

CRITICAL CONCEPTS

INDICATIONS:

This procedure is applicable for every reparable rotator cuff tear.

CONTRAINDICATIONS:

There is no specific contraindication to this procedure if the tear is reparable.

PITFALLS:

Margin convergence repair is not the preferred method for repairing u-shaped rotator cuff tears because most u-shaped types of tears are chronic and represent an advanced stage of the L-shaped tear. Surgeons should evaluate the mobility of the posterior leaf of the tear before applying the margin convergence technique.

The rotator interval slide as a method to increase mobility in large retracted tears is not preferred because the tendinous fibers intermingle with each other and work as a horizontal link at the distal end of the rotator cuff tear. If surgeons violate this structure, the risk of postoperative failure may be increased.

AUTHOR UPDATE:

Through our clinical experience, we believe that, normally, the posterior leaf of the torn rotator cuff is more mobile compared with the anterior or central leaf. Furthermore, we have wondered why the integrity of the repair is relatively poor after the use of the margin convergence suture technique and the rotator interval slide in larger tears. Therefore, we are very reluctant to use the margin convergence technique for large u-shaped tears or to use the rotator interval slide as a mobilization procedure for large retracted tears.

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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

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Key Words. Mesenchymal stem cells • Synovium • Meniscus • Luciferase • LacZ • Cell transplantation

ABSTRACT

Osteoarthritis in the knees, which can be caused by meniscal defect, constitutes an increasingly common medical problem. Repair for massive meniscal defect remains a challenge owing to a lack of cell kinetics for the menisci precursors in knee joint. The synovium plays pivotal roles during the natural course of meniscal healing and contains mesenchymal stem cells (MSCs) with high chondrogenic potential. Here, we investigated whether intra-articular injected synovium-MSCs enhanced meniscal regeneration in rat massive meniscal defect. To track the injected cells, we developed transgenic rats expressing dual luciferase (Luc) and LacZ. The cells derived from synovium of the rats demonstrated colony-forming ability and multipotentiality, both characteristics of MSCs. Hierarchical clustering analysis revealed that gene expression of meniscal cells was closer to that of synovium-MSCs than to that of bone

marrow-MSCs. Two to 8 weeks after five million Luc/LacZ+ synovium-MSCs were injected into massive meniscectomized knee of wild-type rat, macroscopically, the menisci regenerated much better than it did in the control group. After 12 weeks, the regenerated menisci were LacZ positive, produced type 2 collagen, and showed meniscal features by transmission electron microscopy. In in-vivo luminescence analysis, photons increased in the meniscus-resected knee over a 3-day period, then decreased without detection in all other organs. LacZ gene derived from MSCs could not be detected in other organs except in synovium by real-time PCR. Synovium-MSCs injected into the massive meniscectomized knee adhered to the lesion, differentiated into meniscal cells directly, and promoted meniscal regeneration without mobilization to distant organs. *STEM CELLS* 2009;27:878–887

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The meniscus is a wedge-shaped semilunar fibrocartilage that lies between the weight bearing joint surfaces of the femur and the tibia. For symptomatic meniscus injury, a meniscectomy is often performed. This, however, often leads to osteoarthritis [1]. Meniscal suture to preserve its function is limited for its indication, and the result is not always satisfactory due to poor healing of the meniscus. Despite other therapeutic attempts [2],

problems related to its effectiveness and invasion persist. A novel strategy for meniscus injury remains necessary.

Mesenchymal stem cells (MSCs) are postulated to participate in tissue homeostasis, 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. Developmentally, intra-articular tissues are differentiated from common progenitors, referred to as common interzone cells [3]. Synovium-MSCs have high

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chondrogenic potential [4, 5], clinically increase in number in synovial fluid after intra-articular tissue injury to contribute to its repair in part [6] and expand in the presence of pure synovial fluid in tissue cultures of the synovium [7]. Synovial tissue may serve as a reservoir of stem cells that mobilize following injury and migrate to the wound site where, in cooperation with local cells, they participate in the repair response.

Thus, during the natural course of meniscal repair, synovium-MSCs are a potential cell source. Here, we investigated whether intra-articular injected synovium-MSCs enhanced meniscal regeneration in rat massive meniscal defect. Dual colored transgenic (Tg) rats expressing luciferase and LacZ (Luc/LacZ) were created for this study so that the fate of transplanted cells could be traced dynamically and precisely.

MATERIALS AND METHODS

Establishment of Dual Colored Transgenic Rat

Dual colored Tg rats expressing luciferase and Lac-Z were created by cross-breeding ROSA/luciferase Tg Lewis rats [8] with ROSA/LacZ Lewis rats [9]. The expression of luciferase was detected by an *in vivo* bioimaging system, and the expression of LacZ was detected by X-gal staining (detailed later). The F1 hybrids between ROSA/luciferase Tg and ROSA/LacZ Lewis rat neonate were imaged after intraperitoneal injection of D-luciferin (30 mg/kg per body weight) (potassium salt; Biosynth, Postfach, Switzerland, <http://www.biosynth.com>), and then they were stained with X-gal. In the same manner, luciferase and LacZ expressions were examined in various tissues of these rats. Approximately one-fourth of these F1 hybrids expressed luciferase and LacZ in the whole body. We used these "dual colored" F1 hybrids expressing both luciferase and LacZ (Luc/LacZ) for the donor of MSCs.

MSCs Preparation

All experiments were conducted in accordance with the institutional guidelines for the care and use of experimental animals of Tokyo Medical and Dental University and Jichi Medical University. The synovial membranes of bilateral knee joints were excised, minced, and digested for 3 hours at 37°C with type V collagenase (0.2%; Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>), and passed through a 40- μ m filter (Becton Dickinson, Franklin Lakes, NJ, <http://www.bd.com>). Bone marrow was extruded by inserting a 22-gauge needle into the shaft of the femur and tibia bone and flushed out. Synovium and bone marrow cells were cultured in a complete medium (α MEM; Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>; 20% FBS; Invitrogen; 100 units per milliliter penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine; Invitrogen) for 14 days. Then the cells were replated at 100 cells/cm², cultured for 14 days, and frozen at -80°C as passage 1. The stocked cells were rapidly thawed in a water bath at 37°C, plated in a 150 cm² dish, and harvested after 5 days. Then the cells were replated at 100 cells/cm², cultured for 14 days, and collected for further analyses [5]. For colony-forming assay, 100 cells were plated in 60 cm² dishes and cultured for 14 days. The dishes were stained with X-gal, and the same dishes were then stained with 0.5% Crystal Violet.

In Vitro Differentiation Assay

For adipogenesis, the cells were cultured in the adipogenic medium that consisted of a complete medium supplemented with 0.5 μ M dexamethasone, 0.5 mM isobutylmethylxanthine, and 50 μ M indomethacin. After 4 days, the adipogenic cultures were stained with 0.3% Oil Red-O solution or X-gal solution [10].

For osteogenesis, the cells were cultured in the calcification medium in the presence of 100 nM dexamethasone, 10 mM β -glycerophosphate, and 50 μ M ascorbic acid. After an additional 6 weeks, the dishes were stained with 0.5% Alizarin Red solution or X-gal solution.

For *in vitro* chondrogenesis, 8×10^5 cells were placed in a 15 ml polypropylene tube (BD Falcon, Bedford, MA, <http://www.bdbiosciences.com>) and pelleted by centrifugation at 450g for 10 minutes. The pellets were cultured for 21 days in chondrogenic media, which contained 500 ng/ml BMP-2 (Astellas Pharma Inc., Tokyo, Japan, <http://www.astellas.com>), in addition to high-glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10 ng/ml transforming growth factor- β 3 (TGF- β 3) (R&D Systems Inc., Minneapolis, MN, <http://www.rndsystems.com>), 10^{-7} M dexamethasone, 50 μ g/ml ascorbate-2-phosphate, 40 μ g/ml proline, 100 μ g/ml pyruvate, and 50 mg/ml ITS+TMPremix (Becton Dickinson). For histological analysis, the pellets were embedded in paraffin, cut into 5- μ m sections, and stained with 1% Toluidine Blue [11].

Flow Cytometry

Synovium-MSCs at passage 3 were harvested 14 days after plating. One million cells were suspended in 500 μ l phosphate buffered saline (PBS) containing 20 ng/ml fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-coupled antibodies against CD11b, CD45, CD90 (Becton Dickinson), CD34 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, <http://www.scbt.com>), and CD29 (BioLegend, San Diego, CA, <http://www.biolegend.com>). As an isotype control, FITC- or PE-coupled nonspecific mouse IgG (Becton Dickinson) was substituted for the primary antibody. After incubation for 30 minutes at 4°C, the cells were washed with PBS and resuspended in 1 ml PBS for analysis. Cell fluorescence was evaluated by flow cytometry in a FACSCalibur instrument (Becton Dickinson); data were analyzed by using CellQuest software (Becton Dickinson).

Oligonucleotide Microarray

For rat meniscal cells, menisci were minced, digested for 3 hours at 37°C with type II collagenase (0.2%; Sigma), and passed through a 40 μ m filter (Becton Dickinson). Nucleated cells were plated at 100 cells/cm² and cultured in a complete medium. Total RNA was isolated from passage 1 colony-formed cells derived from the synovium, bone marrow [5], and meniscus with the RNeasy Total RNA Mini Kit (Qiagen, Valencia, CA, <http://www1.qiagen.com>).

A comprehensive microarray analysis was performed using 3 μ g of total RNA from each sample and GeneChip Rat 230 2.0 probe arrays (Affymetrix, Santa Clara, CA, <http://www.affymetrix.com>) [12]. Data analysis was performed with GeneSpring software version 7.2 (Agilent Technologies, Palo Alto, CA, <http://www.agilent.com>). To normalize the variations in staining intensity among chips, the "Signal" values for all genes on a given chip were divided by the median value for the expression of all genes on the chip. To eliminate genes containing only a background signal, genes were selected only if the raw values of the "Signal" were more than 30, and expression of the gene was judged to be "Present" by the GeneChip Operating Software version 1.4 (Affymetrix). After elimination, expression data of a total of 14,882 probe sets were employed for further analysis. A hierarchical-clustering analysis was performed using a minimum distance value of 0.001, a separation ratio of 0.5, and the standard definition of the correlation distance. A dendrogram was obtained from a hierarchically clustering analysis using average linkage and distance metric equal to one minus the Pearson correlation applied to the microarray data [13].

