Variation of Mesenchymal Cells in Polylactic Acid Scaffold in an Osteochondral Repair Model

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Objective: To achieve osteochondral regeneration utilizing transplantation of cartilage-lineage cells and adequate scaffolds, it is essential to characterize the behavior of transplanted cells in the repair process. The objectives of this study were to elucidate the survival of mesenchymal cells (MCs). In a polylactic acid (PLA) scaffold and assess the possibility of MC/PLA constructs for osteochondral repair.

Design: Bone marrow from mature male rabbits was cultured for 2 weeks, and fibroblast-like MCs, which contain mesenchymal stem cells (MSCs), were obtained. A cell/scaffold construct was prepared with one million MCs and a biodegradable PLA core using a rotator device. One week after culturing, the construct was transplanted into an osteochondral defect in the medial femoral condyle of female rabbits and the healing process examined histologically. To examine the survivability of transplanted MCs, the male-derived sex-determining region Y (SRY) gene was assessed as a marker of MCs in the defect by polymerase chain reaction (PCR).

Results: In the groups of defects without any treatment, and the transplantation of PLA without cells, the defects were not repaired with hyaline cartilage. The cartilaginous matrix by safranin O staining and type II collagen by immunohistochemical staining were recognized, however the PLA matrix was still present in the defects at 24 weeks after transplantation of the construct. During the time passage, transplanted MCs numbers decreased from 7.8×10^5 at 1 week, to 3.5×10^5 at 4 weeks, and to 3.8×10^4 at 12 weeks. Transplanted MCs were not detectable at 24 weeks.

Conclusions: MCs contribute to the osteochondral repair expressing the cartilaginous matrix, however the number of MCs were decreasing with time (i.e. 24 weeks). These results could be essential for achieving cartilage regeneration by cell transplantation strategies with growth factors and/or gene therapy.

Introduction

OSTEOARTHRITIS (OA) IS ONE OF THE MOST COMMON diseases, with more than 20 million people affected in the United States. It is characterized as degeneration of articular cartilage and underlying subchondral bone.[1] For patients with advanced OA, there are several surgical options, such as chondroplasty, drilling, and microfracture to induce cartilage repair[2–5]; autologous and allogenic osteochondral transplantations as cartilage replacement procedures[6–8]; and artificial joint replacement and arthrodesis as salvage procedures.[9] Though clinical results are generally positive, there are known problems and these procedures do not involve cartilage regeneration.[10–14]

Autologous chondrocyte implantation (ACI) has been reported as a cartilage reconstruction trial.[15] The clinical procedures have been quite successful, and this is currently one of the best surgical options, especially for young, active patients.

However, there are several limitations to this procedure, including the limited availability of harvested cartilage, the dedifferentiation of chondrocytes *in vitro*, degeneration at the harvest site, flow of cell suspension from the transplanted area, and hypertrophy of the periosteal membrane at the transplanted area.[16–20] To resolve these issues, modified trials with various cell sources and scaffolds are being established and additional strategies such as the use of growth factors and/or gene therapy have been attempted. However, the regeneration of genuine, long-lasting, normal cartilage has not yet been successful.

We hypothesized that the survivability and behavior of transplanted cells need to be examined clearly to achieve cartilage regeneration with cell-based treatment. The fate of the cells in the transplanted area has not been explained because of the difficulty in distinguishing transplanted cells from the host cells *in vivo*. Thus, the ability of transplanted cells to survive and contribute to the repair in the

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transplanted area has not been well discussed. This Information could provide the most effective timing for performing additional treatments during the repair process.

Chondrocytes are a suitable source for the repair of cartilage defects that do not penetrate the subchondral bone. However, an osteochondral defect involves both cartilage and subchondral bone (Grade 4 by classification of the International Cartilage Repair Society), and thus chondrocytes may not be adequate for osteochondral repair.[21]

Mesenchymal stem cells (MSCs) have the capacity for self-duplication and the potential to differentiate into several mesenchymal tissues (i.e., bone, cartilage, tendon, muscle, and fat tissues).[22,23] MSCs exist in bone marrow and many other tissues and can be harvested from bone marrow by a minor invasive procedure clinically. MSCs can also be cultured and increased to sufficient numbers *in vitro* while preserving their multipotent differentiation capacity. We previously reported the possibility of using bone marrow–derived mesenchymal cells (MCs), which include MSCs, for osteochondral repair.[24,25] Thus, MCs could be used in osteochondral repair without causing injury to the healthy articular cartilage. Polylactic acid (PLA) scaffolds have been used to occupy the space following treatment of teeth and bones clinically and have been suitable carriers for high-density, viable chondrogenic cells.[26] For these reasons, the combination of MCs and PLA could overcome the formal problems associated with cartilage repair.[27] Thus, the objectives of our study were to elucidate the survivability of MCs in PLA scaffold in an osteochondral defect, and to evaluate the possibility of using MC/PLA constructs for osteochondral repair *in vivo*.

To evaluate the presence of transplanted cells the SRY study was performed. Matrix metalloproteinase (MMP)-1 is an autosomal gene that exists in both male and female cells. The sex-determining region Y (SRY) gene is located on the short arm of the Y chromosome and exists only in male cells. Thus, when male MCs are transplanted into the osteochondral defect of a female femur it is possible to distinguish the male cells from female tissue by detecting the SRY gene using the PCR technique.[28] Because not only the transplanted cells (SRY gene) but also the total number of cells in the tissues (MMP-1 gene) could be calculated by a semi-quantitative method in the SRY study, the relationship between transplanted cells and the surrounding host cells with exact cell numbers could be understood.

Materials and Methods

Animals

Skeletally mature male and female New Zealand White rabbits (9–15 months old and weighing 3.5–4.5 kg) with closed epiphyses were used. All procedures conformed to the guidelines of the University of California Animal Subjects Committee and the American Association for Accreditation of Laboratory Animal Care.

