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Repetitive allogeneic intraarticular injections of synovial mesenchymal stem cells promote meniscus regeneration in a porcine massive meniscus defect model

D. Hatsushika †, T. Muneta †, T. Nakamura †, M. Horie ‡, H. Koga †, Y. Nakagawa †, K. Tsuji §, S. Hishikawa ||, E. Kobayashi ||, I. Sekiya ‡*

† Department of Joint Surgery and Sports Medicine, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan
‡ Center for Stem Cell and Regenerative Medicine, Tokyo Medical and Dental University, Tokyo, Japan
§ Department of Cartilage Regeneration, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan
|| Center for Development of Advanced Medical Technology, Jichi Medical University, Tochigi, Japan

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SUMMARY

Objective: A new strategy is required in order to regenerate a meniscus for extensive defects. Synovial mesenchymal stem cells (MSCs) are an attractive cell source for meniscus regeneration due to their high proliferation and chondrogenic potential. We examined the effect of repetitive intraarticular injections of synovial MSCs on meniscus regeneration in a massive meniscal defect of pigs. We followed up the efficacy using MRI evaluation in addition to macroscopic and histological observations.

Design: Two weeks before the injection of synovial MSCs, the anterior half of the medial menisci was resected in both knees of pigs. Fifty million allogeneic synovial MSCs were injected into the right knee at 0, 2, and 4 weeks and followed up by sequential MRI. The regenerated meniscus, adjacent articular cartilage, and subchondral bone were evaluated by MRI at 2, 4, 8, 12 and 16 weeks. They were also evaluated macroscopically and histologically at 16 weeks ($n = 7$).

Results: The resected meniscus regenerated significantly better in the MSC group than in the control group based on histological and MRI analyses. Macroscopically, the meniscal defect already appeared to be filled with synovial tissue at 2 weeks. Articular cartilage and subchondral bone at the medial femoral condyle were also significantly more preserved in the MSC group based on MRI, macroscopic, and histological analyses.

Conclusions: Intraarticular injections of allogeneic synovial MSCs appeared to promote meniscus regeneration and provide protection at the medial femoral articular cartilage in a porcine massive meniscal defect model.

Introduction

The meniscus is a wedge-shaped fibrocartilaginous structure and plays important roles in load distribution, shock-absorption, and knee joint stability. It has a poor healing potential due to its largely avascular nature, and loss of meniscal function leads to accelerated osteoarthritis[1]. For a massive meniscal defect in a clinical situation, the meniscal allograft is indicated, but it is comparatively invasive, and selecting the proper size of allograft is difficult[2,3]. A novel strategy for meniscus regeneration remains necessary.

It was already reported more than 70 years ago that meniscus lesions did not heal unless they communicated with the synovium and capsule[4]. However, the reparative potential of synovium for meniscus lesion is limited and therefore spontaneous repair of the injured meniscus cannot be expected in many cases. Synovial mesenchymal stem cells (MSCs) are an attractive cell source for articular cartilage and meniscus regeneration. In comparison with other tissue-derived MSCs, synovial MSCs have a high chondrogenic potential[5,6] and remarkable expansion ability with autologous human serum[7].

We previously reported that intraarticular injection of synovial MSCs promoted meniscus regeneration after the anterior half of the

* Address correspondence and reprint requests to: I. Sekiya, Center for Stem Cell and Regenerative Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Tel: 81-3-5803-4017; Fax: 81-3-5803-0170.
E-mail address: sekiya.arm@tmd.ac.jp (I. Sekiya).

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medial meniscus (MM) was resected in rats[8] and rabbits[9]. We hypothesized that this method would also be useful in bigger animals that are more closely related to humans. The purpose of this study was to examine the effect of repetitive intraarticular injections of allogeneic synovial MSCs on the regeneration of the meniscus in a massive meniscal defect model of pigs. We followed up on meniscus regeneration sequentially using MRI, and examined the results macroscopically and histologically at the endpoint. The articular cartilage and subchondral bone were also sequentially evaluated to examine the function of the regenerated meniscus. This study showed the effect of synovial MSCs on meniscus regeneration, and it may lead to the development of a new treatment for meniscus regeneration in clinical situations.

Materials and methods

Animals

All experiments were conducted in accordance with the institutional guidelines for the care and use of experimental animals at Tokyo Medical and Dental University and Jichi Medical University. Thirteen-month-old, skeletally mature Mexican hairless pigs (National Livestock Breeding Center, Ibaraki, Japan) were used. The average weight of these pigs was 32 kg. All pigs were bred under specific pathogen-free conditions and had free access during the study period to food and water in a post-operative care cage (400 cm wide, 1210 cm long and 1090 cm high). To remove donor variation, only one pig was used as a donor for transplantation of allogeneic synovial MSCs, and 10 pigs were used as recipients.

