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Transplantation of aggregates of synovial mesenchymal stem cells regenerates meniscus more effectively in a rat massive meniscal defect

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ABSTRACT

Transplantation of mesenchymal stem cells (MSCs) derived from synovium is a possible therapy for meniscus regeneration. We have previously reported that intraarticular injection of 5 million synovial MSCs promoted meniscal regeneration in rat meniscal defects. However, if a similar cell number per body weight were required, preparation of required human MSCs would not be practical in a clinical situation. The use of aggregates of MSCs may be one of the solutions. Here, we investigated whether the use of aggregates of synovial MSCs regenerated meniscus more effectively in a rat meniscectomized model. The total number of synovial MSCs was adjusted to 25,000 cells, and aggregates consisting of MSCs or 25,000 MSCs suspended in PBS were placed on the meniscal defects. Five million MSCs suspended in PBS were also used as another control. For the regenerated menisci, the area was larger and the histological findings were closer to that of the normal meniscus in the aggregate groups than to that in the suspension groups at 4 weeks. The effects of transplantation of aggregates were still observed at 12 weeks. Luminescence intensity remained higher at 3 weeks and thereafter in the aggregate group than in the suspension group when the same number of luciferase expressing MSCs were transplanted. We confirmed that MSCs transplanted as aggregates existed in the regenerated meniscus focally and partially. Transplantation of aggregates of synovial MSCs regenerated meniscus more effectively in a rat massive meniscal defect.

1. Introduction

The meniscus mainly acts like a shock absorber in the knee joint [1]. For meniscus injury, efforts have been made to preserve the meniscus as much as possible to prevent degenerative arthritis. For meniscal defects after meniscectomy or meniscal degeneration, transplantations of meniscal graft or artificial meniscus have been attempted, however, the invasiveness, durability and safety of the transplant remain controversial [2]. A novel strategy is required for meniscus regeneration.

Mesenchymal stem cells (MSCs), especially those derived from synovium, are an attractive cell source for meniscus regeneration, because synovial MSCs have remarkable proliferation and chondrogenic potentials [3]. We previously reported that intraarticular injection of 5 million synovial MSCs in rat models promoted meniscal regeneration [4] and injection of fewer cells did not [5]. Simply, when the body weights of rats and humans are compared, 100 times more synovial MSCs are required in a clinical situation. However, preparation of 500 million human synovial MSCs is not practical. To address this problem, another strategy is needed.

The use of aggregates of synovial MSCs may be one of the solutions because aggregation of synovial MSCs increased chondrogenesis- and antiinflammatory-related gene expressions [6]. The purpose of this study was to investigate whether the use of aggregates of synovial MSCs regenerated meniscus more effectively in comparison with the use of suspension of synovial MSCs when the same number of the cells was used in a rat massive meniscectomized model. The number of synovial MSCs was adjusted to 25,000 and the effects of the "number per aggregate" and the "number of aggregates" were also examined from viewpoints of meniscal regeneration.

2. Materials and methods

2.1. Isolation and culture of rat synovial MSCs

This study was approved by the Animal Experimentation Committee of Tokyo Medical and Dental University. Synovial

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membranes of bilateral knee joints were harvested from wild type male Lewis rats (Charles River Laboratories Japan, Kanagawa, Japan) and transgenic rats expressing luciferase [7], LacZ or GFP at 12–14 weeks. The minced synovial membranes were digested for 3 h at 37 °C with type V collagenase (0.2%; Sigma–Aldrich, St. Louis, MO), and passed through a 45-μm filter (Becton Dickinson, Franklin Lakes, NJ). The digested cells were cultured in a complete culture medium (αMEM; Invitrogen, Carlsbad, CA; 10% FBS; Invitrogen; penicillin, streptomycin and amphotericin B; Invitrogen) in a normoxic condition for 14 days. The cells were then trypsinized, replated and cultured at 70% subconfluency. The characteristics of the MSCs were previously demonstrated [3,4].

For cell tracking, a fluorescent lipophilic tracer DiI (Molecular Probes, Eugene, OR) was used. The cells were suspended at 1×10^6 cells/ml in αMEM without FBS, and DiI was added at a final concentration of 5 μl/ml. After incubation for 20 min at 37 °C, the cells were washed twice.

2.2. Preparation of aggregates of synovial MSCs

500, 5000 or 25,000 synovial MSCs were suspended in 30 μl complete culture medium, and plated on an inverted culture dish lid. Then, the lid was inverted, and placed on a culture dish (Fig. 1A). The cells were cultured at 37 °C with 5% humidified CO_2 for 3 days in hanging drops.

