Scaffold-Free Tissue-Engineered Allogenic Adipose-Derived Stem Cells Promote Meniscus Healing

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Purpose: To determine whether meniscal tissue could be healed histologically by the implantation of allogenic three-dimensional formed adipose-derived stem cells (ADSCs) in a rabbit model of partial meniscectomy. **Methods:** Forty Japanese white rabbits (aged 15-17 weeks) were assigned to 2 groups. Defects 1.5 mm in diameter were created in the anterior horn of the medial menisci. The defects were left empty in the control group and were filled with cylindrical plugs of allogenic ADSCs extracted from adipose tissue in the experimental group. Macroscopic scoring (range, 0-3), histological scoring (range, 0-12), and immunohistological stainability of type I collagen were evaluated at 2, 4, 8, and 12 weeks postoperatively (n = 5 rabbits for each week). **Results:** Macroscopically, the height of the healing tissue in the experimental group was significantly greater than that of the control group at 2 weeks (3 vs 0, $P = .01$), 4 weeks (3 vs 1, $P = .01$), and 8 weeks (3 vs 2, $P = .02$). Histologically, safranin-O staining was noted at 2 weeks and increased gradually over time in the experimental group. In contrast, the intensity of staining was lower in controls at all weeks. Tissue quality scores were significantly higher in the experimental group than in the controls at all weeks (3 vs 0 at 2 weeks [$P = .00009$], 4.5 vs 2 at 4 weeks [$P = .00023$], 9 vs 5 at 8 weeks [$P = .0047$], 10.5 vs 6 at 12 weeks [$P = .00026$]). The implanted tissue was positive for type I collagen, and stainability was increased gradually over time. **Conclusions:** Three-dimensional scaffold-free allogenic ADSCs implanted into a 1.5-mm avascular meniscal defect survived, adhered to the defect, and promoted histological meniscus healing in a rabbit model. **Clinical Relevance:** ADSC implantation designed to promote meniscal healing may play an important role as a tool for meniscus healing.

Preservation of the menisci is important to prevent the accelerated degeneration of the knee joint. Therefore, arthroscopic meniscal repair is used to preserve meniscus function; however, there are limited operative indications and the outcome is variable.[1,2] In addition, clinical repair techniques such as rasping,[3] fibrin clot,[4] transforming growth factor-beta application,[5] and platelet-rich plasma (PRP) administration[6] have been used in an attempt to increase the healing potential of the menisci. Meniscus allograft transplantation is comparatively invasive and has limited indications.[7]

Recent studies have reported meniscus healing using mesenchymal stem cells (MSCs).[8-11] MSCs are found in adipose tissues,[12] which have been identified as an alternative source of human multipotent stem cells.[13] Adipose-derived stem cells (ADSCs) are relatively easy to isolate in large quantities. Only a small number of studies have reported the use of ADSCs for meniscus healing.[14-16]

Adherent cells are commonly cultured as a 2-dimensional (2D) monolayer using conventional tissue culture techniques. Various 3-dimensional (3D) culture systems have been developed to reflect the in vivo microenvironment more accurately.[17] The spheroid is a 3D structure formed by cell self-assembly to increase cell viability.[18,19] Compared with 2D cultures, 3D cultures are more representative of the in vivo microenvironment.[20,21] We created a columnar cell structure body consisting of spheroid aggregates of ADSCs using the nonscaffold method developed by Ishihara et al.[22]

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The purpose of this study was to determine whether meniscal tissue could be healed histologically by the implantation of an allogenic 3D formed ADSCs engrafted into the meniscal defect, in a rabbit model of partial meniscectomy. We hypothesized that ADSCs would adhere to meniscal defect and promote meniscus healing.

Methods

The experimental protocols were approved by the Institutional Animal Care and Use Committee. This study was approved by the local Advanced Science Research Center. A skeletally mature female Japanese white rabbit (Kitayama Labes, Nagano, Japan) was used as the cell donor.

