Tissue Engineering of Articular Cartilage With Autologous Cultured Adipose Tissue-Derived Stromal Cells Using Atelocollagen Honeycomb-Shaped Scaffold With a Membrane Sealing in Rabbits

Kazunori Masuoka,[1] Takashi Asazuma,[1] Hidemi Hattori,[2] Yasuo Yoshihara,[1] Masato Sato,[3] Kouji Matsumura,[2] Takemi Matsui,[4] Bonpei Takase,[2] Koichi Nemoto,[1] Masayuki Ishihara[2]

[1] Department of Orthopaedic Surgery, National Defense Medical College, 3–2 Namiki, Tokorozawa, Saitama 359-8513, Japan

[2] Division of Biomedical Engineering, Research Institute, National Defense Medical College, 3–2 Namiki, Tokorozawa, Saitama 359-8513, Japan

[3] Department of Orthopaedic Surgery, Surgical Science, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-1193, Japan

[4] Faculty of System Design, Tokyo Metropolitan University, 6–6 Asahigaoka, Hino, Tokyo 191-0065 Japan

Received 11 July 2005; revised 22 September 2005; accepted 26 October 2005
Published online 27 February 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.b.30507

Abstract: Adipose tissue derived stromal cells (ATSCs), which were isolated from adipose tissue of rabbit, have shown to possess multipotential, that is, they differentiate into osteoblasts and adipocytes in plate-culturing and into chondrocytes in an established aggregate culture using defined differentiation-inductive medium. The aim of this study was to evaluate the utility of ATSCs in tissue engineering procedures for repair of articular cartilage-defects using the atelocollagen honeycomb-shaped scaffold with a membrane sealing (ACHMS-scaffold). We intended to repair full-thickness articular cartilage defects in rabbit knees using autologously cultured ATSCs embedded in the ACHMS-scaffold. ATSCs were incubated within the ACHMS-scaffold to allow a high density and three-dimensional culture with control medium. An articular cartilage defect was created on the patellar groove of the femur, and the defect was filled with the ATSCs-containing ACHMS-scaffold, ACHMS-scaffold alone, or empty (control). Twelve weeks after the operation, the histological analyses showed that only the defects treated with the ATSCs-containing ACHMS-scaffold were filled with reparative hyaline cartilage, highly expressed Type II collagen. These results indicate that transplantation of autologous ATSCs-containing ACHMS-scaffold is effective in repairing articular cartilage defects. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 79B: 25–34, 2006

Keywords: regeneration; articular cartilage; adult-derived stem cells; adipose tissue; tissue engineering

INTRODUCTION

Articular cartilages show a limited reparative capacity after injury because of its specific anatomical structure.[1] Injuries of the articular cartilages that penetrate the subchondral bone undergo abnormal repairs and subsequently they form fibrous or fibro-cartilage tissue. These tissues lack the biochemical and mechanical properties necessary to yield a complete and durable repair.[2,3] Initially the repaired tissue may appear and function like hyaline cartilage, but it may deteriorate with time, provoking a degenerative process that is eventually involved in the entire joint.[4,5] This inability to reconstitute the cartilage tissue by an intrinsic healing process has promoted studies of various composite materials in cell-seeded implants in the treatment of cartilage lesions.

Cell-based therapies have been developed and are a promising treatment for cartilage defects. Autogenous chondrocytes-transplantation has proved as a promising method of cartilage repair,[6,7] but it had a few problems such as defects size constraint, donor site morbidity, and restricted monolayer culturing period. Mesenchymal stem cells as alternative cell source have recently received widespread attention be-

Correspondence to: M. Ishihara (e-mail: ishihara@.ndmc.ac.jp)

cause of their potential utility in tissue engineering. The mesenchymal stem cells derived bone marrow have shown to possess multipotential,[8] that is, they can differentiate *in vitro* or *in vivo* into osteoblasts,[9,10] chondrocytes,[11] adipocytes,[12] myotubes,[13] and neuronal cells.[14] The other sources of cells with multipotent differentiation capabilities have been found in various musculoskeletal tissues, including periosteum,[15] synovial tissue,[16] muscle,[17] trabecular bone,[18] and adipose tissue.[19,20] Especially, adipose tissue derived stromal cells (ATSCs) have the potential to differentiate into bone,[21,22] cartilage,[23] fat,[24] myocardium,[25] skin,[26] and neuron.[27,28] Furthermore, no significant difference between ATSCs and human bone marrow derived mesenchymal stem cells (BMSCs) from the same patient was observed on yield of adherent cells, their growth kinetics, cell senescence, differentiation capacity, and gene transduction efficiency.[29]

A new atelocollagen honeycomb-shaped scaffold with a membrane sealing (ACHMS-scaffold) has been reported to allowing a high density and three-dimensional culturing of various cells.[30,31] Furthermore, the previous report indicated the usefulness of the scaffold as a biomaterial for tissue engineering of articular cartilage with allografting of chondrocytes.[32] The main purpose of this work is to evaluate the ACHMS-scaffold for a three-dimensional culturing of ATSCs and its transplantation *in vivo* to critical-sized articular cartilage defects using rabbits.

MATERIALS AND METHODS

Preparation of ACHMS-Scaffold

Preparation of the atelocollagen honeycomb sponge has been described previously[30] and has been obtained from KOKEN, Tokyo, Japan. Briefly, type I atelocollagen from bovine dermis in solution (1%, pH 3) was poured into a shallow flask and exposed to ammonia gas so as to neutralize. The resulting white gel was then rinsed with distilled water to wash out excessive ammonia and salt being produced during the neutralization reaction. The freeze-dried gel resulted in honeycomb-shaped sponge. The pore diameter of the sponge was controlled by changing the concentration of collagen solution and ammonia gas. In this study, we used the scaffold with pores having an average diameter 350 μm . The slices were irradiated with ultraviolet (UV) light to yield an insoluble honeycomb-shaped sponge material.[30] To prepare the collagen-membrane sealing, type I atelocollagen solution (1%, pH 3) was poured into the flask and exposed to ammonia gas to neutralize. The honeycomb-shaped sponges were placed on the produced thin gel and air-dried in a clean bench. After making the membrane insoluble by UV-irradiation, the honeycomb-shaped sponges with membrane sealing at the bottom side were cut into round shapes having a diameter of 6 mm, using a punch. Finally, the round atelocollagen honeycombs with a membrane sealing were treated at 130°C for 20 min in vacuum for sterilization.[31]

