
Meniscal regeneration using tissue engineering with a scaffold derived from a rat meniscus and mesenchymal stromal cells derived from rat bone marrow

Takuma Yamasaki, Masataka Deie, Rikuo Shinomiya, Yasunori Izuta, Yuji Yasunaga, Shinobu Yanada, Patrick Sharman, Mitsuo Ochi

Department of Orthopaedic Surgery, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima, 734-8551, Japan

Received 26 August 2004; revised 31 January 2005; accepted 8 February 2005

Published online 27 July 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.30369

Abstract: The purpose of this study was to regenerate a meniscus using a scaffold from a normal meniscus and mesenchymal stromal cells derived from bone marrow (BM-MSCs). Thirty Sprague-Dawley rat menisci were excised and freeze-thawed three times with liquid nitrogen to kill the original meniscal cells. Bone marrow was aspirated from enhanced green fluorescent protein transgenic Sprague-Dawley rats. BM-MSCs were isolated, cultured for 2 weeks, and 2×10^5 cells were then seeded onto the meniscal scaffolds. Using a fluorescent microscope and immunohistochemical staining, repopulation of enhanced green fluorescent protein positive cells was observed in the superficial zone of the scaffold after 1 week of culture, and then in the

deep zone after 2 weeks. At 4 weeks, expression of extracellular matrices was detected histologically and expression of mRNA for aggrecan and type X collagen was detected. Stiffness of the cultured tissue, assessed by the indentation stiffness test, had increased significantly after 2 weeks in culture, and approximated the stiffness of a normal meniscus. From this study, we conclude that a scaffold derived from a normal meniscus seeded with BM-MSCs can form a meniscus approximating a normal meniscus. © 2005 Wiley Periodicals, Inc. *J Biomed Mater Res* 75A: 23–30, 2005

Key words: meniscus; scaffold; tissue engineering; mesenchymal cell; bone marrow

INTRODUCTION

The meniscus has a key role in the function of the knee joint, and loss of meniscus frequently leads to osteoarthritis and irreversible joint damage.^{1–6} Until now, repair has been recommended for torn menisci, but most degenerated torn menisci have been resected because the intrinsic healing capacity of an injured meniscus is limited.^{2,3} There have been studies that have replaced non-functional menisci with artificial materials, autogenous tissue, or allograft tissue. However, such treatments have had controversial results.^{7–9}

In recent years, meniscal regenerations using tissue engineering have been attempted.^{5,7,10–15} There have been several fundamental biological concerns about this technique including cell source, matrix scaffold, bioreactor considerations, and environmental conditions.⁷ We believe that the two major problems with the strategy of meniscal regeneration are cell source and meniscal scaffold.

Concerning cell source, meniscal chondrocytes, mesenchymal cells, and pluripotential fibroblasts have all been identified as potential sources for the regeneration of meniscal tissue in the hope that these cells could synthesize appropriate extracellular matrices and restore meniscal function.^{5,7,10–15} Among these cell sources, we focused on mesenchymal stromal cells derived from bone marrow (BM-MSCs) because BM-MSCs are believed to include pluripotent progenitor cells and are easily available.^{7,15,16}

As regards scaffold, many different materials have been evaluated as scaffolds for meniscal repair or regeneration, including artificial materials, autogenous tissues, and allograft tissues,^{4,5,7,10,11,13–15,17–24} but on the whole these materials and tissues generally have not yielded successful results. A normal meniscus has an extremely complex biochemical composition,⁴ comprising fibers orientated in various directions, thus enabling the meniscus to effectively respond to the different stresses experienced during load.¹⁴ Therefore, if a similar structure was to be built, regeneration of a more functional meniscus would be expected. We considered a scaffold derived from a normal meniscus to have function and composition approximating a normal meniscus.

Correspondence to: T. Yamasaki; e-mail: tacyama@hiroshima-u.ac.jp

Consequently, we hypothesized that meniscus could be best regenerated using a scaffold derived from a normal rat meniscus seeded with rat BM-MSCs. The purpose of this study was to confirm meniscal regeneration using BM-MSCs under *in vitro* conditions, and to determine the efficacy of the scaffold derived from a normal meniscus.

MATERIALS AND METHODS

Preparation of scaffolds

The protocol of this experimental study was approved by the Ethical Committee of Hiroshima University. Fourteen-week-old male Sprague-Dawley rats were anesthetized with sodium pentobarbital (40 mg/kg body weight). A total of 30 menisci were carefully excised under sterile conditions from the rat knees using the medial parapatellar approach. Each excised meniscus was treated with 10% sodium ethylenediaminetetraacetic acid at 4°C for 3 days for the purpose of decalcification. Each meniscus was then washed twice with phosphate-buffered saline, dried on a clean bench, and freeze-thawed 3 times to kill all the meniscal cells. For freeze-thawing, each meniscus was treated with liquid nitrogen (−196°C) for 10 min and rapidly thawed with sterile saline at 37°C.¹⁸ The freeze-thawed menisci were subsequently preserved at −80°C.