Forty mature female Japanese white rabbits (aged 15-17 weeks) weighing 3.0 to 3.5 kg (mean, 3.2 kg) were used as recipients. The rabbits were divided into 2 groups according to treatment as follows: meniscal defects were left empty for the control group (20 rabbits) and allogenic 3D formed ADSCs were transplanted into the meniscal defect for the experiment group (20 rabbits). The sample size was determined by ethical restrictions on the number of experimental animals allowed and by information reported in a previous study.[23]

Isolation of ADSCs was performed according to a modified method, reported previously.[24] ADSCs were extracted from the adipose tissue of the interscapular fat pad[25] of the donor female; approximately 20 g of adipose tissue was harvested. The tissue was washed with phosphate-buffered saline and minced into small pieces using scissors. Tissue was then digested in 20 mL of 0.12% type I collagenase in phosphate-buffered saline for 45 minutes at 37°C in a water bath, with agitation every 15 minutes. The collagenase activity was neutralized by the addition of 20 mL Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (P/S), and the resulting solution was filtered and collected in a tube. The filtrate was centrifuged at 1,500 rpm for 5 minutes at 25°C, and the supernatant was discarded. The ADSC pellet was resuspended in 15 mL DMEM containing 10% FBS and P/S, and plated into 15-cm-diameter culture dishes at 5×10^5 cells/dish and incubated at 37°C and 5% CO_2 for a week. The culture medium was changed every 2 days to remove nonadherent cells. For subculture, when cells reached 80% to 90% confluence (2×10^6 cells/dish), they were detached with 0.05% trypsin.

Detached cells were then resuspended in DMEM with 10% FBS and P/S. To generate ADSC spheroid, cells were seeded on an ultralow attachment 96-well spheroid plate at 5×10^4 cells/well. Two days after incubation at 37°C and 5% CO_2, the cells aggregated into a spheroid formation approximately 700 μm in diameter.

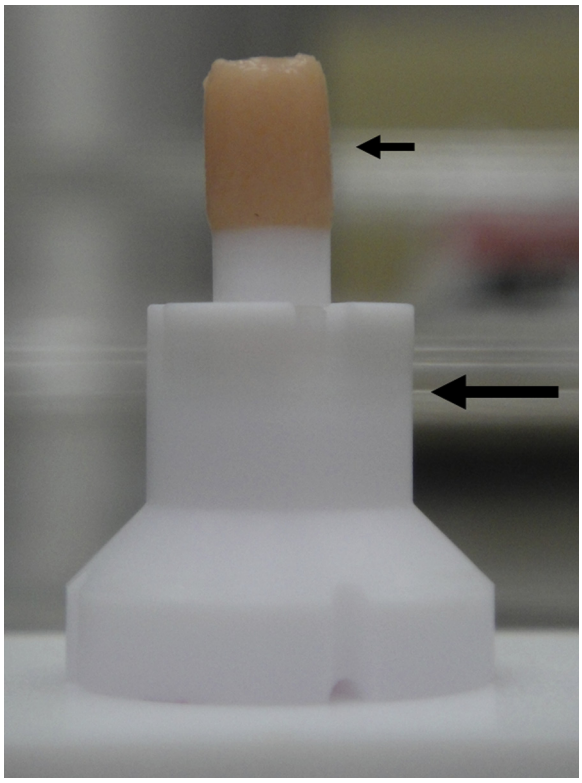

Fig 1. Formation of high-density mesenchymal stem cell, scaffold-free allograft constructs (HDMACs). A handmade cylindrical Teflon mold with an inner diameter of 4.6 mm and height 5 mm (long arrow). The loaded spheroids fused with each other, formatting of cylinder-shaped HDMACs (short arrow).

The spheroids were placed in a handmade tube-shaped Teflon mold with 4.6 mm diameter and 5 mm height (Yasojima Proceed, Japan). At the bottom of the mold, a perforated base disk was added to permit the circulation of medium. Four to five hundred spheroids were added to the mold that was placed into a larger container containing DMEM and the whole setup was incubated at 37°C and 5% CO_2. These loaded spheroids adhere to one another and formed a 3D network of ADSCs aggregated as a large structure after further culturing for several days (Fig 1). We named this structure "high-density mesenchymal stem cell, scaffold-free allograft constructs" (HDMACs).

Surgical Procedure

Anesthesia was achieved with a preoperative intramuscular injection of a mixture of 10 mg of midazolam and 1.0 mg of medetomidine, and a continuous intravenous injection of 5.4 mg/min sodium pentobarbital. Surgery was performed using aseptic techniques. Each animal was placed in the supine position on the operating table. A medial arthrotomy was performed longitudinally using a medial parapatellar surgical approach, and the patella was dislocated laterally. With maximum knee

flexion, the anterior horn of the medial meniscus was exposed and a 1.5-mm-diameter full-thickness circular defect was created in the avascular inner two-thirds[26] of the anterior horn of the medial meniscus of the left knee by biopsy punch.[6,23,27] For the experimental group, the HDMACs were hollowed out by biopsy punch and gently implanted into the defect. The part that protruded from the defect was eliminated carefully using tweezers. In the control group, the meniscal defect was left empty. Joint capsule and skin were sutured as separate layers using nonabsorbable sutures. Rabbits were allowed to move freely in individual cages with free access to water and food. They were euthanized 2, 4, 8, and 12 weeks after implantation to harvest the medial menisci and evaluate healed tissues.