Isolation of ATSCs From Adipose Tissue of Rabbit

Adipose tissue was obtained from male Japanese white rabbits weighing about 3 kg at inguinal part on 14 days before transplantation experiment. ATSCs were prepared as described previously.[19–22] Briefly, 1 g of adipose tissue was washed extensively with 10 ml of phosphated-buffered saline (PBS), and the extracellular matrix was digested with 0.075% collagenase at 37°C for 30 min. After adding Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics (control medium), it was centrifuged at 1200*g* for 10 min. The cell pellet was resuspended in 160 m*M* NH_4Cl and incubated at room temperature for 10 min. After removing cellular remains through a 100-μm nylon mesh, the cells were incubated in control medium in a dish (100 mm in diameter). When the adhered cells such as fibroblast became subconfluent after 5 days of the culture, they were subcultured. On the day before the transplantation (total 13 day-culturing and 2 passages), the cells were released from the culture dish by treatment with trypsin-EDTA solution (1×; 0.2 g EDTA and 0.5 g porcine trypsin per liter of HBSS; Sigma Aldrich Japan, Tokyo) at 37°C for 5 min, and the suspension was centrifuged to pellet the cells. The cells were then resuspended with the control medium and used as ATSCs.

Chondrogenic, Osteogenic, and Adipogenic Differentiations of ATSCs

ATSCs were tested for chondrogenic potential with an established aggregate culture technique, using a defined medium containing TGF-β.[8,33,34] Micromass pellets (2.0×10^5 cells) were cultured in defined medium [DMEM with ITS Premix (Collaborative Biomedical, Bedford, MA), 1 m*M* pyruvate (Life Technologies, Grand Island, NY)], 10 ng/mL TGF-β (R & D systems, Minneapolis, MN), 0.1 μM dexamethasone (Sigma-Aldrich, St. Louis, MO), and 80 μM ascorbate-2-phosphate (Wako Chemicals USA, Richmond, VA) at 37°C in an atmosphere of 5% CO_2 in air and 100% relative humidity. After 21 days of the culture, the pellets were also fixed with 10% buffered formalin for 3 days and embedded in paraffin. Sections about 5-μm thick were cut and stained with toluidine blue.

To evaluate an osteogenic potential of ATSCs,[21,22] the ATSCs were replated onto 6-well plate at a density of 2×10^4 cells and incubated in the control medium for 24 h, after which the medium was changed to osteogenic medium (control medium with 50 μM ascorbate-2-phosphate, 100 n*M* dexamethasone, and 10 m*M* β-glycerophosphate). The cells were cultured in the osteogenic medium for 4 weeks. After that, the cells were fixed in 10% buffered formalin, stained with Alizarin red, observed microscopically, and evaluated for the mineralization.

To examine an adipogenic potential of ATSCs,[19,24] the ATSCs were replated onto 6-well plate at a density of 2×10^5 cells and cultured in the control medium for 5 days, after which the medium was changed to either the control medium or the adipogenic-inductive medium [control medium plus 5

μg/mL insulin, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 60 μM indomethacin (Sigma-Aldrich)]. Three days later, the medium was replaced with a maintenance medium (the control medium plus 5 μg/mL insulin) for 1 day. This cycle of 3 days of inductive medium and 1 day of maintenance medium was repeated three times. After that, the cells were fixed in 10% buffered formalin, stained with Oil red-O (Wako Chemicals, Oosaka, Japan), and observed microscopically.

Transplantation of ATSCs in the ACHMS-Scaffold

Eighteen Japanese white rabbits (male; 16–18-weeks old; average weight, 3 kg) were used in this study. The rabbits were anesthetized with intramuscular injections of 120 mg ketamine and 9 mg xylazine. Adipose tissue was obtained at inguinal part ~14 days before transplantation experiment. The cultured ATSCs were isolated as described above. Briefly, the cells were obtained from 1 g of adipose tissue of each rabbit after the enzymatic isolation and subsequently incubated in DMEM with 10% FBS and antibiotics (control medium). The adhered cells became subconfluent after 5 days, and they were subcultured for 8 days (total 2 passages). The cells were collected and then seeded at a high density (2×10^6 cells/scaffold) onto the round ACHMS-scaffold (diameter, 6 mm; thickness, 2 mm) by centrifugation at 500 rpm (45g) for 5 min. The scaffold containing ATSCs was cultured in control medium at 37°C in an atmosphere of 5% CO_2 in air and 100% relative humidity during overnight. Under the same general anesthesia described above, the knee joint was opened with a medial parapatellar approach. Each patella was dislocated laterally and one full thickness cylindrical defect (4 mm in diameter and 2-mm deep) was created on the patellar groove of the femur in both legs using a low-speed drill (Takagi Corp., Niigata, Japan). The rabbits were classified into the following three recipient groups: (i) the ATSCs-containing scaffold insertion group, in which ATSCs cultured in an ACHMS-scaffold were autografted into the created full-thickness articular cartilage defect, (ii) the scaffold insertion group, in which only the ACHMS-scaffold was inserted, and (iii) the noninsertion group. Before autograft transplantation, ATSCs cultured in the scaffold and the scaffold without cells were cut to adjust to the size of defects. The implanted scaffolds (with and without ATSCs) were inserted into the defects and fixed in the defects without any additional fixation such as fibrin glue. After the operation, all animals were allowed to walk freely without any splint in the cages. These animal experiments were approved and carried out following the guidelines for animal experimentation of the National Defense Medical College, Tokorozawa, Saitama, Japan.