Meniscectomy and MSCs Injection

Wild-type male Lewis rats at 12 weeks of age (Charles River, Yokohama, Japan, <http://www.crj.co.jp>) were used ($n = 27$). Under anesthesia, a straight incision was made on the anterior side of bilateral knee, the anteromedial side of the joint capsule was cut, and the anterior horn of the medial meniscus was dislocated anteriorly with a forceps. The meniscus was then cut vertically at the level of medial collateral ligament, and the anterior half of medial meniscus was excised. The dislocated meniscus was removed and the wound was closed in layers. Immediately after the skin incision was closed, a 27-gauge needle was inserted

at the center of the triangle formed by the medial side of the patellar ligament, the medial femoral condyle, and the medial tibial condyle, toward the intercondylar space of the femur. Then 5×10^6 Luc/LacZ⁺ synovium-MSCs ($n = 14$) or bone marrow-MSCs ($n = 9$) in 50 μ l PBS were injected into the right knee joint. For the control, the same volume of PBS was injected into the left knee. The rats were allowed to walk freely in the cage.

For control of in vivo imaging analysis, 5×10^6 Luc/LacZ⁺ synovium-MSCs were injected into the normal right knee of the wild-type Lewis rats ($n = 4$).

Histology and Detection of LacZ Expression

The whole medial meniscus was collected at 2, 4, 8, and 12 weeks after MSCs injection ($n = 3$ each time point). The samples were fixed with a fixative solution (0.2% glutaraldehyde, 2 mM MgCl₂, and 5 mM EGTA) in PBS for 10–30 minutes at room temperature and washed three times in a washing solution (2 mM MgCl₂, 0.01% sodium deoxycholate, and 0.02% Nonidet P40) in PBS. Then they were treated with an X-gal staining solution (1 mg/ml of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, 2 mM MgCl₂, and 5 mM potassium hexacyanoferrate [III], 5 mM potassium hexacyanoferrate [II] trihydrate) at 37°C for 3 hours. They were subsequently fixed again in 4% paraformaldehyde and decalcified with 0.5 M EDTA (pH 7.5) for 3 days at 4°C, followed by a gradient replacement with 20% sucrose for 24 hours at 4°C, and then evaluated by Toluidine Blue or Eosin staining of paraffin sections.

Immunostaining

Sections were pretreated with 0.4 mg/ml proteinase K (DAKO, Carpinteria, CA; <http://www.dakousa.com>) in Tris-HCl for 15 minutes at room temperature for optimal antigen retrieval. Residual enzymatic activity was removed by washes in PBS, and nonspecific staining was blocked with PBS containing 10% normal horse serum for 20 minutes at room temperature. A primary anti-rat monoclonal antibody against human type II collagen (1 : 200 dilution with PBS containing 1% BSA; Daiichi Fine Chemical, Toyama, Japan; <http://www.daiichi-fcj.co.jp>) was applied to the section which was incubated at room temperature for 1 hour and rinsed again with PBS. Immunostaining was detected by Vectastain ABC reagent (Vector Laboratories, Burlingame, CA; <http://www.vectorlabs.com>), followed by diaminobenzidine staining.

In Vivo Bioluminescent Imaging

A noninvasive bioimaging system IVIS (Xenogen, Alameda, CA; <http://www.caliperls.com>) was used for analysis using IGOR (WaveMetrics, Lake Oswego, OR; <http://www.wavemetrics.com>) and IVIS Living Image (Xenogen) software packages [14]. To detect photons from Luc⁺ cells, undifferentiated MSCs or chondrocyte pellets were suspended in PBS and imaged immediately after the addition of 0.15 mg D-luciferin (potassium salt; Biosynth). Also, for transplanted cell tracking in vivo, D-luciferin was injected into the penile vein of anesthetized rats (30 mg/kg per body weight) under anesthesia with isoflurane. The signal intensity was quantified as photon flux in units of photons per seconds cm² per steradian in the region of interest.

Transmission Electron Microscopy

The regenerated tissues at 12 weeks in the synovium-MSCs treated group and control groups were selected and fixed with 2.5% glutaraldehyde in 0.1 M PBS for 5 hours, washed overnight at 4°C in the same buffer, postfixed with 1% OsO₄ buffered with 0.1 M PBS for 2 hours, dehydrated in a graded series of ethanol, and embedded in Epon 812. Ultrathin sections at 90 nm were collected on copper grids, double-stained with uranyl acetate and lead citrate, and then examined with a transmission electron microscope (H-7100, Hitachi, Hitachinaka, Japan; <http://www.hitachi.co.jp>) [15].

Quantitative Real-Time PCR

Total RNAs were prepared from brain, lung, liver, spleen, kidney, and knee synovium at 3 days after the synovium-MSCs injected rat

by RNAqueous Kit (Ambion, Austin, TX; <http://www.ambion.com>) according to the manufacturer's instructions. For the positive control, total RNAs from various organs of Luc/LacZ Tg rat and expanded MSCs including Luc/LacZ positive cell rates ranged from 0.001 to 100% were used. Also total RNAs from various organs of wild-type rat were prepared as a negative control. They were subjected to *real-time PCR* to measure the level of LacZ.

The primer sets for LacZ, (sense, 5'-GGTGCAGAGAGACAGGAACCAC-3'; antisense, 5'-CCTTCATACTGCACAGGTCTGCT-3') and β -actin (sense, 5'-CCGAGCGTGGCTACAGCTT-3'; antisense, 5'-GGCAGTGGCCATCTCTTGC-3') were synthesized at FASMAC (Kanagawa, Japan; <http://www.fasmac.co.jp>).

First-strand cDNA was synthesized using the I Script cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA; <http://www.bio-rad.com>) with an Oligo (dT) (12–18mers) primer. Real-time quantitative PCR analyses were performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA; <http://www.appliedbiosystems.com>) using SYBR Green I PCR reagents (TOYOBO, Osaka, Japan; <http://www.toyobo.co.jp/e/>) as described previously [12]. To determine the exact copy numbers of the target genes, the quantified concentrations of subcloned PCR fragments of LacZ and β -actin were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 5 ng of total RNA samples were used for each real-time PCR. Data were normalized with β -actin levels in each sample. The copy number is expressed as the number of transcripts per nanogram total RNA.

Statistics

The Mann-Whitney *U* test was used to compare two groups at each period. *p* values less than .05 were considered significant.

RESULTS

Synovium-MSCs Expressing Luc/LacZ Genes

The F1 hybrids between ROSA/luciferase Tg and ROSA/LacZ Lewis rat neonate produced luminescence after intraperitoneal injection of D-luciferin (Fig. 1A), and then they were positive for X-gal staining (Fig. 1B). In the same manner, we next examined luciferase and LacZ expression in various tissues of these rats. Approximately one-fourth of these F1 hybrids expressed luciferase and LacZ in whole body including the synovium (Fig. 1C). We termed these "dual colored" rats as Luc/LacZ Tg rats. MSCs were isolated from the synovium of Luc/LacZ Tg rats. 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. 1D, 1E). Synovium-MSCs from Luc/LacZ Tg rats formed LacZ⁺ single cell-derived colonies consisting of spindle cells (Fig. 1F). They could differentiate into adipocytes (Fig. 1G) and calcified with LacZ expression in vitro (Fig. 1H). The cells could also form cartilage with expressions of both LacZ and luciferase (Fig. 1I). The cells derived from synovium of Luc/LacZ Tg rat demonstrated characteristics of MSCs, and the dual markers were maintained after the differentiation. Flow cytometric analysis demonstrated that the majority of synovium-MSCs expressed CD29 and CD90, and were negative for CD11b, CD34, and CD45 (Fig. 1J).

Hierarchical clustering analysis revealed that the gene expression of meniscal cells was closer to that of synovium-MSCs than that of bone marrow-MSCs (Fig. 2). This indicates that synovium-MSCs may retain a more advantageous character as a MSC source for meniscal regeneration than bone marrow-MSCs.

Meniscal Regeneration After Intra-articular Injection of Synovium-MSCs

To obtain in vivo evidence to support the synovium-MSCs potential, we performed massive meniscectomy in both sides