Isolation and expansion of BM-MSCs

Transgenic Sprague-Dawley rats carrying an enhanced green fluorescent protein (EGFP) gene (EGFP rats), developed by Research Institute for Microbial Diseases of Osaka University, were used as the source of BM-MSCs.²⁵ Ten-week-old EGFP rats were anesthetized with sodium pentobarbital (40 mg/kg body weight). Bone marrow aspirates were obtained from both tibia by penetrating the anteromedial aspect of the proximal tibia with a 23-gauge needle fastened to a 10-mL syringe containing 1 mL of heparinized saline. The aspirates were washed in Dulbecco's modified Eagle medium-high glucose (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Sigma, St. Louis, MO), antibiotic, and antimycotic solution (Gibco). The marrow suspension was seeded on 100-mm dishes and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂. To remove the nonadherent cells, the medium was changed on the fifth day after seeding, and it was changed again every third or fourth day thereafter. After 10–14 days of primary culture, when the proliferating colonies had nearly reached confluence, the adherent cells were harvested with 0.25% trypsin-ethylenediaminetetraacetic acid. The cells were counted, and reseeded at approximately 4×10^5 cells per 100-mm dish. After 7 days of subculture, when the cells had nearly reached confluence, they were harvested as described above.

Seeding of BM-MSCs into the scaffold

The harvested BM-MSCs were resuspended in the complete medium at a concentration of 5×10^6 cells/mL, then 40 μ L of cell suspension (2×10^5 cells) was seeded onto the scaffold in a 48-well plate. After cultivation for 2 h in a humidified atmosphere of 95% air and 5% CO₂ at 37°C, 200 μ L of the medium was added slowly into each well. The medium was changed twice a week, and the tissues were cultured for 1 ($n = 10$), 2 ($n = 10$), and 4 ($n = 10$) weeks, respectively.

As an experimental control, scaffolds without BM-MSCs were cultured for the same time periods.

Histological evaluation

After its designated time in culture, each tissue was fixed with 10% neutral buffered formalin for 12 h. Each specimen was then sectioned horizontally, and the sections were subsequently stained with toluidine blue (TB), and safranin O-fast green (SO) for histological examinations, and anti-EGFP antibody (a-EGFP) (JL-8; BD Biosciences Clontech, Franklin Lakes, NJ) for immunohistochemical staining. The positive patterns of TB and SO were determined by the presence of metachromatism. The sections were also observed with a fluorescent microscope (Leica DM IRB 470 nm ultraviolet, Wetzlar, Germany) to observe EGFP-positive cells.

Analysis of mRNA for extracellular matrices and collagen with reverse transcriptase-polymerase chain reaction (RT-PCR)

Each cultured tissue was digested with 0.25% collagenase at 37°C for 4 h. Total mRNA was extracted with a commercial kit (RNeasy Mini Kit; QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. The purity and amount of isolated RNA were assessed by spectrophotometric measurement at 260 and 280 nm. Total RNA was reverse transcribed to cDNA at 50°C for 50 min in a volume of 20 μ L containing 1 μ L of 10 mM dNTP mix, 1 μ L of 10 mM dithiothreitol, 1 μ L of 50 μ M oligo(dT), 4 μ L of 5X first strand buffer, 1 μ L of RNase OUT, and 200 U of Superscript III (RNase H-free RT) (all from Gibco). After terminating the reaction at 70°C for 15 min, 2 U of RNase H was added to the reaction mixture, followed by incubation at 37°C for 20 min to remove the RNA. The cDNA was diluted 1:500 and then amplified in 50 μ L of a PCR mixture containing 5 μ L of Taq buffer, 4 μ L of MgCl₂, 4 μ L of 10 mM dNTP mix, 1.25 U of Taq polymerase, and primer sets. Primers for aggrecan (sense: 5'-TAGAGAAGAAGAGGGGTTAGG-3', anti-sense: 5'-AGCACTAGGAGCCAGGGTTAT-3', 321 bp), type II collagen (sense: 5'-GGTG GTTA-TGACTTTGGTTAC-3', anti-sense: 5'-CAGGCGTGATGGCTTATTTGT-3', 681 bp), type II collagen (sense: 5'-GAAGCACATCTGGTTTGGAG-3', anti-sense: 5'-TTGG-GGTTGAGGGTTTTACA-3', 447 bp), type X collagen (sense: 5'-ACAAAGAGCGGA-CAGAGACC-3',