Cell isolation and culture

Synovial tissue was taken together with the underlying connective tissue from the suprapatellar pouch, which overlays the non-cartilaginous areas of the femur through an arthrotomy of the knee. Synovial tissue with the underlying connective tissue was digested in 3 mg/mL collagenase D solution (Roche Diagnostics, Mannheim, Germany) in α-minimal essential medium (α-MEM; Invitrogen, Carlsbad, CA) at 37°C for 3 h, filtered through a 70-μm nylon filter (BD Biosciences, Franklin Lakes, NJ). The nucleated cells were plated in 150-cm^2 culture dishes (Nunc, Rochester, NY) at 5×10^5 cells/dish in complete medium (α-MEM supplemented with 10% fetal bovine serum (FBS), 100 U/L penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B [all from Invitrogen]) in 150-cm^2 culture dishes (Nunc), then cultured for 14 days at 37°C, 5% CO_2 with saturated humidity. The medium was changed to remove non-adherent cells every 4–5 days. The adherent cells were harvested with 0.25% trypsin-EDTA (Invitrogen) and cryopreserved as passage 0. Aliquots of 2×10^6 cells in 2 mL of stock medium [α-MEM supplemented with 10% FBS and 5% dimethylsulfoxide (Wako, Osaka, Japan)] were frozen slowly in a Cryo 1°C freezing container (Nunc) and cryopreserved at −80°C. To expand the cells, a frozen vial of the cells was thawed, plated in 60-cm^2 culture dishes at 1×10^6 cells/dish, and cultured for 4 days as passage 1. Then the cells were re-plated in 150-cm^2 culture dishes at 5×10^5 cells/dish and cultured for 14 days as passage 2. Passage 2–4 cells were used for further studies. The number of total cell doublings was 12 at passage 0, 18 at passage 1, 25 at passage 2, 30 at passage 3, and 37 at passage 4.

Colony-forming unit assay

Five hundred cells 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 to observe cell colonies.

In vitro differentiation assay

For chondrogenesis, 2.5×10^5 cells were collected at the bottom of a 15-ml polypropylene tube (BD Biosciences) by centrifugation at 450 × g for 10 min. The pellets were cultured in chondrogenesis medium consisting of high-glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 1 μg/mL bone morphogenetic protein (BMP)-7 (provided from Stryker Biotech, Hopkinton, MA), 10 ng/mL transforming growth factor (TGF)-β3 (R&D Systems, Minneapolis, MN), 100 nM dexamethasone (Sigma–Aldrich, St Louis, MO), 50 μg/mL ascorbate-2-phosphate, 40 μg/mL proline, 100 μg/mL pyruvate and 1:100 diluted ITS + Premix (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL bovine serum albumin and 5.35 mg/mL linoleic acid; BD Biosciences). For microscopy, the pellets were embedded in paraffin and cut into 5 μm sections. They were stained with safranin-o fast green, and immunostained with type II collagen[10–12].

For adipogenesis, cells were cultured for 21 days in adipogenic medium consisting of complete medium supplemented with 100 nM dexamethasone, 0.5 mM isobutyl-methylxanthine (Sigma–Aldrich) and 50 μM indomethacin (Wako). The adipogenic cultures were fixed in 4% paraformaldehyde, then stained with fresh oil red-o solution[13].

For calcification, cells were cultured for 21 days in calcification medium consisting of complete medium supplemented with 1 nM dexamethasone, 20 mM β-glycerol phosphate (Wako) and 50 μg/mL ascorbate-2-phosphate (Sigma–Aldrich). The cells were fixed in 4% paraformaldehyde, then stained with 0.5% alizarin red solution[14].

Meniscectomy

Two weeks before the injection of synovial MSCs, a straight incision was made on the medial side of the knee, the anteromedial side of the joint capsule was cut, and the anterior horn of the MM was exposed under general anesthesia. Then the meniscus was dislocated anteriorly with a forceps, cut vertically at the level of the medial collateral ligament, and the anterior half of MM was excised in both knees.

Intraarticular injection of synovial MSCs

5×10^7 synovial MSCs were suspended in 1 mL of PBS and injected into the right knee three times at 2-week intervals (the first injection day was determined as day 0). The same volume of PBS was injected into the left knee as the control. For the injection, a 23-gauge needle was inserted at the center of the triangle formed by the medial side of the patellar ligament, the medial femoral condyle (MFC), and the medial tibial plateau, toward the intercondylar space of the femur. Immediately after the injection, the medial side of the injected knee was kept down for 10 min so that the injected synovial MSCs could be attached around the defect of the meniscus[15]. After waking up from anesthesia, the pigs were allowed to walk freely without fixation, and without the administration of immunosuppressive drugs to reduce the risk of infection.

Macroscopic examination and histological analysis

After 16 weeks, seven pigs were sacrificed by overdose intravenous injection of KCl under adequately deep general anesthesia. Samples such as MFCs were examined macroscopically for color, integrity, and smoothness, and evaluated using the International Cartilage Repair Society (ICRS) macroscopic score[16]. Specimens of

medial menisci and MFCs were fixed separately in 4% paraformaldehyde for 7 days, then dehydrated with a gradient ethanol series. In order to section finely, the specimens were decalcified with 20% EDTA (pH 7.4) at room temperature for 21 days. The specimens were embedded in paraffin and sectioned into slices 5 μm thick radially for the meniscus and sagittally for the condyles. The sections were stained with safranin-o fast green. To quantify the regeneration of the meniscus, a modified Pauli's scoring system was used[17] (Supplementary Table 1). To quantify the extent of articular cartilage degeneration, the OARSI Osteoarthritis Cartilage Histopathology Assessment scoring system was used[18]. These scores were evaluated by two observers in a blinded manner.

