11]

At 24 weeks, articular cartilage and subchondral bone were better preserved in the MSC group. We propose two possible reasons to account for the results. At 4 and 12 weeks, the size of the meniscus in the MSC group was already larger than that in the control group though the meniscus in the MSC group was still poorly organized histologically. Even immature meniscus within a few months may protect the articular cartilage. As for the other reason, injected MSCs may produce trophic factors to inhibit progression of articular cartilage degeneration.[30–32]

This rabbit model has certain limitations as far as its clinical application. First, rabbit meniscus has a greater spontaneous healing potential than human meniscus, therefore, interspecies differences have to be considered. Second, in this model, an anterior half of the medial meniscus was resected; therefore, we cannot refer to the effects of synovial MSCs on other types of meniscal injuries. Third, we did not perform a biomechanical test for regenerated meniscus. In spite of these limitations, the present study demonstrated a novel treatment for regeneration of a large meniscal defect. This method has a possibility to regenerate meniscus and to restore its functions with low invasion.

ACKNOWLEDGMENTS

We thank Miyoko Ojima for expert help with histology, and Izumi Nakagawa for management of our laboratory.

REFERENCES

1. Bendjaballah MZ, Shirazi-Adl A. Zukor DJ. 1995. Biomechanics of the human knee joint in compression: reconstruction, mesh generation and finite element analysis. Knee 2:69–79.
2. Bolano LE, Grana WA. 1993. Isolated arthroscopic partial meniscectomy. Functional radiographic evaluation at five years. Am J Sports Med 21:432–437.
3. Rangger C, Klestil T, Gloetzer W, et al. 1995. Osteoarthritis after arthroscopic partial meniscectomy. Am J Sports Med 23:240–244.
4. Tenuta JJ, Arciero RA. 1994. Arthroscopic evaluation of meniscal repairs. Factors that effect healing. Am J Sports Med 22:797–802.
5. LaPrade RF, Wills NJ, Spiridonov SI, et al. 2010. A prospective outcomes study of meniscal allograft transplantation. Am J Sports Med 38:1804–1812.
6. Vundelinckx B, Bellemans J, Vanlauwe J. 2010. Arthroscopically assisted meniscal allograft transplantation in the knee: a medium-term subjective, clinical, and radiographical outcome evaluation. Am J Sports Med 38:2240–2247.
7. 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.
8. Pittenger MF, Mackay AM, Beck SC, et al. 1999. Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.
9. Shirasawa S, Sekiya I, Sakaguchi Y, et al. 2006. In vitro chondrogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells. J Cell Biochem 97:84–97.
10. 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.
11. 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.
12. Miyamoto T, Muneta T, Tabuchi T, et al. 2010. Intradiscal transplantation of synovial mesenchymal stem cells prevents intervertebral disc degeneration through suppression of matrix metalloproteinase-related genes in nucleus pulposus cells in rabbits. Arthritis Res Ther 12:R206.
13. Chevrier A, Nelea M, Hurtig MB, et al. 2009. Meniscus structure in human, sheep, and rabbit for animal models of meniscus repair. J Orthop Res 27:1197–1203.
14. Sekiya I, Vuoristo JT, Larson BL, et al. 2002. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc Natl Acad Sci USA 99:4397–4402.
15. 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.
16. 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.
17. 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.
18. Koga H, Muneta T, Ju YJ, et al. 2007. Synovial stem cells are regionally specified according to local microenvironments after implantation for cartilage regeneration. Stem Cells 25: 689–696.
19. Mothe AJ, Tator CH. 2005. Proliferation, migration, and differentiation of endogenous ependymal region stem/progenitor cells following minimal spinal cord injury in the adult rat. Neuroscience 131:177–187.
20. Pauli C, Whiteside R, Heras FL, et al. 2012. Comparison of cartilage histopathology assessment systems on human knee joints at all stages of osteoarthritis development. Osteoarthritis Cartilage 20:476–485.
21. Pritzker KP, Gay S, Jimenez SA, et al. 2006. Osteoarthritis cartilage histopathology: grading and staging. Osteoarthritis Cartilage 14:13–29.
22. Dominici M, Le Blanc K, Mueller I, et al. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317.
23. De Bari C, Dell'Accio F, Vanlauwe J, et al. 2006. Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis. Arthritis Rheum 54:1209–1221.
24. De Bari C, Dell'Accio F, Tylzanowski P, et al. 2001. Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum 44:1928–1942.
25. Huang JI, Zuk PA, Jones NF, et al. 2004. Chondrogenic potential of multipotential cells from human adipose tissue. Plast Reconstr Surg 113:585–594.
26. Murphy JM, Fink DJ, Hunziker EB, et al. 2003. Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum 48:3464–3474.
27. 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.
28. Horie M, Driscoll MD, Sampson HW, et al. 2012. Implantation of allogenic synovial stem cells promotes meniscal regeneration in a rabbit meniscal defect model. J Bone Joint Surg Am 94:701–712.
29. Horie M, Choi H, Lee RH, et al. 2012. Intra-articular injection of human mesenchymal stem cells (MSCs) promote rat meniscal regeneration by being activated to express Indian hedgehog that enhances expression of type II collagen. Osteoarthritis Cartilage 20:1197–1207.
30. Caplan AI, Dennis JE. 2006. Mesenchymal stem cells as trophic mediators. J Cell Biochem 98:1076–1084.
31. He J, Decaris M, Leach JK. 2012. Bioceramic-mediated trophic factor secretion by mesenchymal stem cells enhances in vitro endothelial cell persistence and in vivo angiogenesis. Tissue Eng A 18:1520–1528.
32. Stewart K, Pabbruwe M, Dickinson S, et al. 2007. The effect of growth factor treatment on meniscal chondrocyte proliferation and differentiation on polyglycolic acid scaffolds. Tissue Eng 13:271–280.