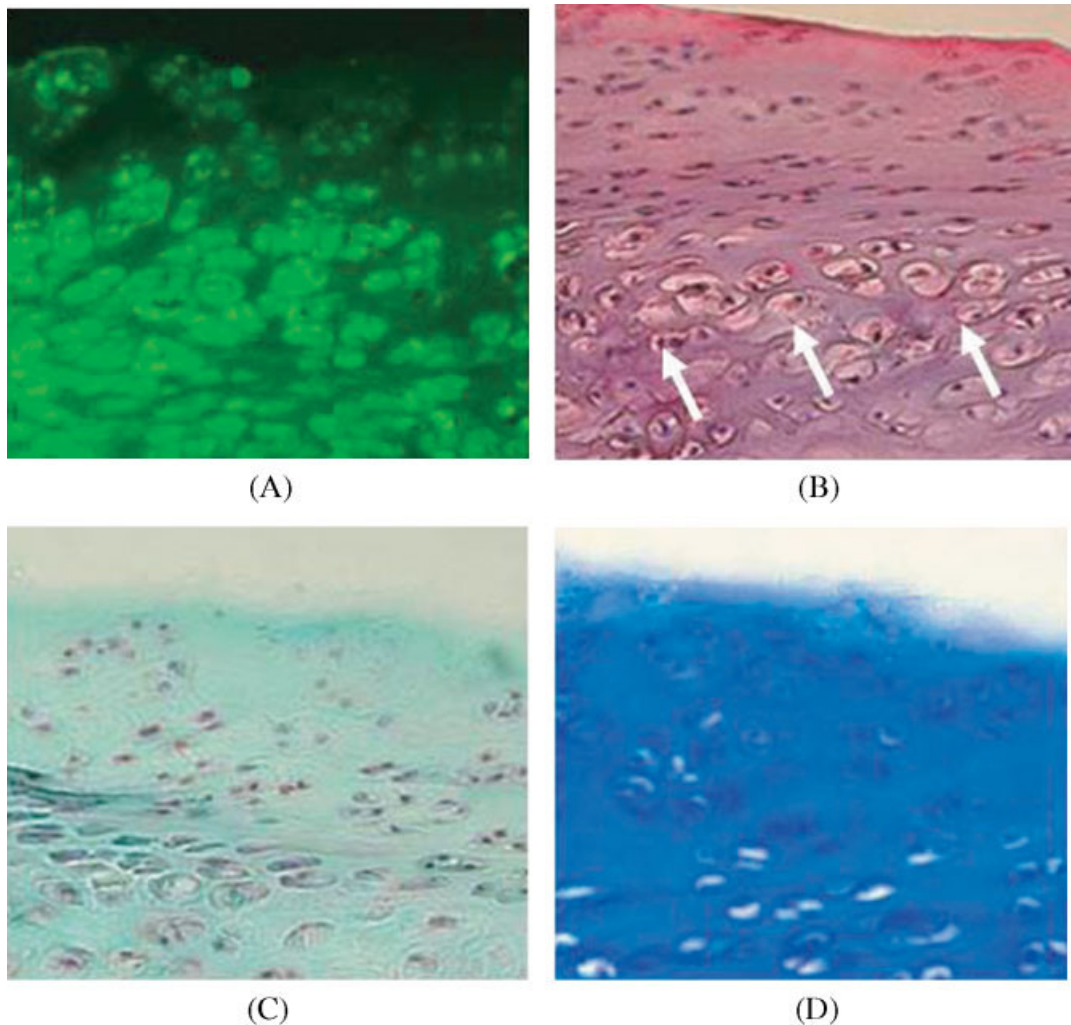


Figure 1. Photomicrographs after 1 week in culture. (A) The cells repopulated in the scaffold retained green fluorescence under the fluorescent microscope (original magnification, $\times 400$). (B) GFP-positive cells were confirmed with a-GFP staining (original magnification, $\times 400$). (C) Expression of extracellular matrices was not detected with SO or (D) TB staining (original magnification, $\times 400$).

anti-sense: 5'-AGAAGGACGAGTGGACATAC-3', 441 bp), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (sense: 5'-GTCTTCACCACC-ATGGAGAAGGCTG-3', anti-sense: 5'-TGAGGTCCACCACCCTGTTGCTGTA-3', 449 bp) were prepared according to published DNA sequences (GenBank).²⁶ PCR was performed in a Minicycler (MJ Research Inc., Watertown, MA), including an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C (type I and X collagen) and 56°C (type II collagen and aggrecan) for 1 min, and extension at 72°C for 1 min. The final cycle included 5 min for extension. The PCR products were analyzed by electrophoresis in 2% agarose gels and stained with ethidium bromide. The 100-bp ladder (Qiagen) was used as a molecular weight marker.