2.3. Meniscectomy

Wild-type male Lewis rats at 12–14 weeks old were used. A straight incision was made on the anterior side of the bilateral knees, and the anterior insertional ligament of the medial meniscus was transected. Then, the anterior half of the medial meniscus was dislocated and resected at the level of the medial collateral ligament.

2.4. Transplantation of aggregates

The total cell number for transplantation per each knee was adjusted to 25,000 cells. "500 cells × 50 aggregates", "5000 cells × 5 aggregates" and "25,000 × 1 aggregate" were placed on the sites of meniscus defects. The suspension of 25,000 cells or 5,000,000 cells was injected into the knee joint in the suspension groups. No cells were transplanted in the untreated group. The rats were allowed to walk freely in each group, and they were sacrificed at 4 weeks ($n = 8$) and 12 weeks ($n = 4$).

2.5. Macroscopic observation

Meniscus was carefully separated from femoral and tibial condyles at 4 and 12 weeks after transplantation. Tibial condyles were stained with India ink to identify location, size and severity of cartilage degeneration. Macroscopic pictures were taken using an Olympus MVX 10 (Olympus, Tokyo, Japan). Quantification for the size of regenerated meniscus was performed using the software Image J (National Institutes of Health, Bethesda, Maryland).

2.6. Histological examination

Meniscus tissue was fixed in 4% paraformaldehyde for 7 days, decalcified in 20% ethylene–diamine–tetra-acetic acid (EDTA) solution for 7 days, respectively and embedded in paraffin wax. The specimens were sectioned in a sagittal plane at 5 μm and stained with safranin-o and fast green. Histological sections were visualized using an Olympus BX 53 microscope (Olympus, Tokyo, Japan). Regenerated meniscus was evaluated using the modified Pauli's score, in which a full score was 18 and a lower score indicated values closer to the normal meniscus [8].

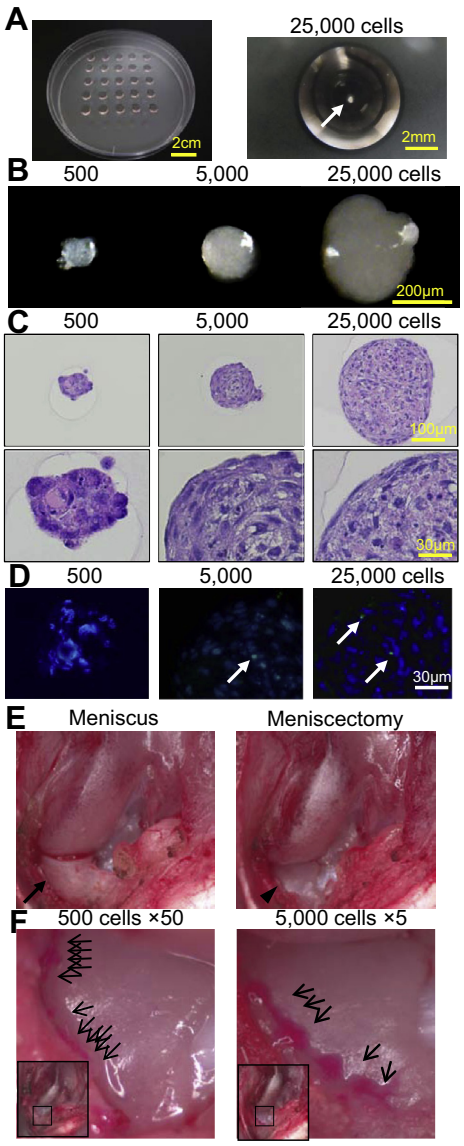

Fig. 1. Preparation, appearance and transplantation of aggregates of rat synovial MSCs. (A) Drops hanging on the cover of dish. Aggregates consisting of 25,000 MSCs are indicated with arrow. (B) Macroscopic images of aggregates consisting of 500, 5000 and 25,000 MSCs 3 days after cultured in hanging drops. (C) Histologies of aggregates stained with hematoxylin and eosin. (D) Aggregates stained with TUNEL (green) and Hoechst (blue). (E) Procedure for removal of meniscus. The medial meniscus (arrow) was exposed, and half of the meniscus was removed (arrow head). (F) Transplantation of aggregates of MSCs on the site of meniscal defects. MSCs were labeled with DiI, and the aggregates are indicated with arrows. (For interpretation of color in this figure legend, the reader is referred to the web version of this article).