There were no complications such as inflammation around the treated joint, and all rabbits were healthy until euthanization.

Macroscopic Evaluation

Macroscopic evaluation was graded in a semiquantitative assessment using the original scoring system of 0 to 3 points based on the height of the thinnest parts of the healing tissue at the meniscal defect as follows: 3 points, greater than two-thirds of the height of the meniscal defect; 2 points, two-thirds to one-third of the height of the meniscal defect; 1 point, less than one-third of the height of the meniscal defect; 0 points, grossly visible full-thickness defects remained.

Table 1. Histological Tissue Quality Score

Category	Points
1. Surface indentation	
Smooth	3
Slight fibrillation or slightly undulating	2
Moderate fibrillation or markedly undulating	1
Severe fibrillation or disruption	0
2. Cellularity of chondrocyte	
Normal cell distribution	3
Moderately normal cell distribution	2
Hypercellularity or hypocellularity	1
No chondrocyte	0
3. Collagen fiber organization	
Collagen fibers well organized, no separations or tears	3
Collagen fibers moderately organized, moderate separations or tears	2
Collagen fiber unorganized, moderate separations or tears	1
Collagen fiber unorganized, severe separations or tears	0
4. Matrix staining (safranin-O)	
Well stained like normal meniscus	3
Moderately stained	2
Slightly moderately stained	1
Slightly stained	0

NOTE. Quantification of histological findings evaluated by modified Pauli's scoring system.[28] The maximum possible score is 12 points. The total score for an unhealed meniscus is 0 points.

Histological Evaluation

Meniscal specimens were fixed in 4% paraformaldehyde, decalcified in 10% ethylenediaminetetraacetic acid solution in accordance with previous studies,[9,23,27] and embedded in paraffin. Specimens were sectioned into 5-μm-thick slices from the center of the meniscal defect in the radial plane, and stained with H&E and safranin-O (2 sections for each). The reparative tissue was evaluated using the modified Pauli's score[28] (Table 1). The quality assessment items were as follows: (1) surface indentation of healed tissue; (2) chondrocyte cellularity; (3) collagen fiber organization; and (4) safranin-O staining intensity. The maximum score was 12 points, 3 for each category. Histological scoring was performed by 2 investigators (J.N. and H.N.) in a blinded manner, and the final score was taken as the average of the 2 investigators' scores.

To detect type I collagen synthesis and localization of healed cells, 3 more paraffin sections from the experimental group were prepared with the healed tissues in the radial plane. The presence of type I collagen was evaluated by staining with goat anti-type I collagen polyclonal antibodies (1:20 dilution; Southern Biotechnology Associates, Birmingham, AL).

To confirm the survival potential and location of transplanted cells, ADSCs were labeled with 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI), which binds to cellular thiols and has long-term stability enabling the tracing of DiI-labeled transplanted cells in the host tissue. Two weeks after transplantation of the labeled HDMACs, frozen sections were prepared in the radial plane. The sections were stained with H&E and the survival of the transplanted cells was determined.

Statistical Analysis

Experimental results are presented as the median and range. The semiquantitative macroscopic scores and histological tissue quality scores were compared between the 2 groups using the Mann-Whitney *U* test. In histological evaluations, interobserver reliability was assessed using 2-way random intraclass correlation coefficients and their 95% confidence intervals were calculated. Differences were considered to be statistically significant when $P < .05$.

Results

No postoperative complications or significant weight changes were observed in this study, and all rabbits were in good health during the experimental period.

Macroscopic Evaluation

The gross findings of the meniscal defects for each group are shown in Figure 2.