Evaluation of stiffness of the cultured tissues

Stiffness of the cultured tissues was measured at 1, 2, and 4 weeks with a tactile sensor system (Biosenser System®;

AXIOM Co., Fukushima, Japan) that could quantitatively assess the degree of softness or hardness of materials via an ultrasonic tactile sensor.²⁷ Stiffness was evaluated at the posterior region of the cultured tissue at 1, 2, and 4 weeks, because the menisci of Sprague-Dawley rats were found to be highly calcified, especially at the anterior region of the menisci.²⁸ As a control, stiffness of scaffolds and of normal rat menisci were measured. As a statistical analysis, all the quantitative data for stiffness were presented as the mean value ± 1 standard deviation. The results were evaluated using Mann-Whitney's *U* test. The level of significance was set at $p < 0.05$.

RESULTS

Histological observation

After 1 week in culture, observation through the light microscope revealed that the scaffold was re-

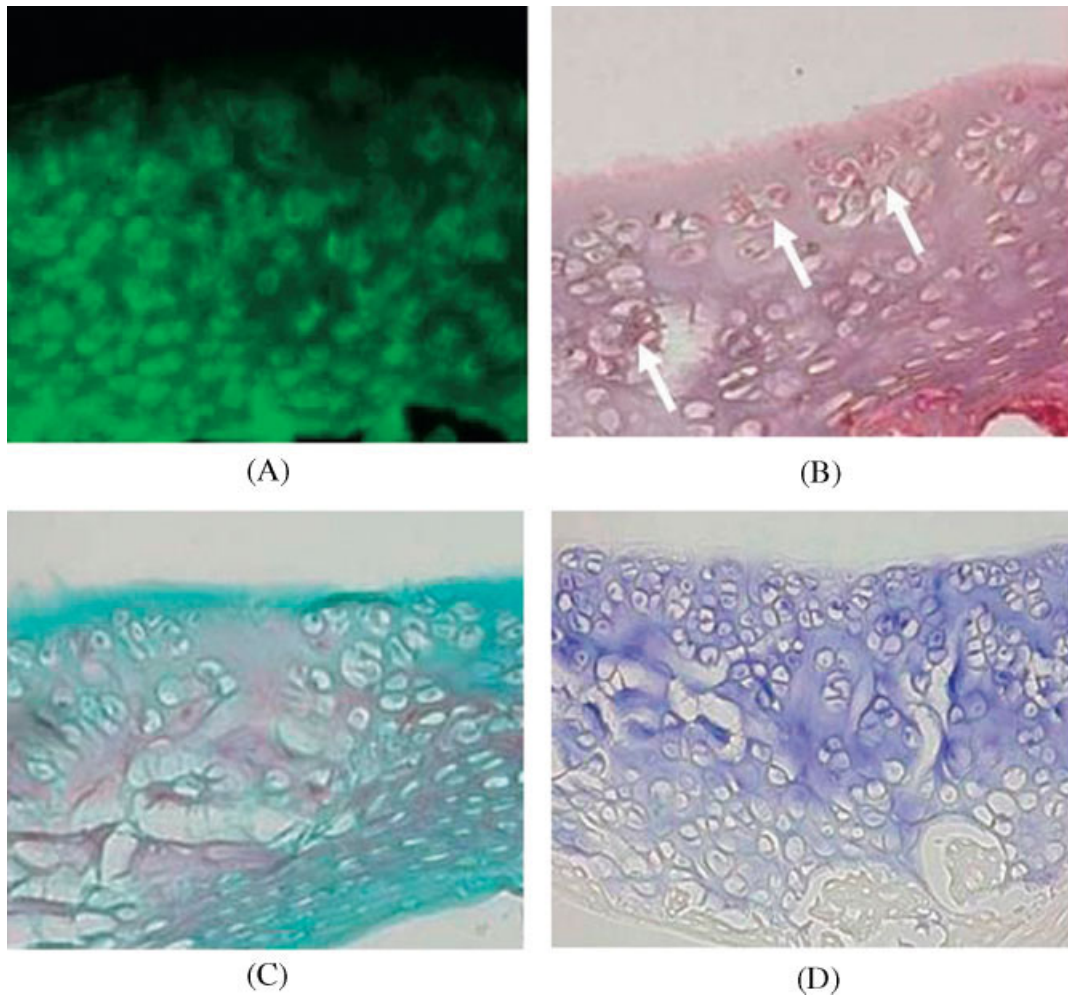


Figure 2. Photomicrographs at 2 weeks in culture. (A, B) Repopulation of GFP-positive cells was observed at the peripheral border of the scaffold (original magnification, $\times 400$). (C) Expression of extracellular matrices was partially detected with SO and (D) TB staining (original magnification, $\times 400$).