炎症と免疫

別　刷

発行：株式会社　先端医学社
〒103-0007 東京都中央区日本橋浜町 2-17-8　KDX浜町ビル

滑膜由来間葉系幹細胞による膝半月板再生

堀江雅史*　宗田　大**　関矢一郎***

膝半月板は自己修復能に乏しく，損傷を放置したり広範囲に切除がおこなわれると，関節軟骨の変性が生じる．修復術の適応には限界があり，低侵襲で半月板を再生させる新たな治療法の開発が期待されている．滑膜由来間葉系幹細胞(滑膜幹細胞)は自己の関節滑膜から分離可能で，自己血清を用いてよく増殖し，軟骨分化能が高いことから，半月板再生の細胞源として利用価値が高い．本研究では，滑膜幹細胞の移植により損傷した半月板の再生が促進されるか動物モデルを用いて検討をおこなった．

はじめに

膝関節内の大腿骨・脛骨間に存在する半月板は，線維性軟骨であり，荷重分散や関節安定性などの重要な機能を担っている．外傷や加齢性の変化によって半月板が損傷した場合，膝関節の痛みやひっかかり，水腫などの症状が生じる．半月板損傷に対する外科的治療としては，修復術や切除術が一般的であるが，修復術の適応となる症例はかぎられることや修復後再断裂の問題がある．また損傷を受けた半月板を切除すると，短期的には半月板の刺激症状は軽快するが，ヒトの半月板は再生能力が低く，長期的には関節軟骨の変性が増悪する問題がある．欧米では半月板の同種移植もおこなわれているが，供給数，治癒環境，手術侵襲などの観点から，一般的な治療にまでは普及しておらず，より良い新たな治療法の開発が望まれている．

〔キーワード〕
半月板
滑膜
間葉系幹細胞
再生
変形性関節症

＊HORIE Masafumi/東京医科歯科大学医学部附属病院整形外科
＊＊MUNETA Takeshi/東京医科歯科大学大学院運動器外科学
＊＊＊SEKIYA Ichiro/東京医科歯科大学大学院軟骨再生学

間葉系幹細胞は成体の間葉組織から採取でき，自己の細胞を使用できる点から，再生医療の細胞源として期待されている[1]．間葉系幹細胞は，骨髄，脂肪，筋肉などのさまざまな間葉系組織から採取可能である．中でも滑膜から分離した間葉系幹細胞(滑膜幹細胞)は，自己血清を用いた培養での増殖能が高く[2]，軟骨分化能が高いことから[3)~5)]，半月板再生の細胞源として利用価値が高い．

2006年にMurphyら[6]は骨髄間葉系幹細胞を動物の膝関節内に投与することにより，切除した半月板が再生されることを報告して以降，間葉系幹細胞を用いた半月板再生に関する報告はいくつかあったが，移植細胞の局在が必ずしも明確ではなく，移植細胞が直接半月板細胞に分化したのか，あるいは関節内投与した細胞が産生する栄養因子により再生が促進されるものなのかといった点については明確ではなかった．また，間葉系幹細胞を関節内投与した後に，移植細胞が増殖するのかしないのか，あるいは関節内にとどまるのか遠隔臓器に移動するのかという点についても不明で

あった．そのため臨床応用にあたり，必要な細胞数や，投与回数，さらには安全性に関する疑問があった．また半月板損傷に対する滑膜由来の間葉系幹細胞の治療効果についてはほとんどわかっていなかった．

われわれは滑膜幹細胞の臨床応用をめざして，半月板再生に関する種々の研究をおこなってきた[7)~9)]．本稿の前半では，ラット半月板広範囲切除モデルにおいて滑膜幹細胞の関節内投与による半月板の再生効果と，移植細胞の動態に関する解析結果について述べる．また後半では，中動物（ウサギ）における半月板無血行野損傷に対する滑膜幹細胞移植の効果について述べる．