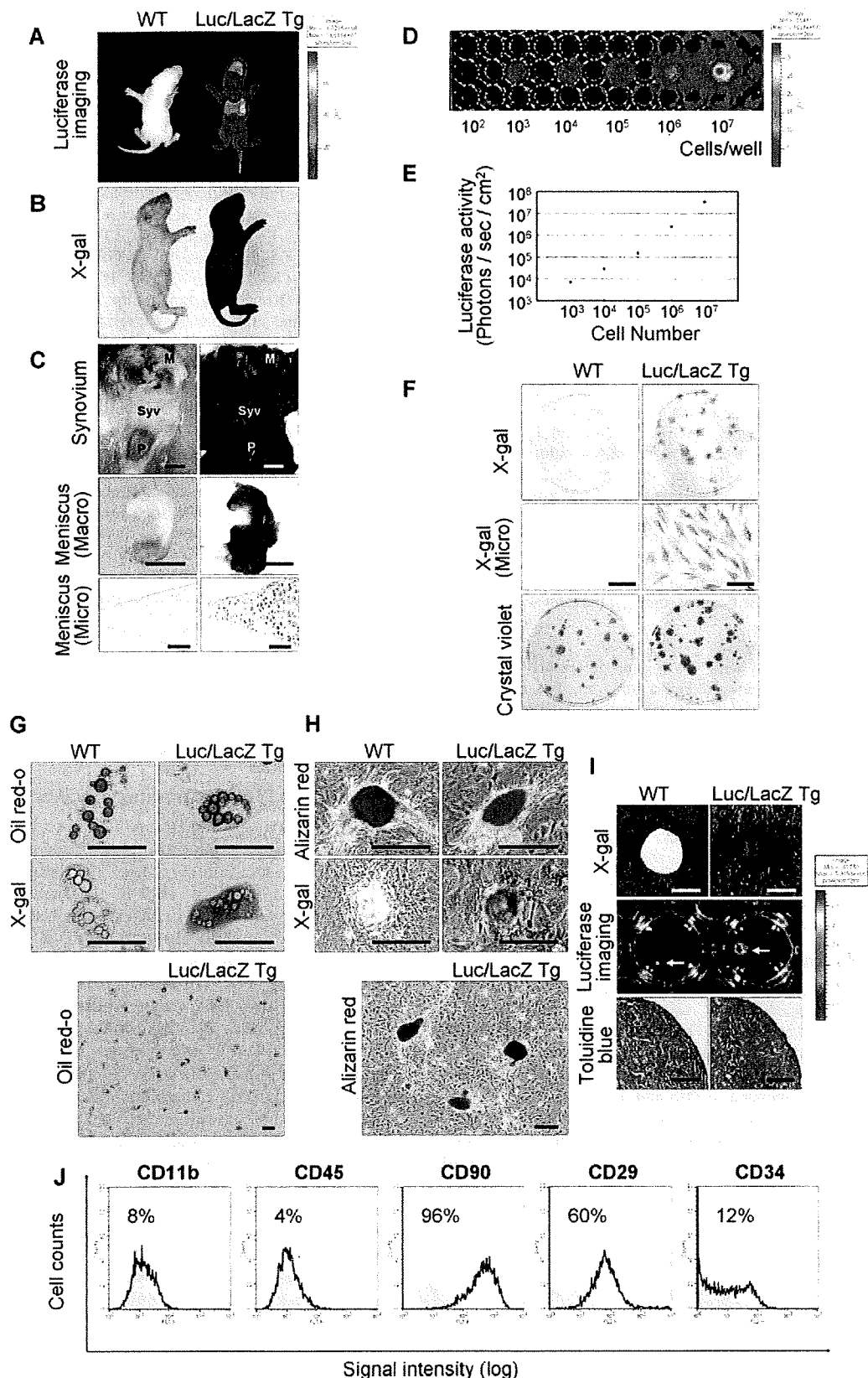


Figure 1. Mesenchymal stem cells (MSCs) derived from the synovium of Luc/LacZ transgenic (Tg) rats expressing dual reporter genes. (A): Luminescent images of wild and Luc/LacZ Tg rats. (B): Wild and Luc/LacZ Tg rats stained with X-gal. (C): Knee synovium and meniscus of wild and Luc/LacZ Tg rats stained with X-gal. Scale bar in upper and middle: 2 mm. Scale bar in lower: 100 μ m. (D): Bioluminescent imaging of varying numbers of synovium-MSCs from Luc/LacZ Tg rats. (E): Quantification for bioluminescent imaging of varying numbers of synovium-MSCs. (F): Colony forming ability of MSCs from Luc/LacZ Tg rat synovium. X-gal positive colony forming cells 14 days after the plating of 100 cells in 60 cm² dishes (top). Microscopic appearances of X-gal positive spindle cells (middle). Scale bars: 50 μ m. Total colonies in the same dishes stained with crystal violet (bottom). (G): Adipogenesis. Scale bars: 25 μ m. (H): Calcification. Scale bars: 100 μ m. (I): Chondrogenesis. Cartilage pellets stained with X-gal (top). Scale bars: 500 μ m. Bioluminescent imaging of cartilage pellets (middle). Histological section stained with toluidine blue (bottom). Scale bar = 100 μ m. (J): Flow cytometric analysis of synovium-MSCs at passage 3. CD11b, CD45, CD90, CD29, and CD 34 expression are shown as an open plot and isotype control expression as a shaded plot. Abbreviations: M, meniscus; P, patella; Syv, synovium; WT, wild-type.

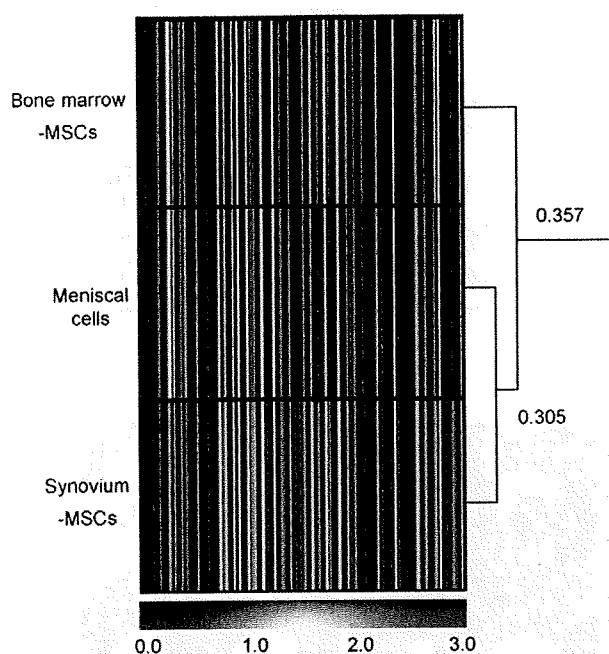


Figure 2. Dendrogram resulting from a hierarchically clustering analysis for gene profile of rat bone marrow-MSCs, synovium-MSCs, and meniscal cells. Gene expression was analyzed with the GeneChip Rat 230 2.0 probe arrays. Data of 14,882 probe sets were analyzed by applying a hierarchical tree algorithm to the normalized intensity. The color code for the signal strength in the classification scheme is shown in the box below. High-expression genes are indicated by shades of red and low expression genes are indicated by shades of blue. The dendrogram at the right provides a measure of the relatedness of gene expression profile in each sample (one minus the Pearson correlation). Abbreviation: MSCs, mesenchymal stem cells.

of the knee of wild rats (Fig. 3A), and Luc/LacZ⁺ synovium-MSCs suspension (5×10^6 in 50 μ l PBS) were injected into the right knee joint immediately after the incision was closed. For the control, the same volume of PBS was injected into the left knee. At 2 weeks, dark blue areas for LacZ were observed around the meniscal defect (Fig. 3B, yellow arrow) and sutured capsule (black arrow), indicating that injected synovium-MSCs intensively adhered to injured sites. Dark blue areas for LacZ were still observed around the meniscal defect and sutured capsule, but not observed in intact synovium, cartilage surface, or cruciate ligaments even at 12 weeks (data not shown). At 4 weeks, the anterior part of the meniscal defect of MSC injection side exhibited better regeneration than the control side (Fig. 3C).

All menisci for the experiment of synovium-MSCs are shown in Figure 3D. At 2 weeks, the regenerated part of the menisci appeared blue after X-gal staining in the MSC injection group, indicating that injected synovium-MSCs contributed to the repair. Square measures of the meniscus in synovium-MSCs injection groups were significantly larger than those in the control groups at 2, 4, and 8 weeks (Fig. 3E). We also evaluated cartilage and observed fibrillation, ones of the degenerative changes on the surface of the medial femoral condyle and medial tibial plateau in the control group at 12 weeks (Fig. 3F).

When we injected the same amount of bone marrow-MSCs, the meniscal defect was more rapidly regenerated than that on the control side at 4 weeks (Fig. 3D). Macroscopically, there were no remarkable differences between the synovium-MSC injection group and the bone marrow-MSC injection group.

Histologically, the contour of the regenerated menisci sharpened and the ultimate forms were closer to the normal

meniscal shape (Fig. 4A). Expression of type II collagen increased in a time-dependent manner. LacZ⁺ MSCs still existed at 12 weeks. In the control group at 12 weeks, regenerated tissue was occupied with less metachromasia, and type II collagen expression was hardly detected. In the bone marrow-MSC injection group, we observed similar features as seen in the synovium-MSC injection group (Fig. 4B).

Electron microscopic analysis of the regenerated meniscus 12 weeks after synovium-MSCs injection demonstrated that round cells with short processes were surrounded by a pericellular matrix, suggesting that meniscal cells were morphologically equivalent to those of the normal meniscus. In contrast, the cell feature in control groups remained to be fibroblastic (Fig. 4C).

Injected MSCs Stay in Knee Joint

The distribution of topically injected synovium-MSCs was evaluated using luciferase-based *in vivo* imaging. When MSCs were injected into the normal knee ($n = 4$), MSC-derived photons were detected around the right knee, to then decrease within 14 days. When injected into meniscectomized knee ($n = 7$), the photons increased in 3 days, then moderately decreased, but could be still observed for 28 days. Substantial luminescence light could not be detected in any other organs of either group (Fig. 5A). Sequential quantification demonstrated that luciferase activities were significantly higher in the meniscectomy group than those in the control group at each time point up to 21 days (Fig. 5B).

To further evaluate whether injected MSCs could migrate to distant organs or not, quantitative real-time PCR was performed. Total RNAs were isolated from the brain, lung, liver, spleen, kidney, and knee synovium of meniscectomized rat at 3 days after the synovium-MSC injection, and were subjected to *real-time PCR* to measure the level of LacZ expression. Injected MSC-derived LacZ gene could be detected only at the injected knee synovium and was not detected in any other organs (Fig. 6). These data confirm the *in vivo* imaging results and indicate that knee-injected MSCs stayed only in the knee joint.

DISCUSSION

A number of reports have previously described the injection of bone marrow-MSCs into the joint for meniscus injury; however, the kinetics and role of injected MSCs remain unknown. In a goat study, a previously removed medial meniscus regenerated 6 months after MSC injection [16]. However, Caplan et al. suggested in their review article that there were too few prelabeled cells to account for the massive regeneration of the meniscus and inferred that the MSCs trophically enhanced the regeneration of the meniscus [17]. In two other articles, cartilage matrix was present around injected bone marrow MSCs in only a small portion of the injured meniscus, but the roles of injected MSCs were not fully demonstrated [18, 19]. To refine our analysis, we created Tg rats expressing dual Luc/LacZ genes.

