defects of Group-A rabbits at four, twelve, and twenty-four weeks following implantation (Figs. 6-A and 6-B). Although the number of CM-DiI-labeled cells decreased with time, a small number of CM-DiI-labeled cells were observed in the regenerated tissue even at twenty-four weeks. Significant fluorescence was not detected elsewhere in the meniscus, suggesting that implanted mesenchymal stem cells preferentially adhered to the site of meniscal injury. No CM-DiI-labeled cells were detected by fluorescence in control specimens.

In Group-B rabbits, GFP-positive synovial mesenchymal stem cells were found within the meniscal defect as early as one day after injury and remained in high concentrations as late as twelve weeks after injury (Figs. 7-A and 7-B). No GFP-positive cells were detected in the contralateral, control menisci, thus supporting the assumption that neither synovial mesenchymal stem cells implanted in the experimental knee nor their progeny directly affect regeneration at the distant injury site in the contralateral knee.

The number of GFP-positive cells within the defect decreased with time from one day to twelve weeks. Even at twelve weeks, however, a population of GFP-positive cells remained in the regenerated tissue and expressed both type-I collagen (Fig. 7-C) and type-II collagen (Fig. 7-D), possibly aiding in the regenerative process.

Discussion

It has been hypothesized for over thirty years that a factor within synovium may enhance meniscal regeneration and healing, but the underlying mechanism remains unclear. Kim and Moon were among the first to document this association when, in 1979, they showed that meniscectomized rabbit knees subjected to complete synovectomy failed to regenerate meniscal tissue to the same extent as knees with intact synovium[36]. More recently, investigators have demonstrated improved healing rates in meniscal tears supplemented with synovial flaps or grafts in animal models[37,38] and synovial rasping in human clinical studies[39,40].

The discovery of mesenchymal stem cells in mature synovial tissue with exceptional potential for proliferation and chondrogenic differentiation suggests that these cells may be the source of regenerative stimulus within synovium[17,18,20,22]. The results of this study provide more convincing evidence that synovial mesenchymal stem cells are capable of stimulating meniscal repair and regeneration.

While the quantity of regenerated tissue was significantly greater in knees supplemented with synovial mesenchymal stem cells at four and twelve-week end points, the defects filled relatively well with tissue by twenty-four weeks in control knees. This may be seen as a shortcoming of the experimental model; however, one must consider not only quantity but also quality of the regenerated tissue. Significantly inferior tissue-quality scores seen in control knees at the twenty-four-week end point suggest that these defects ultimately filled with hypocellular, poorly bonded, fibrous scar tissue as opposed to meniscal fibrocartilage. In contrast, meniscal defects treated with synovial mesenchymal stem cells regenerated with well-bonded tissue closely resembling normal meniscus in cellularity and matrix composition. The results seen in control knees at twenty-four weeks may be analogous to filling of articular cartilage defects with fibrous tissue, as opposed to true regeneration with hyaline cartilage, after failed articular cartilage restoration procedures[41].

Implanted mesenchymal stem cells found within the meniscal defects resemble native meniscal fibrochondrocytes not only in histological appearance but also in the types of collagen they produce. Meniscal fibrochondrocytes, articular chondrocytes, and fibroblasts, as well as the matrices these cells synthesize, can be distinguished by collagen typing[42]. The primary collagen in meniscal fibrocartilage is type-I collagen, but a substantial amount of type-II collagen is present as well[42,43]. In contrast, hyaline cartilage is composed of primarily type-II collagen, and fibrous scar tissue is composed of largely type-I collagen[42,44]. The implanted synovial mesenchymal stem cells in this experiment produced both type-I collagen and type-II collagen, suggesting differentiation into fibrochondrocytes and synthesis of a fibrocartilage matrix.

Much of the recent work surrounding meniscal regeneration has focused on the use of an active biological component added to a tissue scaffold or carrier compound, which functions to contain the biological component within the injured area[30-32,45]. In contrast to prior studies, the improvements in meniscal regeneration in the current model were achieved without the use of additives designed to contain the implanted cells within the defect. Both fluorescently (CM-DiI) labeled and GFP-positive synovial mesenchymal stem cells preferentially adhered to meniscal defects, where they remained for up to twenty-four weeks. On the basis of the work by Koga et al., this adhesion appears to occur via local surface adhesion molecules, including ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1)[46]. In addition, the attachment takes place rapidly, with >60% of synovial mesenchymal stem cells adhering to injured cartilage within ten minutes of application in an ex vivo human cartilage defect model[46]. Thus, the cells themselves seem to have the inherent ability to quickly adhere to the defect and facilitate the regenerative process.

In an effort to determine the optimal source of mesenchymal stem cells for purposes of intra-articular regeneration, prior studies have compared the in vivo regenerative potential of mesenchymal stem cells obtained from a variety of tissue sources[23,34]. Koga et al. found that mesenchymal stem cells derived from bone marrow and synovium have greater in vivo chondrogenic regenerative potential than mesenchymal stem cells derived from adipose and muscle[34]. The chondrogenic and meniscal regenerative potential of mesenchymal stem cells obtained from synovium and bone marrow, however, appears to be more similar[23,34]. Our rationale for selecting synovial mesenchymal stem cells was based largely on in vitro evidence of superior capacity of synovial mesenchymal stem cells for rapid proliferation and synthesis of chondrogenic matrix in rat[20], rabbit[34], and human studies[18]. The current experiment confirmed the superior in vitro proliferation capacity of rabbit synovial mesenchymal stem cells compared with rabbit bone marrow-derived mesenchymal stem cells. This experiment did not, however, directly compare the regenerative potential of

synovial mesenchymal stem cells and bone marrow-derived mesenchymal stem cells or other additional cell types in vivo.

Other approaches for addressing meniscal deficiency and irreparable meniscal tears in humans are under investigation, but an ideal solution has yet to be discovered. Meniscal allograft transplantation, first described in 1989 by Milachowski et al.[47], has shown promising results in select patients[48], but is complicated by concerns regarding processing and storage[49], subclinical host immune response[50], and a low tolerance for graft-recipient size mismatch[51]. The collagen meniscal implant (Collagen Meniscus Implant; ReGen Biologics, Hackensack, New Jersey), a tissue-engineered biologic scaffold fabricated from bovine type-I collagen, has also demonstrated encouraging results compared with partial meniscectomy[45]; however, defect filling is incomplete at second-look arthroscopy[45] and concerns exist regarding the risk of immune reaction to bovine collagen[52].

Furthermore, the above treatment options are designed to treat complete or partial meniscal defects, but neither provides a means to stimulate healing of avascular zone tears. In vitro meniscal tissue-culture experiments offer evidence that proregenerative cytokines such as transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) may potentially provide such a healing stimulus by increasing meniscal cell proliferation and collagen synthesis[53,54]. In vivo evidence in support of the efficacy of these growth factors in meniscal healing is currently lacking, however. For example, the addition of VEGF coating to meniscal suture failed to increase angiogenesis or improve healing compared with conventional suture in avascular meniscal tears in sheep[55].

In the present study, we chose to evaluate the regenerative potential of synovial mesenchymal stem cells without scaffolds or additional growth factors. The ability of synovial mesenchymal stem cells to adhere independently to the site of meniscal injury, differentiate into fibrochondrocytes, and synthesize a new matrix that closely resembles native meniscal fibrocartilage without a scaffold or extrinsic cytokines seems to negate the need for such additional stimulus. In addition, this approach avoids the potential for complications associated with disease transmission and immune reaction and, in this model, results in virtually complete regeneration of the meniscal defect. It is possible, however, that meniscal regeneration stimulated by supplementation with synovial mesenchymal stem cells may be further enhanced by the addition of a tissue scaffold when used for large meniscal defects in subjects with little inherent regenerative capacity.

