Muscle Derived, Cell Based *Ex Vivo* Gene Therapy for Treatment of Full Thickness Articular Cartilage Defects

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ABSTRACT. ***Objective.*** To evaluate the effectiveness of transplanted allogeneic muscle derived cells (MDC) embedded in collagen gels for the treatment of full thickness articular cartilage defects, to compare the results to those from chondrocyte transplantation, and to evaluate the feasibility of MDC based *ex vivo* gene therapy for cartilage repair.

Methods. Rabbit MDC and chondrocytes were transduced with a retrovirus encoding for the ß-galactosidase gene (LacZ). The cells were embedded in type I collagen gels, and the cell proliferation and transgene expression were investigated *in vitro*. *In vivo,* collagen gels containing transduced cells were grafted to the experimental full thickness osteochondral defects. The repaired tissues were evaluated histologically and histochemically, and collagen typing of the tissue was performed.

Results. The MDC and chondrocyte cell numbers at 4 weeks of culture were 305 ± 25% and 199 ± 25% of the initial cell number, respectively. The initial percentages of LacZ positive cells in the MDC and chondrocyte groups were 95.4 ± 1.9% and 93.4 ± 3.4%, and after 4 weeks of culture they were 84.2 ± 3.9% and 76.9 ± 4.3%, respectively. *In vivo*, although grafted cells were found in the defects only up to 4 weeks after transplantation, the repaired tissues in the MDC and chondrocyte groups were similarly better histologically than control groups. Repaired tissues in the MDC group were mainly composed of type II collagen, as in the chondrocyte group.

Conclusion. Allogeneic MDC could be used for full thickness articular cartilage repair as both a gene delivery vehicle and a cell source for tissue repair. (J Rheumatol 2002;29:1920–30)

Key Indexing Terms:
CARTILAGE GENE THERAPY MUSCLE DERIVED CELLS
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Articular cartilage has very limited intrinsic healing capacity, thus injured cartilage cannot fully regenerate and leads to secondary arthritis[1,2]. Numerous methods have been attempted to enhance the repair of full thickness articular cartilage defects, including abrasion arthroplasty[3,4], microfracture[5], transplantation of chondrocytes[6,7], perichondrium[8] and periosteum[9], and osteochondral graft[10]. However, no treatment has yet regenerated long-lasting hyaline cartilage. In 1994, Brittberg, *et al* reported that cartilaginous defects in the knee were treated successfully with transplantation of chondrocytes cultured in monolayer[6]. However, concerns were raised about this type of chondrocyte transplantation using monolayer culture: one is the maintenance of the chondrocyte phenotype during the prolonged monolayer culture period, and the other is the risk of transplanted chondrocyte leakage from the grafted site during range of motion and weight bearing on the joint. One solution for these concerns is using a 3-dimensional culture system without altering their phenotype[11-14]. Recently, Katsube, *et al* reported transplantation of chondrocytes cultured in type I collagen gels[7]. They showed better repair of the defects histologically by transplanting chondrocytes cultured in collagen gels than by transplanting chondrocytes cultured in monolayer or periosteal graft only, or foregoing treatment altogether. We believe one of the best procedures available for the treatment of full thickness articular cartilage defects is transplantation of chondrocytes embedded in collagen gels.

Another important approach for the repair of full thickness articular cartilage defects is the use of growth factors that can enhance cartilage healing. Several growth factors, including transforming growth factor-ß1 (TGF-ß1)[15,16], insulin-like growth factor-1 (IGF-1)[17,18], and bone morphogenetic protein 2 (BMP2)[19,20] have been found capable of enhancing chondrocyte proliferation and extracellular matrix synthesis *in vitro* and *in vivo*. Although some studies have revealed that

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direct injection of those therapeutic proteins into the joint has some beneficial effect on cartilage healing, their relatively short half-life *in vivo* often necessitates high dosage of protein or repeated injections. Gene therapy represents one potential solution to these problems and is a promising method to deliver large amounts of therapeutic proteins into cartilage defects for an extended period. Chondrocyte transplantation is an especially good candidate for *ex vivo* gene therapy for cartilage repair. Chondrocytes are the logical cell of choice for cartilage, as they can be harvested from the host before transplantation. Subsequently, the cells can be genetically engineered *in vitro* prior to implantation.