Immunohistochemistry

For type II collagen, after sequential pretreatments with proteinase K, hydrogen peroxidase, and gout serum, sections were covered with rabbit anti-human type II collagen antibody (1:1000 dilution; Abcam, Cambridge, UK) and incubated at 4°C overnight. After extensive washing with PBS, the sections were reacted with the secondary antibody of biotinylated goat anti-rabbit IgG (1:200 in dilution; Vector Laboratories, Burlingame, CA) for 30 min at room temperature. For type I collagen, sections were covered with mouse anti-human type I collagen antibody (1:800 dilution; Abcam) and incubated at 4°C overnight. After washing, the sections were reacted with the secondary antibody of biotinylated horse anti-mouse IgG (1:200 in dilution; Vector Laboratories) for 30 min at room temperature. Immunostaining was detected with the Vectastain ABC reagent (Vector Laboratories) followed by diaminobenzidine staining. The sections were counterstained with hematoxylin.

Magnetic resonance imaging evaluation

MRI analysis was performed before meniscectomy, 3 h, 2 weeks, 4 weeks, 8 weeks, 12 weeks and 16 weeks after the first injection of synovial MSCs. An MRI system at 1.5 T (Magnetom Essenza, Siemens Healthcare Sector, Erlangen, Germany) was used with a 4-Channel Special-Purpose Coil (Siemens). The coil was positioned in the medial side of the knees in a standardized fashion using the MFCs as a landmark. The regenerated meniscus and articular cartilage were evaluated a using a fast-spin echo proton density sequence, T1, and quantitative T2 mapping by sagittal plane.

For quantification of MRI analysis for the regenerated meniscus, the mean T2 value at the center of the regenerated region in the T2 mapping images was calculated with Image J software (version 1.43, National Institutes of Health, Bethesda, MD). For quantification of MRI analysis for the MFC, a new scoring system, the Whole-Organ Magnetic Resonance Imaging Score (WORMS), which we modified, was used[19] (Supplementary Table 2). The score was evaluated by two investigators blinded to treatment category. The inter observer variability was examined with interclass correlation coefficients (ICC) by IBM SPSS statistics 20. The ICC between the two measures was 0.995.

Statistical methods

The StatView 5.0 program (SAS Institute, Cary, NC) was used for statistical analyses. Comparisons between the MSC group and the control group were analyzed using the Wilcoxon singled-ranks test. A *P*-value of <0.05 was considered statistically significant.

Results

Properties of synovial MSCs

Fourteen days after 500 synovial cells were cultured in three dishes of 60-cm^2, 130, 134, and 137 colonies were observed [Fig. 1(A)]. The pellet of the colony-forming cells differentiated into

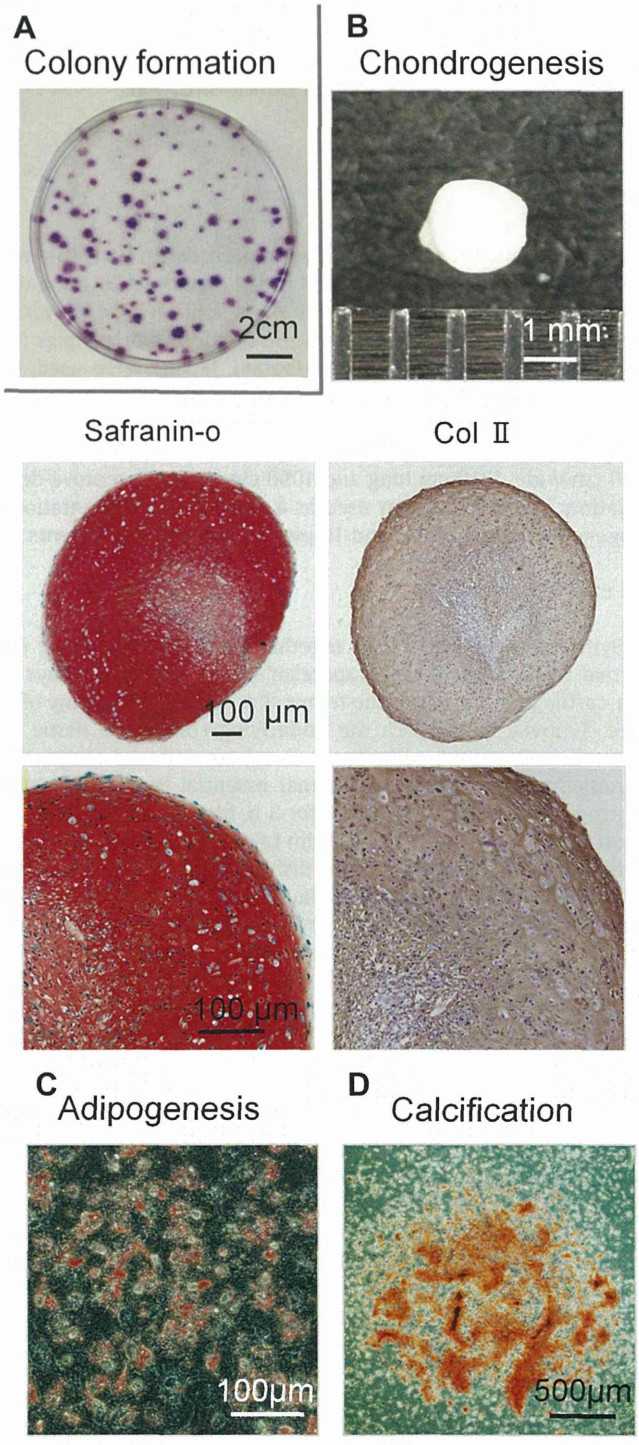

Fig. 1. Representative images for colony formation and multi differentiation of synovial MSCs. (A) Colony formation stained with crystal violet. (B) Macroscopic and histological images for chondrogenesis. (C) Adipogenesis stained with oil red-o. (D) Calcification stained with alizarin red.

cartilage, where the matrix appeared red with safranin-o staining, indicating the existence of proteoglycans, and was positive for type II collagen [Fig. 1(B)]. The colony-forming cells differentiated into adipocytes stained with oil red-o [Fig. 1(C)] and calcified stained with alizarin red [Fig. 1(D)].