There are three main factors involved in the interaction between injected synovium-MSCs and meniscal defect: (a) an increase and decrease in the number of the cells, (b) an adherence to the meniscal defect, and (c) differentiation and maintenance.

In *in vivo* imaging analysis, synovium-MSCs injected into the meniscectomized knee transiently increased in number unlike the situation in which the same population of the cells was injected into the intact knee. This indicates that meniscus injury or incision of the articular capsule can produce some cytokines/chemokines to proliferate synovium-MSCs. In a rabbit model,

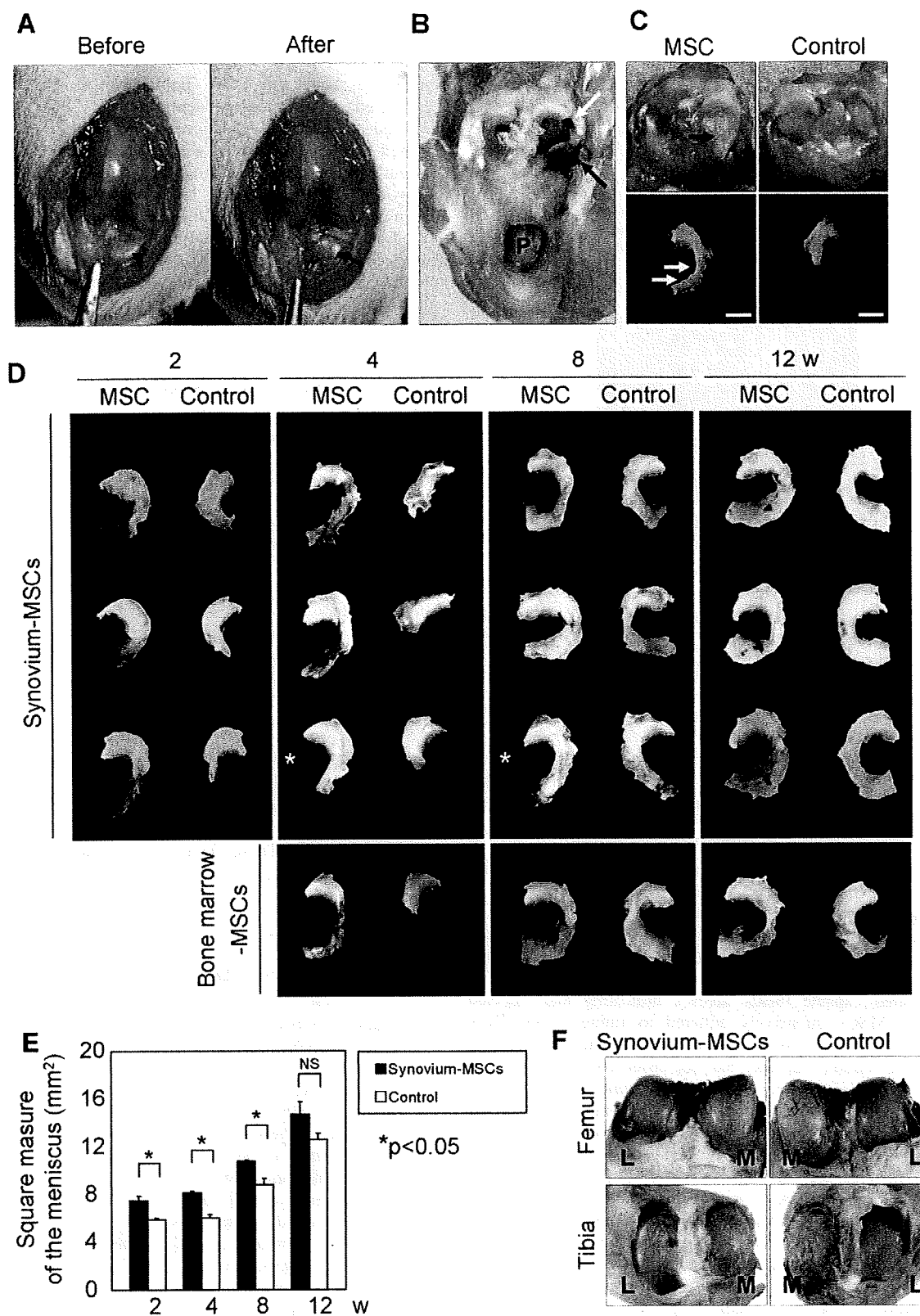


Figure 3. Macroscopic observation of meniscal regeneration after intra-articular injection of synovium-MSCs derived from Luc/LacZ transgenic (Tg) rats. (A): Surgical procedure for massive meniscectomy of a wild rat. The medial meniscus was dislocated anteriorly (left, arrowheads), the anterior half of the meniscus was excised, and tibial cartilage was exposed (right, arrow). (B): Macroscopic findings of the meniscectomized knee 2 weeks after the injection of synovium MSCs. The knee was stained with X-gal. LacZ positive areas were revealed around the meniscal defect (yellow) and sutured capsule (black). (C): Representative macroscopic findings of the tibial joint 4 weeks after synovium-MSCs injection. In the MSC injection group (left), the anterior part of meniscal defect has regenerated (arrow), whereas in the control group, the meniscal defect remains unchanged. Scale bar: 2 mm. (D): Macroscopic findings of the regenerated meniscus at 2, 4, 8, and 12 weeks. All menisci were stained with X-gal except those denoted by an asterisk. Representative macroscopic observation of meniscal regeneration after intra-articular injection of bone marrow-MSCs derived from Luc/LacZ Tg rats are shown in the lower part. (E): Sequential quantification for area of the meniscus. Values are averages with standard deviations ($n = 3$ for each group). $*p < .05$ between the synovium-MSC group and the control group at each period by Mann-Whitney U test. (F): Representative macroscopic findings of the joint surface of femur and tibia 12 weeks after the synovium-MSC group and the control group. The cartilage was stained with India ink. Abbreviations: L, lateral; M, medial; MSCs, mesenchymal stem cells; NS, not significant; P, patella.

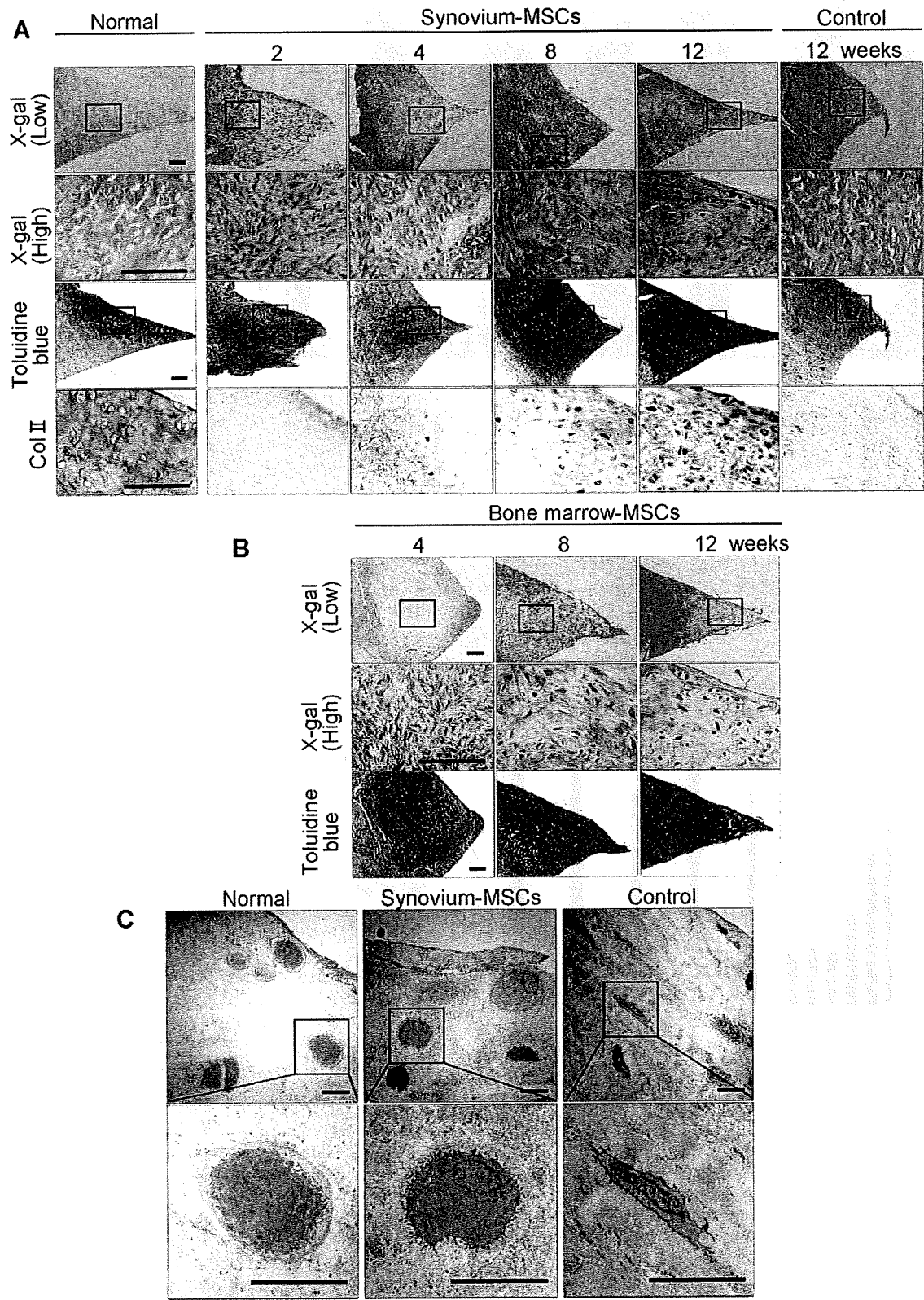


Figure 4. Histological observation of meniscal regeneration after the intra-articular injection of MSCs derived from Luc/LacZ transgenic rats. (A): Representative sections of normal meniscus and regenerated tissues in the synovium-MSC injection group stained with X-gal (and eosin as counter staining), toluidine blue, and immunostained with collagen type 2. Scale bar = 100 μ m. (B): Representative sections of regenerated tissues in the bone marrow-MSC injection group stained with X-gal (and eosin as counter staining), toluidine blue. Scale bar: 100 μ m. (C): Transmission electron microscopy imaging of typical cells in normal meniscus, regenerated part of meniscus both in the synovium-MSC injection group, and the control group at 12 weeks. Scale bar = 10 μ m. Abbreviation: MSCs, mesenchymal stem cells.