1．ラット半月板部分切除後に滑膜幹細胞を関節内へ投与すると半月板の再生が促進される[7)]

滑膜幹細胞を関節内投与することによって，広範囲に切除した半月板が再生されるのかどうかを調べるためラット半月板切除モデルを作成し検討をおこなった．ここでは移植細胞の動態を詳細に追跡するため，レポーター遺伝子であるLuciferaseとLacZ遺伝子を全身性に同時発現するトランスジェニックラット[10)]の滑膜組織から滑膜幹細胞を分離培養して以下の実験に用いた．

方法：野生型ラットの両膝関節を切開して，半月板の前方半分を切除摘出（**図1A**）したのちに，LuciferaseとLacZ遺伝子を同時に発現する滑膜幹細胞500万個を含む浮遊液を片側の膝関節へ投与した（mesenchymal stem cells：MSC群）．比較のため，反対側の膝には細胞投与をおこなわず（control群），それぞれの半月板の修復過程を12週まで観察した．

結果：2週後，膝関節を展開しX-galで染色すると，半月板欠損部と，切開後縫合した関節包部に，LacZ陽性細胞が観察された（**図1B**）．このことは，関節内注射した滑膜幹細胞が組織損傷部に高率に生着することを示している．12週までの半月板を観察すると，2，4，8週で幹細胞を注射したものは，control群よりも半月板が大きく再生していた（**図1C**）．また，再生した部位にはLacZ陽性の組織が含まれており，関節内投与した細胞が損傷部へ接着し，半月板を構成する細胞に直接分化することがわかった（**図1C**）．12週では，control群でも幹細胞を注射したものと肉眼的には同程度の大きさであったが，組織学的に詳細に検討すると，幹細胞を移植した膝の半月板は，軟骨組織に特徴的なⅡ型コラーゲンを発現していたが，control群ではⅡ型コラーゲンを発現しておらず，幹細胞の投与により半月板の質が向上することが示された（**図2A**）．さらに電子顕微鏡による観察をおこなうと，幹細胞移植後の半月板は生来の半月板に近い細胞形態であることがわかった（**図2B**）．

Luciferase陽性の滑膜幹細胞を正常膝（半月板損傷のない膝）の関節内に注射すると，発光強度が時間経過とともに減少し，7日以降では観察できなくなった．他方，半月板を切除したものに同様の検討をおこなうと，発光強度は漸増し，3日でピークに達し，その後漸減した（**図3A，B**）．正常膝では発光は早期に減弱していったのに対して，半月板損傷のある膝では，Luciferase発光強度が増す，あるいはLuciferase陽性細胞の数が一過性に増加したという点は興味深い．また関節内に注射した滑膜幹細胞が肺などの膝関節以外の臓器に移動する現象は認められなかった．

本研究によって，滑膜幹細胞を関節内に投与すると切除した半月板の再生が促進されることが示された．LuciferaseとLacZのダブルトランスジェニックラットの滑膜由来の幹細胞を使用することにより，注射した細胞が損傷組織に生着し，直接半月板様細胞に分化する機序が存在すること，関節内注射した間葉系幹細胞は一過性に増殖するがその後漸減すること，注射した細胞は他臓器へ移動しないことが明らかになった．