Two weeks after implantation in the control group, 1 of 5 specimens had less than two-thirds of the height of the healing tissue (2 points), 1 specimen had less than

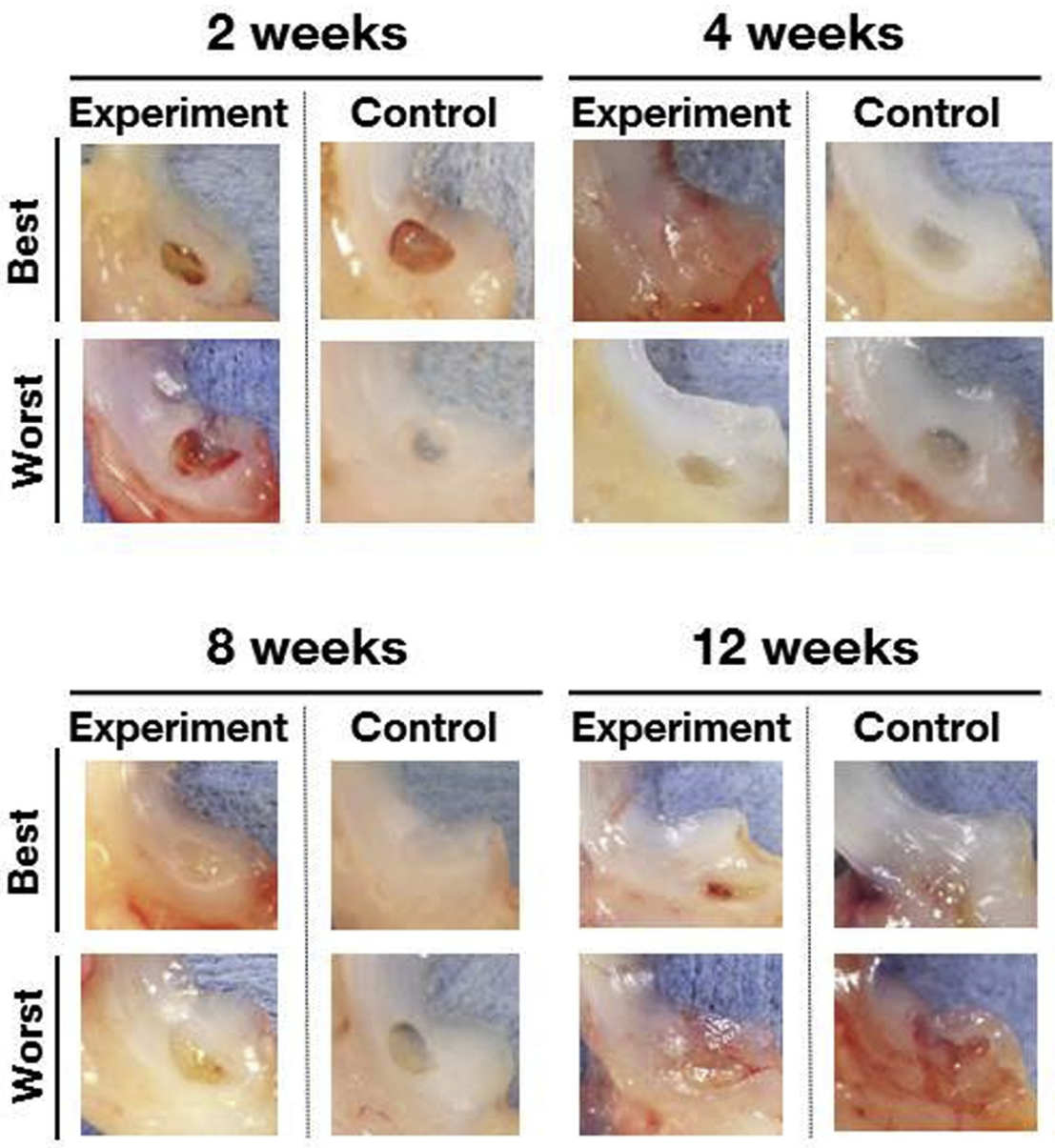

Fig 2. Macroscopic findings of the control and experimental groups. Features of the anterior horn of the medial meniscus are shown. The "best" and "worst" specimens of each week were selected as representatives.