Macroscopic Evaluations

All evaluations were conducted at 4 or 12 weeks after the operation. At the end of the study period, the rabbits were sacrificed with an overdose intravenous anesthesia. The distal parts of the femur were harvested and observed. Each defect was evaluated and scored according to a gross appearance grading scale, consisting of four categories with a total score ranging from 0–8 points as described by Cook et al. (Table I).[35]

TABLE I. Grading Scale for Gross Appearance

Description	Points
Intraarticular adhesions	
None	2
Minimal/fine loose fibrous tissue	1
Major/dense fibrous tissue	0
Restoration of articular surface	
Complete restoration	2
Partial restoration	1
No restoration	0
Erosion of cartilage	
None	2
Defect site/site border	1
Defect site and adjacent normal cartilage	0
Appearance of cartilage	
Translucent	2
Opaque	1
Discolored or irregular	0
Maximum total	8

This table was extracted from Cook et al.'s grading scale for gross appearance (ref. 35).

Histological Evaluations

After macroscopic evaluation, the distal part of the femur was fixed with 10% buffered formalin for a period of 7 days. Each specimen was decalcified with 10% EDTA in distilled water (pH 7.4) for 3 weeks and embedded in paraffin. Sections about 6-μm thick were sagittally cut through the grafted area, stripped from paraffin, and stained with toluidine blue.

Immunohistochemical staining with type II collagen was performed as described previously.[32] Briefly, sections were, after stripping from paraffin, pretreated with 0.1 mg/mL actinase E (Kaken Pharmaceutical) in PBS at 37°C for 30 min. After incubating the section with 10% pig serum at room temperature for 30 min to reduce the degree of nonspecific background staining, the sections were incubated overnight with 50 μg/mL mouse anti-human type II collagen monoclonal antibody (Daiichi Fine Chemical Corp., Toyama, Japan) in PBS containing 0.1% bovine serum albumin at 4°C. The sections were then incubated with biotinylated rabbit anti-mouse immunoglobins (Dako Co., Carpinteria, CA) at a dilution of 1:500 for 30 min and followed with peroxidase-conjugated streptavidin (Dako) at dilution of 1:500 for 30 min at room temperature. Finally, the sections were immuno-stained by incubating with 20 mg diaminobenzidine and 5 μL hydrogen peroxide (30%) in 100 mL PBS for 5 min at room temperature. The other sections were incubated with PBS instead of specific primary antibodies and stained as a negative control.

The sections were evaluated microscopically and scored according to a histological grading scale, consisting of five

categories with a total score ranging from 0–14 points as described by Wakitani et al. (Table II).[11]

Statistical Analysis

Macroscopic and histological results were analyzed using the Kruskal–Wallis one-way test and subsequently used with the Wilcoxon signed-rank test. *p*-values less than 0.05 were accepted as statistically significant.

RESULTS

Structure of ACHMS-Scaffold

The upper surface of the ACHMS-scaffold is honeycomb-shaped, as shown in Figure 1(A,B). The lower surface is sealed with the same atelocollagen membrane. Scaffolds having a diameter of about 6 mm were used in the present study. The honeycomb-shaped parts consisted of thin multiple collagen membranes of less than 1 μm in thickness. It is elastic and, during the entire cell culture procedures, never deforms or collapses upon handling with forceps. Scaffolds can be easily cut with scissors or sharp knives into a desirable shape, which can be maintained during the cell culturing.

Adipose Tissue Harvest and Cell Isolation

After isolation of cells with enzymes, as described above, more than 2×10^5 nucleated cells adhered to the culture plates were obtained from 1 g of adipose tissue and were cultured in control medium described above. After 5 days, the attached cells became subconfluent and fibroblast-like [Figure 2(A)]. They were then detached with trypsin-EDTA solution and were subcultured for 8 days (2 passages). After the cell culture, the cells were collected and used as ATSCs. The cell number of ATSCs after incubation increased to at least 25 times more than that of the originally plated cells. There was no significant difference in cell yield between adipose tissue from each rabbit.

TABLE II. Histological Grading Scale for Defect Cartilage

Category	Points
Cell morphology	
Hyaline cartilage	0
Mostly hyaline cartilage	1
Mostly fibrocartilage	2
Mostly noncartilage	3
Noncartilage only	4
Matrix staining (metachromasia)	
Normal (compared with host adjacent cartilage)	0
Slightly reduced	1
Markedly reduced	2
No metachromatic stain	3
Surface regularity	
Smooth (>3/4)	0
Moderate (>1/2–3/4)	1
Irregular (1/4–1/2)	2
Severely irregular (<1/4)	3
Thickness of cartilage	
>2/3	0
1/3–2/3	1
<1/3	2
Integration of donor with host adjacent cartilage	
Both edges integrated	0
One edge integrated	1
Neither edge integrated	2
Maximum total	14

This table was extracted from Wakitani et al.'s histological grading scale (ref. 11).