Synovial MSCs promote meniscus regeneration

For analysis of meniscus regeneration, meniscectomy was performed 2 weeks before transplantation of allogeneic synovial MSCs; then they were injected three times at 0 weeks, 2 weeks, and 4 weeks. The regenerated meniscus was harvested 16 weeks after the first injection [Fig. 2(A) and (B)].

Macroscopically, the anterior half of the MM, where the native meniscus was previously removed, appeared to regenerate both in the MSC and control groups [Fig. 3(A)]. Histologically, the regenerated meniscus in the MSC group was better stained with safranin-o than that in the control group in all seven pigs [Fig. 3(B)]. Representative sections for type I and II collagen indicated that the positive area for each of them was larger in the regenerated meniscus transplanted with synovial MSCs than that in the regenerated meniscus without synovial MSCs. The positive area for type II collagen almost corresponded to the red area in the sections stained with safranin-o in regenerated menisci in the MSC group, in the control group, and the normal meniscus. The modified Pauli's histological score[17] (Supplementary Table 1) was significantly better in the MSC group than in the control group [Fig. 3(C)].

The meniscal defect was already filled with synovial tissue 2 weeks after injection, and the synovial tissue became smooth at 4 and 6 weeks [Fig. 4(A)], though the sequential macroscopic observation was performed only in the MSC group ($n = 1$). Sequential MRI analysis showed the process of meniscus regeneration in the MSC group [Fig. 4(B)]. The signal intensity in the resected area increased rapidly 3 h after meniscectomy, then decreased gradually over the course of time in both groups. The resected area appeared less disorganized in the MSC group than in the control group at 8, 12, and 16 weeks. Quantification analyses demonstrated that the T2 value in the regenerative meniscus was significantly lower than in the control group from 2 weeks through 16 weeks [Fig. 4(C)].

Injected MSCs prevent articular cartilage degeneration

Macroscopically, articular cartilage on the MFC appeared to be better preserved in the MSC side than in the control side in all seven pigs [Fig. 5(A)]. The ICRS score for macroscopic observation[16] was significantly better in the MSC group than in the control group. Histologically, cartilage and bone also appeared to be better preserved in the MSC group in each pig [Fig. 5(B)]. The OARSI score for histological observation[18] was significantly better in the MSC group than in the control group.

Based on sequential MRI observations of MFCs, the articular cartilage and subchondral bone lesions worsened in the control group over time [Fig. 5(C)]. Contrarily, in the MSC group, cartilage was better preserved at each time point. The MRI score

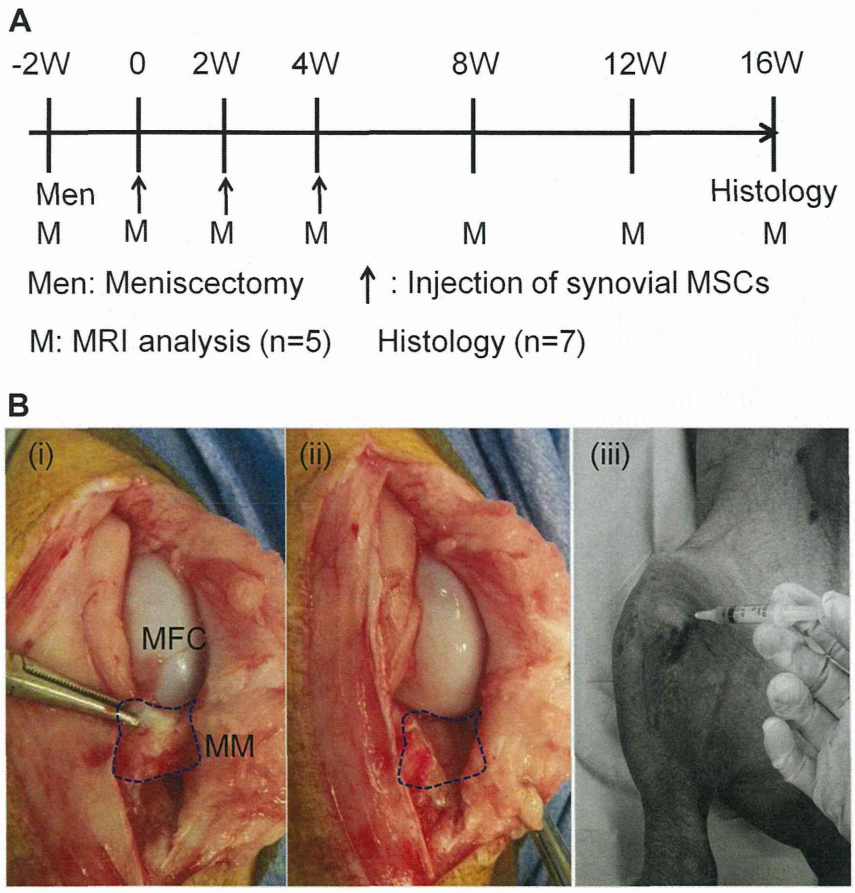

Fig. 2. Experimental set up. (A) Schematic drawing for meniscectomy, injection of MSCs, MRI, and histological analyses. (B) Meniscectomy and injection of MSCs. (i) Medial capsule was incised longitudinally to expose MM. (ii) Anterior half of the MM was removed. (iii) 2 weeks after the meniscectomy, suspension of synovial MSCs was injected into the knee joint. Area where the meniscus was originally located is indicated with dotted line.