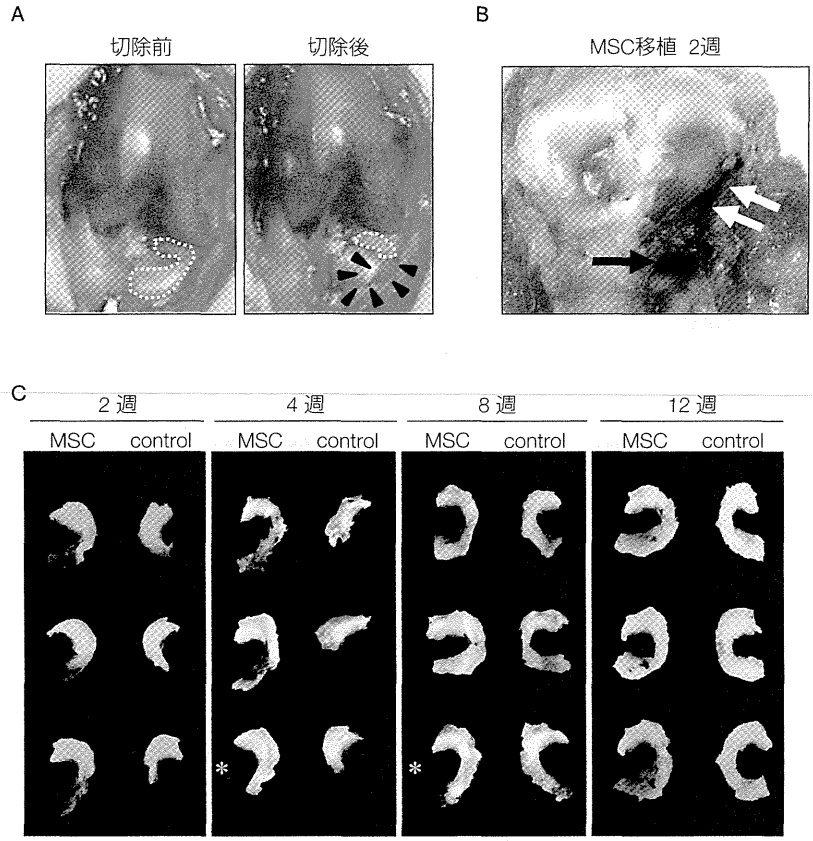

図 1. ラット半月板部分切除モデルと滑膜幹細胞投与後の肉眼像(Horie M *et al*, 2009[7]より引用)

A：ラット半月板の切除前と切除後の肉眼像．膝関節を切開後に内側半月板を露出させ(左)，前方 1/2 を切除した(右)．点線部は半月板を，黒矢印部は切除がおこなわれた部位を示す．

B：MSC 移植後 2 週の膝肉眼像(X-gal 染色)．LacZ 陽性組織(青)が半月板再生部(白矢印)と関節切開部(黒矢印)に認められた．

C：半月板肉眼像(n＝3)．2，4，8 週では MSC 群において半月板は control 群にくらべてより大きく再生していた．LacZ 陽性組織(青)が 12 週まで再生部分に認められた．

2．ウサギ半月板無血行での部分欠損作成後に滑膜幹細胞を局所移植すると半月板の再生が促進される[8]

半月板の無血行野における損傷は，血行野における損傷にくらべて治癒率が悪い．本研究ではウサギ半月板の無血行野における部分損傷モデルを用いて，滑膜幹細胞移植による半月板の再生効果を調べた．

方法：ウサギ内側半月板無血行野に直径 1.5 mm の円柱状欠損を作成(**図 4**)した後に，Green Fluorescent Protein (GFP) あるいは蛍光色素(CM-DiI)で標識した滑膜幹細胞 200 万個を含む PBS 浮遊液を半月板欠損部に局所投与した(MSC 群)．比較のため，反対側の膝には細胞投与をおこなわず(control 群)，それぞれの半月板の修復過程を 24 週時まで観察し，形態・組織学的解析をおこなった．

結果：肉眼的観察においては，control 群では，4 週，12 週では，5 例中 4 例において欠損部が残存したままであったのに対して，MSC 群では 4 週時に欠損部が残存したものは 1 例のみであり，