populated with round cells and spindle-shaped cells. Repopulation with round cells that were similar to the meniscal chondrocytes was observed at the free edge of the scaffold. Observation through the fluorescent microscope showed that both the round cells and the spindle-shaped cells retained green autofluorescence, demonstrating the presence of EGFP cells [Fig. 1(A)]. a-EGFP staining proved these cells to be EGFP positive [Fig. 1(B)]. Therefore, the round cells were thought to originate from EGFP rat BM-MSCs. However, SO and TB staining scarcely detected metachromatism in extracellular matrices [Fig. 1(C,D)]. At 2 weeks, repopulation of EGFP-positive cells was also observed at the peripheral border of the scaffold [Fig. 2(A,B)], and expression of extracellular matrices was partially detected histologically [Fig. 2(C,D)]. At 4 weeks, both repopulation of EGFP-positive cells and expression of extracellular matrices had expanded from the free edge to the peripheral border of the scaffold [Fig. 3(A-D)]. In the control tissue, neither repopulation

of EGFP-positive cells nor expression of extracellular matrices was detected, and the histological findings were regarded as a negative control [Fig. 4(A-D)].

Expression of cartilage-specific genes by RT-PCR

To investigate the chondrogenic differentiation of cultured BM-MSCs in the scaffold, mRNA expression of aggrecan core protein and type I, II, and X collagen was assessed using RT-PCR. After 1 and 2 weeks in culture, none of the assessed genes except the GAPDH house-keeping gene were expressed. At 4 weeks, expression of mRNAs for aggrecan and type X collagen (a specific gene for hypertrophic cartilage or osteogenic differentiation) was detected, but mRNAs for type I collagen (a specific gene for fibrocartilage) and type II collagen (specific genes for hyaline-like cartilage) were not detected [Fig. 5(A)]. In contrast, no assessed genes were expressed