In the case of gene therapy and tissue engineering applications for cartilage repair, transplanted cells have 2 different functions: a cell source for the repaired tissue and a gene delivery vehicle. For both roles, chondrocytes are not the only cells that can be used for this purpose. Pluripotent stem cells derived from bone marrow that can differentiate into various lineages can potentially be used for cartilage repair[21,22]. Recently, it has been reported that skeletal muscle also contains a population of cells that display stem cell characteristics[23-25]. Therefore, skeletal muscle may represent a source for the isolation of cells that can be used in gene therapy for cartilage repair.

We evaluated the effectiveness of transplanted allogeneic muscle derived cells (MDC) embedded in collagen gels for the treatment of full thickness articular cartilage defects, to compare the results to those from chondrocyte transplantation, and to evaluate the feasibility of *ex vivo* gene therapy for the repair of articular cartilage.

MATERIALS AND METHODS

Experimental rabbits were kept in the Rangos Research Center Animal Facility of Children's Hospital of Pittsburgh in accord with the policies and procedures detailed in the *Guide for the Care and Use of Laboratory Animals* (US Department of Health and Human Services). The research protocol was reviewed and approved by the Animal Research and Care Committee at Children's Hospital of Pittsburgh and the University of Pittsburgh.

Isolation of MDC and chondrocytes. Twelve-week-old New Zealand white (NZW) rabbits weighing about 2.5 kg (range 2.3–2.7 kg) were used in this study. The quadriceps femoris muscles were removed from the animals' hind limbs, and muscle tissue was isolated from other connective tissue and minced into small pieces. Muscle tissue was digested by serial 1 h incubations at 37˚C with 0.2% collagenase type XI (Gibco BRL, Grand Island, NY, USA), dispase (grade II, 240 units, Gibco BRL), and 0.1% trypsin, all diluted in Hanks' balanced salt solution (HBSS, Gibco BRL). After the slurry was filtered with a 70 µm cell strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA), the filtrate was plated on a collagen coated flask for 1 h at 37˚C to allow dissociated cells to adhere. The MDC were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% penicillin (100 IU/ml)/streptomycin (100 µg/ml)/amphotericin B (0.25 µg/ml). The cell suspension was then aspirated and replated on a collagen coated flask for 1 h. During the first several passages of the primary cultures, the MDC were purified by this preplate technique to eliminate fibroblastic cells[26,27]. After the purified cells were kept in monolayer culture for several days to produce a confluent cell population, the cells were detached from the flasks with 0.25% trypsin. Isolated cells were collected by centrifugation (2000 g, 5 min). These cells were washed 3 times with culture medium before the cell numbers were counted using a hemocytometer with the trypan blue dye exclusion test.

Articular cartilage slices were first taken from the knee, hip, and shoulder joints of the animals, and then detached from adherent connective tissue before being cut into small pieces. Chondrocytes were isolated by enzymatic digestion. First, cartilage slices were minced and washed 3 times with sterile 0.9% sodium chloride. Then they were treated with 0.25% trypsin in sterile saline for 30 min, followed by 0.25% collagenase type II (Gibco BRL) for 6 h at 37˚C in a culture tube. The cell suspension was then centrifuged at 2000 g for 5 min. Cells were washed 3 times with culture medium before they were counted.

Immunohistochemical staining of MDC for desmin. To investigate the myogenicity index of the MDC, a monoclonal antibody specific for desmin was used. Desmin is expressed in myogenic cells in contrast to fibroblastic cells[23,26,27]. After 1.0×10^5 MDC were plated in a 20 mm culture dish and incubated 24 h at 37˚C, the cultured cells were fixed with cold methanol (–20˚C) for 1 min and blocked with 5% horse serum for 1 h. Primary antibody (1/200 monoclonal mouse anti-desmin; Sigma, St. Louis, MO, USA) was diluted in phosphate buffered salt solution (PBS). The primary antibody was applied at room temperature overnight. After biotinylated secondary antibodies were applied for 1 h at room temperature, the cells were rinsed with PBS. Then they were incubated 1 h at room temperature with 1/300 streptavidin conjugated with Cy3 fluorochrome. The cells were visualized by fluorescence microscopy, and the percentage of positive cells was calculated by counting positively stained cells in 10 randomly chosen 20× fields.

Preparation of retro-LacZ virus. The retroviral vector MFG-NB used for these studies contained a modified LacZ gene (nls-LacZ) that included a nuclear localization sequence cloned from the simian virus (SV40) large tumor antigen, which is transcribed under the control of the long terminal repeat (LTR). The titer of this retroviral vector was 1×10^7 colony forming units (cfu)/ml.