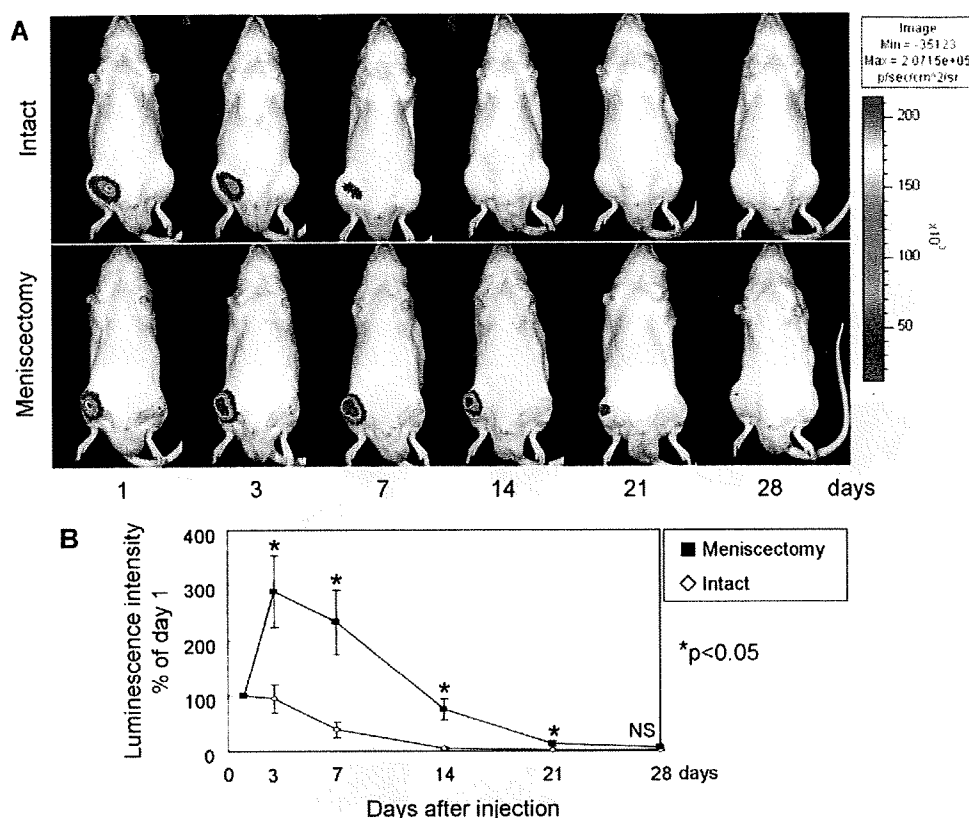


Figure 5. In vivo imaging analysis. (A): Imaging of photons from Luc⁺ cells. Five million synovium-mesenchymal stem cells (MSCs) derived from Luc/LacZ transgenic rats were injected into the intact knee or the meniscectomized knee. Luciferin was injected into the penile vein at indicated points to monitor luminescence driven by synovium-MSCs. (B): Sequential quantification of luminescence intensity. Average percentages of the value at 1 day are shown with standard deviations. **p* < .05 between meniscectomy group (*n* = 7) and intact group (*n* = 4) at each period by Mann-Whitney *U* test. Abbreviations: Max, maximum; Min, minimum; NS, not significant.

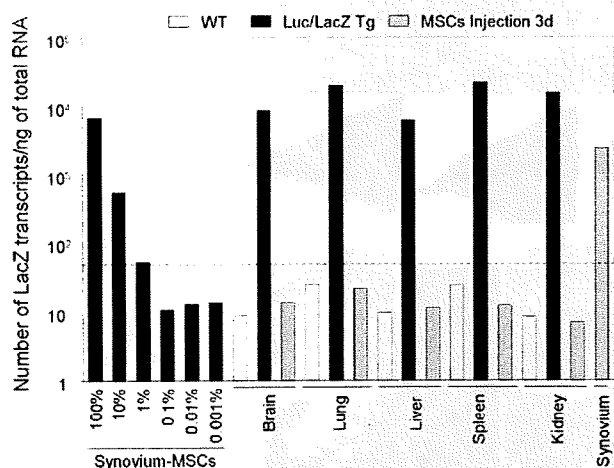


Figure 6. Real-time PCR analysis. The mRNA levels of LacZ obtained from expanded synovium-MSCs and various organs (brain, lung, liver, spleen, kidney, and knee synovium) were determined by SYBR green-based real-time quantitative RT-PCR. The copy number is expressed as the number of transcripts per nanogram of total RNA. The dashed line shows the minimum detection limit (50 copies per nanogram) which was determined by these dilution series. Abbreviations: MSCs, mesenchymal stem cells; Tg, transgenic; WT, wild-type.

meniscal lesions expressed TGF- β , interleukin-1 α , and platelet derived growth factor (PDGF) [20]. Human synovium-MSCs have PDGF receptor α and β , and neutralizing PDGF decreases the proliferation of synovium-MSCs in vitro [21]. PDGF may affect the number of transplanted synovium-MSCs.

We could detect MSC-derived photons in the knee joint up to 28 days after the injection but we could not detect it at a longer time point. We have two speculations about this

result. One is that a luciferase-based in vivo imaging system cannot detect a small number of Luc⁺ cells, as shown in Figure 1D. More than 1,000 cells are needed to detect the light emission. The other is due to blood circulation. The photons are produced only when luciferase is exposed to luciferin substrate and we injected luciferin intravenously. If the Luc⁺ cells had existed in the hypovascular area, they could not have been detected. However, in vivo imaging analysis has a great advantage in tracking cells in vivo because we did not have to sacrifice animals, and we could observe the same recipient throughout the observation periods.

Those synovium-MSCs which seem unnecessary for meniscal repair decreased in number and finally fell below measurable limits based on an in vivo imaging system. Possibly, synovium-MSCs participate in intra-articular tissue homeostasis and repair as do MSCs in mesenchymal tissues throughout the body. This phenomenon differs markedly from ES cells, which form teratomas in the mouse knee joint and subsequently destroy the joint [22]. Induced pluripotent stem cells may hold attraction for future applications, but teratoma formation cannot be overlooked [23].

In vivo imaging analysis suggests that the injected cells did not mobilize out of the injected joint. To confirm this result, we performed real-timePCR to detect Lac Z transcripts. We determined that LacZ transcripts were not detected in brain, lung, liver, spleen, or kidney 3 days after the injection, although the minimum detection limit was 50 copies per nanogram RNA which corresponded to 1% LacZ⁺ cells. Detection of LacZ⁺ cells in all sections through whole tissues will provide data from another point of view; however, it will be very arduous work.

Injected synovium-MSCs intensively adhered to injured sites of meniscus and synovium. We previously reported that injected synovium-MSCs efficiently adhered to the defect of

articular cartilage [24] and anterior cruciate ligament [6]. The mechanisms that guide the homing of injected cells are not well-understood, but stromal cell-derived factor-1 and monocyte chemotactic protein-1 are candidates to explain them [17].

Undifferentiated synovium-MSCs, attached around the meniscal defect, differentiate into meniscal cells. The environment of the meniscal defect is surrounded by femur and tibia cartilage, synovial tissue, the remaining meniscus, and synovial fluid. The space is also influenced by mechanical stress. This environment itself will provide sufficient signals to induce and maintain meniscal differentiation of synovium-MSCs. Similarly, undifferentiated synovium-MSCs implanted onto an articular cartilage defect differentiate into cartilage cells [25].

In our massive meniscectomized model, bone marrow-MSCs also promoted meniscus regeneration. There were no notable differences of regenerated meniscus in morphology between the synovium-MSC group and the bone marrow-MSC group, although gene profile of synovium-MSCs is closer to that of meniscal cells than that of bone marrow-MSCs. The situation seems to be similar in chondrogenesis as was the case in our previous studies. The gene profile of synovium-MSCs is closer to that of chondrocytes than that of bone marrow-MSCs [26]. Transplanted bone marrow-MSCs onto the cartilage defect differentiated into chondrocytes at a similar level to that synovium-MSCs which were transplanted in the same way [27]. In contrast, *in vivo* chondrogenic assay demonstrated that synovium-MSCs produced more cartilage matrix than bone marrow-MSCs [4, 5]. An *in vivo* model with more sensitivity may distinguish the difference.

Although reparative potential of synovium- and bone marrow-MSCs is similar, synovium-MSCs have an advantage in that they have a higher proliferation potential. In rats, the colony number per nucleated cell was approximately 1/100 in synovium, whereas it was 4/10⁵ in bone marrow. Rat synovium-MSCs expanded much faster than bone marrow-MSCs [5]. Also, human synovium-MSCs proliferated much faster than bone marrow-MSCs when cultured with autologous human serum [21].

For adipogenesis, it usually takes 3 weeks for human bone marrow MSCs to differentiate into adipocytes [10]. In this study, rat bone marrow- and synovium-MSCs differentiated into adipocytes in only 4 days, which was similar to our previous report [5]. Although the content of the adipogenic differentiation medium is similar, the duration to induce sufficient accumulation of lipid vesicles is totally different between MSCs in humans and rats. This indicates the species specificity of MSCs.