Harvest and culture of bone marrow cells

Mature male rabbits ($n = 6$) were given intramuscular injections of 35 mg/kg ketamine and 5 mg/kg xylazine. After the medications had taken effect, the rabbits received intravenous injections of 97.8 mg/kg sodium pentobarbital via a lateral ear vein and were sacrificed. Bone marrow was harvested from

bilateral femurs and tibias by a sterile surgical technique and suspended in phosphate buffered saline (PBS; Gibco, Grand Island, NY) containing 2% bovine serum albumin (BSA; Serologicals Proteins, Kankakee, IL). After centrifuging for 5 minutes to remove serum ingredients, bone marrow was treated with sterile water for 10 seconds to disrupt red blood cells, resuspended into 2% BSA in PBS, and centrifuged again for 5 minutes. The precipitated bone marrow cells from one rabbit were cultured in eight 100 mm diameter tissue culture dishes (Falcon, Franklin Lakes, NJ) with 10 mL culture medium of 10% fetal bovine serum (FBS) and antibiotic-antimyconic (Gibco) in Dulbecco's modified Eagle's medium (DMEM; Gibco) at 37°C in a humidified atmosphere of 5% CO_2. The medium was completely changed, first 24 hours after the seeding of cells, and every three days thereafter, to remove as many floating cells as possible. Two weeks after culturing, the remaining cells adhering to the bottom of the dish were detached by a 5 minutes treatment of 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, St. Louis, MO) and counted using a hemocytometer.

Formation of MC/PLA constructs

Two weeks after culturing, the remaining adherent fibroblast-like cells were regarded as MCs (containing MSCs), and these achieved about 80% confluence.

Cylindrical cores (3.7 mm in diameter×3 mm deep) were prepared from cubes of porous D, D-L, and L-PLA (Drylac Cube, Kensey Nash, Exton, PA) to create MC/PLA constructs. To improve the nutrition supply to the center of the cores, a 1 mm diameter channel was created in the center of the cores.

Cultured MCs were detached and counted as described above. A PLA core and 1 million cells in 500 μL culture medium were rotated at 100 rpm in a glass vial for 2 hours on the rotator device.[29] Following that, the constructs were cultured for 1 week at 37°C in a humidified atmosphere of 5% CO_2.

Surgical procedure

Female rabbits ($n = 52$) were given intramuscular injections of 35 mg/kg ketamine and 5 mg/kg xylazine, and a facemask delivering 1–2% isoflurane was used during surgery. An anterior midline incision was made through the skin of the left knee, and the articular surface of the femur was exposed using a medial parapatellar retinacular approach. The knee joint was immobilized in a deeply flexed position, and a cylindrical full-thickness osteochondral defect 3.7 mm in diameter (wider than 2/3 of the condyle) and 3 mm in depth was created in the central weight-bearing surface of the medial femoral condyle using a surgical drill bit. Three groups were then prepared: Group 1 was a defect alone without treatment ($n = 12$), Group 2 was a defect filled with the PLA core alone without cells ($n = 12$), and Group 3 was a defect filled with the MC/PLA construct ($n = 28$). All rabbits were allowed to move freely after these procedures in a temperature-controlled environment with a 12-hour light/dark cycle. Intramuscular buprenorphine was administered for at least 72 hours for postoperative pain control.

Gross morphology

At 1, 4, 12, and 24 weeks after surgery, the rabbits ($n = 3$ in each group at each time period) were sacrificed, the knee

joints were exposed as described above, and the distal portions of the femurs were harvested.

Histology

After evaluating gross morphology, the samples were fixed in 10% buffered neutral formalin for 1 week at room temperature and decalcified with 30% formic acid for approximately 4 weeks under vacuum (20–25 psi) with shaking. The samples were rinsed with water for 1 hour to remove formic acid, dehydrated with ascending alcohol grades, cleared with a xylene substitute at room temperature, infiltrated in paraffin at 60°C under vacuum (15 psi) and pressure (7 psi), and then embedded into paraffin blocks.

The samples were cut into 10 μm sections (2035 Jung Biocut; Leica, IL) and deparaffinized and stained either with Safranin O/Fast Green or Hematoxylin/Eosin. The sections were assessed for surface regularity, existence of PLA matrix, matrix staining of the cartilaginous tissues, reconstruction of the subchondral bone in the deeper layer, and integration of the repair tissue with the adjacent tissues. The extent of inflammatory response and osteophyte formation were also evaluated. Macroscopic and histologic evaluations were performed by two other observers.

Immunohistochemical staining

Sections for staining the slides were deparaffinized by heating at 57°C for 1 hour. The slides were then put into a xylene substitute for 10 minutes twice, followed by placing in 100% ethanol for 1 minute, 70% ethanol for 1 minute, 25% ethanol for 1 minute, and Tris-buffered saline (TBS) for 1 minute to rehydrate serially. For recovering antigenicity, the slides were treated with decal antigen retrieval solution for 30 minutes (Bio Genex, San Ramon, CA). Afterward they were rinsed with 100% methanol twice, then with 70% methanol and TBS, and then treated with 3% tritonX-100 in TBS for 10 minutes. The slides were then treated with 1% H_2O_2 in water for 30 minutes, rinsed with TBS three times, and reacted with primary monoclonal type II collagen antibody (Medicorp, Montreal, Quebec, Canada) at a dilution of 1:100 of 0.1% tritonX-100 and 1% BSA in TBS overnight at room temperature. The slides were rinsed with TBS thrice and incubated with biotinylated antimouse secondary IgG antibody (Sigma) at a dilution of 1:500 in TBS for 1 hour at room temperature. Following a rinse with TBS three times, the sections were incubated with peroxidase-linked avidin (Sigma) at a dilution of 1:500 in TBS for 1 hour. The slides were then rinsed with TBS three times. Diaminobenzidine (Sigma) and Hematoxylin were used for color development and counter staining.