Limitations of this study include those that are inherent to the animal model and those that result from the experimental design. Although the capacity for meniscal regeneration is lower in rabbits than rats, it is likely greater than that in humans[27,33]. In light of this, caution is encouraged when extrapolating the results of this study to humans.

With regard to the experimental design, the results of this study portray a simplified picture of the events occurring at a molecular level, as the present study did not evaluate the influence of synovial mesenchymal stem cells on the production or local concentration of proregenerative cytokines such as TGF-β, IGF-1, or PDGF, which promote proteoglycan synthesis and increase cellularity within the meniscus[53,54]. It is likely that the regenerative process involves a complex interplay between the intact meniscus adjacent to the defect, the implanted synovial mesenchymal stem cells, and alterations in local cytokine concentrations, and this was not fully elucidated in the present study. Finally, although the degree of tissue bonding was considered as a component of the tissue quality score, this experiment did not include a precise quantitative evaluation of the integration between regenerated and native meniscal tissue. Nor did it evaluate the biomechanical characteristics of the regenerated tissue, which are critical to meniscal function. Such an evaluation would be more appropriate in a model with a discrete linear tear or larger meniscal defect.

In conclusion, synovial mesenchymal stem cells implanted into the rabbit knee are capable of adhering to sites of meniscal injury, differentiating into type-I and type-II collagen-producing cells with appearance similar to native meniscal fibrochondrocytes, and enhancing both the quality and quantity of regenerated meniscal tissue. In addition to providing a potential explanation for the association between synovial stimulation and meniscal healing, these results may generate further stimulus for exploring the utility of synovial mesenchymal stem cells in the treatment of meniscal injury in large animal models or humans, with potential applications ranging from supplementing suture repair of avascular zone meniscal tears, to stimulating regeneration of large meniscal defects.

Appendix

(eA) A table showing the histological tissue quality scoring system and an expanded Materials and Methods section describing the tissue harvesting and mesenchymal stem cell preparation, in vitro differentiation assays, and tissue quality analysis are available with the online version of this article as a data supplement at jbjs.org. ■

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Update

This article was updated on May 16, 2012, because of a previous error. The legend for Figures 7-A and 7-B that had previously read "Representative macroscopic appearance (**Fig. 7-A**) and histological sections (**Fig. 7-B**) of the meniscal defect one day to twelve weeks after the implantation of GFP-positive green fluorescent protein under fluorescence" now reads "Representative macroscopic appearance (**Fig. 7-A**) and histological sections (**Fig. 7-B**) of the meniscal defect one day to twelve weeks after the implantation of GFP-positive synovial mesenchymal stem cells under fluorescence."

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Arthroscopic, histologic and magnetic resonance imaging analyzes of cartilage repair after a minimally invasive method of transplantation of allogeneic synovial mesenchymal stromal cells into cartilage defects in pigs

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Abstract

Background aims. Transplantation of synovial mesenchymal stromal cells (MSC) may induce repair of cartilage defects. We transplanted synovial MSC into cartilage defects using a simple method and investigated its usefulness and repair process in a pig model. *Methods.* The chondrogenic potential of the porcine MSC was compared *in vitro*. Cartilage defects were created in both knees of seven pigs, and divided into MSC treated and non-treated control knees. Synovial MSC were injected into the defect, and the knee was kept immobilized for 10 min before wound closure. To visualize the actual delivery and adhesion of the cells, fluorescence-labeled synovial MSC from transgenic green fluorescent protein (GFP) pig were injected into the defect in a subgroup of two pigs. In these two animals, the wounds were closed before MSC were injected and observed for 10 min under arthroscopic control. The defects were analyzed sequentially arthroscopically, histologically and by magnetic resonance imaging (MRI) for 3 months. *Results.* Synovial MSC had a higher chondrogenic potential *in vitro* than the other MSC examined. Arthroscopic observations showed adhesion of synovial MSC and membrane formation on the cartilage defects before cartilage repair. Quantification analyzes for arthroscopy, histology and MRI revealed a better outcome in the MSC-treated knees than in the non-treated control knees. *Conclusions.* Leaving a synovial MSC suspension in cartilage defects for 10 min made it possible for cells to adhere in the defect in a porcine cartilage defect model. The cartilage defect was first covered with membrane, then the cartilage matrix emerged after transplantation of synovial MSC.

Key Words: *cartilage repair, mesenchymal stromal cells, pig, synovium*

Introduction

Cartilage injuries are a common clinical problem and if left untreated may cause osteoarthritis, one of the leading causes of disability (1). Stem cell therapy for cartilage repair may be one possible strategy for improvement of cartilage injury. The candidate therapeutic cells are mesenchymal stromal cells (MSC), which can be isolated from various mesenchymal tissues (2,3). We have reported previously the superiority of human synovial-derived MSC for cartilage repair (4–6) and *in vitro* expansion with autologous human serum (7).

Various methods have been used to transplant MSC into cartilage defects, such as intra-articular injection (8,9) and the use of scaffolds (10). We have demonstrated recently that leaving the knee immobilized for 10 min immediately after delivering a suspension of synovial MSC into the defect results

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in approximately 60% of the cells adhering to the defect to promote cartilage repair in rabbits (11). This 'local adherent technique' can be performed less invasively and without scaffolds compared with other methods.

We hypothesized that this method will also be useful in animals that are more closely related to humans. The purpose of the present study was to examine the usefulness of the local adherent technique with synovial MSC in pigs. The knee joints of pigs are similar to those of humans in terms of size (12) and cartilage-specific properties (13). In this study, synovial MSC were transplanted into the cartilage defect of pigs using the local adherent technique, and repaired cartilage was examined sequentially arthroscopically, histologically and by delayed gadolinium-enhanced magnetic resonance imaging (MRI) of cartilage (dGEMRIC) (14,15).

Methods

Animals

All experiments were conducted in accordance with the institutional guidelines for the care and use of experimental animals of the Tokyo Medical and Dental University (Tokyo, Japan) and Jichi Medical University (Tochigi, Japan). Nine male and six female Mexican hairless pigs (National Livestock Breeding Center, Ibaraki, Japan) were used. They were 13 months old, on average 33.5 kg in weight, and skeletally mature, with the growth plates closed. 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 (40 cm in width, 121 cm in length and 109 cm in height). One wild-type pig and one transgenic green fluorescent protein (GFP) pig (16) were used as donors for synovial MSC for transplantation. Two other pigs were also used as sources for MSC for *in vitro* proliferation and differentiation assays. These four pigs were killed on the day when the tissues were harvested. Twelve other wild-type pigs were used as recipients. For GFP observation, two pigs were killed on the day MSC were transplanted, and for observation of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR, USA) two pigs were killed at 7 days after transplantation. For arthroscopic, histologic and MRI analyzes, three pigs were killed at 1 month, and five pigs were killed at 3 months, after transplantation.