one-third of the height of the healing tissue (1 point), and 3 specimens had grossly visible meniscal defects remaining (0 points), with an overall score of 0 (0-2). Four weeks after implantation, 1 specimen had less than two-thirds of the height of the healing tissue (2 points), 2 specimens had less than one-third of the height of the healing tissue (1 point), and 2 specimens had the defects remaining (0 points), with an overall score of 1 (0-2). Eight weeks after implantation, 1 specimen had more than two-thirds of the height of the healing tissue (3 points), 2 specimens had less than two-thirds of the height of the healing tissue (2 points), and 2 specimens had defects remaining the defects (0 points), with an overall score of 2 (0-3). At 12 weeks after implantation, 3 specimens had more than two-thirds of the height of the healing tissue (3 points), 1 specimen had less than two-thirds of the height of the healing tissue (1 point), and 1 specimen had defects remaining (0 points), with an overall score of 3 (0-3). In contrast, 2 weeks after implantation in the experimental group, 3 of 5 specimens had more than two-thirds of the height of the healing tissue (3 points), and 2 specimens had less than two-thirds of the height of the healing tissue (2 points), with an overall score of 3 (2-3). Four weeks after implantation, 4 specimens had more than two-thirds of the height of the healing tissue (3 points), and 1 specimen had less than two-thirds of the height of the healing tissue (2 points),

Table 2. Semiquantitative Analysis of Macroscopic Findings

	Score		
	Experiment	Control	*P* Value
2 wk	3 (2-3)	0 (0-2)	.01
4 wk	3 (2-3)	1 (0-2)	.01
8 wk	3 (3-3)	2 (0-3)	.02
12 wk	3 (3-3)	3 (0-3)	.13

NOTE. Data are presented as the median (range).

with an overall score of 3 (2-3). All specimens taken later (8 weeks after implantation) had more than two-thirds of the height of the healing tissue at any time point, giving an overall score of 3 (all 3) (Table 2).

Histological Evaluation

Representative sections of the reparative meniscal tissue stained with safranin-O at 2, 4, 8, and 12 weeks for the experimental and control groups are shown in Figure 3A and a nondefect meniscus section stained with safranin-O is shown in Figure 3B. In the experimental group, safranin-O staining was noted at 2 weeks and increased gradually over time. In contrast, in the control group, although the defect was partially repaired via spontaneous healing, the healed tissue had less safranin-O staining than that in the experimental group at all weeks.

Furthermore, compared with the control group, the histological tissue quality score was significantly higher in the experimental group across all time points ($P < .05$), and increased in a time-dependent manner (Table 3). The interobserver reliability was 0.85 and 95% confidence intervals were 0.76 to 0.93.

Immunohistochemical Evaluation

Type I collagen expression in the healing tissue of the meniscal defect for the experimental group is shown in Figure 4A, and type I collagen expression for a nondefect meniscus is shown in Figure 4B. Healed tissue was positive for type I collagen and the extent of staining increased gradually over time, approaching that of the nondefect meniscus by 12 weeks after implantation in the experimental group. However, collagen expression was barely detectable in the controls.

DiI Labeling

Two weeks after implantation of HDMACs labeled with DiI, macroscopically the meniscal defect was filled. In the radial histological sections, DiI-positive areas could be detected and were confined to the meniscal defect (Fig 5).

The Power Analysis

The post hoc power analysis was performed and the results showed that the power of testing was 0.7 in statistical tests.

Discussion

Allogenic ADSCs implanted into avascular meniscal defects survived and adhered to the meniscal defects.