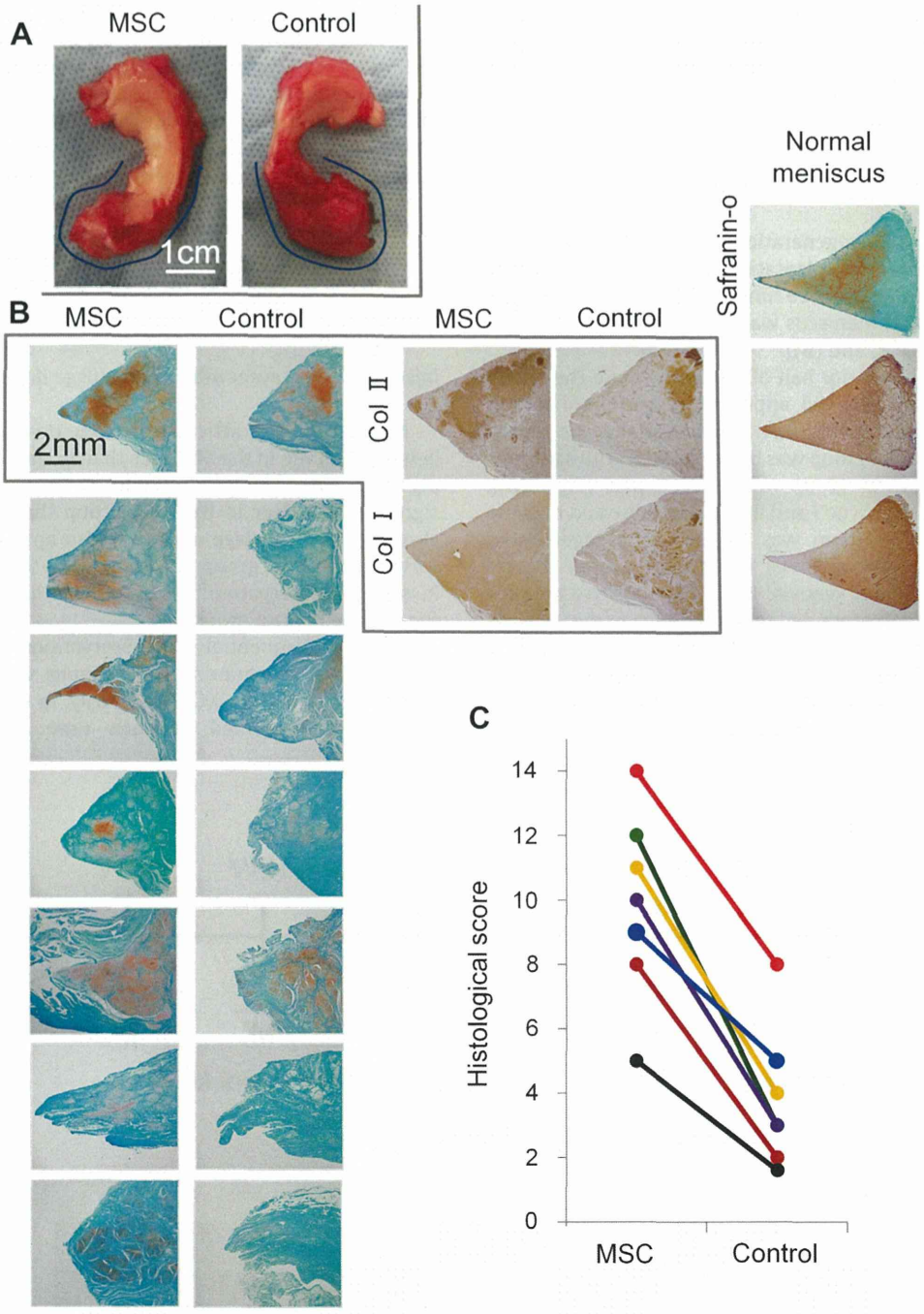

Fig. 3. Analyses of the regenerated meniscus. Meniscectomy was performed 2 weeks before, synovial MSCs were injected three times at 0, 2, and 4 weeks, then the regenerated meniscus was harvested 16 weeks after the first injection. (A) Macroscopic observation of the medial menisci harvested from the same pig. Regenerated area is indicated with a blue line. (B) Radial sectioned histologies for the normal and regenerated meniscus stained with safranin-o and immunostained with type I/II collagen. To remove individual variability, the regenerated menisci in the MSC and control groups of the same pig are shown in the same row. For type I/II collagen, representative sections were shown. The sections derived from the same pig were surrounded with a gray line. (C) Quantification of histology evaluated by modified Pauli's scoring system (Supplementary Table 1) ($n = 7$, $P < 0.05$ by Wilcoxon signed-ranks test).

(Supplementary Table 2) was significantly better in the MSC group than in the control group at 8 weeks and thereafter [Fig. 5(D)].

Discussion

The cells derived-from porcine synovium formed colonies, differentiated into chondrocytes and adipocytes, and were calcified when cultured in the appropriate differentiation medium, though they were not derived from a single cell. As MSCs are defined by adherence to culture dish, colony formation, and trilineage differentiation[20], our results showed that colony-forming cells derived from porcine synovium had characteristics of MSCs.