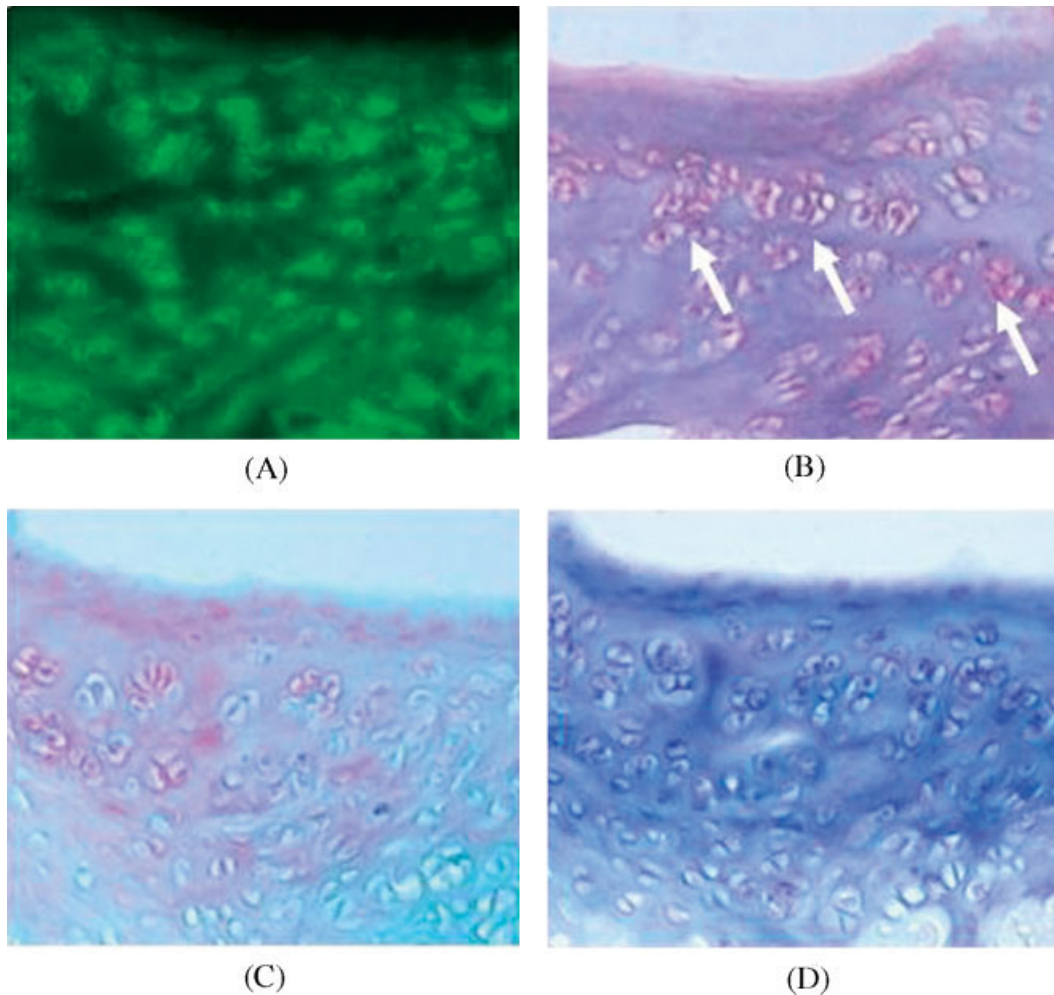


Figure 3. Photomicrographs at 4 weeks in culture. (A, B) Repopulation of GFP-positive cells and expression of extracellular matrices had expanded from the free edge to the peripheral border of the scaffold with (C) SO or (D) TB staining (original magnification, $\times 400$).

in the control tissue [Fig. 5(B)]. Gene expression of the cells from the meniscus of a normal rat was also assessed as a positive control, and mRNAs for aggrecan and type II collagen were detected [Fig. 5(C)].

Measurement of stiffness of the cultured tissues

Stiffness of the cultured tissues was measured to examine whether stiffness would change with increasing time cultivating BM-MSCs in the scaffold. The mean stiffness of normal menisci in Sprague-Dawley rats was 12.41 ± 2.16 g/mm², and the mean stiffness of the scaffolds we prepared was 9.18 ± 0.60 g/mm² (Fig. 6). The mean stiffness of the cultured tissues at 1, 2, and 4 weeks was 8.93 ± 0.67 , 11.56 ± 1.36 , and 11.72 ± 1.04 g/mm², respectively (Fig. 6). There was a significant difference in the mean value of stiffness of the cultured tissues between 1 and 2 weeks ($p < 0.01$), but not between 2 and 4 weeks. Stiffness of the cul-

tured tissue approximated to that of a normal meniscus after 2 weeks in culture. Control scaffolds showed no significant change in stiffness during 4 weeks in culture.

DISCUSSION

The current study demonstrated that the scaffold we prepared has the potential for cellular repopulation and adequate stiffness, similar to that of the normal meniscus. In previous studies, other meniscal scaffolds such as a type I collagen sponge, type I and II collagen-glycosaminoglycan copolymers, and synthetic biodegradable polymers containing polyglycolic acid and polylactic acid using cell seeding had been reported, and efficacy of these scaffolds was emphasized histologically.^{7,11,12,14,15} However, none of those scaffolds was evaluated for mechanical strength or