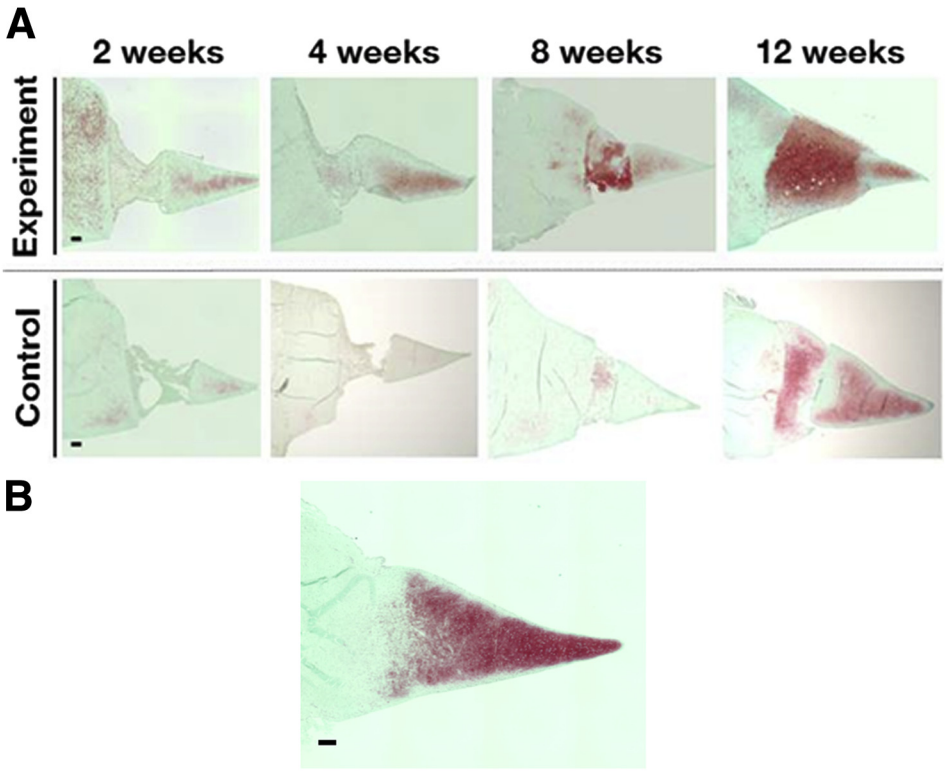

Fig 3. Histological findings with safranin-O staining. (A) Representative sections of healed menisci stained with safranin-O at 2, 4, 8, and 12 weeks after implantation of high-density mesenchymal stem cell, scaffold-free allograft constructs. Scale bar: 200 μm. (B) Representative sections of a normal rabbit meniscus stained with safranin-O. Scale bar: 200 μm.

Table 3. Histological Tissue Quality Score (0-12 Points)

	Score		
	Experiment	Control	*P* Value
2 wk	3 (2-5)	0 (0-2)	.00009
4 wk	4.5 (4-8)	2 (1-4)	.00023
8 wk	9 (4-12)	5 (1-7)	.00470
12 wk	10.5 (8-12)	6 (4-11)	.00026

NOTE. The difference between the experimental and control groups was significant at all time points ($P < .05$). Data are presented as the median (range).

The gross findings revealed higher semiquantitative scores in the experimental group than in controls. The resulting tissue showed increased safranin-O staining over time and a higher histological tissue quality score than that of controls. This tissue was positive for type I collagen, which also increased gradually over time. The survival of implanted cells was confirmed by DiI labeling with DiI-positive areas detected in the implant site.

Many attempts to improve the healing of the meniscal avascular zone using MSCs have been reported. Horie et al.[23] reported that implanted allogenic synovial MSCs adhered to the injury site and differentiated into cells similar to native meniscal fibrochondrocytes, enhancing both the quality and quantity of regenerated meniscal tissue in a rabbit model meniscal avascular zone injury. Hatsushika et al.[9,29] reported that intra-articular injection of synovial MSCs appeared to promote meniscus regeneration and protected articular cartilage in a massive meniscus defect model. Katagiri et al.[30] reported that transplantation of aggregates of synovial MSCs regenerated the meniscus more effectively than intra-articular injection of synovial MSCs. Duygulu et al.[11] reported that intra-articular administration of autologous bone marrow aspirate improved healing of meniscal tears in a sheep model as determined by both light and electron microscopic findings. Zellner et al.[31] compared the outcomes of PRP and bone marrow stem cells (BMSCs) in a rabbit model. They observed that the combination of biodegradable composite matrices and BMSCs was more effective than the combination of composite matrices and PRP in the repair avascular meniscal tear. Shen et al.[10] conducted intra-articular injection of meniscus-derived stem cells to treat massive meniscus defects in rabbits. They reported that allogenic meniscal stem cells promoted meniscus regeneration, with effective protection of the joint surface and maintenance of the joint space width. However, only 3 reported that transplanted ADSCs facilitated meniscal repair in the rabbit.[14-16]

The outcomes of all of these studies are concordant with that in the present study, wherein MSCs used in the absence of a scaffold provided a beneficial healing effect in the meniscal avascular zone.

Supplementation of artificial scaffolds with MSCs has also facilitated meniscal repair,[32,33] but scaffolds potentially influence the surrounding microenvironment, which could affect the regional specification of implanted MSCs.[34] In addition, nonphysiological scaffolds might disturb the physiologic circulation of signaling molecules such as growth factors, which might influence cellular differentiation.[22]

ADSCs have several advantages as a source of tissue stem cells that led us to adopt them for use in this study.