In our model, the MM was cut vertically at the level of the medial collateral ligament and the anterior half of MM was resected. Before we started this study, we had thought that the remaining part of the meniscus might be pushed out of the load-bearing area of the knee joint because destabilization of the medial meniscus (DMM) model, in which the MM ligament was

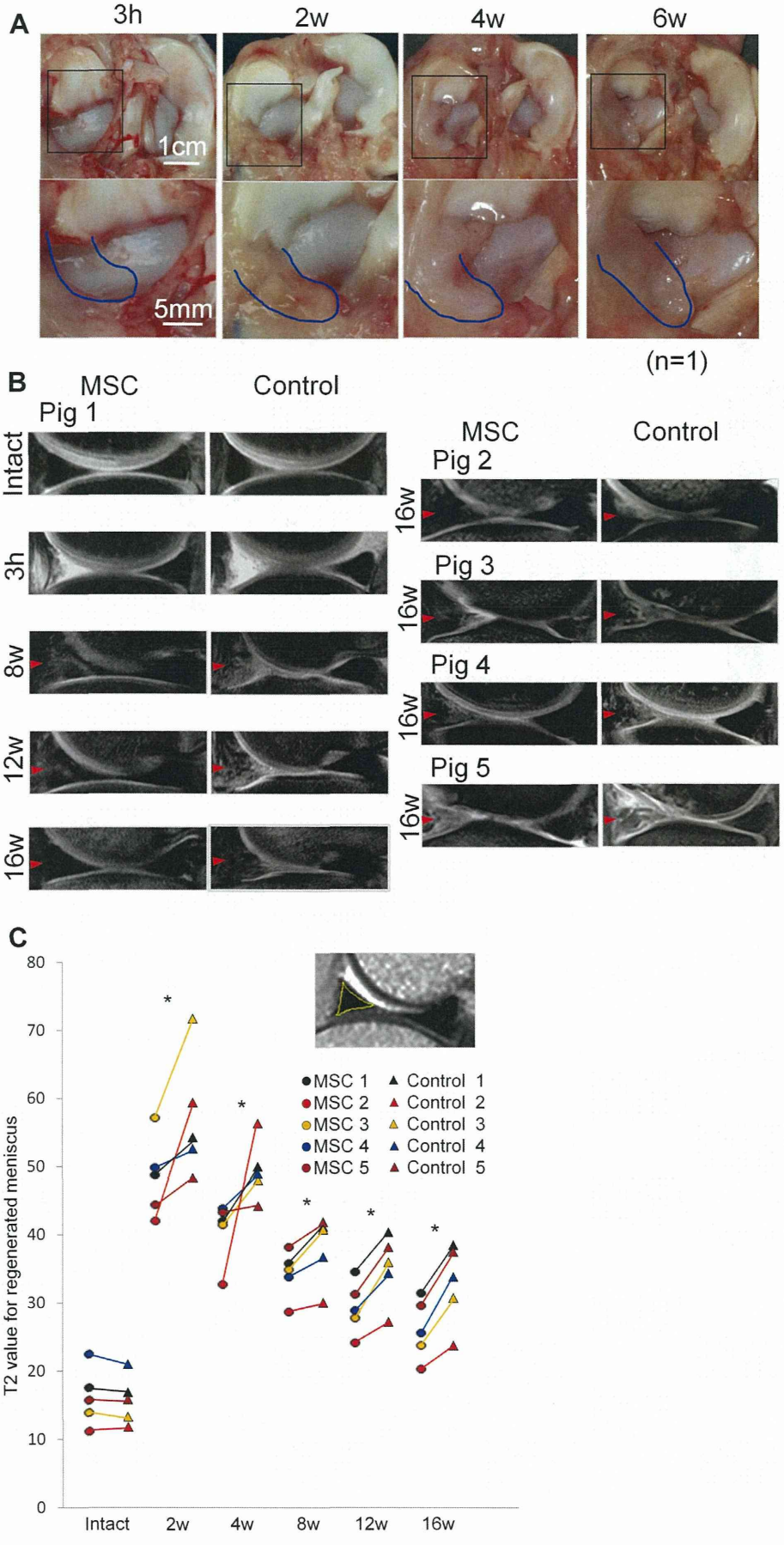

Fig. 4. Sequential observation for the regenerated meniscus. (A) Macroscopic images of the tibial side of the knee joint. Meniscectomy was performed 2 weeks beforehand, then synovial MSCs were injected only once. The bellower figures were indicated by the boxes in the upper figures. Area where the meniscus was removed is shown with a blue line. The pictures at 6 weeks were reversed horizontally to facilitate visualization. (B) Sequential sagittal MRI of fast-spin echo proton density sequence images at the center of the regenerated meniscus. Regenerated meniscus is indicated with a red arrow head. (C) Evaluation of T2 value for the regenerated meniscus ($n = 5$, $^*P < 0.05$ by Wilcoxon signed-ranks test). T2 image and evaluated area are also shown.