Transduction of cells and preparation of collagen implants. MDC and chondrocytes were seeded at a density of 1.5×10^6 cells in 75 cm^2 flasks (Becton Dickinson), allowed to grow to 50% confluence, and washed twice with sterile PBS. Then they were incubated with 5 ml of retroviral suspension (1×10^7 cfu/ml) for 4 h in 5% CO_2/95% air at 37˚C in the presence of 8 µg/ml polybrene (Sigma) with gentle swirling of the vector suspension every 15 min. After this initial incubation, 5 ml fresh DMEM was added to each flask, and cells were incubated an additional 24 h at 37˚C. At 24 h postinfection, the viral suspension was removed from each flask, and cells were washed twice with PBS. After MDC and chondrocytes were isolated through 0.25% trypsin, the isolated MDC and chondrocyte suspension was centrifuged at 2000 g for 5 min. These cells were washed 3 times with culture medium before being counted. MDC and chondrocytes were embedded in bovine type I collagen gels (Vitrogen 100, Celtrix, Santa Clara, CA, USA) containing the culture medium at a concentration of 2×10^6 cells/ml, and then 125 µl of the collagen cell medium mixture was placed in a 20 mm diameter culture dish (Becton Dickinson). The cell-collagen composites were incubated at 37˚C for 30 min to allow the collagen to become gel before they were overlaid with 2.0 ml of culture medium. Cell cultures were incubated in 5% CO_2/95% air at 37˚C and replaced with fresh DMEM every 3 days. For the *in vivo* study, the same volumes of collagen gels with and without cells were prepared and cultured the same way.

Number of MDC and chondrocytes in vitro. At 1, 2, 3, and 4 weeks postculture, MDC and chondrocytes in collagen gels were collected by incubation with 0.25% collagenase in DMEM at 37˚C for 30 min. The cell suspension was collected, and viable cells were counted using a hemocytometer in conjunction with the trypan blue dye exclusion test. At each time point we examined 2 groups of 6 samples, determining the growth curves of chondrocytes and MDC.

Percentage of LacZ positive cells after transduction. After we counted the MDC and chondrocytes at each time point, the cell suspensions were centrifuged at 2000 g for 5 min. The collected cells were seeded in a 20 mm culture dish at a concentration of 1.0×10^5 cells per dish before they were incubated at 37˚C for 24 h to allow them to adhere to the culture dish. After 24 h incubation, the cells were washed twice with PBS, fixed with 1.5% glu-

taraldehyde (Sigma) for 1 min, and rinsed twice with PBS. To detect LacZ gene expression by the transduced cells *in vitro*, the cells were incubated in 2 ml of X-gal substrate [0.4 mg/ml 5-bromo-chloro-3-indolyl-b-D-galactoside (Boehringer-Mannheim, Indianapolis, IN, USA), 1 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$/5 mM $K_3Fe(CN)_6$] in PBS for 24 h. The percentage of LacZ positive cells was determined by counting the number of LacZ positive cells (blue cells) and negative cells (colorless cells, white) using microscopy. We counted the cells of 10 different areas (at least 100 cells per area) and calculated the average percentage of LacZ positive cells.

Surgical procedure. After 3 weeks of culturing MDC and chondrocytes in collagen gels, they were carefully detached from the culture dishes and washed 3 times in the culture medium for transplantation. After 3 weeks of culturing, the average cell number in the collagen gels increased 214 ± 24% in the MDC group and 172 ± 27% in the chondrocyte group. The collagen of gels maintained its 3-dimensional structure after *in vitro* culturing. Seventy-two 12-week-old NZW rabbits were anesthetized by intramuscular injection of ketamine HCl (Abbott Laboratories, North Chicago, IL, USA) at a dose of 8 mg/kg body weight and xylazine (Xyla-ject, Phoenix Pharmaceuticals, St. Joseph, MO, USA) at a dose of 8 mg/kg body weight; anesthesia was maintained with inhalation of 1% to 3% halothane gas using a mask. Both knee joints were approached through medial parapatellar incision, and each patella was dislocated laterally. Full thickness articular cartilage defects (6 × 4 mm wide × 3 mm deep) were created in the trochlear groove of the femur using a Surgairtome (Linvatec, Largo, FL, USA) with a 3 mm stainless steel burr, which was operated by nitrogen gas. The animals were divided into 3 groups. In the MDC and chondrocyte groups, the defects were filled with MDC or chondrocytes cultured in collagen gels. In the control group, defects were filled with collagen gel without cells. The defects were covered with autologous periosteum that had been obtained from the ipsilateral medial proximal tibia. The periosteum was sutured to each corner of the defect with 6-0 polypropylene sutures (Proline, Ethicon, Somerville, NJ, USA) with the cambium layer of the periosteum facing to the patella (Figure 1). All rabbits were allowed free cage activity after surgery.