Assessment of MC viability in the defect

The rabbits in Group 3 ($n = 4$ in each time period) were sacrificed and all the tissues inside the defect area were harvested with a curette. Genomic DNA was isolated from the tissues using phenol and chloroform extraction aided by sonication (Sonifier 350; Branson Ultrasonic, Danbury, CT) and ethanol precipitation.

PCR was performed with Taq DNA polymerase (Promega Express, Philadelphia, PA) and oligonucleotide primer pairs (Sigma) based on the sequences of the rabbit MMP-1 cDNA (sense: 5′-GGTACCAAGAGAAAGGGAGGCAAGAC-3′, antisense: 5′-CTCGAGCAGATCCTTCTAATGCCTGGAC-3′) and SRY cDNA (sense: 5′-TGAACGCATTCATGGTGTGGT-3′, antisense: 5′-AGTCTTTGCGCCTCCTGGAA-3′). PCR conditions included an initial denaturation step at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C (25 seconds each), annealing at 55°C (1 minute), and extension at 72°C (1 minute) for both MMP-1 and SRY. PCR products were analyzed by 1.5% agarose gel electrophoresis and viewed by ethidium bromide staining. The predicted sizes of PCR-amplified products were 375 bp for MMP-1 and 157 bp for SRY genes (Fig. 1).

To estimate cell numbers in the samples, PCR was first performed with several known quantities of male-derived MCs, that is, 1×10^6, 5×10^5, 2.5×10^5, 1.25×10^5, 6.13×10^4, and 3.06×10^4. PCR products were electrophoresed through 1.5% agarose gels (Fig. 2A, B), and the strength of these bands was quantified using the National Institutes of Health Image Analysis Software (NIH, Bethesda, MD). Then the linear functions of each two adjacent points (i.e., 1×10^6 and 5×10^5, 5×10^5 and 2.5×10^5) were calculated and the standard lines of PCR-amplified MMP-1 and SRY sequences were generated (Fig. 2C, D). The intensity of the bands from all samples was measured by NIH software, and the cell numbers were estimated using one of the linear functions from the standard lines as a semi-quantitative method.

Accuracy of PCR semi-quantitative method

In order to evaluate the accuracy of this cell counting method, the cell numbers in the construct (time zero) were calculated by counting the cells directly in cell suspension using a hemocytometer and then subtracted from the original 1 million cells. We also calculated the cell number in the construct by MMP-1 and SRY with PCR method (all the cells
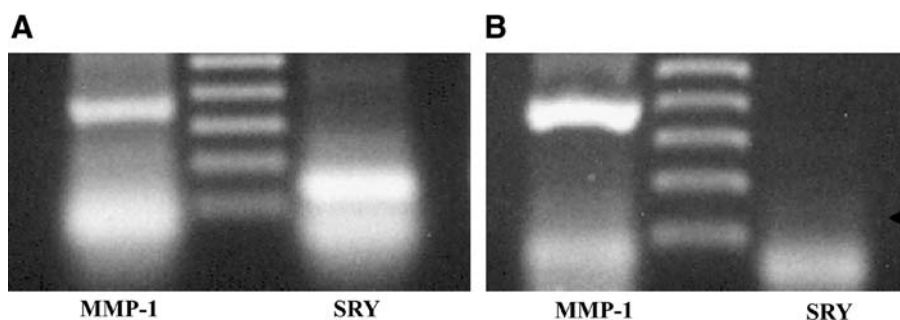

FIG. 1. The polymerase chain reaction (PCR) of matrix metalloproteinase (MMP)-1 (left lanes) and sex-determining region (SRY) (right lanes) genes was performed with male (**A**) and female (**B**) mesenchymal cells (MCs). Both MMP-1 and SRY genes exist in male-derived MCs. MMP-1 genes exist in female-derived MCs, but SRY genes do not.
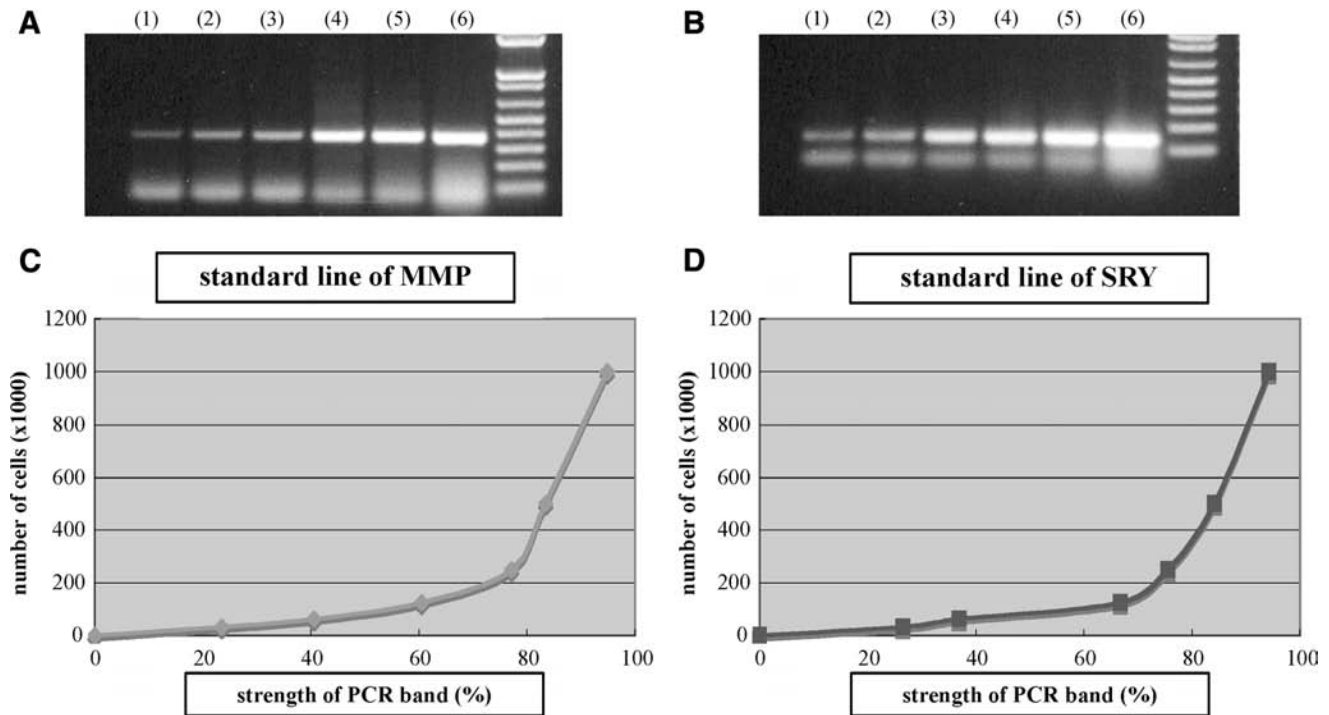
MMP-1   SRY   MMP-1   SRY