For calcification, we found that rat MSCs already calcified in 3 weeks, which was similar to our previous report [5]. In this study, we expected that the calcified area would increase after an additional 3 weeks; therefore, we observed calcification for a total of 6 weeks. Seemingly, the calcified area did not increase during the last 3 weeks (data not shown).

We previously created a 1 mm diameter cylindrical defect in the anterior part of medial meniscus in rats to examine the effect of synovium-MSCs injected intra-articularly. Contrary to our expectation, the cylindrical defect was filled spontaneously, and there was no effect on injected synovium-MSCs through 2-12 weeks [28]. To avoid spontaneous healing, we resected the anterior half of medial meniscus for this study. Meniscal size also increased in the control group, and the difference of meniscal size between the two groups disappeared at 12 weeks; however, the synovium-MSC injected groups showed better results from the standpoint of type II collagen expression and electron microscopic features.

For clinical application, interspecies differences have to be considered. The inherent healing capacity of the meniscus has been shown to be lacking in the inner third and is very limited in the middle third of this poorly vascularized tissue

in humans [29] and dogs [30]. We used a rat model, and rat meniscus had a greater spontaneous healing potential. To demonstrate the effectiveness of intra-articular injection of synovium-MSCs for meniscus regeneration, further experimental studies in larger animals are needed.

Native meniscus play an important role in knee stability and shock absorption [31], and this property is linked to the biphasic microstructure of the meniscus [32]. The extracellular matrix of the meniscus is composed mainly of collagen, with smaller quantities of proteoglycans, matrix glycoproteins, and elastin [33]. In this study, we showed that in the synovium-MSC injection group, the menisci regenerated much better than it did in the control group morphologically, and synovium-MSC injection prevented cartilage degeneration at 12 weeks after the meniscectomy as shown in Figure 3F. However, our data lack the details about biomechanical and biochemical properties of regenerated tissues, and it is still uncertain whether the regenerated menisci function in a normal manner and prevent secondary osteoarthritic change in the long term. Therefore, future studies should include biomechanical and biochemical analysis of the regenerated menisci.

Recently, we reported that human synovium-MSCs increased in synovial fluid after intra-articular ligament injury and that exogenous synovium-MSCs adhered to the injured ligament in a rabbit model [6, 34]. We also demonstrated that autologous synovial fluid enhanced migration of MSCs from synovium of osteoarthritis patients in a tissue culture system seemingly to delay the progression of the cartilage degeneration [7]. We speculate that synovial tissue may serve as a reservoir of stem cells that mobilize following intra-articular tissue injury and migrate to the site to participate in the repair response.

According to our speculation, in the case of meniscus injury, MSCs are mobilized from synovium into synovial fluid, and these cells adhere to the injured meniscus. However, the number of MSCs suspended in the synovial fluid and attached to the site is too low to repair or regenerate the injured meniscus, explaining poor spontaneous healing potential of meniscus. Intra-articular injection of synovium-MSCs can boost natural healing ability for meniscal regeneration.

Intravenous infusion of synovium-MSCs may be another route for administration. Although promising results were reported with *i.v.* infusion of bone marrow-MSCs in animal disease models [35], contrary views are also reported showing that a large fraction of intravenously infused bone marrow-MSCs are trapped in the lung [36]. Cell therapy for meniscal injury has an advantage in that intra-articular injection is possible instead of systemic injection.

The meniscal-deficient knee is a common problem faced by orthopedic surgeons. Although repair of meniscal lesions is possible in selected cases, the poor healing capacity of the tissue often dictates meniscectomy, the most common treatment for meniscal injury. Although meniscectomy provides pain relief and return to function, the loss of meniscal tissue results in long-term dysfunction and secondary osteoarthritis. Currently, there are multiple strategies for addressing this objective, including meniscal allografts, biologic scaffolds for tissue-engineered replacement tissue, and biologic stimuli for meniscal tissue regeneration. In this study, we focused on meniscal regeneration by using synovium-MSCs. Our method has a possibility of regenerating meniscus with less invasion in comparison with meniscal transplantation.

CONCLUSION

Synovium-MSCs injected into the massive meniscectomized knee adhered to the lesion, differentiated into meniscal cells

directly, and promoted meniscal regeneration without mobilization to distant organs.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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1. 高分子

4) 糖鎖による軟骨細胞機能制御の可能性

- 軟骨変性に伴う N- 結合型糖鎖の変化より -

岩崎倫政・松橋智弥・瓜田 淳
高畑雅彦・三浪明男・西村紳一郎

要 旨

軟骨組織変性を基本病態とする変形性関節症の発症早期より N- 結合型糖鎖に変化が生じた。この変化は、本疾患の発症病因である軟骨細胞のアポトーシスや細胞外マトリクス分解酵素の分泌に関与していると考えられる。更なる研究により、糖鎖による軟骨細胞の機能制御や関節疾患への関与などが明らかになっていくものと考えられる。これらが明らかになると、糖鎖生物学および糖鎖工学的アプローチによる新たな治療法の開発や、再生医療用マテリアルの開発が可能になるであろう。

キーワード

糖鎖, N- 結合型糖鎖, 軟骨, 軟骨細胞, 運動器組織, 変形性関節症

❖ はじめに

高齢化社会に突入した現代において、高齢者の運動機能障害は大きな社会問題となっている。運動機能障害をきたす運動器疾患のうち最も頻度の高いものは変形性関節症（osteoarthritis：OA）をはじめとする関節疾患である。2006 年の WHO の報告では、日本を含む西側先進諸国における OA の発症頻度は 65 歳以上の全人口の 75% に達し、そのうちの 85% の患者に何らかの日常生活動作の制限を認めるとしている。さらに、この報告では各国の経済活動に及ぼす OA の影響についても言及しており、労働制限の原因疾患として、OA は循環器系疾患に次ぐものであり、各国の GNP の約 1～2.5% の損失原因になるとしている。しかし、その病因については、いまだ十分には解明されてはおらず、決定的な治療法もないのが現状である。OA をはじめとする関節疾患の病因を解明し、その発症および進行を予防する研究を行うことは、医学的見地からのみならず社会・経済的側面からも極めて重要である。

OA をはじめとする多くの関節疾患において治療ターゲットとなる関節軟骨は、血管および神経組織

をもたず、少数の軟骨細胞と豊富な細胞外マトリクス（extracellular matrix：ECM）成分により構成される極めてユニークな組織である。したがって、軟骨組織の恒常性維持には、軟骨細胞-ECM 間の相互作用（interaction）が極めて重要であると考えられている。この相互作用を理解することが、OA をはじめとする関節疾患の病因を解明することにつながり、さらには軟骨細胞の機能調節機構の解明にも結びつくと予想される。

糖鎖科学はポストゲノム時代の新たな生物科学として、近年その重要性が非常に高まっている。主要な糖鎖遺伝子のクローニングの成果を基盤として、様々な生物学的局面における糖鎖のもつ多彩な機能が明らかになり、疾患との直接的な関連性も証明されてきた¹⁾²⁾。なかでも、細胞表面に局在する糖鎖は「細胞の顔」として、接着、細胞マーカー、シグナル伝達など重要な生理機能を有する。われわれは糖鎖がもつこのような多彩な機能に注目し、軟骨細胞の恒常性維持には糖鎖が重要な役割を果たしているだろうとの仮説のもと、軟骨組織の糖鎖解析と軟骨変性に伴う糖鎖構造の変化を明らかにしてきた。本稿において、その成果を紹介し

表③のような細胞への刺激応答の制御の知見をもとに、前駆細胞、細胞の足場となる多孔質材料、細胞の増殖・分化を促す細胞増殖因子などのシグナル因子をともに移植して体内での組織再構築を図っている。しかしながら、足場材料には細胞との親和性や適切な形状・強度、また正常組織に置換するための生体内分解性など制御すべき多くの性質がある。シグナル因子に関しても、そのままの投与では生体内半減期が短く（数分～数時間）、効果が限定的であり、細胞に適切な濃度・時間で作用させるための技術が必要とされている。

これらの問題に対するタンパク質を用いた足場材料やシグナル因子担体の設計は、材料の細胞に対する親和性の高さおよび分解産物の安全性などから多くの検討が行われている。各論は他稿に詳しいが、われわれのグループでも、I型コラーゲンスポンジを用いた骨、皮膚、毛、歯周・脂肪組織の再生誘導やスポンジの力学補強による再生効率の向上、ゼラチンハイドロゲルを用いたシグナル因子の徐放とそれによる種々の組織の再生誘導および複数の因子の徐放による効果を報告している^{6)~8)}。また、細胞増殖因子の徐放が可能なゼラチンからなるスポンジを作製し、細胞足場とシグナル因子徐放化の両方の機能をもつECMに近い次世代足場を開発している⁹⁾。これらの研究は、タンパク質を用いた足場材料設計は細胞周辺環境として有用であることを示している。加えて、フィブリンを用いた足場材料と細胞増殖因子の複合化や細胞増殖因子を固定したコラーゲンスポンジを用いた血管新生の促進などの報告もある^{5) 10) 11)}。

V. 今後の展望

これまで述べてきたように、タンパク質は遺伝子で蓄積された情報を的確に発現して機能を果たすことで、生体内で重要な役割を担っている。さらに、タンパク質などからなるECMおよびシグナル因子による細胞周辺への構造構築と表面への適切な呈示が細胞の種々の応答に対して大きな役割をもつことも明らかとなっている。また、周囲の環境による細胞応答の制御に関しては、ここ数年のうちにさらに様々な知見が報告されている。Zhangら¹²⁾、Calviら¹³⁾は多分化能をもつ幹細胞と呼ばれる細胞が体内に存在し、体内でニッチ(niche)と呼ばれる微小環境にとどまることでその性質を維持していることを報告している。加えて、培養表面に関する細胞応答に関しても、力学強度¹⁴⁾や接着面積¹⁵⁾、ゲル内での培養による三次元化¹⁶⁾などによって、増殖や分化状態が変化することも近年報告されている。このような報告はすべて生体内と同様の環境を細胞に提供した場合に適切な分化挙動が得られるといったものであり、これらの知見を生かして人工ECMとして細胞応答を制御できる材料をいかに作製するかがわれわれに求められている。また今後、更なる生物医学研究の進歩によって、ECMタンパク質、シグナル因子、受容体タンパク質との詳細な相互作用の解明が期待される。それらの知見をもとに新しい人工タンパク質をデザインする、あるいは細胞の周辺環境を構築するといったこともわれわれには必要となってくるであろう。