Macroscopic examination. After initial anesthesia with the described dose of ketamine HCl and xylazine, the rabbits were killed with an intracardiac injection of pentobarbital sodium (Nembutal, Abbott) at 2, 4, 8, 12, and 24 weeks after the operation. Six defects of each group were examined macroscopically for color, integrity, contour, and smoothness, and were photographed. Synovitis of the knee was also investigated.

Histological evaluation. After macroscopic examination, the distal femurs were dissected and fixed with 10% buffered formalin for 1 week. Then they were decalcified with decalcifying solution (Decalcifier II, Surgipath, Richmond, IL, USA) for 2 days and embedded in paraffin. Sagittal sections 5 µm thick were obtained from the center of each defect and were stained with safranin O-fast green. Sections were evaluated for the quality of the repaired tissue according to the histological grading scale described by O'Driscoll, *et*

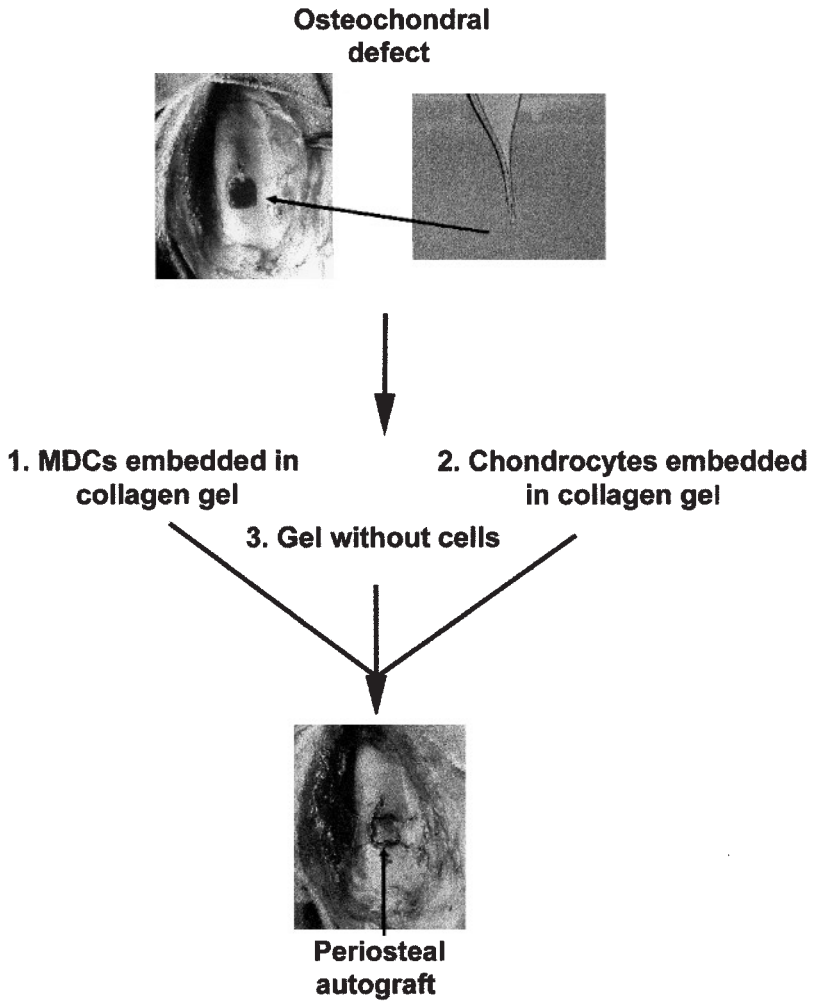

Figure 1. The experimental design. MDC: muscle derived cells.

al[28]. This histological evaluation was performed by 2 of the authors in blind fashion.