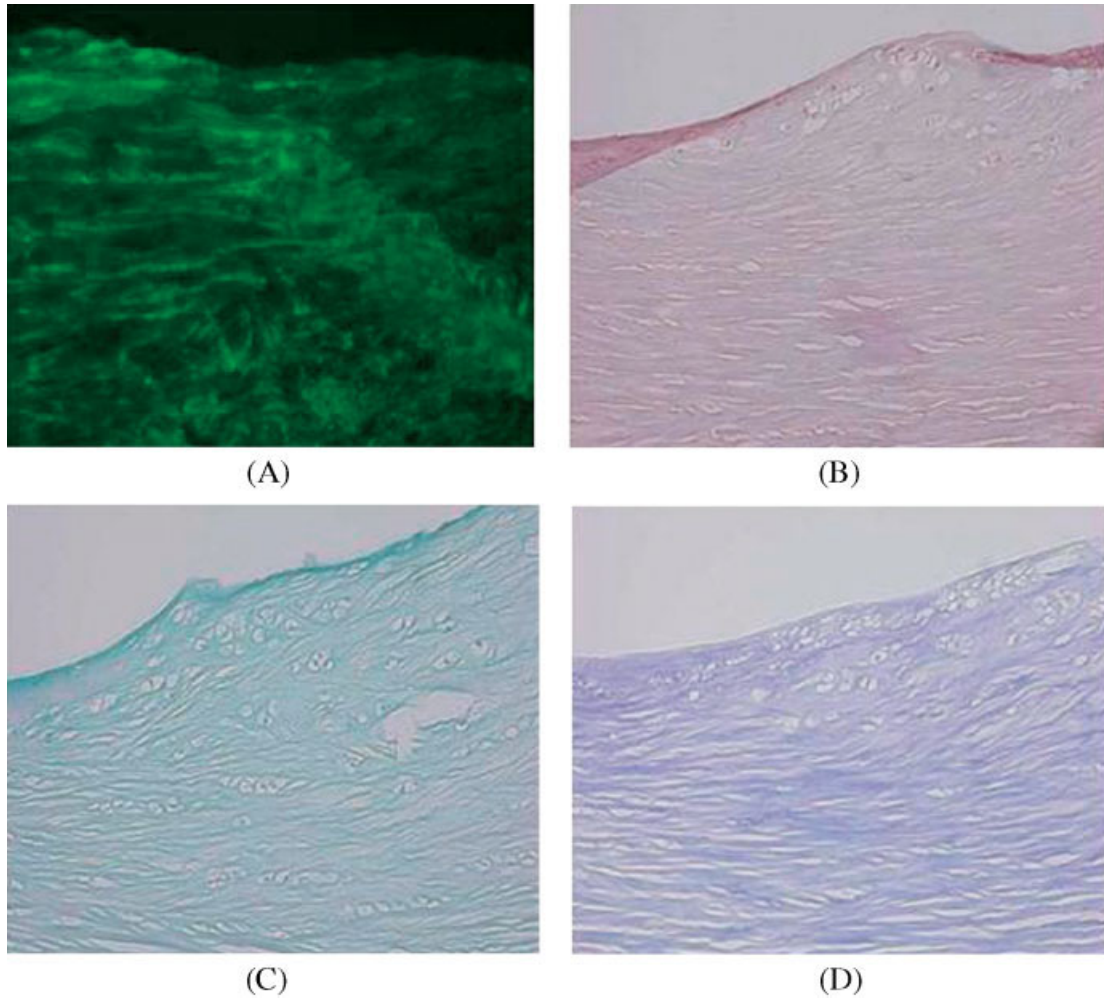


Figure 4. Photomicrographs of the control tissue at 4 weeks in culture. (A, B) Neither repopulation of GFP-positive cells was observed, nor expression of extracellular matrices detected with (C) SO or (D) TB staining (original magnification, $\times 200$).

stiffness of the regenerated tissues. We considered it important to evaluate the regenerated tissue not only histologically and genetically, but also either physio-

logically or mechanically for clinical applications. When a live allograft tissue is transplanted *in vivo*, immunoreaction to the tissue must be considered. Me-

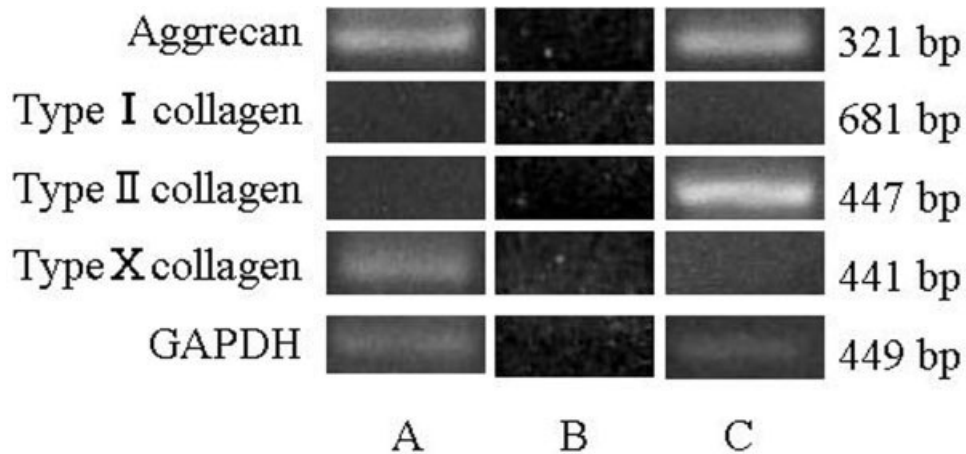


Figure 5. Analysis of expression of mRNA for aggrecan, type I, II, and X collagen, and GAPDH with RT-PCR. (A) After 4 weeks in culture. Expression of aggrecan and type X collagen gene was detected. (B) The control tissue after 4 weeks in culture. None of the assessed genes were expressed. (C) A positive control using a normal meniscus. Expression of aggrecan and type II collagen was detected.