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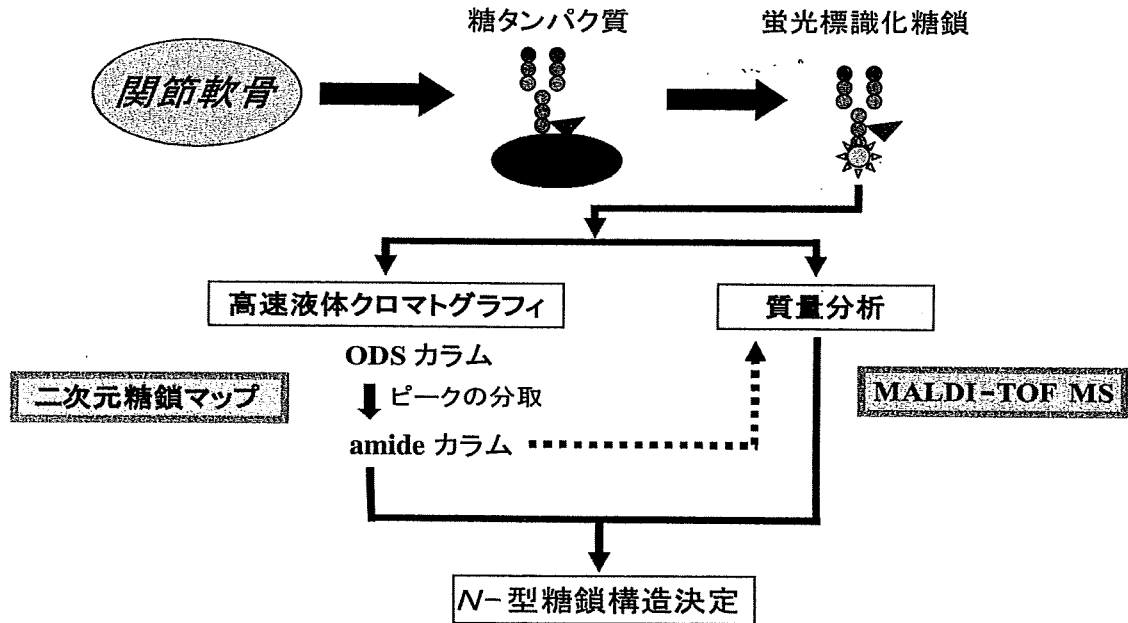
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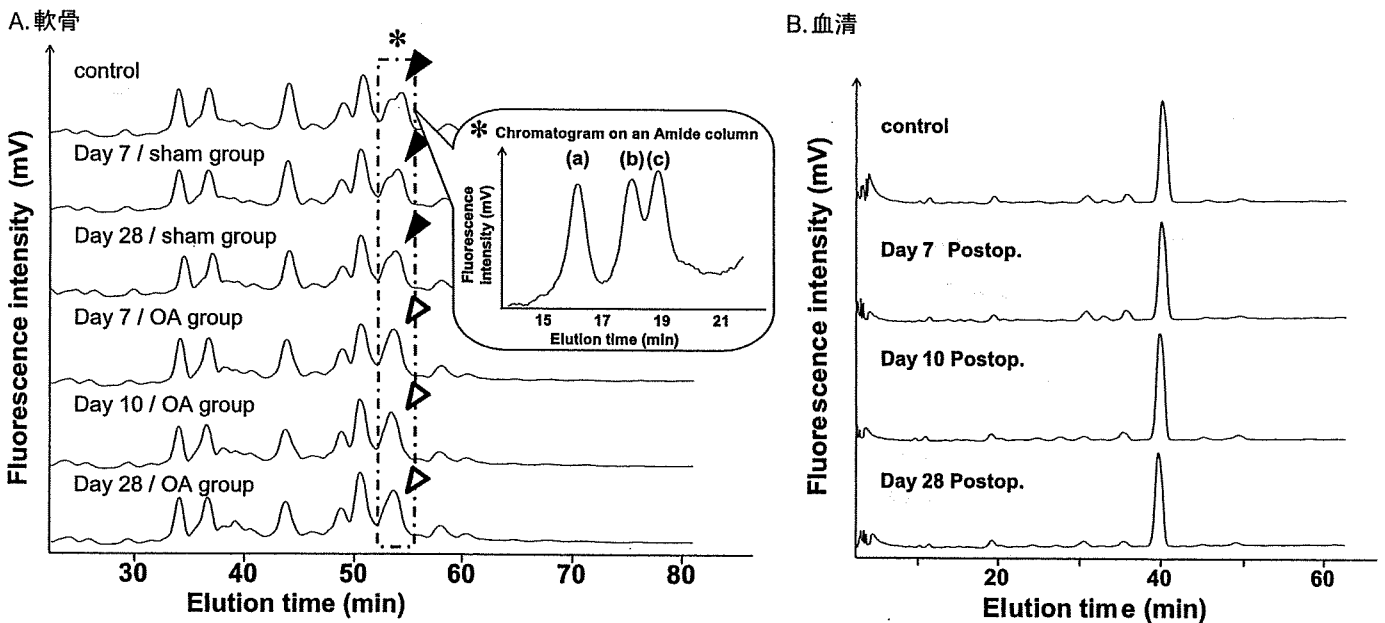
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図① 軟骨組織中の N- 結合型糖鎖解析方法



図② 軟骨および血清中の糖鎖構造解析結果 (文献 8 より)



A. 術後 7 日目より OA グループにおいて軟骨中の N- 結合型糖鎖構造の変化を示すピークパターンの変化を認める。
B. 血清中の N- 結合型糖鎖構造には変化を認めない。

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つの複合型 N- 結合型糖鎖が同定された。1 つは 1A1-210.4b (図③ A) であり、これは OA 軟骨中で発現が増加していた。一方、210.41b (図③ B) は OA 組織中では発現が減少していた。なお、血清中の N- 結合型糖鎖は正常と OA 軟骨間に有意な変化は認めなかった (図② B)。

3. 軟骨組織中の N- 結合型糖鎖の機能

上述したように軟骨組織の OA 変化の極めて早期より N- 結合型糖鎖に変化が生じていることが明らかとなった。一般に OA の発症初期には、その発症誘因となる何らかの刺激に軟骨細胞が反応し、アポトーシスや ECM 分解酵素の分泌が生じるとされている。これらが引き金となり、ECM の分解が促進され、軟骨組織の変性が生じていくとされる⁹⁾⁻¹¹⁾。したがって、今

糖鎖がもつ生体内での軟骨細胞の機能調節について言及したい。

I. N-結合型糖鎖

糖鎖は、グルコースやガラクトースなどの糖が鎖状に連なった物質で、細胞表面やタンパク質（糖タンパク質）、脂質（糖脂質）の先端に結合し存在する。動物が作り出す糖タンパク質は大きく2つに大別される。アスパラギン（N）残基の酸アミドに結合するN-結合型糖鎖と、セリンあるいはスレオニン残基の水酸基に結合するO-結合型糖鎖である。このうち、N-結合型糖鎖は見かけ上著しい多様性があり、主にマンノースとN-アセチルグルコサミンからなる高マンノース型、ガラクトース、シアル酸、フコースが結合した複合型、さらには混成型、哺乳動物にはこれまで見出されていないキシロース含有型のサブグループがある（表1）³⁾。われわれは、軟骨組織の糖鎖の中で、このN-結合型糖鎖をターゲットにした解析を行ってきた。

1. 軟骨組織中のN-結合型糖鎖解析（図1）

生後15週齢の成熟日本白色家兎の膝正常軟骨組織および前十字靱帯切断により生じさせたOA（変性）

軟骨を経時的に採取し、解析を行った。採取した軟骨片をタンパク質消化酵素のトリプシン、キモトリプシン、プロナーゼ、糖鎖遊離酵素のN-グリコシダーゼFにて処理してN-結合型糖鎖を切り出す。その切り出したN-結合型糖鎖をゲル濾過することにより精製する。精製したN-結合型糖鎖を2-アミノピリジンにてビリジルアミノ（PA）化して蛍光標識化し、それをゲル濾過にて精製する⁴⁾⁵⁾。そして、精製した蛍光標識化糖鎖を二次元高速液体クロマトグラフィー（HPLC）法にて解析する⁶⁾。具体的にはODSカラムにて疎水性分画して変化のあった糖鎖を分取し、それをamideカラムにて親水性分画する。それぞれの分析で得られた溶出時間をグルコース・ユニット値に変換し、データベース検索により候補となる糖鎖を絞り込む。最終的には、分取した糖鎖の質量分析による解析などを行うことにより糖鎖を同定する⁷⁾。

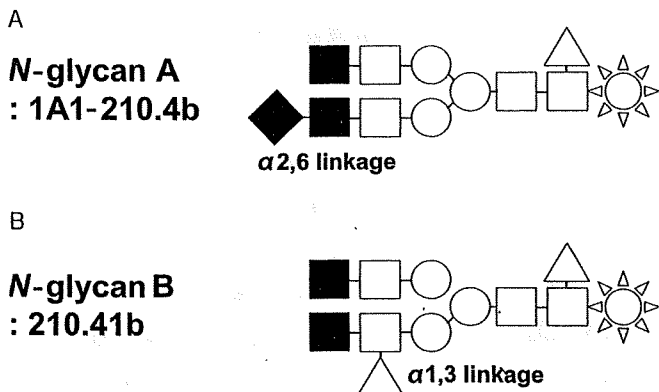
2. OA変化に伴う軟骨組織中の糖鎖変化⁸⁾

OA軟骨においては、明らかな組織学的変化が生じる以前の術後7日目よりN-結合型糖鎖構造の変化を示すピークパターンの変化が認められた（図2A）。この変化をさらに解析することにより、変化している2