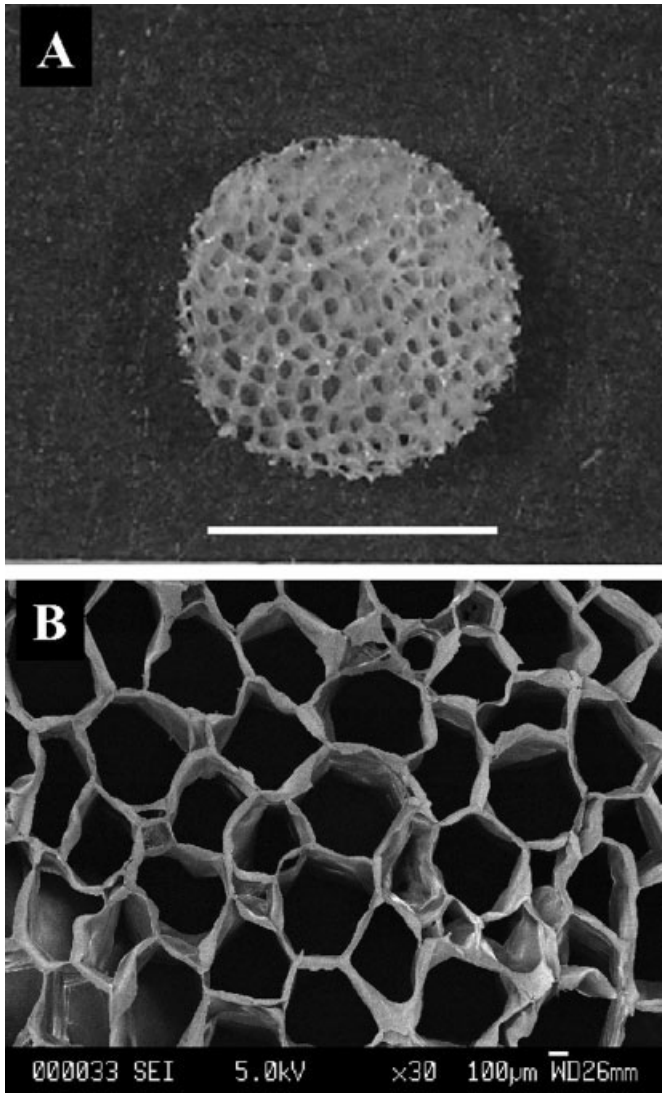

Figure 1. Appearance of the honeycomb-shaped atelocollagen scaffold with membrane sealing (ACHMS-scaffold). Upper panel A: microscopic appearance of the ACHMS-scaffold (original magnification, ×4). Bar represents 5 mm. Lower panel B: scanning electron microphotographs of the chondrocyte-seeded ACHMS-scaffold (original magnification, ×100). Bar represents 100 μm.

Chondrogenic, Osteogenic, and Adipogenic Differentiations of ATSCs

In the chondrogenic assay, ATSCs were cultured in the aggregate culture, using a defined medium containing TGF-β.[8,33,34]

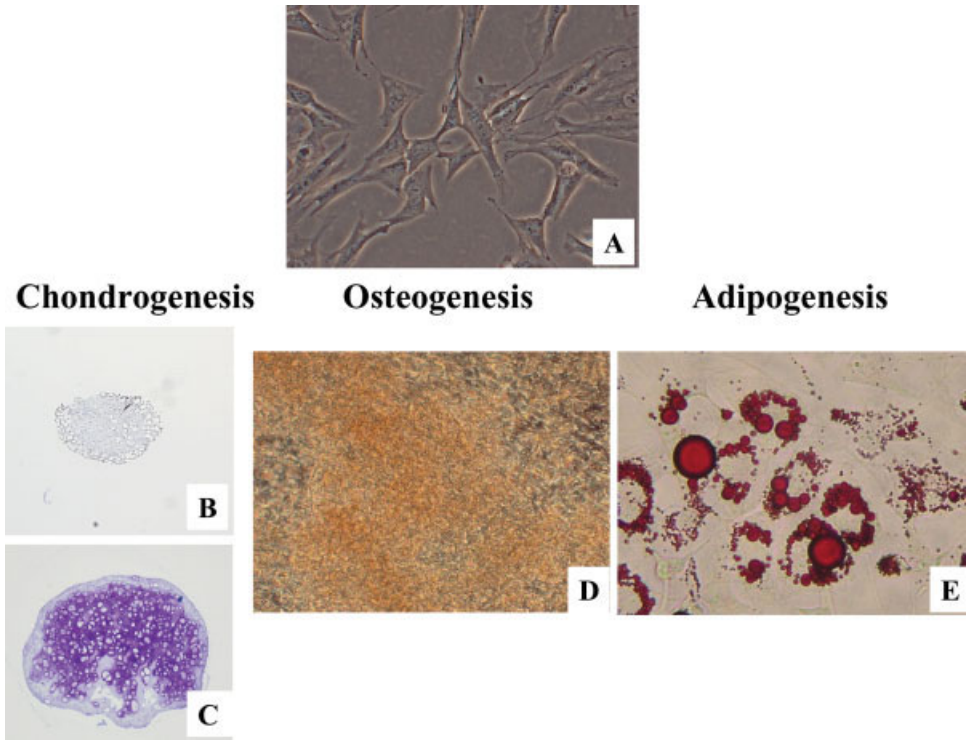

Figure 2. Multipotent differentiation of adipose-derived stromal cells *in vitro*. A: Phase-contrast light micrograph of adipose-derived stromal cells (original magnification; ×200). B: Toluidine blue staining (original magnification; ×100) of adipose-derived stromal cells in pellet cultures in control medium after 21 days. C: Toluidine blue staining (original magnification, ×100) of adipose-derived stromal cells in pellet cultures after chondrogenic induction *in vitro* in chondrogenic medium after 21 days. D: Alizarin red staining of adipose-derived stromal cells after osteogenic induction *in vitro* in osteogenic medium after 4 weeks (original magnification, ×200). E: Oil red O staining of adipose-derived stromal cells after adipogenic induction *in vitro* in adipogenic medium after 3 weeks (original magnification, ×200).

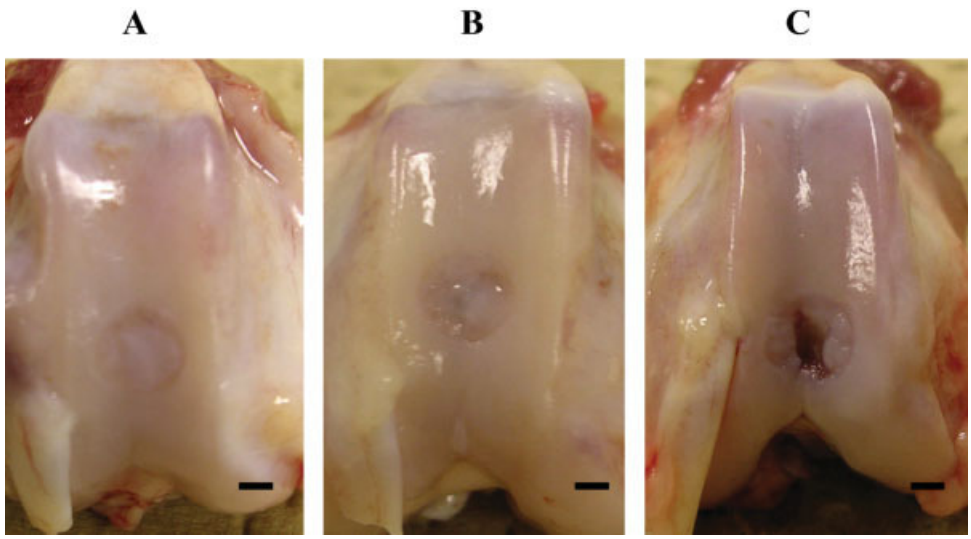

Figure 3. Macroscopic appearance of osteochondral defect repair at postoperative 12 weeks. A: ATSCs-containing ACHMS-scaffold insertion group. B: ACHMS-scaffold insertion group. C: Noninsertion group. Bar represents 1.5 mm.