Cell isolation and culture

Synovial tissue was harvested from the suprapatellar pouch, which overlays the non-cartilaginous areas of the femur, through an arthrotomy of the knee. The tissue was digested in 3 mg/mL collagenase D solution (Roche Diagnostics, Mannheim, Germany) in α-minimal essential medium (αMEM; Invitrogen, Carlsbad, CA, USA) at 37°C for 3 h, filtered through a 70-μm nylon filter (Becton-Dickinson and Co., Franklin Lakes, NJ, USA) and the nucleated cells plated in a 150-cm^2 culture dish (Nalge Nunc International, Rochester, NY, USA) in complete culture medium [αMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B (all from Invitrogen)] and incubated at 37°C with 5% humidified CO_2. The medium was changed to remove non-adherent cells every 4–5 days and then cultured for 14 days as passage 0 without refeeding. To cryopreserve the cells, they were resuspended at a concentration of 2×10^6 cells/mL in αMEM with 5% dimethylsulfoxide (Wako, Osaka, Japan) and 10% FBS. Aliquots of 2 mL were frozen slowly in a Cryo 1°C freezing container (Nalge Nunc International) and cryopreserved at –80°C. To expand the cells, a frozen vial of the cells was thawed, plated in 60-cm^2 culture dishes, and incubated for 4 days. Then the cells were replated at 5×10^5 cells/150-cm^2 culture dish (passage 2) and cultured for an additional 14 days. The nucleated cells derived from periosteum, muscle and adipose tissue were isolated and expanded in the same manner as those from synovium.

Bone marrow was aspirated from the tibial tuberosity. Periosteum was peeled off from the tibia. Muscle was obtained from the quadriceps. Adipose tissue was prepared from the subcutaneous fat around the knee. Nucleated cells from the bone marrow were isolated with a density gradient (Ficoll-Paque; Amersham Biosciences, Uppsala, Sweden).

Colony-formation assay

Nucleated cells derived from synovium were plated at 0.5, 5, 50 and 500×10^3 cells/60-cm^2 dish, cultured for 14 days, and stained with crystal violet. The optimal initial cell density was determined based on the following criteria: (a) the colony size was not affected by contact inhibition, and (b) the greatest number of colonies was obtained. We then harvested the cells plated at optimal densities from the remaining dishes and expanded them as mentioned above.

In vitro proliferation assay

Synovial MSC were plated at 5×10^3 cells/60-cm^2 dish in complete culture medium and passaged every 14 days. Cells from each passage were harvested and counted with a hemocytometer, and the total accumulated cell number was calculated.

In vitro differentiation assay

For chondrogenesis, 250 000 cells were placed in a 15-mL polypropylene tube (Becton-Dickinson and Co.) and centrifuged 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 (Stryker Biotech, Hopkinton, MA, USA), 10 ng/mL transforming growth factor (TGF)-β3 (R&D Systems, Minneapolis, MN, USA), 100 nM dexamethasone (Sigma-Aldrich Corp., St Louis, MO, USA), 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 Discovery Labware, Bedford, MA, USA). For microscopy, the pellets were embedded in paraffin, cut into 5-μm sections, and stained with toluidine blue (17–19).

For adipogenesis, cells were cultured in adipogenic medium, which consisted of complete medium supplemented with 100 nM dexamethasone (Sigma-Aldrich Corp.), 0.5 mM isobutyl-methylxanthine (Sigma-Aldrich Corp.) and 50 μM indomethacin (Wako), for 21 days. The adipogenic cultures were fixed in 4% paraformaldehyde and then stained with fresh Oil Red O solution (20).

For calcification, cells were cultured in calcification medium, which consisted of a complete medium of 1 nM dexamethasone, 20 mM β-glycerol phosphate (Wako) and 50 μg/mL ascorbate-2-phosphate (Sigma-Aldrich Corp.), for 21 days. The cells were fixed in 4% paraformaldehyde and stained with 0.5% Alizarin Red solution (21).

DiI labeling

Synovial MSC were resuspended at 1×10^6 cells/mL in αMEM without FBS, and a fluorescent lipophilic tracer, DiI, was added at a final concentration of 5 μL/mL. After incubation for 20 min at 37°C and two washings with phosphate-buffered saline (PBS), DiI-labeled cells were resuspended in 100 μL culture medium (22).

Experimental set-up

The first pig was used for anatomical study and harvesting mesenchymal tissues to stock the MSC for further analyzes. When pigs for the *in vivo* study were prepared, cryopreserved synovial MSC were thawed and expanded 2 weeks before transplantation. On the day of transplantation surgery, all colony-forming cells were harvested and suspended in 100 μL culture medium and transplanted as described. Four pigs were used for an early adhesion assay with transplantation of GFP porcine synovial MSC ($n = 2$) (Figure 2A) and DiI-labeled MSC ($n = 2$). Other pigs were analyzed by arthroscopy every month, and two pigs were killed at 1 month after treatment for histologic, macroscopic and MRI analyzes. Five pigs were killed at 3 months after treatment and analyzed by histology and MRI (Figure 2D).

Transplantation of synovial MSC into the cartilage defects

All pigs underwent general anesthesia, and the medial femoral condyle was approached through a medial parapatellar incision. Full-thickness osteochondral defects (8 × 8 mm square and 2 mm deep; approximately 1.5 mm cartilaginous and 0.5 mm bony part) were created with various sizes of drills in the weight-bearing area of the medial femoral condyles in both knees, 10 mm below the terminal ridge. When the defects were created, bleeding was not observed, and a procedure to stop bleeding from the bottom of the defect was not required.

The right knee of each pig was treated with MSC and the left knee served as a vehicle internal control. The MSC were harvested and collected from the culture dishes several hours before transplantation, and harvested MSC were suspended in a 50-mL conical tube containing 40 mL culture medium. Just before the transplantation, the tube was centrifuged for 5 min at 1500 r.p.m., and the supernatant was removed. Centrifuged MSC were suspended in 100 μL culture medium. The transplanted cell number was a maximum of 5.3×10^7, a minimum of 2.2×10^7, and on average 3.8×10^7.

The cartilage defect was faced upward, and its position was held manually. A suspension of prepared MSC in 100 μL culture medium was placed into the defect through an 18-gauge needle. Culture medium alone (100 μL) was placed into the defects in the left knee in the same manner. After 10 min, the incisions were closed without washing the inside of the knee joint. After the anesthetic wore off, the pigs were allowed to walk freely without fixation. To reduce the risk of infection, we avoided the use of an immune suppressor.

For killing the pigs, an overdose intravenous injection of KCl was used under adequately deep general anesthesia. For macroscopic analyzes, all samples at 1 month ($n = 3$) and 3 months ($n = 5$) were evaluated with the International Cartilage Repair Society (ICRS) macroscopic score (23) (see the supplementary tables).

Arthroscopy

All knees were observed with arthroscopy (Linvatec 8180A camera console surgical video equipment, with LIS8430 for the light source; Zimmer Inc., Warsaw, IN, USA) at 1, 2 and 3 months after transplantation. An arthroscope, a probe and a shaver system were inserted through longitudinal incisions at the medial and lateral sides of the patella tendon. All arthroscopic observations were evaluated by Oswestry arthroscopy score (23) (see the supplementary tables). For arthroscopic observation of GFP MSC, a newly developed fluorescence arthroscope (Olympus Medical Systems Corp., Tokyo, Japan) was used.