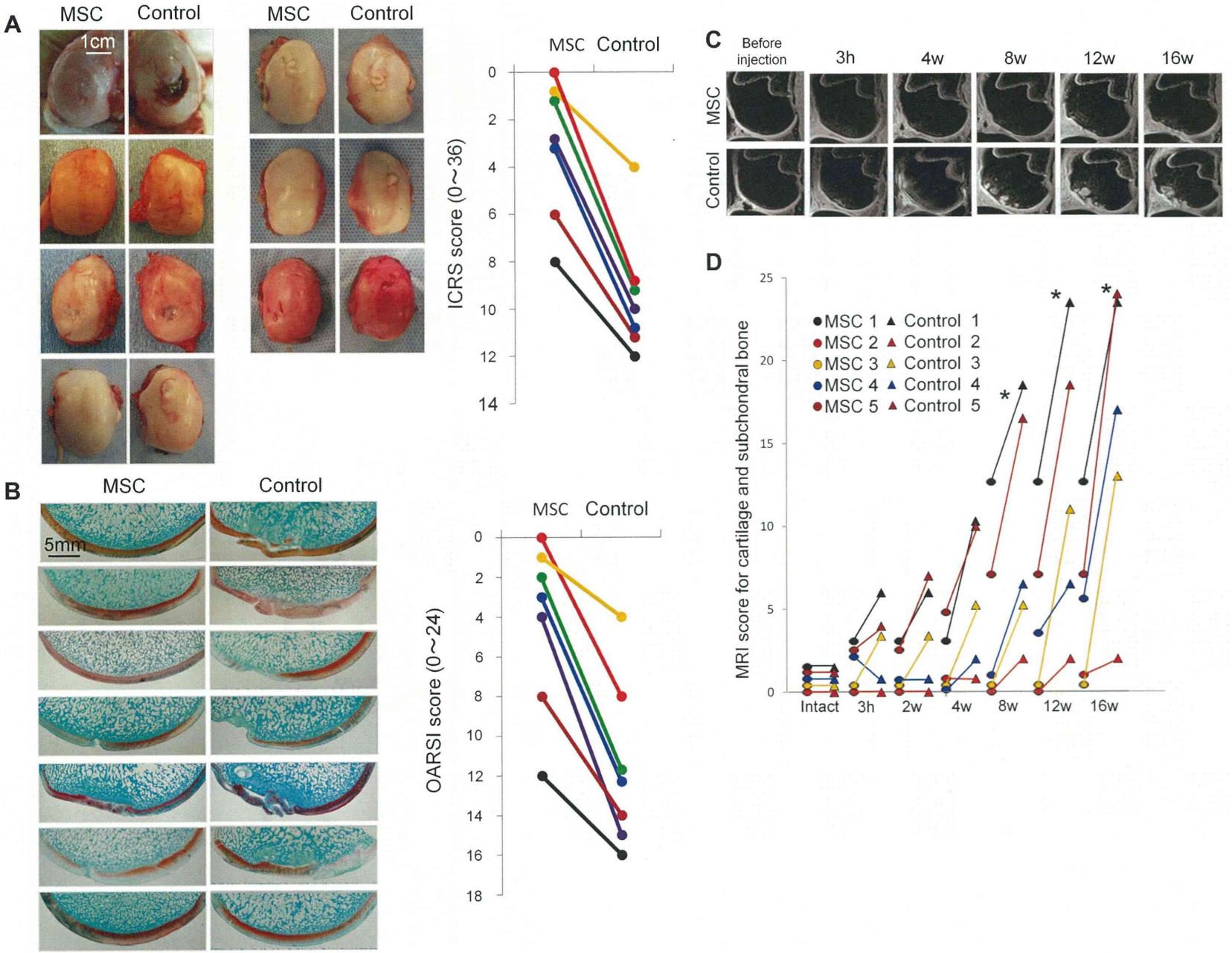

Fig. 5. Analyses of the MFC. Meniscectomy was performed 2 weeks beforehand, synovial MSCs were injected three times at 0, 2, and 4 weeks, then regenerated meniscus was harvested 16 weeks after the first injection. (A) Macroscopic observation and the ICRS score ($n = 7$, $P < 0.05$ by Wilcoxon signed-ranks test). (B) Histological observation and the OARSI score ($n = 7$, $P < 0.05$ by Wilcoxon signed-ranks test). For histology, radial sectioned slides at the center of the MFCs stained with safranin-o are shown. (C) Sequential sagittal MRI fast-spin echo proton density sequence images at the center of the MFC. (D) Quantification of MRI analysis for the MFC by modified WORMS[19] (Supplementary Table 2; $n = 5$, $^*P < 0.05$ by Wilcoxon signed-ranks test).

dissected, is known as an osteoarthritis model in mice[21]. However, the remaining part of the meniscus did not extrude markedly as shown in Fig. 4(A). In this study, we observed the repair process for only 18 weeks after the anterior half of MM was resected, and the remaining part of the meniscus may be pushed out of the load-bearing area of the knee joint after a longer-term follow-up. We previously examined similar models in rats[8,22–24] and rabbits[9]. The remaining part of the meniscus did not extrude markedly within our observation period in all studies we examined.

In this study, allogeneic synovial MSCs were used in order to avoid donor variation of synovial MSCs. Autologous synovial MSCs may survive longer than allogeneic ones. However, donor site morbidity and delay in growing sufficient cells may become problematic with autologous synovial MSCs. Comparison of autologous synovial MSCs with allogeneic synovial MSCs for meniscus regeneration in a pig model will be a subject of our future investigation.

We showed that the meniscal defect was first filled with synovial tissue; then the meniscus regeneration progressed. The induction of synovial tissue to the meniscus defect and/or the formation of synovial tissue in the meniscus defect might be an important initiation for meniscus regeneration. Exogenous synovial MSCs had the possibility to promote this process.