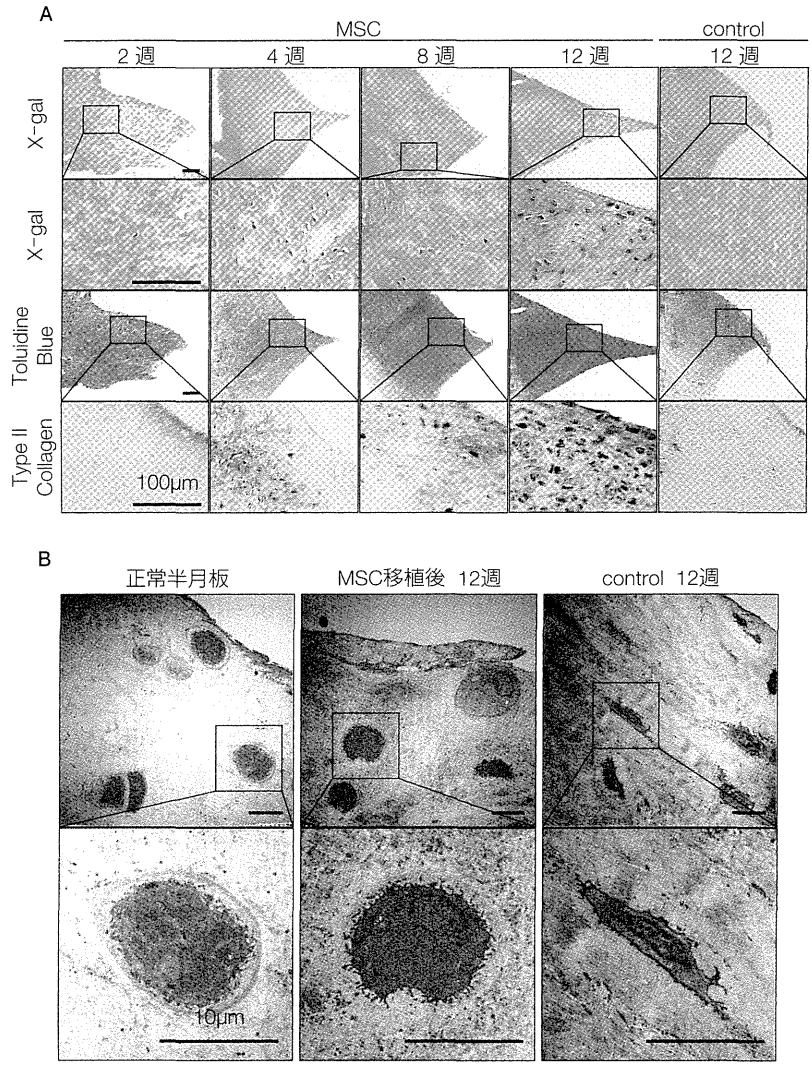

図 2. 滑膜幹細胞投与後の半月板組織像(Horie M *et al*, 2009[7]より引用)

A：半月板組織像．MSC 群では経時的にトルイジンブルー染色性，Ⅱ型コラーゲン発現が上昇し，形態的にも正常の半月板に類似した組織再生が認められた．LacZ 陽性細胞(青)は 12 週時まで認められた．

B：12 週時の半月板電子顕微鏡像．MSC 群では，再生組織には正常半月板細胞に類似した円形の半月板様細胞が認められた．コントロール群では紡錘形の線維芽細胞が大部分を占めていた．

12 週以降は全例で欠損部が再生組織で覆われていた(**図 5**)．

組織学的には，control 群では，4 週時は欠損部への細胞流入は疎であり，12 週時になると部分的に軟骨に分化した再生組織が確認できたが，再生組織と残存半月板との境界は結合せずに間隙が残存したままであった．24 週時になると欠損部は再生組織でほぼ充填されたが，大部分は線維性組織であり，間隙は結合しないまま残存した．一方 MSC 群では，4 週時には欠損部は高密度の細胞と豊富な細胞でほぼ充填され，12 週時にはサフラニン染色陽性の基質成分に富んだ軟骨様組織に分化し，境界部の結合性も良好であった．また 24 週時はさらに成熟した軟骨様組織に分化していた

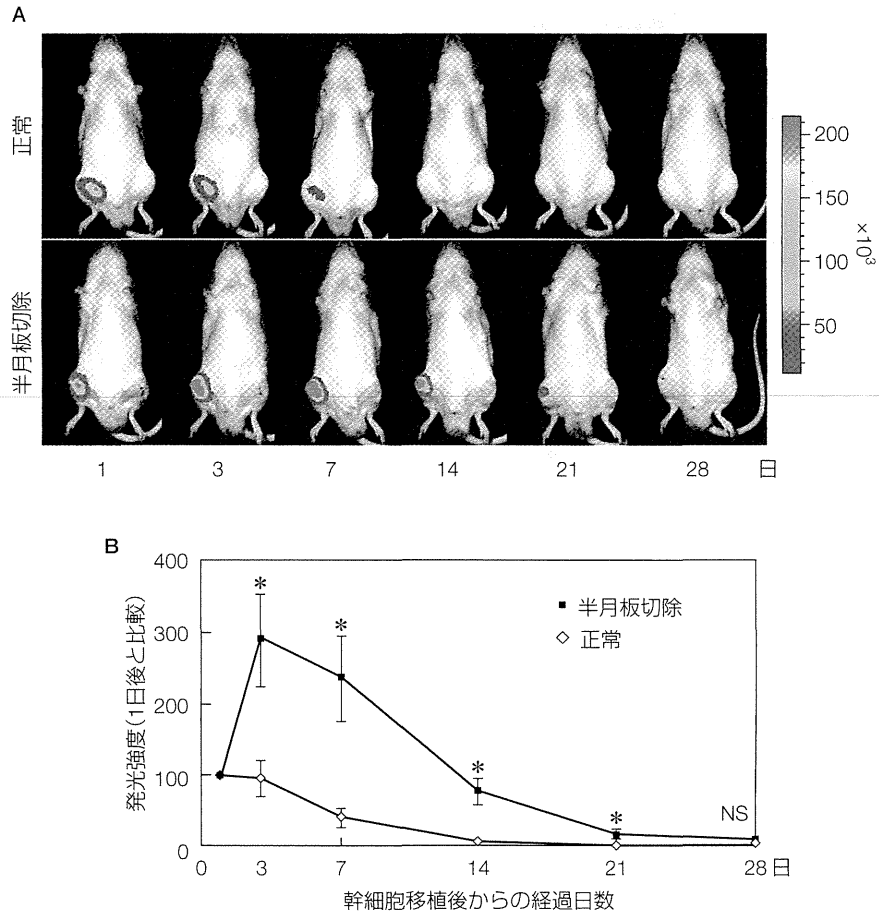

図 3. *In vivo* イメージングによる移植細胞の追跡(Horie M *et al*, 2009[7]より引用)