Histologic analyzes

The samples were cut into a thickness of a 1.5-cm square with 5 mm containing a defect, fixed in 4% paraformaldehyde, and decalcified with 0.5 M ethylene diamine tetra acetic acid (EDTA; pH 7.5) for 3 days at 4°C. Paraffin sections were stained with Safranin O. All samples were evaluated with a modified Wakitani score (11) (see the supplementary tables).

dGEMRIC

Before histologic analyzes, medial femoral condyles were collected and pre-contrast MRI was performed. An MRI system at 1.5 Tesla (Signa HDx; GE Healthcare, UK) was used with a custom-made microimaging coil. Each specimen was pre-treated with 0.5 mM gadopentate dimeglumine ($Gd\text{-}DTPA^{2-}$; Magnevist®; Schering, Berlin, Germany) in 0.9% normal saline overnight at 4°C with continuous stirring. The next day the samples were removed from refrigeration, and post-contrast MRI was performed at room temperature. R1 was defined as the reciprocal of the T1 value. The R1 measurement was performed using a fast-spin echo inversion-recovery (FSE-IR) sequence (2400 ms repetition time, 18 ms echo time, six inversion times of 50–2000 ms, 30×30 mm field of view, 1.0-mm section thickness, 512×512 matrix). The difference between the pre-Gd-enhanced R1 value and the post-Gd enhanced R1 value (ΔR1) indicated the glycosaminoglycan (GAG) concentration (14). Color-coded ΔR1-calculated heat maps of the cartilage were generated using MATLAB (Mathworks, Natick, MA, USA) with a mono-exponential curve fit. Blue represents a high content of GAG, and red a low content. For R1 measurements, the region of interest (ROI) for repaired tissue was defined as the area where both sides were connected between native and repaired cartilage; the bottom was the interface between bone and repaired cartilage, and the top was the superficial surface of the repaired cartilage. The ROI for native cartilage was drawn over the full-thickness weight-bearing areas of the femoral condyle at both sides of the repair site, about 3 mm from the lateral edge of the repair site (14,15).

Statistical analyzes

To assess differences, Wilcoxon rank-sum tests were used except for MRI analysis. For MRI analysis, the paired *t*-test was used. A value of $P < 0.05$ was considered significant.

Results

Characteristics of porcine synovial cells as MSC

The initial cell-plating density to produce the optimal colony number was determined to be 5×10^3 cells/60-cm^2 dish (Figure 1A). Three cell lineages derived from three different pigs maintained their proliferation potential over 20 passages (Figure 1B). Colony-forming cells derived from porcine synovium displayed a trilineage potential, differentiating into chondrocytes and adipocytes, and osteocytes, when cultured in their respective differentiation media (Figure 1C). *In vitro* chondrogenesis assays demonstrated that cartilage pellets of colony-forming cells derived from synovium were the heaviest among those derived from the other mesenchymal tissues (Figure 1D). These results indicated that colony-forming cells derived from porcine synovium had similar characteristics to those of MSC, and the highest chondrogenic potential compared with cells derived from the other tissues examined.

Local adherent technique for transplantation of MSC

After expanding for 14 days (Figure 2A), colony-forming cells derived from synovium of the tg-GFP pig expressed GFP (Figure 2B). A drop of MSC suspension through a needle (Figure 2Ci) could be detected with the GFP arthroscopy system (Figure 2Cii). After placement of the MSC suspension for 10 min, the bottom of the cartilage defect looked foggy (Figure 2Ciii) and GFP MSC were still detected in the cartilage defect (Figure 2Civ), even though the irrigation fluid was flushed from the tip of the arthroscope (see the supplementary movies). DiI-labeled MSC were also traced (Figure 2D,E) and remained in the cartilage defect at 7 days (Figure 2F), but they could not be found at 1 and 3 months.

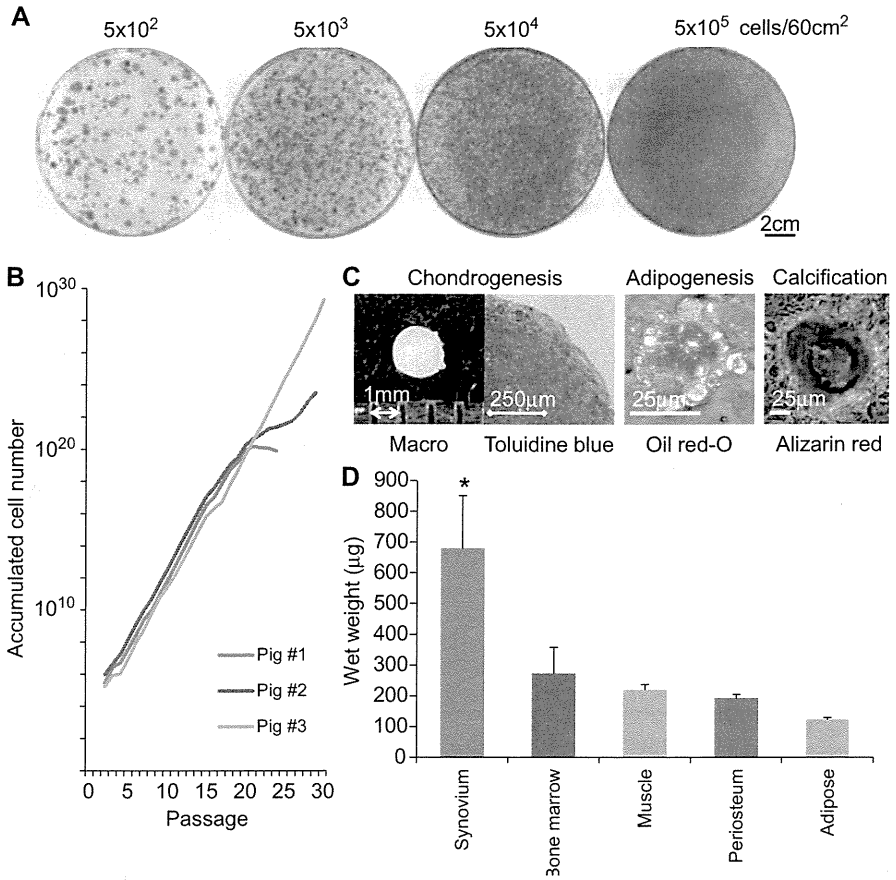

Figure 1. Characteristics of porcine synovial MSC. (A) Colony formation. (B) Proliferation. (C) *In vitro* chondrogenesis, adipogenesis and calcification. (D) Comparison of the chondrogenic potential among MSC derived from various mesenchymal tissues. $*P<0.05$ ($n=5$) between synovium and each of the other tissues by Wilcoxon rank-sum test.

Arthroscopic and macroscopic observation

At 1 month, a thin membrane covered the cartilage defects only in the MSC-treated knees (Figure 3A). At 2 months, a thicker white membrane covered the defects in the MSC-treated knees, while the cartilage defects were enlarged in the control knees. At 3 months, the defects were covered with cartilage tissue in the MSC-treated knees. In contrast, the defects were further enlarged in the control knees. Arthroscopic observation was easier in the MSC-treated knees at all time-points because intra-articular adhesion and synovial hypertrophy were less in the MSC-treated knees compared with the control knees. The Oswestry arthroscopy score improved over the course of time, and a significant difference between the two groups was observed at 3 months (Figure 3B). Similar results were obtained with the macroscopic evaluation (Figure 3C). The ICRS score for macroscopic observation was significantly higher in the MSC-treated knees than in the control knees (Figure 3D). We found no complications throughout this cell transplantation study in the knees examined.