Immunohistochemical analysis. *Immunostaining for LacZ*. At 2, 4, 8, and 12 weeks after the operation, 4 tissues of each time point present within the defects were excised sharply en bloc. The tissues were placed in a freezing medium (Triangle Biomedical Science, Durham, NC, USA), flash-frozen at –196˚C in liquid nitrogen, and stored at –80˚C until they were sectioned and stained. After being sectioned with a cryostat into slices 8 µm thick, the tissues were washed with PBS twice, fixed with 1.5% glutaraldehyde for 1 min, and rinsed twice with PBS. To detect grafted LacZ positive cells, the sections were incubated in X-gal substrate in PBS for 2 h. After X-gal staining, sections were counterstained with hematoxylin-eosin. The LacZ positive cells (blue stained cells) were examined using a light microscope (Nikon).

Collagen typing of repaired tissue. At 24 weeks after the operation, we examined the collagen composition of the repaired tissues by electrophoretic analysis of cyanogen bromide derived peptides (CNBr). Repaired tissues in the defects were excised, while surrounding normal cartilage and subchondral bone were carefully avoided. The tissues were freeze-dried, weighed, and digested with CNBr at a concentration of 20 mg CNBr/mg tissue in 70% formic acid. Tissues were digested with the CNBr for 18 h at room temperature. CNBr digests were diluted with water (1/10) and dried. Equal aliquots of CNBr derived peptides diluted in electrophoresis buffer based on original dry weights were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12.5% polyacrylamide gel. CNBr derived peptides from type I collagen purified from bovine bone and type II collagen purified from rabbit articular cartilage were loaded on gel as standards. The intensity of the CB 3,5 peptide from type I collagen and CB 10 from type II collagen was determined by densitometric scanning of the protein bands using a Biorad GS 800 imaging densitometer. The relative ratio of type I α2(I) CB 3,5 and type II collagen CB 10 was used to estimate the relative ratio of type I and type II collagen in the samples.

Statistical analysis. All numerical data were expressed as the mean ± standard deviation. The Mann-Whitney U test was used to compare the percentage of LacZ positive cells between the MDC group and chondrocyte group *in vitro*. For the histological grading scale between the 3 groups *in vivo*, one-way analysis of variance was used. If a statistical difference existed, Scheffe's post-hoc test was used. A p value < 0.05 was regarded as statistically significant.

RESULTS

Immunohistochemical staining of MDC for desmin. Immunostaining for desmin revealed that the percentage of desmin positive cells in primary MDC before purification was 6.7 ± 1.5%, indicating there was a small population of myogenic cells in primary rabbit MDC. Indeed, the majority of MDC that rapidly adhere to the flasks are desmin negative fibroblasts[26,27]. After purification of the MDC by preplating, the percentage of desmin positive cells (myogenic cells) increased to 38.7 ± 2.9% (data not shown).

Number of MDC and chondrocytes in vitro. As for the culture in collagen gels, chondrocytes could maintain their round phenotypes throughout the culture periods for up to 4 weeks. MDC also maintained their round shape at the early time periods of culture, but the numbers exhibiting polygonal fibroblast-like morphology increased with cell culturing. However, myotube or myofiber formations were not observed in the collagen gel culture. Detachment or shrinkage of the gels was not observed. Cell numbers in collagen gels in both groups increased with culturing, as shown in Figure 2A. At 4 weeks of culturing, the cell number in the MDC group was 305 ± 25% of the initial cell number; the number in the chondrocytes group was 199 ± 25%, showing significant difference between the groups.