A：バイオイメージング像．Luciferase 陽性の間葉系幹細胞を正常膝または半月板切除膝に投与した．半月板切除膝ではより長期間発光が認められた．赤は発光強度が強く，青は発光強度が弱いことを示す．

B：Luciferase 発光の定量評価．正常膝では幹細胞由来の発光は早期に減弱したが，半月板切除膝では一過性に上昇しゆるやかに減弱した．

(**図 6A**)．

組織学的定量評価では，欠損部の修復面積は 4 週，12 週時において MSC 群が有意に大きく再生しており(**図 6B**)，組織学的スコアを用いた質的評価では，12 週，24 週時において MSC 群が有意に良好な点数を示した(**図 6C**)．

細胞追跡をおこなうために，CM-DiI で標識した滑膜幹細胞を投与し，蛍光顕微鏡で観察すると，投与した CM-DiI 陽性の細胞は欠損部に集積し 24 週時まで残存していた．また経時的に CM-DiI 陽性の細胞数は減少していくことがわかった(**図 7A**)．さらに GFP 陽性の滑膜幹細胞を用いて同様のモデルで観察をおこなうと，細胞投与後 12 週時には，GFP 陽性の細胞はⅠ型・Ⅱ型コラーゲン陽性の半月板様細胞に直接分化していた(**図 7B**)．

本研究によって，ウサギ半月板無血行野での半月板欠損に対して，滑膜幹細胞を局所投与すると

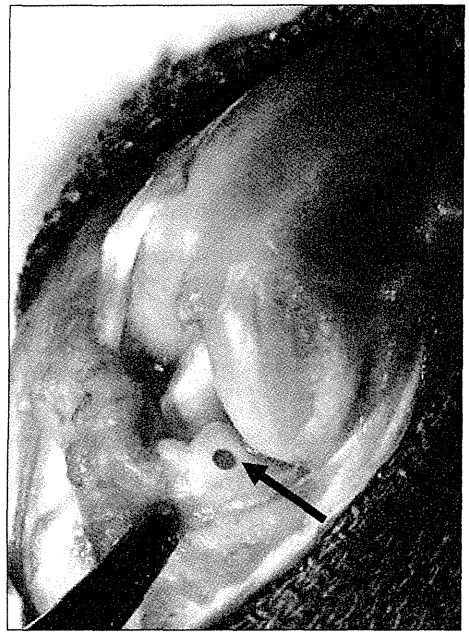

図 4. ウサギ半月板部分欠損作成後の肉眼像
(Horie M *et al*, 2012[8]より引用)
ウサギ内側半月板無血行野に直径 1.5 mm の円柱状欠損を作成した(矢印).

半月板の再生が促進されることが示された.

おわりに

これまでわれわれは,滑膜幹細胞の移植により損傷した半月板の再生が促進されることを動物モデル(ラット・ウサギ)で示してきた.臨床応用のためには,よりヒトの構造に近い大動物(ブタなど)での検証や,再生した半月板の強度の検証などが必要であり,現在解析を進めている.

本細胞治療のメリットは,自分自身の滑膜を利用できること,自己血清を用いて細胞を大量に増やせること,関節鏡を用いて低侵襲に細胞を移植できること,などが挙げられる.われわれは自家滑膜間葉系幹細胞を用いて,膝の関節軟骨損傷に対する再生治療をすでに臨床応用している.2008年より開始し,多くの例で軟骨が再生することを確認している.