Histologic analyzes

At 1 month, membranous tissue completely covered the defects only in the MSC-treated knees (Figure 4A). At 3 months, newly synthesized cartilage matrix was observed in every sample in the MSC-treated knees. In contrast, there was no cartilage matrix in the control knees (Figure 4B). Furthermore, cartilage defects were further enlarged in the control knees. Higher magnified observations demonstrated a columnar arrangement of chondrocytes with lacunae in the repaired cartilage in the MSC-treated knees (Figure 4C,D). The modified Wakitani score for histologic analysis of cartilage repair was significantly higher in the MSC-treated knees than in the control knees at 3 months (Figure 4E).

dGEMRIC

The cartilage defects showed predominantly red (lower glycosaminoglycan concentration) in both the MSC and control knees at 1 month (Figure 5A). At 3 months, they changed to blue (higher glycosaminoglycan concentration) in the MSC-treated

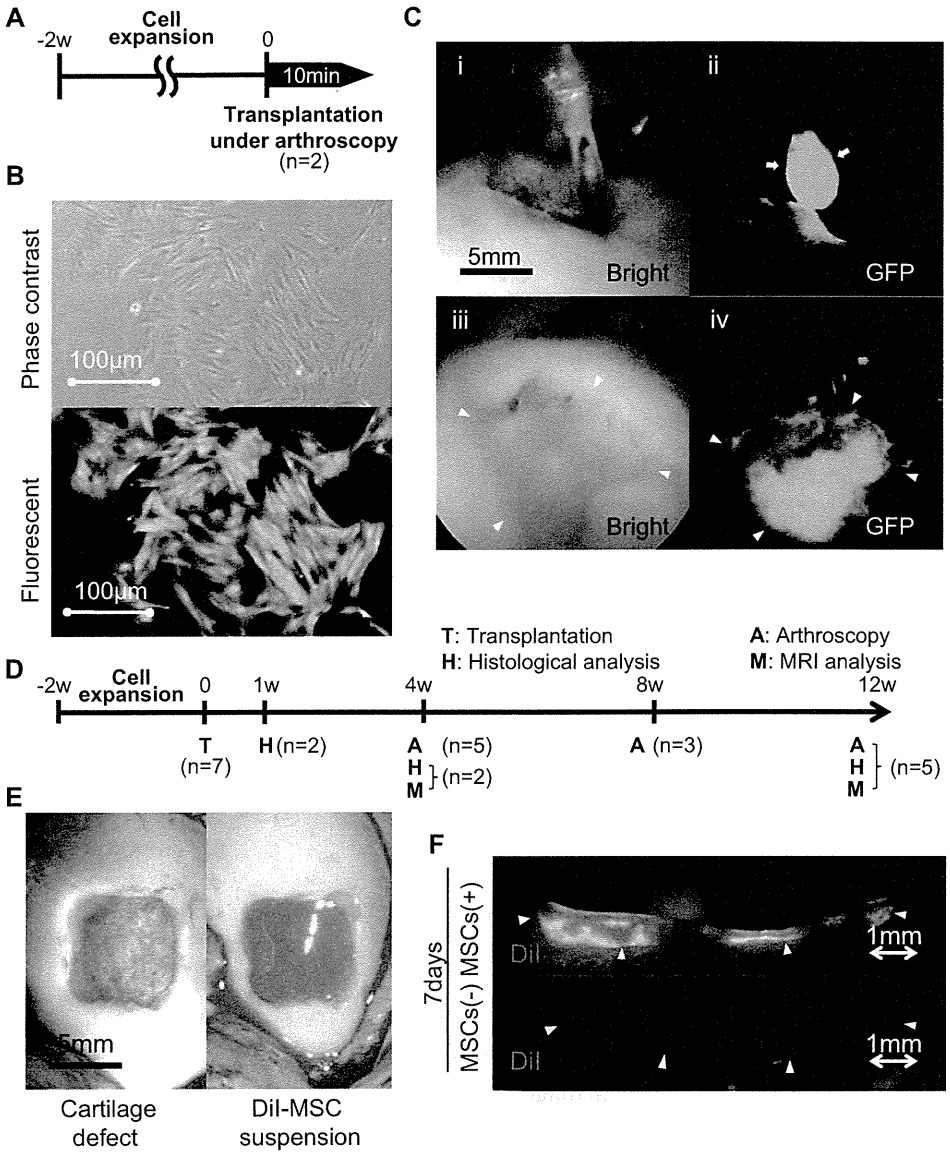

Figure 2. Experimental set-up and local adherent technique for MSC transplantation. (A) Schematic drawing for arthroscopic transplantation and detection of GFP MSC. (B) Synovial MSC from the transgenic GFP pig used to visualize delivery and adhesion of cells in the defect under phase–contrast and fluorescent illumination. (C) Arthroscopic view during transplantation of GFP MSC into the cartilage defect. Arrows indicate the MSC suspension leaving the needle. Arrowheads indicate the margin of the cartilage defect. (D) Schematic drawing for histologic, MRI and other arthroscopic analyzes. (E) Full-thickness cartilage defect (left) and DiI-labeled MSC suspension dropped into the defect (right). (F) Fluorescent images of cartilage defect sections 7 days after transplantation of DiI-labeled MSC.

knees, while remaining red in the control knees. The average R1 value for ROI (Figure 5B) was higher in the MSC-treated knees than in the control knees (Figure 5C).

Discussion

One of the principal findings of the study was the high chondrogenic potential of MSC from synovium in pigs. In this study, *in vitro* chondrogenesis assays demonstrated that cartilage pellets of MSC from synovium were heavier than those from bone marrow, muscle, periosteum and adipose tissue in pig. We have reported similar results previously in humans (4), rats (5) and rabbits (22). These findings suggest that MSC derived from synovium have a high chondrogenic potential irrespective of animal species.

The *in vitro* chondrogenic potential was evaluated by the weight of the pellet. During *in vitro* chondrogenesis of MSC, the pellets increased in size and weight. In contrast, the DNA yield per pellet decreased over time. The radioactivity per DNA in the cells, assessed by pre-labeling with 3H-thymidine, was stable during *in vitro* chondrogenesis of MSC. Consequently, the increase in pellet size could be attributed to the production of extracellular matrix (ECM) and not

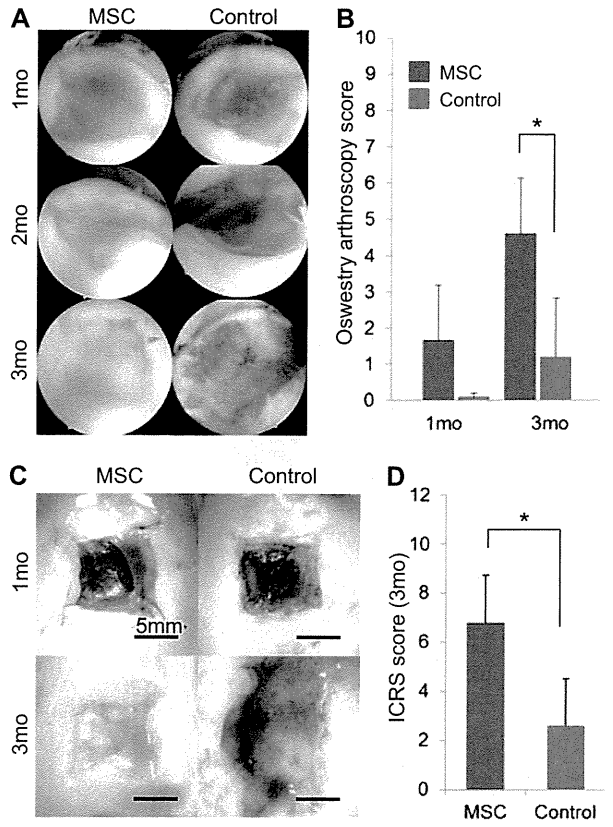

Figure 3. Arthroscopic and macroscopic analyzes of cartilage defects with and without transplanted MSC. (A) Sequential arthroscopic view at 1, 2 and 3 months. (B) Quantification of arthroscopic view of cartilage defect. $*P<0.05$ by Wilcoxon rank-sum test. (C) Representative macroscopic features. (D) Quantification of macroscopic features of cartilage defect. $*P<0.05$ by Wilcoxon rank-sum test.