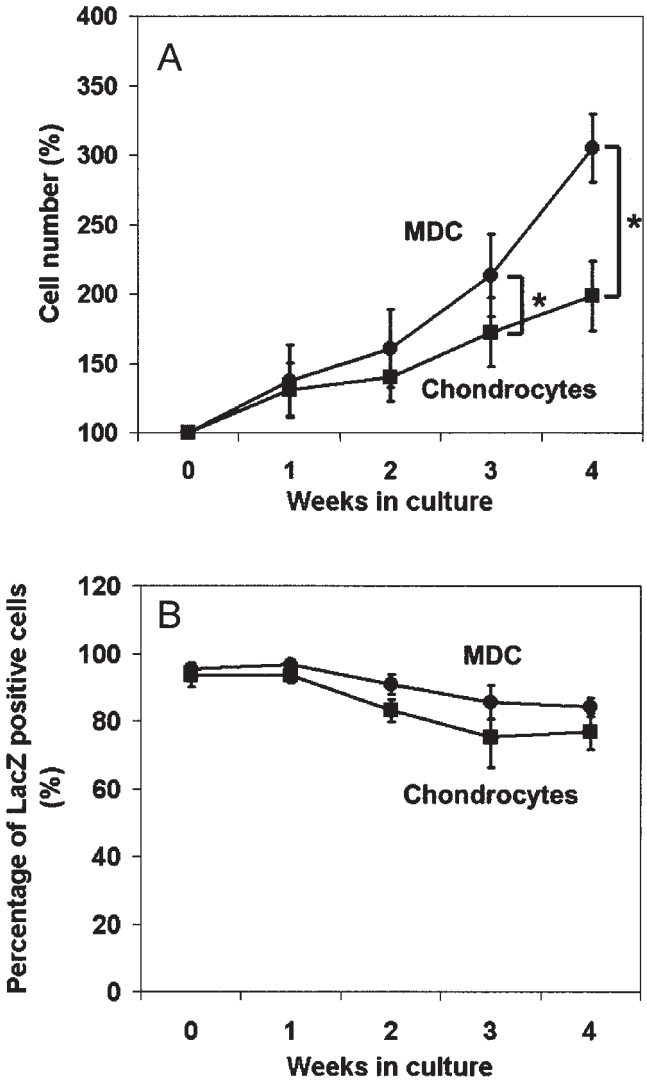

Figure 2. A. The chronological change of cell numbers in the MDC and chondrocyte groups compared to the initial cell number (shown as percentage). At 3 and 4 weeks of culture, the cell number in the MDC group is significantly higher than that in the chondrocyte group. B. The chronological change of the percentage of LacZ positive cells. The initial percentages of LacZ positive cells in the MDC and chondrocyte groups are 95.4 ± 1.9% and 93.4 ± 3.4%, and at 4 weeks of culture, they are 84.2 ± 3.9% and 76.9 ± 4.3%, respectively.

Percentage of LacZ positive cells after transduction. The percentage of LacZ positive cells in both groups is shown in Figure 2B. At the onset of culture, percentages of LacZ positive cells in the MDC and chondrocytes were 95.4 ± 1.9% and 93.4 ± 3.4%, respectively. Although the LacZ positive cells decreased at each time point, the percentages of LacZ positive cells in the MDC and chondrocyte groups were still 84.2 ± 3.9% and 76.9 ± 4.3% after 4 weeks of culturing, respectively. There was no significant difference between the MDC and chondrocyte groups.

Macroscopic examination. Macroscopic findings of each group are shown in Figure 3. In the MDC group, the gross appearance of the grafted areas at 4 and 8 weeks revealed that they were filled with white, glossy, and smooth repaired tissues. At 12 weeks, the repaired tissues were still white, glossy, and smooth, and the margin of the repaired tissues seemed to be integrated into the surrounding recipient normal cartilage. At 24 weeks, the white and glossy repaired tissue covering the defects was still observed, but appeared to be thinner and patchy compared to the results at 12 weeks.

In the chondrocyte group, the gross appearance of the repaired tissues at all sampling times was similar to that of the repaired tissues in the MDC group. At early time points after transplantation (4 and 8 weeks), the repaired tissues covering the defects were white, glossy, and smooth. At 12 and 24 weeks after transplantation, the regenerated cartilage became thinner and patchy compared to earlier time points.

In the gel control group, the repaired tissues in the defects were similar to those in the MDC and chondrocyte groups at 4 and 8 weeks. However, at 12 weeks, the surfaces of the repaired tissues were irregular and had lost their glossy appearance. At 24 weeks, the repaired tissues were irregular and had a bony consistency.

In these 3 groups, there was no severe osteoarthritic change, but mild osteophyte formation was observed on the edge of the trochlear groove where stitches for fixation of the periosteum were placed.

Histological evaluation. Histological findings of each group are shown in Figures 4 and 5. In the MDC group, at 4 weeks after transplantation, the defects were filled with repaired tissues with smooth surfaces. The repaired tissues contained round chondrocytic cells as well as flat fibroblastic cells. The areas stained intensely by safranin-O contained round cells with chondrocytic morphology. The bone marrow had clearly infiltrated the base of the defects. At 8 weeks, the repaired tissues were stained more intensely by safranin-O. Most of the cells in the repaired tissues were round and had a chondrocytic morphology. The tissues were well integrated into the subchondral bone without cell infiltration from bone marrow. At 12 weeks, the histological appearance was similar to that at 8 weeks. However, the repaired tissues became slightly thinner when compared to the results at 8 weeks. The margins of the