FIG. 2. PCR of MMP-1 gene (**A**) and SRY gene (**B**) was performed with different concentrations of MCs: (1) 3.06×10^4, (2) 6.13×10^4, (3) 1.25×10^5, (4) 2.5×10^5, (5) 5×10^5, and (6) 1×10^6. The strength of each PCR band from the known cell numbers was calculated using NIH software, and the standard lines of MMP-1 (**C**) and SRY (**D**) genes were drawn. The X-axis shows the strength of the PCR bands (%), and the Y-axis shows the number of cells ($\times 1000$).

were MCs at this time point). These three numbers represented the same measures at this time point, because all cells in the PLA were considered MCs.

Statistical analysis

The variation of cell numbers with time was statistically analyzed using the two-tailed Student's t-test assuming equal variances. All results were presented as means with standard deviations. Significance was defined as a p-value < 0.05.

Results

Gross assessment

The rabbits in all groups showed no symptoms of infection or swelling in the knee joints.

Group 1: At 1 week postsurgery, the margins of the defects could be recognized and the surfaces depressed. At 4 weeks the margins were detectable and the surfaces depressed; however, the defects were filled with white opaque tissue. At 12 weeks the surfaces did not depress and the defects were filled with white opaque tissue. At 24 weeks the surfaces were depressed in two of the three samples, and the defects were filled with white opaque tissue (Fig. 3-1, 3-4, 3-7, 3-10).

Group 2: At 1 week the defect areas were defined and the surfaces slightly depressed. At 4 weeks the surfaces were still slightly depressed and the defects covered with white opaque tissue. At 12 weeks the surfaces did not depress but were covered with white opaque tissue. At 24 weeks the margin of the defects was recognizable; however, the surfaces did not depress and the defects were still covered with white opaque tissue (Fig. 3-2, 3-5, 3-8, 3-11).

Group 3: At 1 week the margins were recognized and the surfaces slightly depressed. At 4 weeks the surfaces did not depress and the defects were covered with white opaque tissue. At 12 weeks the surfaces did not depress and the color of the tissues covering the defects became similar to the surrounding tissue. At 24 weeks the margins became unclear, the surfaces did not depress, and the color of the surface was similar to that of the surrounding tissue (Fig. 3-3, 3-6, 3-9, 3-12).

Histological evaluation

Group 1: At 1 week postsurgery the margins could be recognized and the surfaces depressed and irregular. Some areas of the defects appeared to be filled with fibrous tissue; however, the defects were not repaired with cartilaginous and/or osseous tissue. At 4 weeks the surfaces were still depressed. Most parts of the defects were filled with fibrous tissue. The repaired tissue was not stained with Safranin O. At 12 weeks the surface became smooth and the deeper layer of the defects was reconstructed with bone tissue, but the superficial layer was not repaired with cartilage tissue. At 24 weeks the deeper layer of the defects was remodeled with bone, though the defects were not repaired with cartilage tissue. There was integration between the defect area and the host tissue (Fig. 4-1, 4-4, 4-7, 4-10).