TABLE III. Results of Grading Scale for Gross Appearance

	Time after surgery (wk)	*n*	Scores on gross grading scale				
			A	B	C	D	*Total*
ATSCs-containing scaffold	4	6	2.0 (2)	1.5 (1–2)	1.3 (1–2)	0.5 (0–1)	5.3 (4–6)*
	12	6	2.0 (2)	1.7 (1–2)	1.3 (1–2)	1.2 (1–2)	6.2 (5–7)*
Scaffold	4	6	2.0 (2)	1.3 (1–2)	1.3 (1–2)	0.7 (1–2)	5.3 (4–6)*
	12	6	2.0 (2)	1.3 (1–2)	1.5 (1–2)	1.2 (1–2)	6.0 (5–7)*
Noninsertion (control)	4	6	1.7 (1–2)	0.7 (0–1)	0.5 (0–1)	0.2 (0–1)	3.0 (1–5)
	12	6	1.8 (1–2)	0.8 (0–2)	1.0 (0–2)	0.5 (0–1)	4.2 (2–6)

Each data represents mean and range (parentheses) of the scales.
* $p < 0.05$, *versus* noninsertion (control).

During the culture, the size of the pellet increased [Figure 2(C)]. After 21 days of the culture, the pellet sections demonstrated the formation of cartilage-like tissue as indicated by the toluidine blue staining when compared with that of the control [Figure 2(B)]. These results suggest that ATSCs possess the capacity to differentiate toward the chondrogenic phenotype cells.

ATSCs were incubated in the control medium for 24 h, after which the medium was changed to the osteogenic medium. The cells cultured in osteogenic medium were changed morphologically from elongated fibroblast-like appearance to more cuboidal shape. After 4 weeks, all dishes were stained with Alizarin red, indicating the formation of a calcium-rich matrix [Figure 2(D)].

To determine the potential of ATSCs to differentiate toward adipogenic phenotype cell, ATSCs were cultured with adipogenic induction medium. After cyclic induction with adipogenic medium, ATSCs had cytoplasmic lipid droplet accumulation and became adipogenic phenotype cells. Histological examination revealed the formation of small lipid droplets in some cultures after one or two cycles of treatment. During the culture, the number of lipid-droplet-containing cells increased, and after four cycles of adipogenic-inductive treatment, lipid-droplet-containing cells were present in all cell samples. The lipid-containing cells were stained with Oil Red-O [Figure 2(E)], and these results revealed that ATSCs have the capacity to differentiate to adipogenic phenotype cells.

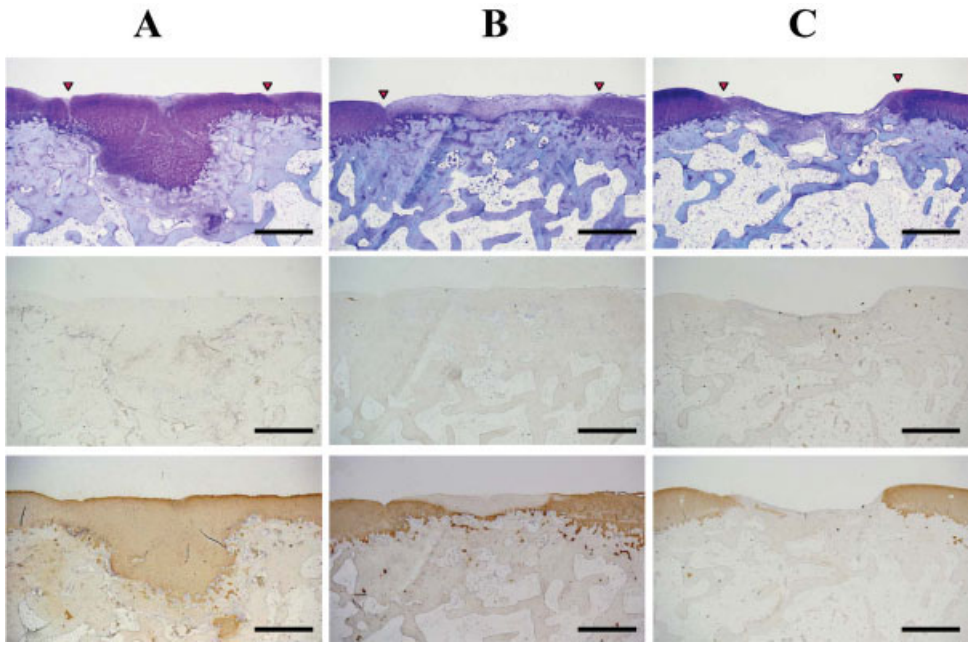

Figure 4. Histological observations of osteochondral defect repair at postoperative 4 weeks. A: ATSCs-containing ACHMS-scaffold insertion group. B: ACHMS-scaffold insertion group. C: Noninsertion group. Upper (first line) panels: Toluidine blue-staining of the implanted scaffold (bar lengths, 1 mm). Middle (second line) panels: negative control-immunostaining (PBS instead of the Type II collagen-antibody) of the implanted scaffold (bar length, 1 mm). Lower (third line) panels: type II collagen-immunostaining of the implanted scaffold (bar length, 1 mm). Each microphotograph is a representative of six tissue samples stained with toluidine blue and anti-type II collagen in each group, and the triangles show defected area of the cartilage.