2.7. Immunohistochemistry

Paraffin-embedded sections were deparaffinized in xylene and pretreated with 0.4 mg/ml proteinase K (DAKO, Carpinteria, CA) in Tris–HCl buffer for 15 min. Endogenous peroxidases were quenched by using 0.3% hydrogen peroxidase in methanol for 15 min. Nonspecific antigen was blocked by preincubation with PBS containing 10% normal horse serum (Vector Laboratories, Burlingame, CA) or rabbit serum (Vector Laboratories) for 20 min. A primary anti-rat monoclonal antibody against human type II collagen (1:200 in dilution, Daiichi Fine Chemical, Toyama, Japan) or a primary anti-rat polyclonal antibody against goat CXCR4 (1:500

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

2.8. LacZ staining

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

2.9. TUNEL staining

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

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

2.10. In vivo bioluminescent imaging

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

2.11. Fluorescent microscopic examination

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

2.12. Statistical analysis

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

3. Results

3.1. Appearance of aggregates of rat synovial MSCs

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

3.2. Meniscal regeneration by transplantation of aggregates of synovial MSCs

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

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

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

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

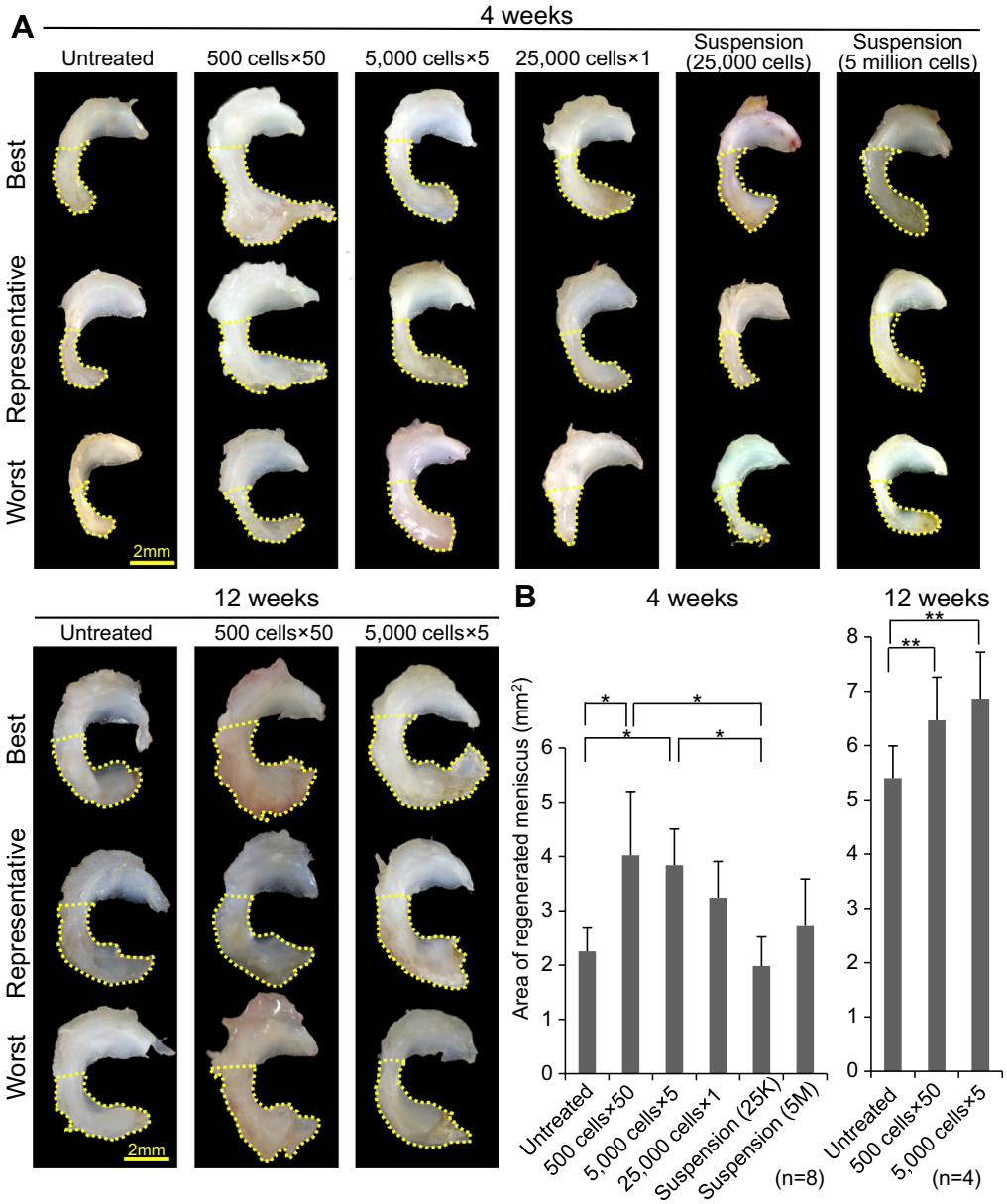

Fig. 2. Macroscopic analysis of regenerated menisci after transplantation of aggregates. (A) Macroscopic findings of the regenerated menisci at 4 and 12 weeks. Regenerated areas are surrounded with yellow dotted lines. (B) Quantification for areas of the regenerated menisci. Values are averages with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