Group 2: At 1 week the margins were discernable and the surfaces slightly depressed. The defects were occupied with PLA matrix, but the defect areas were not repaired with cartilage tissue. At 4 weeks the surfaces were still slightly depressed and PLA was confirmed to exist in the defect areas. The repaired tissues slightly increased compared with that of the 1-week model, but the defects were not repaired
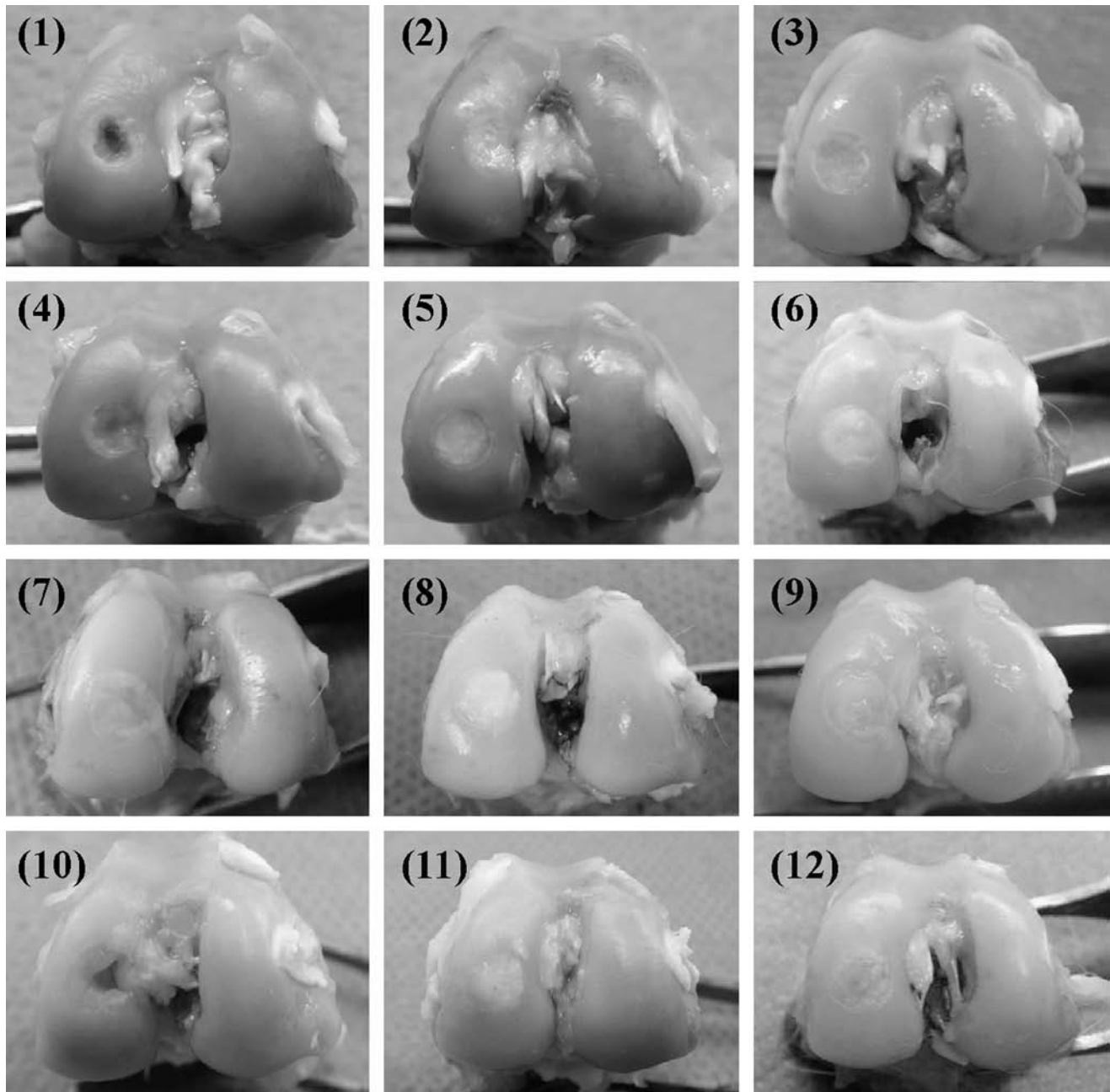
FIG. 3. Macroscopic findings of the defects: Group 1 (1, 4, 7, and 10) are defect alone, Group 2 (2, 5, 8, and 11) are PLA core alone without MCs, and Group 3 (3, 6, 9, and 12) are MC/PLA construct. (1–3) are at 1 week, (4–6) at 4 weeks, (7–9) at 12 weeks, and (10–12) at 24 weeks after surgery.

with cartilage and bone tissue. The integration of PLA with the host was not achieved. At 12 weeks the surfaces became smooth and PLA still existed in the defects. The deeper layer of the defects was remodeled with bone, however the defects were not repaired with cartilage tissue. At 24 weeks PLA still existed in the defects, but the defects were not repaired with cartilage tissue. The integration between the defect area and the host tissue was present (Fig. 4-2, 4-5, 4-8, 4-11).

Group 3: At 1 week the surfaces were slightly depressed, the defects were occupied with PLA including fibrous tissues, and the defects were not repaired with cartilage tissues. At