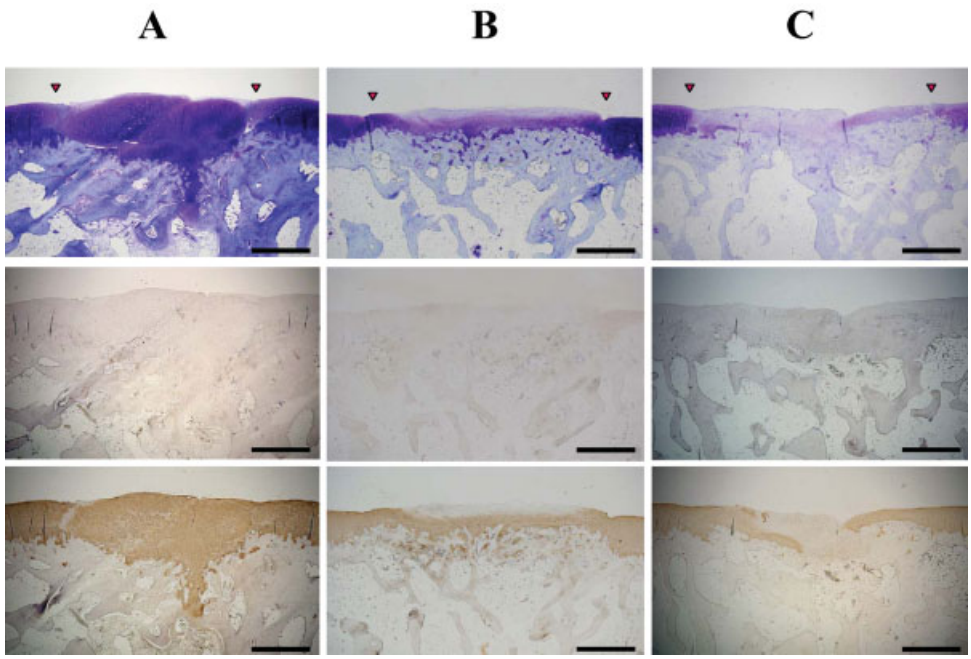

Figure 5. Histological observations of osteochondral defect repair at postoperative 12 weeks. A: ATSCs-containing ACHMS-scaffold insertion group. B: ACHMS-scaffold insertion group. C: Noninsertion group. Upper (first line) panels: Toluidine blue staining of the implanted scaffold (bar lengths, 1 mm). Middle (second line) panels: negative control-immunostaining (PBS instead of the Type II collagen-antibody) of the implanted scaffold (bar length, 1 mm). Lower (third line) panels: type II collagen-immunostaining of the implanted scaffold (bar length, 1 mm). Each microphotograph is a representative of six tissue samples stained with toluidine blue and anti-type II collagen in each group, and the triangles show defected area of the cartilage.

Macroscopic Observations

No sign of arthritis, for example cartilage erosion or severe synovial proliferation, was observed in any operated knee. The defects in the cells-containing scaffold insertion group [Figure 3(A)] and scaffold insertion group [Figure 3(B)] were filled with smooth and white tissue when compared with the noninsertion group [Figure 3(C)]. The borders of reparative tissue were also visible and the colors of tissue were slightly different from that of the surrounding normal cartilage [Figure 3(A,B)]. But the defects in the non-insertion group remained empty or covered in reddish tissue [Figure 3(C)]. The global macroscopic scores (Table I) obtained in the cells-containing scaffold insertion group ($n = 6$) and the scaffold insertion group ($n = 6$) were statistically better than those in the noninsertion groups ($n = 6$) (Table III).

Histological Observation

In the cells-containing scaffold insertion group, the defects were completely filled with reparative tissue when compared with those of the noninsertion group. The reparative tissue showed a smooth surface being connected with adjacent normal cartilage at 4 and 12 weeks after operation [Figures 4(A) and 5(A)]. In contrast, the surface of noninsertion group [Figures 4(C) and 5(C)] was irregular and the thickness of the tissue was smaller than that of the cell-containing scaffold insertion group. But, that of the reparative tissue in the scaffold insertion group [Figures 4(B) and 5(B)] was as smooth as that in the cells-containing scaffold insertion group. The intensity of toluidine blue and anti-type II collagen declined and the areas of staining were also found smaller in the scaffold insertion group and noninsertion group. Furthermore, the means in Wakitani's score (Table II) of the cell-containing scaffold insertion group exhibited significantly better than those of the other two groups at 12 weeks (Table IV).

DISCUSSION

It is well known that many adult tissues contain lineage committed progenitor cells for tissue maintenance and repair. Many studies have demonstrated that culture-expanded BMSCs can be directed into the chondrogenic lineage *in vitro*[8,33,34] and *in vivo*,[11] resulting in a hyaline cartilage production. The addition of chondrogenic supplements (ITS Premix, 1 m*M* pyruvate, 10 ng/mL TGF-β, 0.1 μM dexamethasone, and 80 μM ascorbate-2-phosphate) to BMSC cultutres in a three-dimensional matrix was found capable of inducing rapid chondrogenesis.[8,33,34] Similarly, ATSCs isolated human adipose tissue were shown to be induced to chondrogenic differentiation[19,20] and to possess the ability to produce cartilage matrix proteins.[23] The present results show

TABLE IV. Results of Histological Grading Scale

	Time after surgery/(wk)	*n*	Scores on histologic grading scale					
			A	B	C	D	E	*Total*
ATSCs-containing scaffold	4	6	1.2 (1–2)	1.3 (1–2)	1.3 (1–2)	0.2 (0–1)	1.7 (1–2)	5.7 (4–7)*
	12	6	1.0 (0–2)	1.2 (0–2)	0.7 (0–1)	0.3 (0–1)	1.3 (1–2)	4.5 (3–7)**
Scaffold	4	6	1.7 (1–2)	1.7 (1–2)	1.3 (1–2)	1.2 (0–2)	1.5 (1–2)	7.3 (4–10)
	12	6	1.5 (1–2)	1.7 (1–2)	1.2 (0–2)	1.2 (0–2)	1.5 (1–2)	7.0 (5–9)
Noninsertion (control)	4	6	2.2 (1–3)	2.3 (1–3)	2.3 (2–3)	1.3 (0–2)	1.8 (1–2)	10.0 (7–13)
	12	6	2.0 (1–3)	2.0 (1–3)	1.5 (0–3)	1.2 (0–2)	1.5 (1–2)	8.2 (7–12)