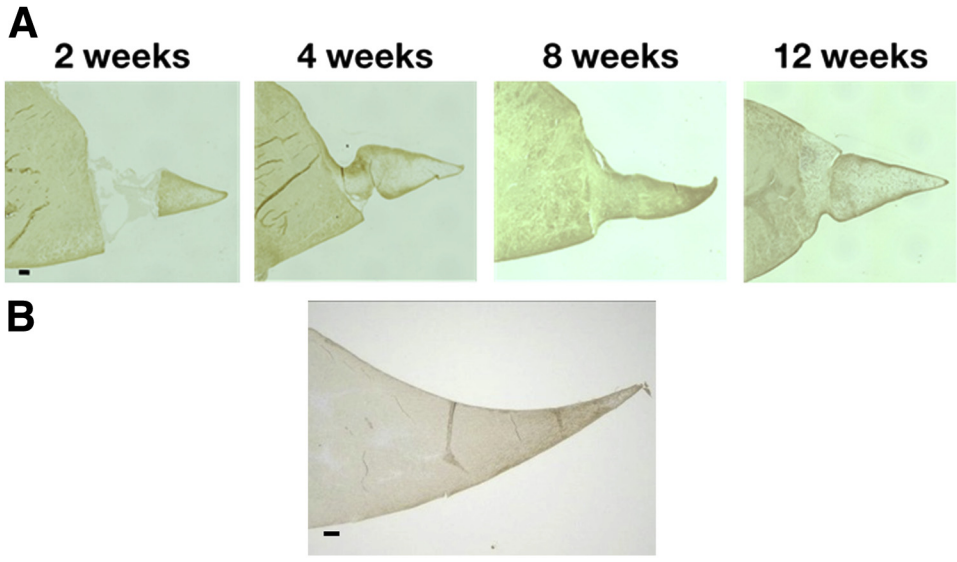

Fig 4. Immunohistological findings with type I collagen in the experimental group. (A) Representative sections of the regenerated meniscus stained with type I collagen at 2, 4, 8, and 12 weeks after implantation of high-density mesenchymal stem cell, scaffold-free allograft constructs (HDMACs). Implanted HDMACs were not stained well at 2 weeks after implantation. After 4 weeks, more staining is seen in the healed meniscal tissue and the stainability gradually increased over time. Scale bar: 200 μm. (B) Representative sections of a normal rabbit meniscus stained with type I collagen. Scale bar: 200 μm.