4 weeks the surfaces were slightly depressed and PLA existed in the defects. The deeper layer of the defects was not repaired with bone tissue; however, the superficial layer of the defects was stained with Safranin O, indicating the existence of cartilaginous matrix. At 12 weeks the surface became smooth and PLA existed in the defects. The layer of cartilaginous tissue became deeper than that of the 4-week model, and the integration of PLA with the host was not achieved. Integration between the defect area and the host tissue was developing. At 24 weeks the surface was smooth, and PLA still existed in the defects. The deeper layer of the defects was repaired with
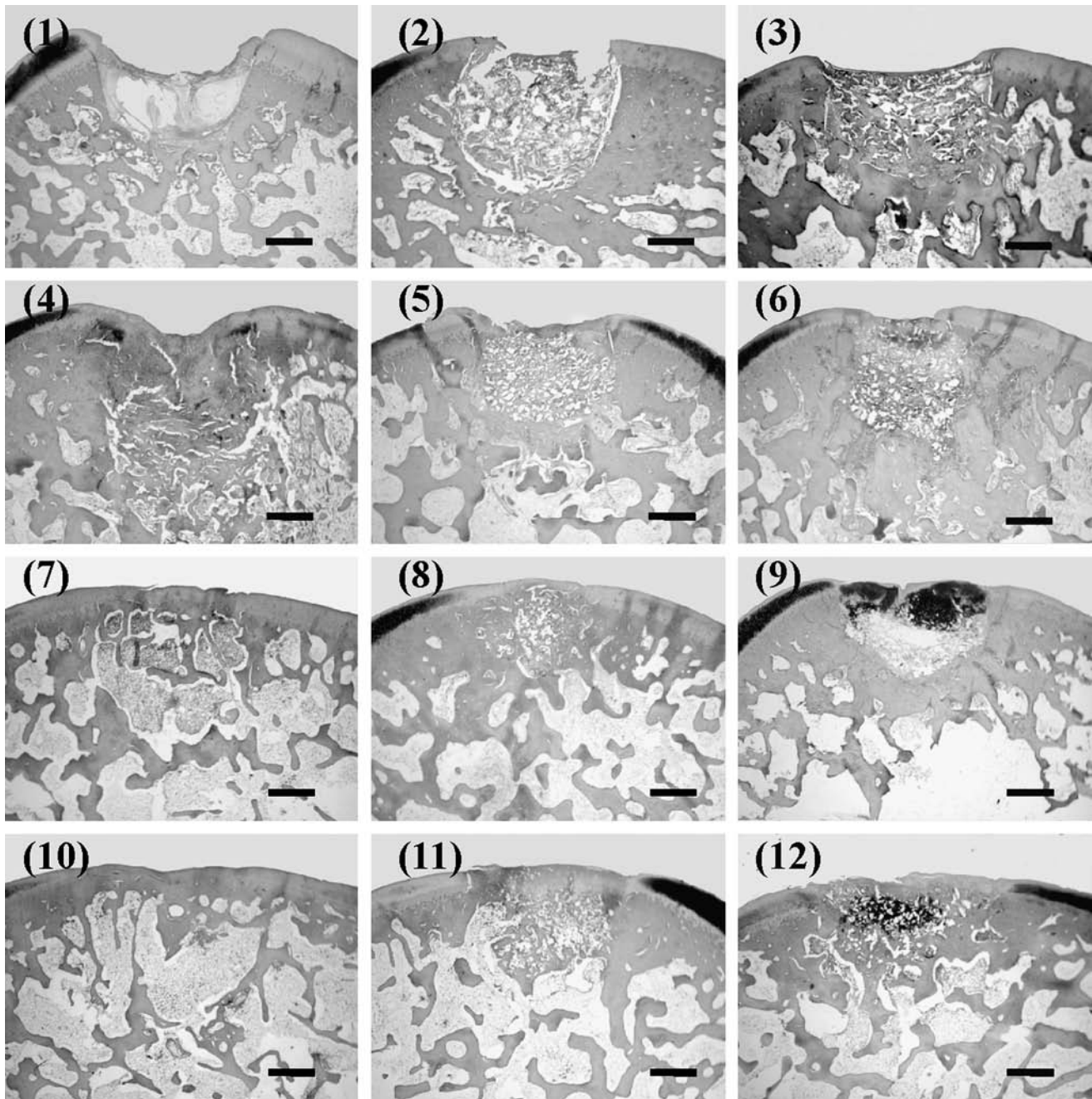
FIG. 4. Histological findings of the defects stained with Safranin O/Fast Green (scale bar: 1 mm). (1), (4), (7), and (10) correspond to Group 1; (2), (5), (8), and (11) correspond to Group 2; and (3), (6), (9), and (12) correspond to Group 3. (1)–(3) are at 1 week, (4)–(6) at 4 weeks, (7)–(9) at 12 weeks, and (10)–(12) are at 24 weeks after surgery.

bone tissue and the superficial layer was filled with cartilaginous tissue. Integration between the defect area and the host tissue was achieved (Fig. 4-3, 4-6, 4-9, 4-12).

Immunohistochemical staining

The sections at 24 weeks in Group 3 were used for immunohistochemical staining. The cartilage layer was stained with type II collagen, but the subchondral layer did not stain in the surrounding host area. Some areas of the superficial layer, which were not stained with Safranin O, stained with type II collagen similar to the surrounding cartilage layer. A few areas of the deeper layer stained with Safranin O also stained with type II collagen (Fig. 5).

Accuracy of PCR semi-quantitative method

At time zero, just after the creation of the construct, all the cells in the construct were considered MCs. The cells were counted directly by MMP-1 and SRY with PCR method. These three methods showed no significant differences, and therefore the accuracy of the PCR semi-quantitative method was confirmed.
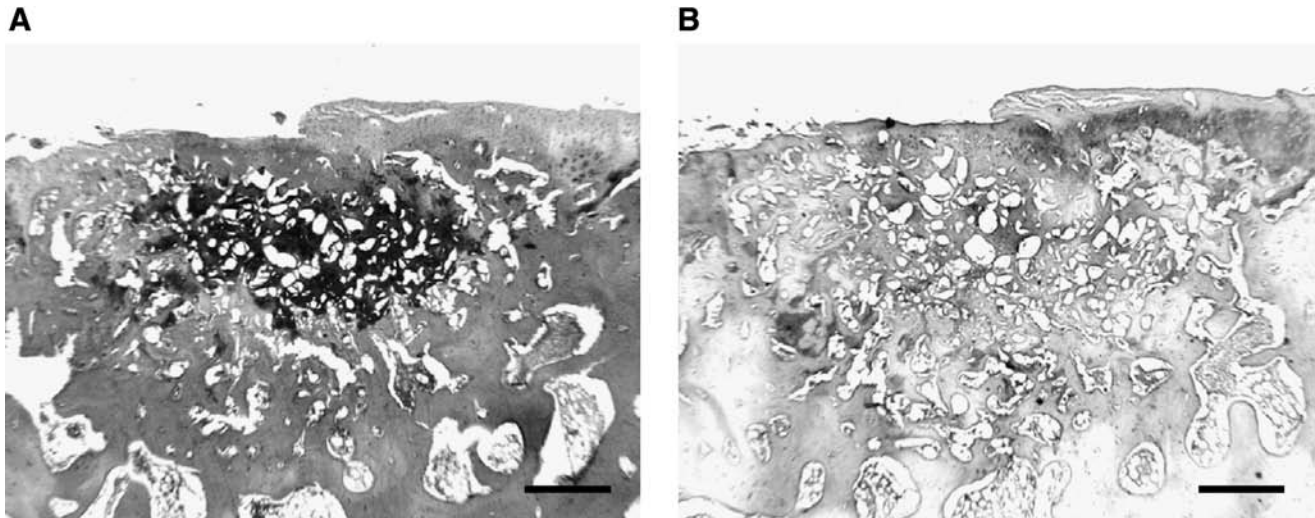
A

B

FIG. 5. Higher magnification of the section of Group 3 stained with Safranin O/Fast Green at 24 weeks (**A**), and immunohistochemical staining of type II collagen (**B**). Scale bar: 500 μm.