Each data represents mean and range (parentheses) of the scales.
*$p < 0.05$, *versus* noninsertion (control).
**$p < 0.01$, *versus* scaffold and noninsertion (control).

that ATSCs from rabbits also possess the ability to differentiate into chondrocytes, osteoblasts, and adipocytes *in vitro* (Figure 2) and produce reparative hyaline cartilage when implanted with a three-dimensional scaffold (ACHMS-scaffold) into rabbit-articular cartilage defects (without addition of any chondrogenic supplements). These findings demonstrate that ATSCs using the ACHMS-scaffold may provide a novel and abundant autologous cell source for developing tissue engineering approaches for repair or replacement of articular cartilage defects.

The usefulness of an ACHMS-scaffold in the *in vitro* mass cultures of anulus fibrosus cells[31,36,37] and chondrocyte cells[32] in tissue engineering procedures has been reported for three-dimensional culture. Collagen is frequently used as scaffold in various mammalian cell cultures. In particular, three-dimensional cell cultures are commonly carried out using collagen in gels, sponges, films, and sandwich structures of these.[38,39] In general, a scaffold for cartilage tissue engineering is expected to fulfill the prerequisites concerning structure and cytocompatibility and should enable a preformed shape prior to transplantation. Furthermore, the scaffold should be noncytotoxic and nonantigenic, should guarantee a uniform cell distribution, maintain cell viability and phenotype, and allow the synthesis of extracellular matrix components as is required for the development of solid connective tissue.[38,39] The present study demonstrates the applicability of the ACHMS-scaffold in terms of stability, cytocompatibility, uniform and high density seeding of ATSCs, and accumulation of cartilage-like matrix components *in vivo*. The present *in vivo* results also indicate the usefulness of this ACHMS-scaffold as a biomaterial for *in vitro* mass cultures of ATSCs, for differentiation of ATSCs to chondrocytes, and for tissue engineering of cartilage formation *in vivo*.

In the present study, articular cartilage defects implanted with ATSCs-containing ACHMS-scaffolds *in vivo* showed improved histological findings [Figures 4(A) and 5(A), and Table IV). The autografted ATSCs were differentiated slowly to chondrocytes in the articular cartilage defects and produced an extracellular matrix similar to that of hyaline cartilage. Since three-dimensionally cultured ATSCs *in vitro* were not differentiated to chondrocytes without chondrogenic supplements in the culture medium (Figure 2B), the differentiation factors should exist in the articular cartilage defects. The goal of regenerating articular cartilage is not only to achieve anatomic morphology, but also to restore its function. The autografted ATSCs exhibited a proliferation and differentiation activity in the articular cartilage defects, resulting in the formation of hyaline cartilage. Furthermore, our preliminary results showed that autografted ATSCs from adult rabbits (about 10-months old) still have a similar potential to produce the hyaline cartilage (data not shown). However, the mechanical strength properties of regenerated articular cartilage and *in vivo* studies on large size animals (i.e. sheep or pig) remain to be investigated.

Other observations regarding the intrinsic healing capacity of osteochondral defects in rabbits constitute the standard for healing time.[40] Shapiro et al. described impressive positive results with early repair (12 weeks) of 3-mm osteochondral defects, but an increasing extent of degenerative changes was observed after 24 weeks in their animal model.[41] Therefore a reasonable follow-up period of at least 24 weeks is necessary. Since we evaluated the cartilage regeneration at 4 and 12 weeks in the present study, we plan to carry out the 24-week follow-up study for cartilage regeneration using ATSCs and ACHMS-scaffold.

In summary, we carried out tissue-engineering using autograft implantations so as to regenerate articular cartilage. The autografted ATSCs with ACHMS-scaffolds exhibited a differential activity to chondrocytes showing *in vivo* extracellular matrix production. These results suggest a possibility to achieve regeneration of articular cartilage through autografting of ATSCs-containing ACHMS-scaffold.

REFERENCES

1. Hunter W. Of the structure and disease of articulating cartilages. Clin Orthop Relat Res 1995;317:3–6.
2. Furukawa T, Eyre DR, Koide S, Glimcher MJ. Biochemical studies on repair cartilage resurfacing experimental defects in the rabbit knee. J Bone Joint Surg Am 1980;62:79–89.
3. Coletti JM, Akeson WH, Woo SLY. A comparison of the physical behavior of normal articular cartilage and the arthroplasty surface. J Bone Joint Surg 1972;54A:147–160.