これまで治療困難であった半月板損傷に対して,本手法が有効な治療手段となることをめざし今後

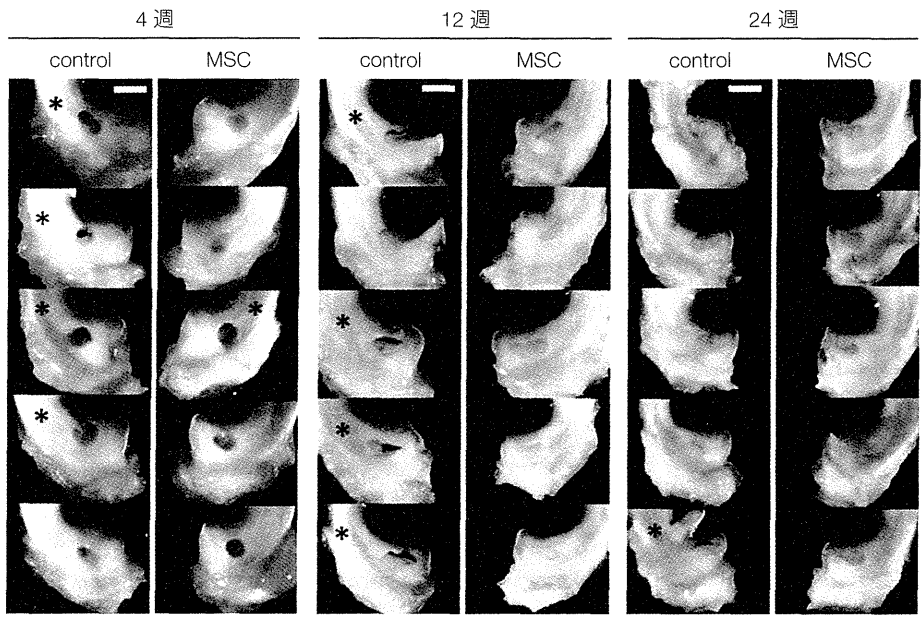

図 5. 半月板肉眼像(n=5) (Horie M *et al*, 2012[8]より引用)
MSC 群では早期に欠損部の再生が認められた.＊は肉眼的に確認できる欠損部が残存した例を示す.

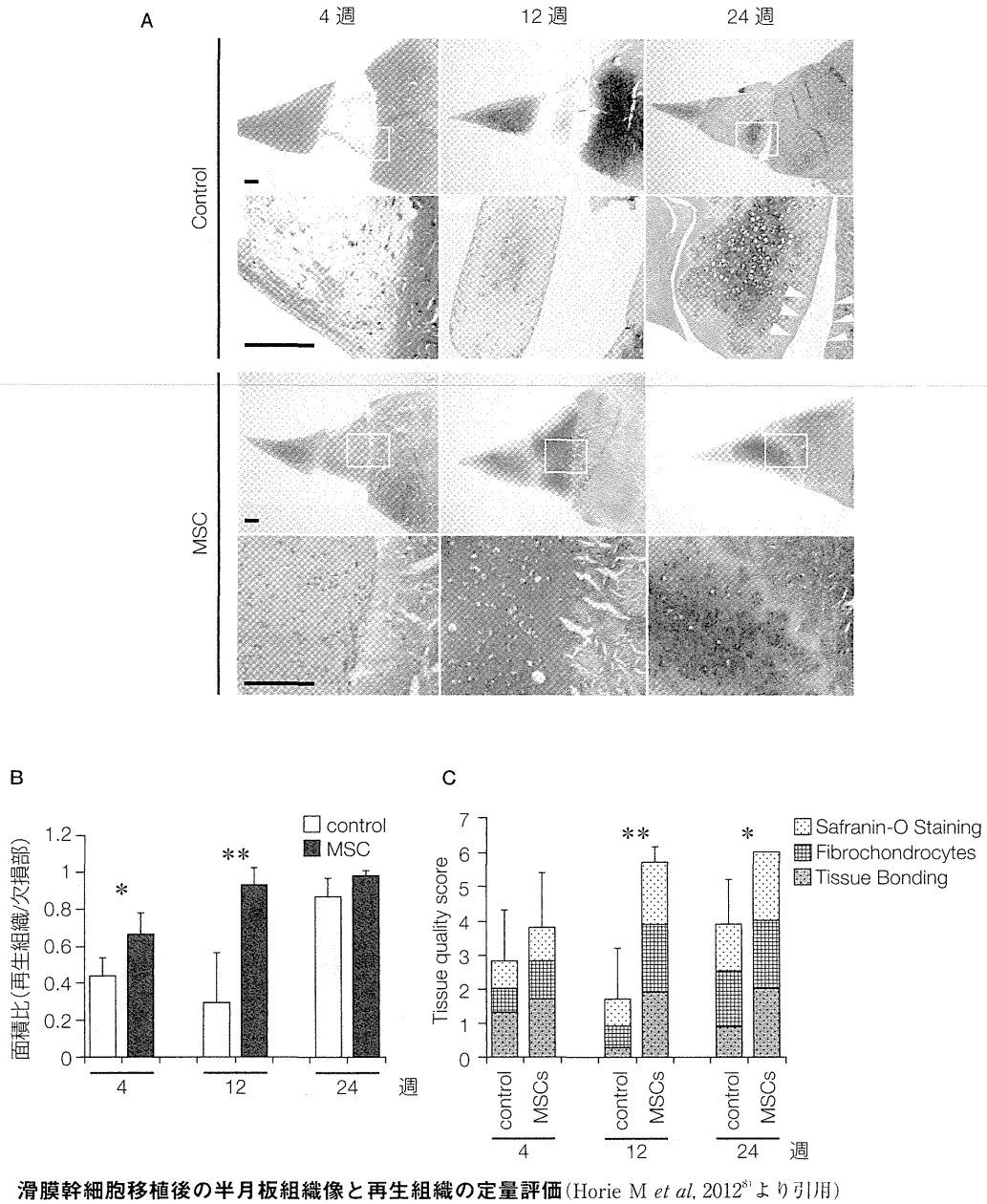

図 6. 滑膜幹細胞移植後の半月板組織像と再生組織の定量評価(Horie M *et al*, 2012[8]より引用)