to the proliferation of the cells (19,24). Pellet weight is always correlated with the expression of cartilage-related mRNA, such as COL2A1, with proteoglycan staining by Safranin O, type II collagen by immunostaining, and protein expression of chondroitin 4-sulfate by enzyme-linked immunosorbent assay (ELISA) (4–7,17–19,25). Furthermore, the results of *in vitro* chondrogenesis reflected the results of *in vivo* chondrogenesis in that undifferentiated MSC were transplanted into cartilage defects, and cartilage matrix production by MSC was evaluated after 4 weeks in rabbits (6). All the results demonstrate that the weights of the pellets are quantitative indicators for chondrogenesis of MSC.

In vitro chondrogenesis appears to be most successful when a combination of dexamethasone, TGF-β and BMP is used in MSC derived from bone marrow (18), synovium (19), muscle (26), periosteum (27) and adipose tissue (28). However, our current results do not exclude the possibility that a different combination of growth factors may induce a more effective chondrogenesis dependent on MSC sources.

To track the cells, we used both GFP and DiI systems. The use of GFP cells is advantageous in that dead GFP cells are not detected. In this study, GFP synovial MSC were derived from the Jinhua pig, and the recipients used were Mexican hairless pigs. This was a major mismatch transplantation model, because Jinhua and Mexican hairless pigs have a high independency of gene profile as a result of inbreeding (29). Therefore, the analysis of transplantation of GFP cells was limited for the observation of arthroscopic transplantation of synovial MSC, because we wanted to avoid the possibility of an immune reaction after adherence of the cells. The use of GFP cells is disadvantageous in that GFP is often undetectable after processing for histology, especially in the case of paraffin embedding (30). To solve these problems, we used the DiI system to track the transplanted cells.

For histologic and other analyzes, we created cartilage defects and left the suspension of MSC on the defects for 10 min in an open arthrotomy. For GFP analysis, after the cartilage defects were created in an open arthrotomy, the joint capsule and skin were sutured, then the suspension of MSC was placed on the defects through the needle while we observed the defect with an arthroscope, and the suspension was left for 10 min. Fluorescence arthroscopy demonstrated that GFP MSC remained in the cartilage defects, even though the irrigation fluid was flushed from the tip of the arthroscope. This indicates that the method we used makes it possible to transplant MSC into the cartilage defects through a small incision by arthroscopy, with minimal invasiveness. Although a GFP-detecting endoscopy system for the airway has been reported previously (31), this system still seems to be unpopular. Our study is the first report demonstrating GFP cells in joints with arthroscopy.

In this study, the number of MSC adhering to the cartilage defect was not quantified. In our previous *ex vivo* study using human and rabbit samples, a suspension of synovial MSC was placed on the full-thickness defect of the articular cartilage fragment, and approximately 60% of the cells were attached to the defect within 10 min (11). A recent study reported that the addition of magnesium to the cell suspension increased the number of synovial MSC attached to the cartilage defect *in vitro* and *in vivo* (32). In our pig study, the medium for MSC suspension contained 1 mM magnesium, and we estimated that more than 60% of the cells adhered to the cartilage defect.

The cartilage defect we created might be better called an osteochondral defect rather than a cartilage defect. We tried to create a full thickness cartilage

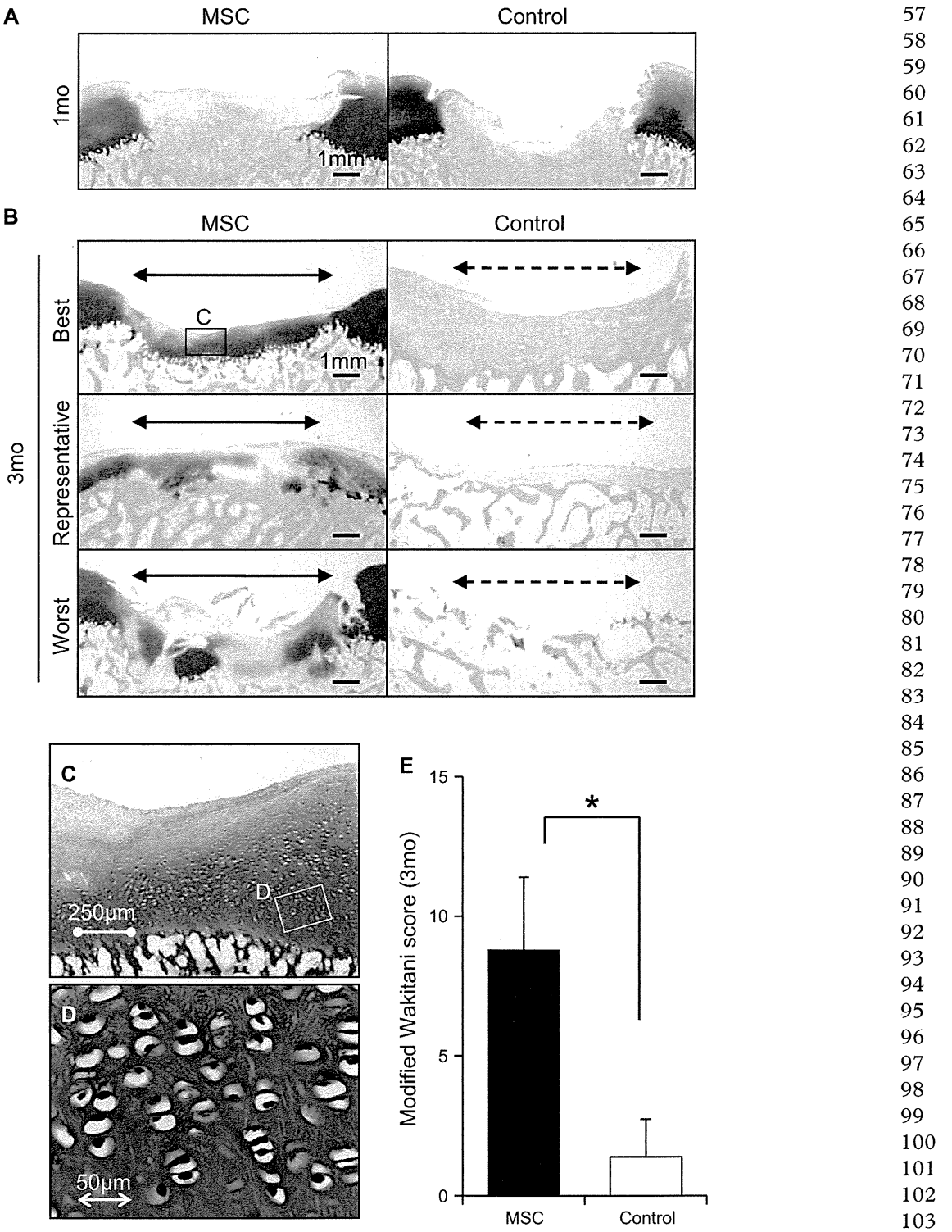

Figure 4. Histologic analyzes of cartilage defect transplanted with MSC. (A) Representative sections stained with Safranin O at 1 month. Red indicates extracellular matrix, and blue indicates cancellous bone. (B) Example sections of the best, representative and worst outcomes in the MSC-treated knees at 3 months and in the control from the opposite sides. Borders of the original defect are shown by both arrowheads. (C) Magnified histology of the indicated area. (D) High magnification of the indicated area. (E) Quantification of histologies of cartilage defect. $*P < 0.05$ by Wilcoxon rank-sum test.