4. Buckwalter JA, Mankin HJ. Articular cartilage. 2: Degeneration and osteoarthrosis, repair, regeneration, and transplantation. J Bone Joint Surg Am 1997;79A:612–632.
5. Hunziker EB. Articular cartilage repair: Basic science and clinical progress. A review of the current status and prospects. Osteoarthritis Cartilage 2002;10:432–463.
6. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994; 331:889–895.
7. Ochi M, Uchio Y, Kawasaki K, Wakitani S, Iwasa J. Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee. J Bone Joint Surg Br 2002;84B:571–578.
8. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–147.
9. Boo JS, Yamada Y, Okazaki Y, Hibino Y, Okada K, Hata K, Yoshikawa T, Sugiura Y, Ueda M. Tissue-engineered bone using mesenchymal stem cells and biodegradable scaffold. J Craniofac Surg 2002;13:231–239.
10. Dong J, Uemura T, Shirasaki Y, Tateishi T. Promotion of bone formation using highly pure porous β-TCP combined with bone marrow-derived osteoprogenitor cells. Biomaterials 2002;23: 4493–4502.
11. Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI, Goldberg VM. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Joint Surg Am 1994;76:579–592.
12. Beresford JN, Bennett JH, Devlin C, Leboy PS, Owen ME. Evidence for inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. J Cell Sci 1992;102:341–351.
13. Ferrari G, Cusella-DeAngelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 1998;279:1528–1530.
14. Woodbury D, Reynoldsk B, Black IB. Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J Neurosci Res 2002;96:908–917.
15. Nakase T, Nakahara H, Iwasaki M, Kimura T, Kimata K, Watanabe K, Caplan AI, Ono K. Clonal analysis for developmental potential of chick periosteum-derived cell: Agar gel culture system. Biochem Biophys Res Commun 1993;195:1422–1428.
16. De-Bari C, Dell'Accio F, Tylzanowsky P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum 2001;44:1928–1942.
17. Lee JY, Qu-Petersen Z, Cao B, Kimura S, Jankowski R, Cummis J, Usas A, Gates C, Robbins P, Wernig A, Huard J. Clonal isolation of muscle-derived cells capable of enhancing muscle regeneration and bone healing. J Cell Biol 2000;150:1085–1100.
18. Noth U, Osyczka AM, Tuli R, Hickok NJ, Danielson KG, Tuan RS. Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells. J Orthop Res 2002; 20:1060–1069.
19. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng 2001;7:211–228.
20. Zuk PA, Zhu M, Ashjian P, De-Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Franser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002;13:4279–4295.
21. Hattori H, Sato M, Masuoka K, Ishihara M, Kikuchi T, Matsui T, Takase B, Ishizuka T, Kikuchi M, Fujikawa K, Ishihara M. Osteogenic potential of human adipose tissue-derived stromal cells as an alternative stem cell source. Cells Tissues Organs 2004;178:2–12.
22. Hattori H, Masuoka K, Sato M, Ishihara M, Asazuma T, Matsui T, Takase B, Kikuchi M, Nemoto K, Ishihara M. Bone formation using human adipose tissue-derived stromal cells and a biodegradable scaffold. J Biomed Mater Res. Forthcoming.
23. Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F. Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. Biochem Biophys Res Commun 2002;290:763–769.
24. Toriyama K, Kawaguchi N, Kitoh J, Tajima R, Inou K, Kitagawa Y, Torii S. Endogeous adipocyte precursor cells for regenerative soft-tissue engineering. Tissue Eng 2002;8: 157–165.
25. Nishida M, Li TS, Hirata K, Yana M, Matsuzaki M, Hamano K. Improvement of cardiac function by bone marrow cell implantation in a rat hypoperfusion heart model. Ann Thorac Surg 2003;75:768–773.
26. Bogaerdt AJ, Zuijlen PPM, Galen M, Lamme EN, Middelkoop E. The suitability of cells from different tissues for use in tissue-engineered skin substitutes. Arch Dermatol Res 2002; 294:135–142.
27. Safford KM, Hicok KC, Safford SD, Halvorsen, YD, Wilkinson WO, Gimble JM, Rice HE. Neurogenic differentiation of murin and human adipose-derived stromal cells. Biochem Biophys Res Commun 2002;294:371–379.
28. Ashjian PH, Elbarbary AS, Edmonds B, De-Ugarte D, Zhu M, Zuk PA, Lorenz HP, Behaim P, Hedrick MH. In vitro differentiation of human processed lipoaspirate cells into early neural projenitors. Plast Reconstr Surg 2003;111:1922–1931.
29. De-Ugart DA, Morizone K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, Dragoo JL, Ashjian PH, Thomas B, Benhaim P, Chen I, Fraser J, Hedrick MH. Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs 2003;174:101–109.
30. Itoh H, Aso Y, Furuse M, Noishiki Y, Miyata T. A honeycomb collagen carrier for cell culture as a tissue engineering scaffold. Artif Organs 2001;25:231–237.
31. Sato M, Asazuma T, Ishihara M, Kikuchi T, Masuoka K, Ichimura S, Kikuchi M, Kurita A, Fujikawa K. An atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS-scaffold) for the culture of annulus fibrosus cells from an intervertebral disc. J Biomed Mater Res 2003;64A: 248–256.
32. Masuoka K, Asazuma T, Ishihara M, Sato M, Hattori H, Ishihara M, Yoshihara Y, Matsui T, Takase B, Kikuchi M, Nemoto K. Tissue engineering of articular cartilage using an allograft of cultured chondrocytes in a membrane-sealed atelocollagen honeycomb-shaped scaffold (ACHMS-scaffold). J Biomed Mater Res Br 2005;75B:177–184.
33. Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM, Johnstone B. The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. J Bone Joint Surg Am 1998;80:1745–1757.
34. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 1988;238:265–272.
35. Cook SD, Patron LP, Salkeld SL, Rueger DC. Repair of articular cartilage defects with osteogenic protein-1 (BMP-7) in dogs. J Bone Joint Surg Am 2003;85A:116–123.
36. Sato M, Asazuma T, Ishihara M, Ishihara M, Kikuchi T, Kikuchi M, Kurita A, Fujikawa K. An experimental study of the regeneration of the intervertebral disc with an allograft of cultured annulus fibrosus cells using a tissue-engineering method. Spine 2003;28:548–553.

37. Sato M, Kikuchi M, Ishihara M, Asazuma T, Kikuchi T, Masuoka K, Hattori H, Fujikawa K. Tissue engineering of the intervertebral disc with cultured annulus fibrosus cells using atelocollagen honeycomb-shaped scaffold with a membrane seal (ACHMS scaffold). Med Biol Eng Comput 2003;41:365–371.
38. Pachence JM. Collagen-based devices for soft tissue repair. J Biomed Mater Res 1996;33:35–40.
39. Riesle J, Hollander AP, Langer R, Freed LE, Vunjak-Novakovic G. Collagen in tissue-engineered cartilage: Types, structures, and crosslinks. J Cell Biochem 1998;71: 313–327.
40. Rudert M. Histological evaluation of osteochondral defects: Consideration of animal models with emphasis on the rabbit, experimental setup, follow-up and applied methods. Cells Tissues Organs 2002;171:229–240.
41. Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. J Bone Joint Surg Am 1993;75:532–553.