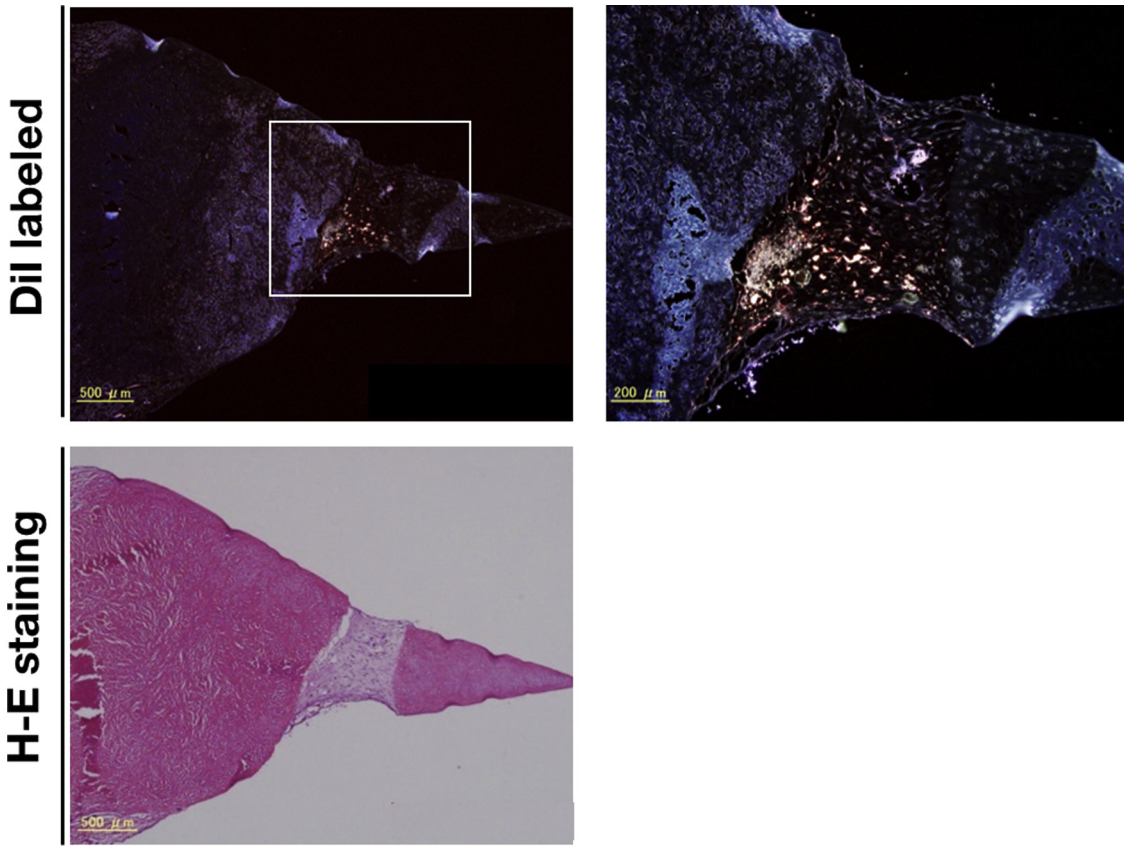

Fig 5. DiI labeling cell tracking. High-density mesenchymal stem cell, scaffold-free allograft constructs labeled with DiI were implanted into the meniscal defect. The labeled cells were partially detected and remained in the meniscal defect 2 weeks after implantation.

Harvesting of ADSCs is less invasive than that of BMSCs; many more stem cells can be harvested at one time, and ADSCs can be easily isolated in large quantities with good viability.[24,35,36] In addition, a previous study indicated that ADSCs have multipotency properties similar to those of BMSCs.[37] Moreover, the use of ADSCs has been shown clinically in cases of breast reconstruction and augmentation,[38,39] tracheal fistula,[40] Crohn's fistula,[41] and cranial bone defects.[42]

HDMACs can be made without a scaffold and using any differentiation agents, such as growth factors, and can be implanted into the meniscal defect. Furthermore, HDMACs composed of spheroid aggregates are thought to be structurally stable because the intracellular microenvironment of the spheroids is similar to that in vivo, and spheroids can be maintained over long periods with self-differentiation and structural self-assembly.[43-45]

Although the potential for ADSCs to differentiate into various tissues seems to be limitless, it is not clear how transplanted ADSCs promote meniscal healing and repair. Tissue healing might be promoted by paracrine effects because ADSCs secrete various growth factors that can regulate neovascularization and immunosuppression.[46-48] Ishihara et al.[22] reported that after implantation of an undifferentiated MSC 3D construct in an osteochondral defect in rabbits, the MSCs differentiated into bone and cartilage and maintained the original border between them. They concluded that the implanted undifferentiated MSCs spontaneously differentiated into articular cartilage and subchondral bone. We did not investigate the differentiation mechanisms of ADSCs. Therefore, we could not comment on whether the healing tissue in the meniscus defects originated from the ADSCs themselves or from cells recruited locally.

Limitations

This study had some limitations. The meniscus structure and healing capacity differ between rabbits and humans.[26] Therefore, the results obtained from this animal model cannot be assumed to be directly applicable to humans. The post hoc power analysis showed that this study did not have a strong power of testing, and the small sample size limits the statistical rigor of the findings and efficacy conclusions. However, we did study multiple time points and thus had information regarding changes with time, and the blinded manner avoided biases in assessment. In addition, we did not evaluate the mechanical properties of the healing tissue

or the effect on the cartilage of the tibia and femur. Although meniscal healing was detected histologically, we are thus unable to comment on whether the healing tissue at the meniscal defect plays a role in meniscal function. In addition, we did not investigate the viability of cells in the HDMACs. Clinically speaking, cylindrical meniscal defects do not necessarily reflect meniscal injuries, because the defects in this model were completely surrounded by meniscal tissue and mechanically protected. Moreover, we did not perform statistical analysis of the representative sections of the healed or normal meniscus. Also, we did not conduct quantitative assessments, statistical analysis, or grading scales in our evaluation of immunohistochemical results or DiI-labeled HDMACs. Furthermore, the absence of a comparator decreased the validity of the present study because we could not assess the superiority of 3D scaffold-free HDMACs to other methods of ADSC implantation.

Finally, we did not investigate the mechanisms underlying meniscal healing.

Conclusions

Three-dimensional scaffold-free allogenic ADSCs implanted into a 1.5-mm avascular meniscal defect survived, adhered to the defect, and promoted histological meniscus healing in a rabbit model.

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