defect, but it was not technically easy to do with precision. Therefore, we preferred to create the osteochondral defect in order to be sure all the cartilage was removed, because any remaining cartilage would affect the outcome of this study. We also thought that if we could repair an osteochondral defect with our

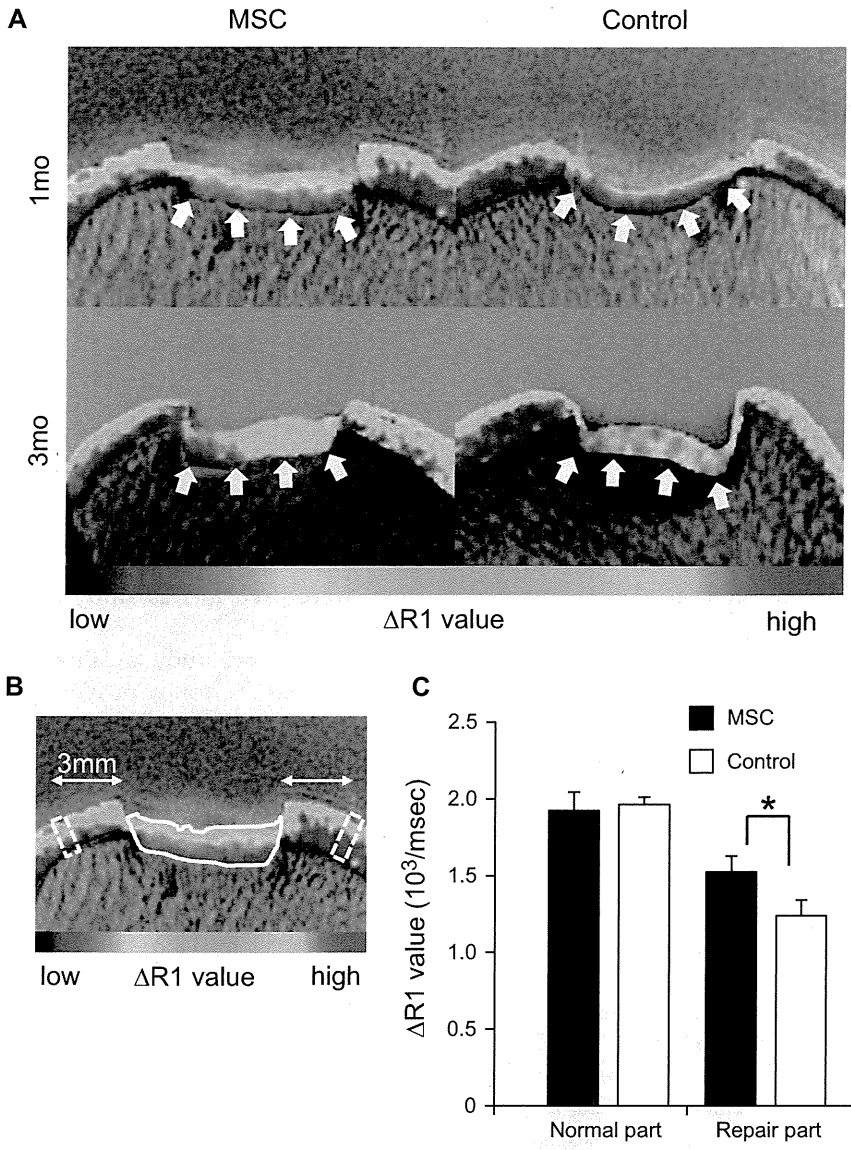

Figure 5. Evaluation with dGEMRIC. (A) Representative images. Arrows indicate the bottoms of the repair tissue. (B) ROI for repaired cartilage (solid-line area) and for native cartilage (dotted-line areas). (C) Quantification of R1 values at 3 months. $*P<0.05$ by paired t-test.

method, we could also repair a full thickness cartilage defect through further abrading of the full thickness cartilage defect to create an osteochondral defect.

By penetrating the subchondral bone, host bone marrow MSC would have migrated into the defect. Because bone marrow MSC also have chondrogenic potential (33), the effect of bone marrow MSC would not have been negligible in our study. However, we were able to demonstrate the higher effect of synovial MSC, because the control defects were not repaired at all. The depth of the osteochondral defect may have affected the result of the repair. Chang *et al.* (34) compared the histologic score of the spontaneous repair of the defect between a 2-mm and 5-mm depth of osteochondral defect in pigs for 36 weeks, and the score of the 2-mm defect was better than that of the 5-mm osteochondral defect. In our study, a 2-mm osteochondral defect consisting of 1.5 mm in the cartilage and 0.5 mm in the subchondral bone was created, and the influence of the subchondral bone defect would have been less than that when the subchondral bone was penetrated deeper.

In this study, DiI-labeled cells were detected at 1 week, but not at 4 and 12 weeks. The process of cartilage repair was observed within at least 3 months. These findings suggest that transplantation of synovial MSC secretes some trophic factors to enhance cartilage repair rather than directly differentiating into chondrocytes. According to our recent report, in a co-culture of rat nucleus pulposus cells and human synovial MSC, a species-specific microarray revealed that gene profiles of the nucleus pulposus were altered markedly, with suppression of genes related to matrix degradative enzymes and inflammatory cytokines (35).

Identification of the trophic factors by synovial MSC in a cartilage defect model is required in a future study.

We have shown that transplantation of synovial MSC into cartilage defect promotes cartilage repair in pigs. To the best of our knowledge, only Ando *et al.* (36) have previously reported the effect of transplantation of synovial MSC into cartilage defects in a pig model. They cultured synovial MSC at a high density in growth medium containing ascorbate 2-phosphate, to form a complex of the cultured cells and the extracellular matrix. After detaching the tissue-engineered construct by application of shear stress using gentle pipetting, the constructs were implanted into the cartilage defect (36). Comparing Ando *et al.*'s study (36) and ours, our method is simpler, and we provide several kinds of novel information during the process of cartilage repair.

We have reported previously that placing a synovial MSC suspension on the osteochondral defect for 10 min promotes cartilage regeneration in rabbits. Histologic analyzes demonstrated that the osteochondral defect was initially filled with cartilage matrix at 4 weeks, then the border between the bone and cartilage moved upward, and finally the thickness of the regenerated cartilage became similar to that of the neighboring cartilage in rabbits (11,32). In the pig study, after transplantation of synovial MSC, the cartilage defect was first covered with a membrane at 4 weeks, then the cartilage matrix emerged, although the repair of the subchondral bone was not observed. These findings may indicate different processes of cartilage repair between rabbits and pigs.