Variation of MC numbers in PLA from in vitro *to* in vivo *conditions*

The repaired tissues were harvested from the transplanted areas in Group 3. These tissues were soft and easy to separate with curette from the host tissues in the 1-, 4-, and 12-week groups. In the 24-week group, the repaired tissues connected tightly to the adjacent tissues, and it was impossible to harvest all the repaired tissues in the defect. In order not to contaminate the host tissues, the repaired tissues were harvested only from the center area of the defects in this group.

PCR was performed for harvested tissues at different time periods *in vivo*, as well as on the day of MC/PLA construct creation (time zero), and 1 week after culturing of the construct *in vitro*. The strength of all PCR bands were calculated using NIH imaging software and applied to the standard lines of MMP-1 and SRY genes. Then the number of total cells and transplanted MCs in the harvested tissues were estimated.

At time zero, total number of cells was 9.3×10^5 and increased up to 9.8×10^5 during 1 week culturing *in vitro*. The constructs were transplanted and the total cell numbers were 9.8×10^5 at 1 week, 1.0×10^6 at 4 weeks, 1.1×10^6 at 12 weeks, and 4.2×10^5 at 24 weeks. MC numbers were 7.8×10^5 at 1 week after transplantation, 3.5×10^5 at 4 weeks, and 3.8×10^4 at 12 weeks. MCs were not detected at 24 weeks (Fig. 6). The ratio of the number of MCs divided by the total number of cells in the defect was 79% at 1 week after transplantation, 34% at 4 weeks, and 4% at 12 weeks. The ratio at 24 weeks was not calculated, because transplanted MCs were not detected in the defects at this time (Fig. 7).
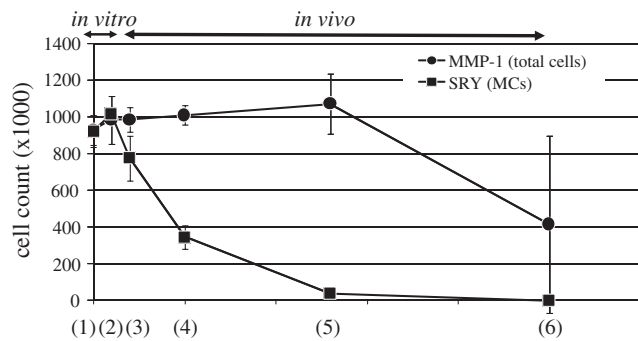
Discussion

The survivability of MCs in a PLA scaffold in an osteochondral defect and the possibility of osteochondral repair with MC/PLA construct were evaluated in the *in vivo* conditions. Transplanted MCs survived and contributed to the osteochondral repair by expressing cartilaginous
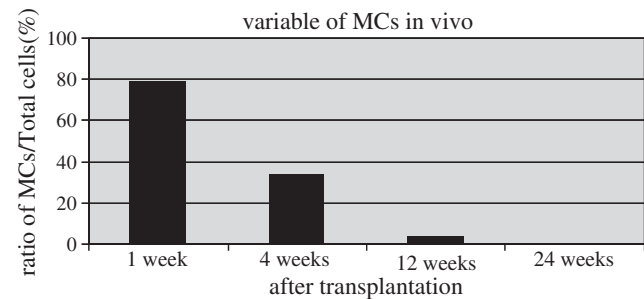
FIG. 6. The varying cell numbers of MCs (line with squares) and total cells (line with circles) in the defects from *in vitro* to *in vivo* conditions. The data was presented as mean ± SD. The X-axis shows the time periods: (1) time zero, (2) the construct 1 week after culturing, (3) 1 week after transplantation, (4) 4 weeks after transplantation, (5) 12 weeks after transplantation, and (6) 24 weeks after transplantation. (1) and (2) represent *in vitro*, and (3)–(6) represent *in vivo* conditions. The Y-axis shows the cell numbers (×1000).

FIG. 7. The varying ratio of transplanted MCs to total cells with time after transplantation of the construct *in vivo*. The Y-axis shows the ratio of MCs to total number of cells. The ratio at 24 weeks was zero, because transplanted MCs were not detected in the defects.

matrix. The MCs, however, were replaced by host cells with time and disappeared within 24 weeks after the transplantation.

Several chondrogenic lineage cell sources have been reported, such as chondrocytes, MCs, periosteal cells, and perichondrial cells.[15,30–32] Periosteum was used to affix transplanted cells in the transplanted area originally. The scaffolds commonly used are PLA, polylactic-glycolic acid (PLGA), collagen gel, fibrin glue, and atelocollagen gel.[28,33–36] Although chondrogenic cell/scaffold constructs could facilitate transplantation of a large number of cells in the defect, regeneration of cartilage had not been fully achieved. Additional growth factors and gene therapies have been described to improve repair tissue close to hyaline cartilage. But it is essential to elucidate the behavior and survivability of transplanted cells when cell-based treatment is performed. Therefore, we tried to evaluate the role of transplanted cells without advanced treatments in this study.