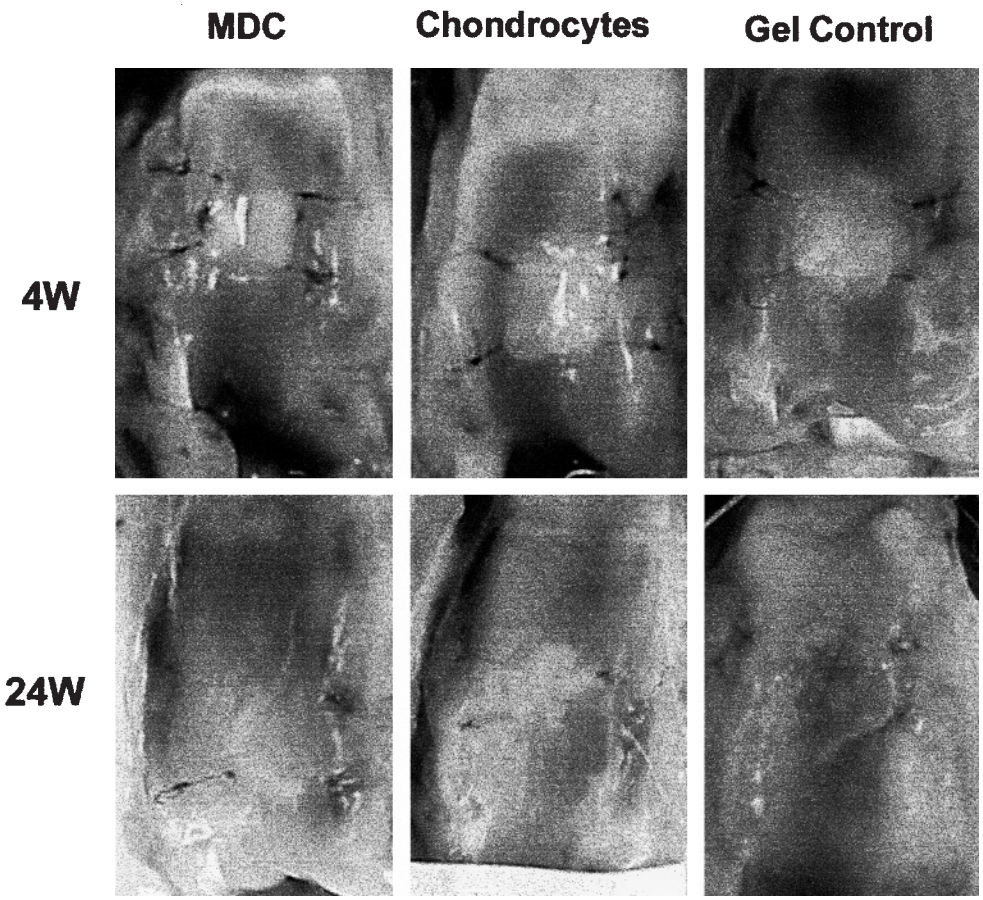

Figure 3. Macroscopic findings at 4 and 24 weeks after surgery in each group. At 24 weeks after operation, the white and glossy tissues covering the defects were observed in the MDC and chondrocyte groups. However, in the gel control group, the repaired tissues were irregular and had bony consistency.