After placement of the MSC suspension, consisting of on average 38 million cells in 100 μL, for 10 min, although the inside of the knee joint was filled with irrigation fluid flushed from the tip of the arthroscope, the bottom of the cartilage defect looked foggy through conventional light arthroscopy (Figure 2Ciii). This was possible because the cartilage defect was mostly covered with synovial MSC. The color of the suspension of synovial MSC was similar to that of the cartilage defect after placement of the MSC suspension for 10 min, which supports our speculation. For clinical application, we can guess the existence of MSC without labeling, by arthroscopic observation if a high concentration of MSC suspension is prepared.

dGEMRIC requires more effort than conventional MRI because it requires twice as many imagings both before and after contrast agent administration. However, dGEMRIC can provide information about the thickness of repaired cartilage and glycosaminoglycan concentration (14,15). In this study, we confirmed the usefulness of dGEMRIC for cartilage repair. To the best of our knowledge, this is the first study to analyze porcine cartilage repair by dGEMRIC and to compare its histologic results.

Although transplantation of synovial MSC induced cartilage repair compared with control knees, cartilage repair was not yet complete at 3 months. We can suggest three reasons for this. First, 3 months was too short a time to mature the cartilage defect in this model. Even in our rabbit study, it took 6 months to repair the cartilage defect after transplantation of synovial MSC (22). In porcine studies by others, it seems that cartilage repair was not complete at 6 months after bone marrow MSC transplantation (37–39). Because of the limitation of our animal facility, we could not perform observations for more than 3 months in this study. Second, we created the cartilage defect in both knees, and all pigs were free in the cage. Therefore, both knees could not avoid bearing weight. Third, allogeneic synovial MSC were used in this study to prevent variability of porcine MSC.

However, this study is valuable because we have demonstrated the ability of synovial-derived MSC to repair cartilage in the porcine knee relative to vehicle-treated knees. Furthermore, the potential problems in this study, as mentioned above, can be overcome if and when this therapy is applied in humans, because weight bearing can be controlled on the treated knee, and autologous cells can be prepared to expand in autologous human serum (7).

In conclusion, an *in vitro* chondrogenesis assay revealed that MSC from synovium had a higher chondrogenic potential than that from other mesenchymal tissues in pig, as has been found in other species (4,5,22). Through the use of transgenic porcine GFP-expressing synovial MSC and a new fluorescence arthroscopy system, we were able to visualize the actual delivery and adhesion of the cells in the cartilage defect. We utilized dGEMRIC to obtain detailed serial images of cartilage repair produced by MSC. Sequential arthroscopic, histologic and MRI analyzes demonstrated that the cartilage defect was first covered with a membrane, and then the cartilage matrix emerged after transplantation of synovial MSC (Figure 6).

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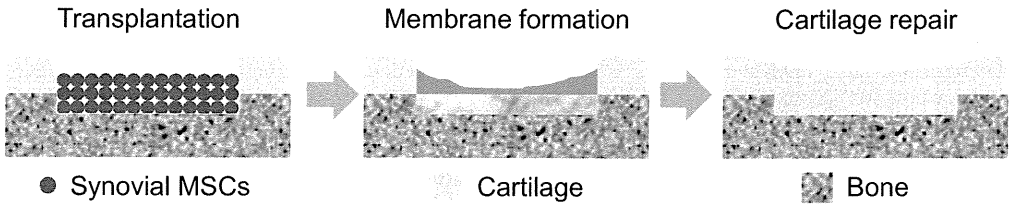

Figure 6. Diagram of the process of cartilage repair. At about 1 month, a membranous layer formed over the defect, and by 3 months cartilage had formed to repair the defect.

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Author contributions: TN, IS, TM and EK designed the experiments; TN and IS wrote the manuscript; TN performed experiments and collected and analyzed data; IS, TM and EK supervised the project and provided study materials and financial support; DH and MH assisted with the porcine experiment; KT proofread the manuscript; TK provided the GFP transgenic pig; AW performed magnetic resonance imaging, and analyzed data; SH, YF and HT provided and managed the pigs.

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Supplementary material available online

Supplementary movies 1–2.
Supplementary tables 1–3.

Supplementary material for Nakamura T *et al.* Arthroscopic, histologic, and MRI analyzes of cartilage repair after a minimally invasive method of transplantation of allogeneic synovial mesenchymal stem cells into cartilage defects in pigs, *Cytotherapy*, 2012

Supplemental movie 1. Arthroscopic view of dripping suspension of GFP pig synovial MSC into the cartilage defect in pig. Irrigation fluid was drained out of the knee joint.

Supplemental movie 2. Arthroscopic view of defect 10 min after the suspension of GFP pig synovial MSC which remained on the cartilage defect. The knee joint was filled with irrigation fluid that was flushed from the tip of the arthroscope.

Supplemental tables. Oswestry arthroscopy score, ICRS macroscopic score, and modified Wakitani score.

Supplementary Table 1. Oswestry arthroscopy score.

OAS	*Points*
Graft level with surrounding cartilage	
Level	*2*
Raised	*1*
Below	*0*
Integral en wdh surrounding cadbge	
Complete	*2*
Minor disruption (<25% of area)	*1*
Major disruption (>25% of area)	*0*
Appearance of surface	*2*
Smooth	*2*
Fine tronds	*1*
Severe 1ronda. 1ibnlla1on	*0*
Color of graft	
Pearly, hyaline-Ike	*2*
White	*1*
Yellow bone	*0*
Stillness on probing	*2*
Normal compared to adjacent cartilage	*2*
Softer	*1*
Very soft/hard	*0*
Total	*0–10*

Supplementary Table 2. ICRS macroscopic score.

Cartilage repair assessment ICRS	*Points*
Degree oi deled lepair	
In level with sunoundng cartilage	*4*
75% repair oi deled deplh	*3*
50% repair oi deled depth	*2*
25% repair oi deled depth	*1*
0% lepair oi deled depth	*0*
Integral on to border zone	
Complete integral on wit h Surrounding cartilage	*4*
Demarcating border<1 mm	*3*
3/4th of grafl integrated, 1/4th with a notable border>1 mm width	*2*
1/2 of graft integrated with surrounding cartilage, 1/4 with a notable border>1 mm	*1*
From no contact to 1/4th oi graft integrated with smounding cartilage	*0*
Macroscopic appearance	
Intact smoot n surface	*4*
fibrillated surface	*3*
Small, scattered tissures or cracs	*2*
Several, small or few but large fissures	*1*
Tdal degeneration of grafted area	*0*
overall repair assessment	
Code 1: normal	*12*
Grade II: nearly normal	*11–8*
Grade III:abnormal	*7–4*
Giade IV: severely abnormal	*3–1*

Supplementary Table 3. Modified wakitani score.

Category	*Points*
Cell morphology	
Hyaline cartilage	*4*
Mostly hyaline cartilage	*3*
Mostly fibrocartilage	*2*
Mostly non-cartilage	*1*
Noncartilage only	*0*
Matrix-staining (metachromasia)	
Normal (compared with host adjacent cartilage)	*3*
Slightly reduces	*2*
Markedly reduced	*1*
No metachromatic stain	*0*
Surface regularity[a]	
Smooth (> 3/4)	*3*
Moderate (1/2 to 3/4)	*2*
Irregular {1/4 to 1/2}	*1*
Severely Irregular (<1/4)	*0*
Thickness of cartilage[b]	
2/3 to 4/3	*3*
5/3 to 4/3	*2*
1/3 to 2/3 or > 5/3	*1*
< 1/3	*0*
Integration of donor witti host adjacent cartilage	
Both edges integrated	*2*
One edge integrated	*1*
Neither edge integrated	*0*
Total maximum	*15*

[a]Total smooth area of the reparative cartilage compared with the entire area of the cartilage defect. [b]Average thickness of the reparative cartilage compared with that of the surrounding cartilage.