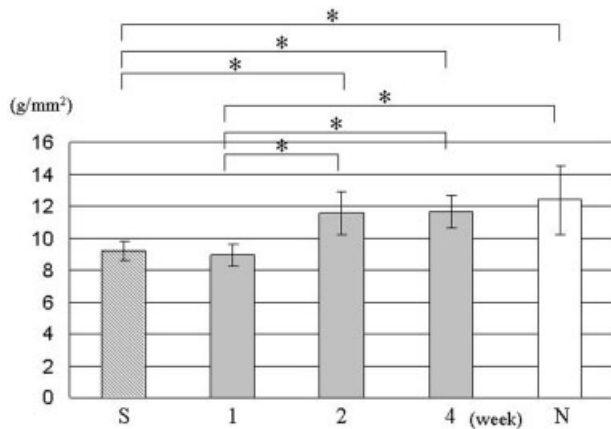


Figure 6. Measurement of stiffness of the cultured tissue. The mean stiffness of the cultured tissue increased over 4 weeks ($*p < 0.01$). The mean stiffness of the scaffold is also shown (S) and was significantly lower than a normal meniscus (N) ($*p < 0.01$). The tissue after 2 weeks in culture was significantly more stiff than the scaffold ($*p < 0.01$), and approximated a normal meniscus.

meniscal replacement using a cryopreserved or deep-frozen allograft has been reported without immunosuppressive agents in previous studies.^{17,18,23} Thus, the scaffold we prepared is thought not to be inferior to other meniscal scaffolds as regards the immunoreactive problem.

This study also revealed that BM-MSCs had the potential to repopulate the scaffold and to differentiate into chondrocyte-like cells. Tissue engineering for meniscal repair or regeneration using BM-MSCs has already been attempted. Port et al.¹³ reported on meniscal repair supplemented with exogenous fibrin clot and BM-MSCs in a goat model, but the addition of BM-MSCs in conjunction with the fibrin clot did not enhance meniscal healing. Meanwhile, Walsh et al.¹⁵ described meniscal regeneration in the rabbit partial meniscectomy model using BM-MSCs with a bovine type I collagen sponge as a scaffold, and the regenerated tissue was more abundant compared with the sponge alone. The concentration of BM-MSCs we prepared was the same as that of BM-MSCs Walsh et al. described, and higher than that of BM-MSCs Port et al. described. We considered it important to seed adequate BM-MSCs to the scaffold to regenerate a meniscus.

Furthermore, in this study, we proved that meniscal regeneration using the scaffold derived from the deep-frozen meniscus and BM-MSCs *in vitro* is possible, and also that the regenerated tissue has more appropriate stiffness than has been obtained via other methods, approximating the normal meniscus.

The period of cellular repopulation of a scaffold has been described as 1 or 2 weeks with the cells derived from the adjacent synovium or meniscal cells.^{12,18} We observed cellular repopulation with BM-MSCs at 1

week, and expression of extracellular matrices derived from BM-MSCs increased over 4 weeks in culture. BM-MSCs derived from a GFP rat were available to distinguish the origin of the cells which repopulated the scaffold under the fluorescent microscope. Observation through the fluorescent microscope during the period in culture demonstrated fading of emission of EGFP with some specimens, so a-EGFP staining was also performed to confirm GFP-positive cells. Thus BM-MSCs were proved to repopulate the scaffold in the same period as meniscus cells and remain viable in the scaffold. To our knowledge, this is the first investigation in which the scaffold derived from a deep-frozen meniscus was used, and in which BM-MSCs were observed to repopulate the scaffold and to synthesize extracellular matrices in the early phase of the culture. A normal meniscus consists of fibrocartilage which contains a large amount of type I collagen and only a very small amount of type II collagen.^{29–31} However, in our experiment, we could not detect type I and type II collagen of rats. We suspect this might be attributable to a methodological problem because we also failed to detect type I collagen in a normal meniscus.

The scaffold we prepared, which has a similar stiffness to a deep-frozen meniscus, is significantly softer than a normal meniscus. By seeding BM-MSCs to the scaffold, the cultured tissue obtained as much stiffness as a normal meniscus after 2 weeks in culture. These results indicate that transplantation of the regenerated meniscus using our method would be superior to a deep-frozen meniscus allograft as regards protection of joint cartilage against mechanical damage in the knee.

In summary, this study shows the efficacy of a scaffold derived from a normal meniscus repopulated with BM-MSCs in the early phase of the culture *in vitro*, and the potential of differentiation of BM-MSCs into chondrocyte-like cells. We consider that further investigation regarding transplantation of the tissue we built *in vivo* is needed in the future.

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