MCs and a PLA core have been applied for osteochondral reconstruction. MCs including MSCs have the capacity to differentiate into both cartilage and bone under adequate conditions. PLA is a biocompatible, biodegradable, and nonimmunologic reactive material *in vivo*.[29] The benefits of chondrogenic cell/PLA constructs for cell survival, increase in cell number, and expression of cartilaginous matrix *in vitro* were demonstrated.[26]

Articular cartilage tissues have a limited intrinsic healing potential.[37] The natural healing capacity of a full-thickness osteochondral defect was, however, demonstrated in a rabbit model with a small-size defect.[38] The defect was 3.7 mm in diameter and 3.0 mm in depth in our experiment, and could be applied as an unrepaired defect model as the defect was not repaired with hyaline cartilage in Group 1.

Recently, the potential of an immunoresponse by small polymeric particles released from the PLA has been described.[39] However, no inflammatory reaction was observed in the knee joints of Group 2. The PLA was also confirmed to not have the capacity to repair an osteochondral defect.

Twenty-four weeks after transplantation of the MC/PLA construct in Group 3, the defect was filled with PLA matrix, glycosaminoglycan (GAG), and type II collagen. When compared with serial sections having positive areas of GAG and type II collagen, there was coexistence in some areas. Thus, it is assumed that MCs could maintain their capacity to differentiate into cartilage lineage cells in the PLA *in vitro*, and once MCs are transplanted into the defect some MCs could express one or both of the cartilage matrices *in vivo*.

To detect the presence of transplanted cells, MSC/gelatin constructs were transplanted into osteochondral defects, and MSCs were detected 2 weeks later by labeling with fluorescent dye.[40] The chondrocytes were also detected in the defect up to 14 weeks after ACI using fluorescent dye.[41] Emans *et al.*[42] demonstrated transplantation of chondrocyte or periosteal cell/scaffold constructs into an osteochondral defect and examined cell viability by fluorescent labeling 5 days after transplantation. However, the intensity of the fluorescent dye may decrease with time and may not be adequate for a long-term study. Mierisch[43] performed the transfection of a fluorescent protein gene to chondrocytes and traced the transplanted cells in the defect 4 weeks after ACI. This method, however, includes difficulty with the ratio of transfection efficiency.

By contrast, we previously succeeded in viewing transplanted cells using transgenic rats *in vivo*. Transgenic rat–derived MCs were transplanted into osteochondral defects of wild-type rats, and the fate of transplanted cells was evaluated by *in situ* hybridization technique[24] or with a confocal laser scanning microscope.[25] The cells could be traced as long as they survived. Nishimori *et al.*[44] also demonstrated transplantation of MSCs into osteochondral defects using a transgenic rat model and reported the existence of transplanted cells up to 4 weeks after transplantation. In our models, however, osteochondral defects were repaired with hyaline-like cartilage and the transplanted cells were detected up to 24 weeks after transplantation. The number of cells in the transplanted area was not determined because cell viability was assessed with two-dimensional sections.

Ostrander *et al.*[28] demonstrated the SRY study for examining the fate of donor cells in their rabbit model. They transplanted a perichondrial cell/PLA construct, and the survivability of the transplanted cells was evaluated up to 4 weeks after transplantation. The results showed that more than 87% of the transplanted cells survived during this time in the defect and, though the number of transplanted cells decreased, the total number of cells increased with time. They concluded that a decline in the percentage of donor cells was possible only with an influx of host cells. Even though our results showed that MCs stayed longer than the perichondrial cells, MCs disappeared within 24 weeks. However, a decline in donor cell numbers does not necessarily mean failure of the treatment.[32] These authors preincubated the construct before transplantation and demonstrated enhanced survivability of perichondrial cells in the PLA. Based on their result, in our study the construct was cultured 1 week before transplantation and number of MCs increased *in vitro*. They discussed the importance of a marginal influx of host cells into the implanted construct and a marginal efflux of donor cells into surrounding tissues for integration between host and donor. They concluded that donor cells decreased and host cells increased at the same time in the defect with integration.

We focused on the total number of cells in the defect, detected by MMP-1, as well as the transplanted cells, detected by SRY, and thus the cell movement between donor and host could be assessed in this model. Total cell number did not change significantly up to 12 weeks; however, MCs were decreasing in number. PLA was confirmed to provide a suitable condition for cell survival in the defect.[28] The efflux of the transplanted MCs into surrounding tissue was also viewed histologically.[25] Based on these results, MCs were thought to survive in the PLA and to be replaced by an influx of host cells, with an assumed efflux of MCs into surrounding tissue. Because of this process, the integration of repair tissue with the adjacent tissue was well developed at 24 weeks after transplantation, even though the tissues in the defect area were easily harvested by curettage until 12 weeks. In order not to contaminate MCs in the host tissue, the tissues in the center area of the defects were harvested at this time period. MCs could not be detected in the 24-week samples, and therefore MCs were thought to have differentiated into cartilage and bone lineages in the defect in the *in vivo* condition, efflux to the surrounding tissues, and be replaced by host cells while expressing cartilage and bone matrices.

In this study we succeeded in examining the variation of MCs *in vivo* by a semi-quantitative method. Cartilage recon-

struction trials with cell/scaffold construct transplantation for cartilage repair have been performed in the clinical area. However, it is still unclear how many cells survive in the defects and how many cells should be transplanted. The results of this study could provide the answers to these questions, as it seems that transplanted cells decrease in the early time period. If further treatments are added to improve regenerated tissue, it is suggested they be done *in vitro* or in the early stage *in vivo*. The SRY study is a useful method to evaluate the survivability of transplanted cells *in vivo*, and MC/PLA constructs could be clinically used for osteochondral repair.

Conclusion

Transplanted MCs can survive in PLA scaffolds in an osteochondral defect and promote repair of the defect. However, the number of cells decrease with time *in vivo.* These results are essential to understanding the behavior of autologous cell transplantation for tissue engineering strategies.

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Disclosure Statement

No competing financial interests exist.

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