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

3.4. Transplanted aggregates remain in the knee joint

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

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

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

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

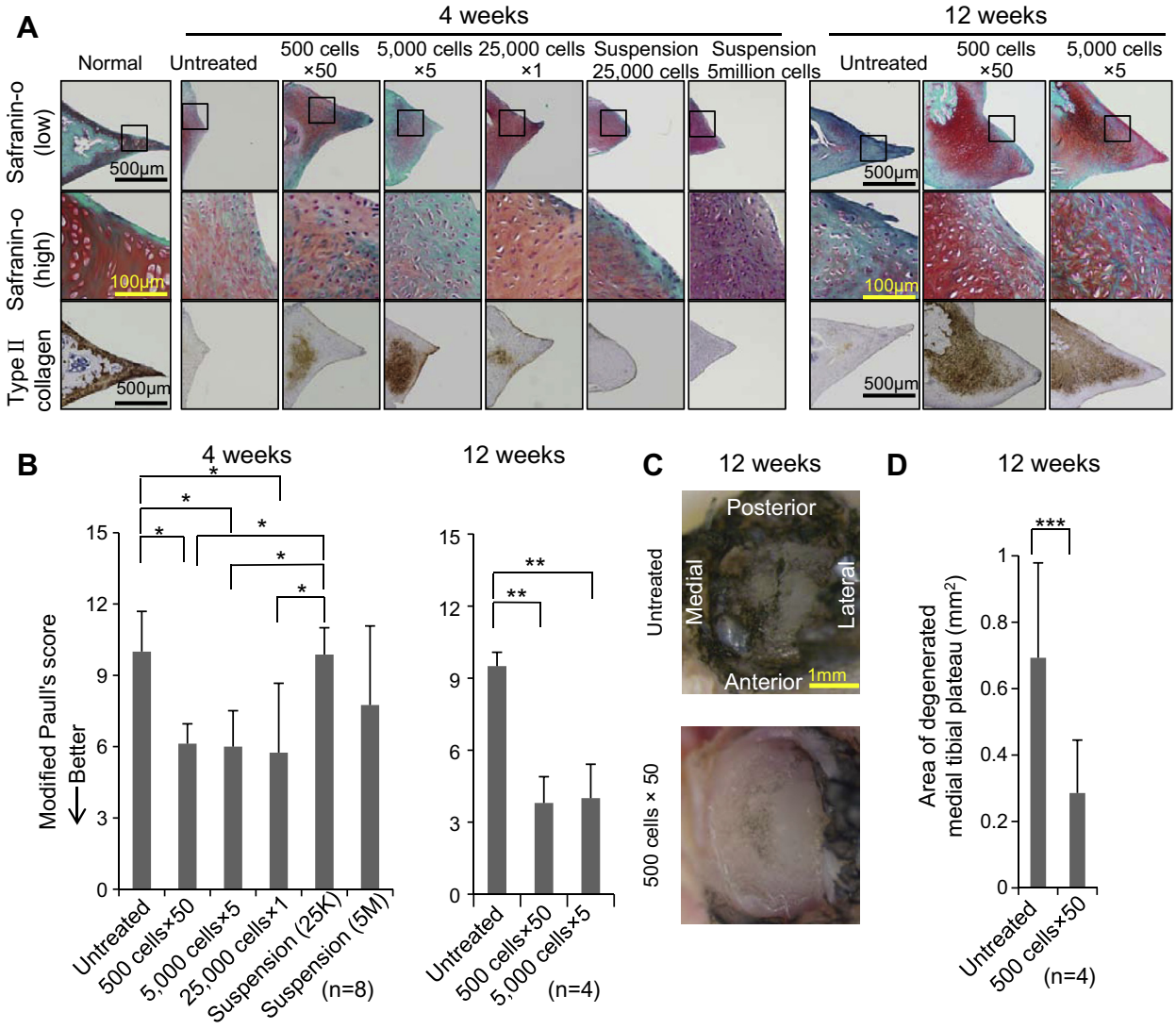

Fig. 3. Histological observation of regenerated menisci after transplantation of aggregates. (A) Representative sections of normal meniscus and regenerated menisci at 4 and 12 weeks. (B) Modified Pauli's score for histology. Values are averages with standard deviations ($n = 8$ at 4 weeks and $n = 4$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