表1 N-結合型糖鎖のサブグループと構造（文献3より）

サブグループ	糖鎖構造
複合型	
高マンノース型	
混成型	
キシロース含有型	

図③ 軟骨中で変化していた複合型 N- 結合型糖鎖 (文献 8 より)



A. OA 軟骨中で発現が増加していた 1A1-210.4b

B. OA 組織中では発現が減少していた 210.41b

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回同定した N- 結合型糖鎖は, OA 変化を生じさせる軟骨細胞の機能調節に関与していると推測される。現在, レクチン染色による N- 結合型糖鎖の局在, 軟骨細胞中の関連する糖鎖関連酵素遺伝子のノックダウンにより同定した糖鎖の機能解析を進めている。

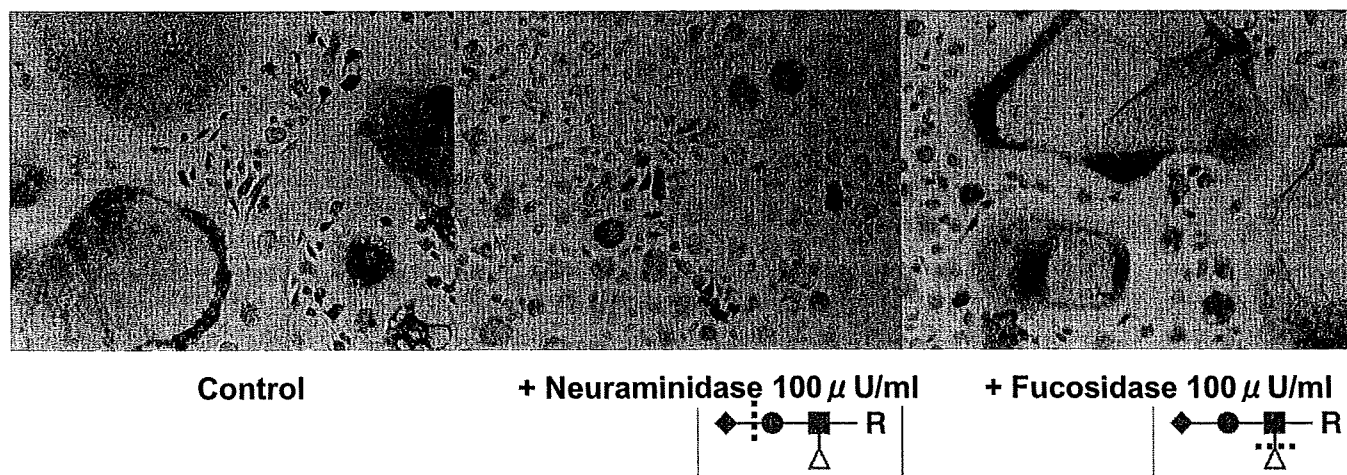
II. 糖鎖による運動器組織細胞の機能制御

われわれはウサギ軟骨 OA (変性) モデルを用いて, 軟骨中の糖鎖が発症の極めて早期より変化し, 軟骨細胞のアポトーシスや ECM 分解酵素の分泌を促進して

いる可能性を示した。誌面の都合上, 詳細は記載しないが, マウス軟骨変性モデルを用いて, 軟骨変性に伴い軟骨細胞のある種の糖鎖関連酵素遺伝子の発現が変化し, それに関連する N- 結合型糖鎖にも変化が生じたことを明らかにした。さらに, われわれは同じ運動器組織細胞である破骨細胞に対するシアリル糖鎖の機能解析も行ってきた¹²⁾。

シアリ酸は糖鎖先端に存在するアミノ糖の一種であり, 様々な細胞表面に糖タンパクや糖脂質の一部として存在する。シアリ酸はその構造的多様性や結合様式によって細胞間認識や細胞接着などに関与すると考えられており, ウイルスと細胞の融合や受精, 赤血球の貪食などにおいても重要な役割を果たすことが報告されている。われわれは, このシアリ酸を含むシアリル糖鎖が骨の新陳代謝に関与する破骨細胞にも豊富に発現しており, その分化制御機構に関わる可能性があることを明らかにしてきた。血球系幹細胞を起源とするマクロファージ系細胞である破骨前駆細胞には, ガラクトースと $\alpha(2,3)$ 結合または $\alpha(2,6)$ 結合するシアリ酸がともに発現しており, これらは RANKL 刺激後に発現が増加し, 細胞膜へ集積する傾向がみられた。さらに, 分化の最終段階である細胞融合した多核成熟破骨細胞では, $\alpha(2,6)$ 結合シアリ酸の発現が減少することがわかった。この細胞表面に発現するシアリ酸を外因性シアリダーゼで除去すると破骨前駆細胞は TRAP 陽性単核細胞までは分化したが, 多核巨細胞を形成できないことがわかった (図④)。また, シアリ酸転移酵素の 1 つである β galactoside $\alpha(2,6)$ sialyltransferase-I

図④ 骨髓マクロファージから M-CSF と RANKL を用いた破骨細胞分化系における細胞表面糖鎖除去による影響 (文献 12 より)



コントロールやフコシダーゼ (フコース切断酵素) で処理した細胞では, 酒石酸抵抗性酸ホスファターゼ陽性の多核巨細胞 (成熟破骨細胞) が形成されているが, ノイラミニダーゼ (シアリ酸切断酵素) で処理した細胞は, 多核巨細胞の形成が阻害されている。 (× 200 倍)

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(ST6Gal-I) 遺伝子の発現をノックダウンした細胞でも同様に多核破骨細胞の形成が阻害されたことから、シアリル糖鎖が破骨細胞の分化、特に細胞融合過程において重要な役割を果たしている可能性が示唆された。

このように、糖鎖は生体内において軟骨や骨などの運動器組織細胞の機能制御を担っている可能性があり、運動器疾患の発症にも大いに関与していることが予想される。

❖ おわりに

今後は、軟骨細胞レベルでの N- 結合型糖鎖解析およびその機能の解明を進め、最終的には糖鎖関連酵素

遺伝子の組織特異的遺伝子破壊動物を作製し、生体内での軟骨組織に対する糖鎖機能の解明を行っていく予定である。糖鎖生物学のアプローチを用いた OA の病因解明に関する研究は、これまでほとんど行われていない。OA 研究に糖鎖生物学を融合させることにより、今まで明らかにされなかった新たな知見が得られ、予防法や治療法の開発につながっていくと考えられる。さらに、糖鎖を基盤マテリアルに導入することにより、軟骨細胞の接着性や増殖性の制御が可能な機能性 scaffold マテリアルが開発され、軟骨再生医療への応用も可能になると考える。

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The Use of Laser Capture Microdissection on Adult Human Articular Cartilage for Gene Expression Analysis

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Abstract

The integrity of articular cartilage is maintained by chondrocytes, the sole type of cell that resides within the tissue. The non-calcified region of articular cartilage can be divided into three zones based on histological features, in which the chondrocyte metabolism is known to differ obviously among the zones. In pathological cartilage, the chondrocyte metabolism may change dramatically, which could play a pivotal role in the progression of the disease. Since such change in metabolism differs obviously from site to site within cartilage, it is crucial to determine the chondrocyte metabolism in respective regions. To this end, we have employed laser capture microdissection (LCM) to analyze chondrocyte metabolism in various regions of pathological and control cartilage. In this report, we describe our protocol for LCM on adult human cartilage tissue. With this protocol, a specific site of cartilage tissue was successfully obtained by LCM for gene expression analysis.

Key Words: Laser capture microdissection; articular cartilage; mRNA; osteoarthritis; chondrocyte; metabolic change; regional difference.

1. Introduction

Articular cartilage is a thin layer of connective tissue which covers bone surfaces within synovial joints. It is a unique tissue for the capacity to withstand repetitive loads, allowing frictionless motion of the joint. Articular cartilage is primarily composed of water and extracellular matrix, with a small volume of cells. The matrix of articular cartilage is composed of several types of collagen and proteoglycans (1). Chondrocytes are the only kind of cell that resides within cartilage, and since no other types of cells are seen in cartilage, chondrocytes are considered to be entirely responsible for the integrity and turnover of cartilage matrix.

Based on histological features, articular cartilage can be divided into four layers of superficial (tangential), middle (transitional), deep (radial) zones and calcified cartilage underneath (1-3) (Figure 1). Among them, calcified cartilage is unique in that the matrix contains an abundance of calcium apatite crystals and is clearly distinguished from the above three zones by a tide mark. In the three non-calcified zones, the metabolic activity of chondrocytes is known to differ considerably among the zones (1, 2, 4-6).

Osteoarthritis (OA) is a disease that primarily affects articular cartilage. In OA, the cartilage matrix is lost gradually over years, eventually devastating functional joints. OA is an age-related disease. With the aging of society, OA has become the most prevalent joint disease in developed countries (7). OA of a knee joint is particularly an issue, in view of its prevalence, severity of symptoms, and association with disability (8, 9).

Focal loss of articular cartilage is a unique feature in the pathology in OA. In OA joints, cartilage is most severely degenerated in weight-bearing areas, but may remain almost intact in non-weight-bearing areas. Reflecting such regional differences, the chondrocyte metabolism differs considerably from site to site within cartilage. Since chondrocytes become catabolic and promote cartilage degeneration in OA, it is critically important to determine chondrocyte metabolism at respective sites within OA cartilage. Conventionally, such regional differences can be known only by histological methods, such as immunohistochemistry or in situ hybridization.

Laser capture microdissection (LCM) is an innovative technology that enables acquisition of specific sites of the tissues based on histological features. We have employed this method and successfully analyzed gene expression levels of chondrocytes in respective regions of OA and control cartilage. In this section, we describe in detail our LCM protocol for adult human articular cartilage tissue. The use of LCM on cartilage