A：半月板組織像(サフラニン染色)．MSC 群では早期に欠損部の再生が認められた．24 週になると control 群でも欠損部は再生組織で満たされたが，線維性組織が大部分を占め，間隙は結合しないまま残存した(矢印)．

B：半月板欠損部の再生面積比(再生組織/欠損部)．4 週，12 週時において MSC 群が有意に大きく再生していた．
$^{*}P<0.05$　$^{**}P<0.01$

C：組織学的スコアによる質的評価．再生組織のサフラニン染色性，細胞形態，組織結合性の 3 項目について，各項目 0～2 点とし(6 点満点)評価した．12 週，24 週時において MSC 群が有意に良好な点数を示した．
$^{*}P<0.05$　$^{**}P<0.01$

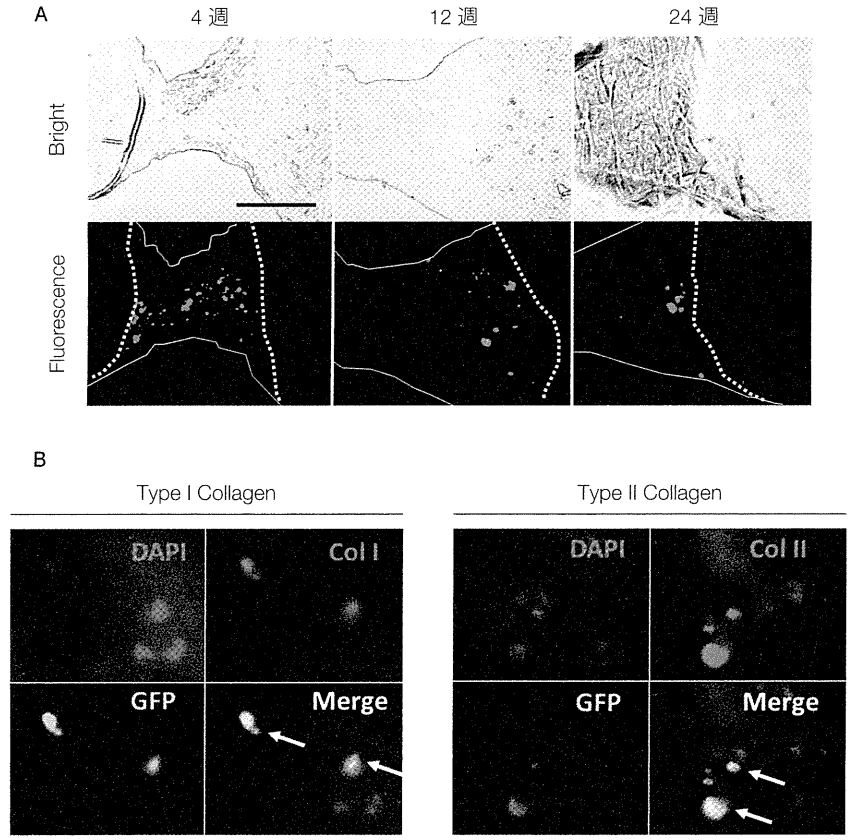

図 7. 移植細胞の組織学的追跡(Horie M *et al*, 2012[8]より引用)
A：CM-DiI 陽性の滑膜幹細胞移植後の蛍光顕微鏡像．CM-DiI 陽性の細胞(赤)は欠損部に集積し 24 週時まで残存していた．また経時的に CM-DiI 陽性の細胞数は減少した．
B：GFP 陽性の滑膜幹細胞移植後 12 週の免疫染色(Ⅰ型・Ⅱ型コラーゲン)．GFP 陽性細胞(緑)は，Ⅰ型・Ⅱ型コラーゲン陽性細胞(赤)に直接分化した．Col Ⅰ:Ⅰ型コラーゲン，Col Ⅱ:Ⅱ型コラーゲン．

も更なる研究を進めていく．

謝辞

本研究は「再生医療の実用化ハイウェイ」と「グローバル COE プログラム」の支援による．

文　献

1) Pittenger MF *et al*：Multilineage potential of adult human mesenchymal stem cells. *Science* **284**：143-147, 1999
2) Nimura A *et al*：Increased proliferation of human synovial mesenchymal stem cells with autologous human serum：comparisons with bone marrow mesenchymal stem cells and with fetal bovine serum. *Arthritis Rheum* **58**：501-510, 2008
3) Sakaguchi Y *et al*：Comparison of human stem cells derived from various mesenchymal tissues：superiority of synovium as a cell source. *Arthritis Rheum* **52**：2521-2529, 2005
4) Yoshimura H *et al*：Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res* **327**：449-462, 2007
5) Koga H *et al*：Comparison of mesenchymal