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

3.5. Aggregates of synovial MSCs disperse shortly

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

4. Discussion

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

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

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

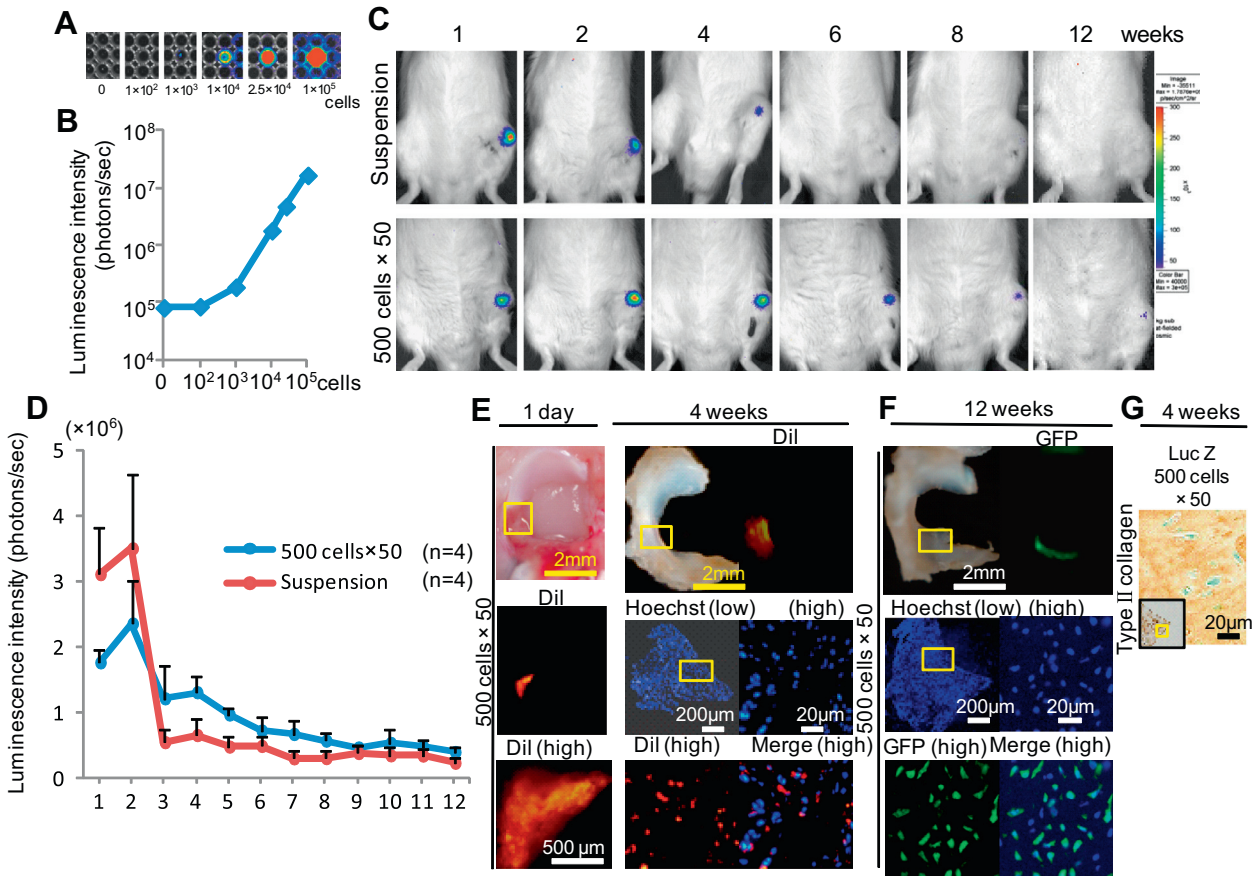

Fig. 4. Tracking the transplanted MSCs. (A) In vitro bioluminescent imaging of varying numbers of synovial MSCs from transgenic rats expressing luciferase. (B) Quantification for bioluminescent imaging. (C) Imaging of photons from MSCs expressing luciferase. 25,000 MSCs suspended in PBS were injected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

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

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

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

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

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

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

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

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

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

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

Acknowledgments

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Appendix A. Supplementary data

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

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