Though there were no significant differences in the size of the regenerated meniscus between the two groups at 16 weeks based on macroscopic observation, the histological features in the MSC group were better than those in the control group. In MRI analysis, quantification of T2 values in the regenerated meniscus was closer to the native values in the MSC group than in the control group, particularly at 16 weeks. These findings demonstrated that the resected meniscus regenerated better in the MSC group. Intra-articular injections of synovial MSCs promoted meniscus regeneration.

Articular cartilage and subchondral bone were better preserved in the MSC group. We propose two possible explanations for the results observed. First, even an immature meniscus may protect the articular cartilage, because the T2 value of the meniscus in the MSC group was already closer to the normal value than the control group at 4 weeks. Second, injected MSCs may produce trophic factors to inhibit progression of articular cartilage degeneration.

To the best of our knowledge, only Murphy *et al.* and Faqeh *et al.* have previously reported the effect of injection of MSCs on retardation of articular cartilage degeneration in massive meniscectomized models of large animals. Murphy *et al.* used goats and removed the whole MM and the resected anterior cruciate ligament[25]. In their study, 10 million bone marrow MSCs were injected into the knee joint. Faqeh *et al.* used sheep and also removed the entire MM and anterior cruciate ligament[26]. They precultured bone marrow MSCs in chondrogenic media containing TGFβ3 and IGF-1; then 10 million bone marrow MSCs were injected into the joints. Both Murphy *et al.* and Faqeh *et al.* demonstrated that degeneration of the articular cartilage was reduced in the MSC group. Although they demonstrated that the MM was markedly regenerated, only one sample was shown, and quantification analysis for the regenerated meniscus was not demonstrated in either paper.

Pigs are useful animals for preclinical study. The immune system of pigs is similar to that of humans. Furthermore, inbred pigs have a genetically defined and fixed major histocompatibility complex, making reproducible studies of immunologic mechanisms possible[27]. With respect to proliferation and extracellular matrix synthesis of chondrocytes, human chondrocytes shared more similarity with porcine than with ovine or equine chondrocytes[28]. Though there are multiple reports on the use of dogs and non-human primates, their use is limited by ethical concerns, especially in Japan. While larger animals may more closely approximate the human clinical situation, they carry greater logistical, financial, and ethical considerations. Therefore, the use of minipigs was most suitable for this study.

We injected synovial MSCs not once but three times because we thought a single injection might be insufficient for meniscus regeneration. One account of a similar strategy is based on the papers by Murphy *et al.* and Faqeh *et al.* as mentioned above. The greater the number of times synovial MSCs are injected, the better the resected meniscus will regenerate. However, too many repetitive injections may be associated with some adverse events such as synovitis, ectopic tissue formation, and tumorigenesis. An analysis to determine the optimal number of injections is required as a next step.

To investigate MSC trophic factors, we previously performed a similar study in a rat model[22]. Human MSCs were injected into the rat knee joint after the anterior half of the meniscus was resected, and the changes in transcription in both human and rat genes were assayed by species-specific microarrays and real-time RT-PCRs. Human MSCs injection increased expression of rat type II collagen, and inhibited osteoarthritis progression. Human MSCs were activated to express high levels of a series of genes including BMP2 and parathyroid hormone-like hormones.

In this study, we analyzed knee joints by MRI in addition to macroscopic and histological evaluation. According to the regulatory science recommended by the FDA, evaluation of quantitative imaging, such as MRI, was required for identifying new predictors of efficacy in non-clinical and clinical evaluations[29]. We followed up on the efficacy and safety of injected synovial MSCs using sequential MRI evaluation.

In terms of clinical application, we propose five limitations in this study. First, the type of defect is an uncommon type. We removed only the anterior half of the MM. Most types are bucket handle tears and, after a partial meniscectomy, part of the inner meniscus is then removed, leaving the rim intact. Second, we used a model in which a spontaneous repair took place. Apparently a neomeniscus is formed relatively rapidly and this is also the case in other models of large animals such as goats and sheep. Interspecies differences have to be considered[30]. Third, we injected synovial MSCs 2 weeks after the resection. This suggests that the space for the resected meniscus was still preserved. In a clinical situation, patients complaining of symptoms after meniscectomy usually have no space for the resected meniscus. Therefore, some procedures are required to regain the space for meniscus regeneration. Fourth, we did not perform a biomechanical test for the regenerated meniscus and we do not know the properties of the regenerated meniscus. Fifth, for analyses of sequential macroscopic observation, the sample number in each experiment was low and quantification with the associated statistics was lacking. In spite of these limitations, the present study demonstrated a novel treatment for regeneration of a massive meniscal defect. This method has the possibility to regenerate the meniscus and to restore its functions with low invasiveness.

Conclusion

Repeated intraarticular injections of synovial MSCs appeared to promote meniscus regeneration and protected articular cartilage in a pig massive meniscal defect model.

Author contributions

Daisuke Hatsushika: Conception and design, collection of data, data analysis, and manuscript writing. Takeshi Muneta: Conception and design, administrative support. Tomomasa Nakamura: Data analysis, collection of data. Masafumi Horie: Conception and

design. Hideyuki Koga: Conception and design. Yusuke Nakagawa: Collection of data and data analysis. Kunikazu Tsuji: Conception and design. Shuji Hishikawa: Collection of data and provision of study material. Eiji Kobayashi: Financial support, and provision of study material. Ichiro Sekiya: Conception and design, financial support, manuscript writing, final approval of manuscript.

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Conflict of interest

No conflict of interest for any of the authors.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2014.04.028.

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