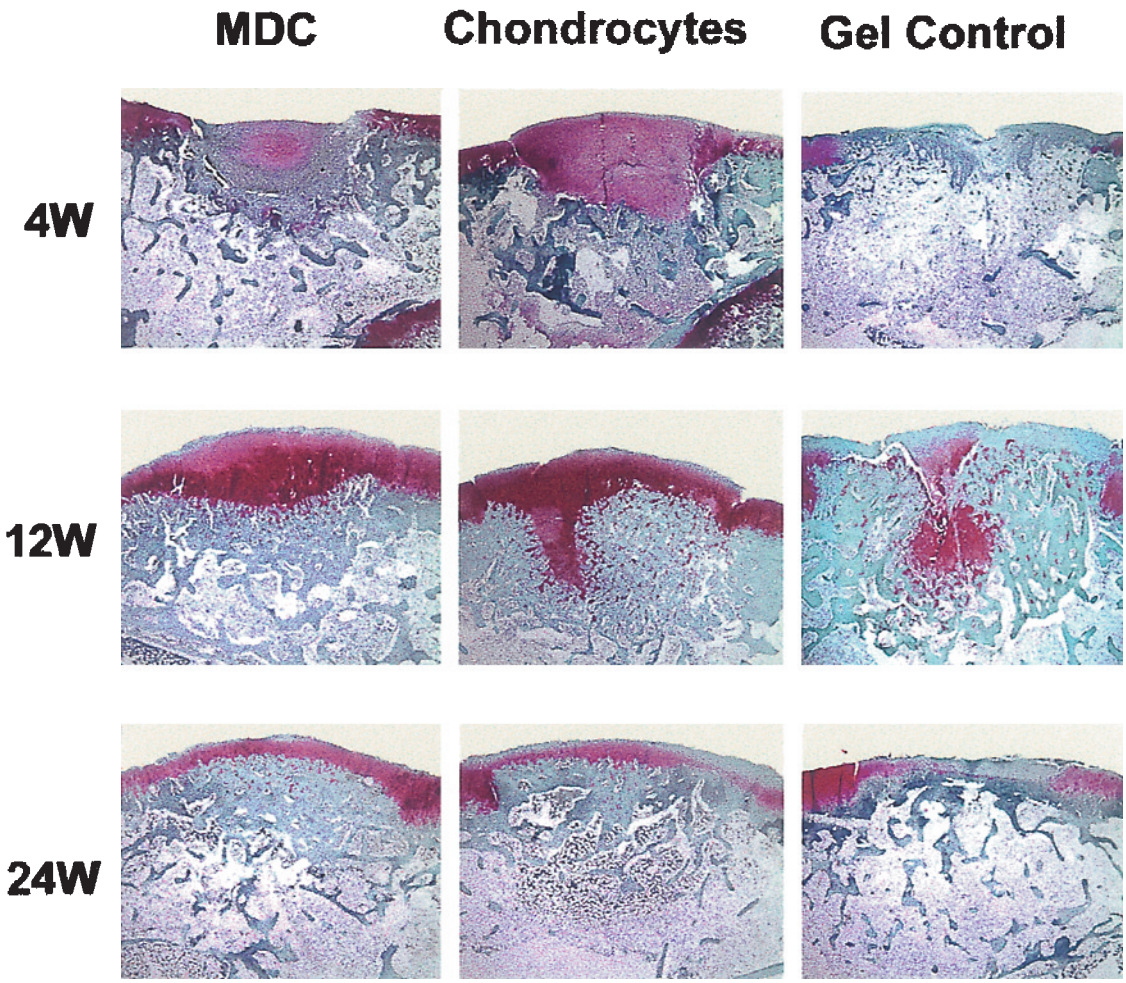

Figure 4. Representative histological findings at 4, 12, and 24 weeks after operation in each group. At 24 weeks, in the MDC and chondrocyte groups, the repaired tissues were thin compared to those at 12 weeks, showing that the subchondral bone had invaded the repaired tissues. However, the repaired tissues were stained intensely by safranin-O and surfaces were relatively smooth. The gel control group had no apparent healing at 24 weeks postsurgery (original magnification ×20).

repaired tissues were completely integrated into the surrounding articular cartilage in 2 of 6 specimens. At 24 weeks after transplantation, the repaired tissues became thinner than observed at 12 weeks, indicating that subchondral bone was invading the repaired tissues. The tissues were less stained by safranin-O and had slightly irregular surfaces compared to observations at 12 weeks. Complete integration between the tissues and surrounding articular cartilage was seen in 3 of 6 specimens.

In the chondrocyte group, the time course of the histological appearance of the repaired tissues was similar to that in the MDC group. At 4 weeks after transplantation, the repaired tissues contained both round chondrocytic and flat fibroblastic cells. At 8 weeks, most cells in the repaired tissues were round and chondrocytic. However, after 12 weeks, safranin-O staining was not seen through the full thickness of the cartilage, as the upper one-third of cartilage showed no safranin-O staining. The repaired tissues were thicker at 4 and 8 weeks compared to 12 weeks after transplantation. Complete integration between the repaired tissues and the surrounding articular cartilage was seen in 3 of 6 specimens at 12 weeks and 2 of 6 specimens at 24 weeks.

In the gel control group, the histological appearance at 4 and 8 weeks after transplantation was similar to those in the MDC and chondrocyte groups. Repaired tissues were stained more intensely by safranin-O at 8 weeks than at 4 weeks, and they contained round chondrocytic cells. However, the repaired tissues at 12 weeks became thinner, with irregular surfaces, compared to earlier time points. Matrix staining by safranin-O was also weak and irregular in distribution at 24 weeks after transplantation, showing degradation of the repaired tissue.

Histological scoring for repaired tissue. Chronological changes of histological grading scores are shown in Figure 6.

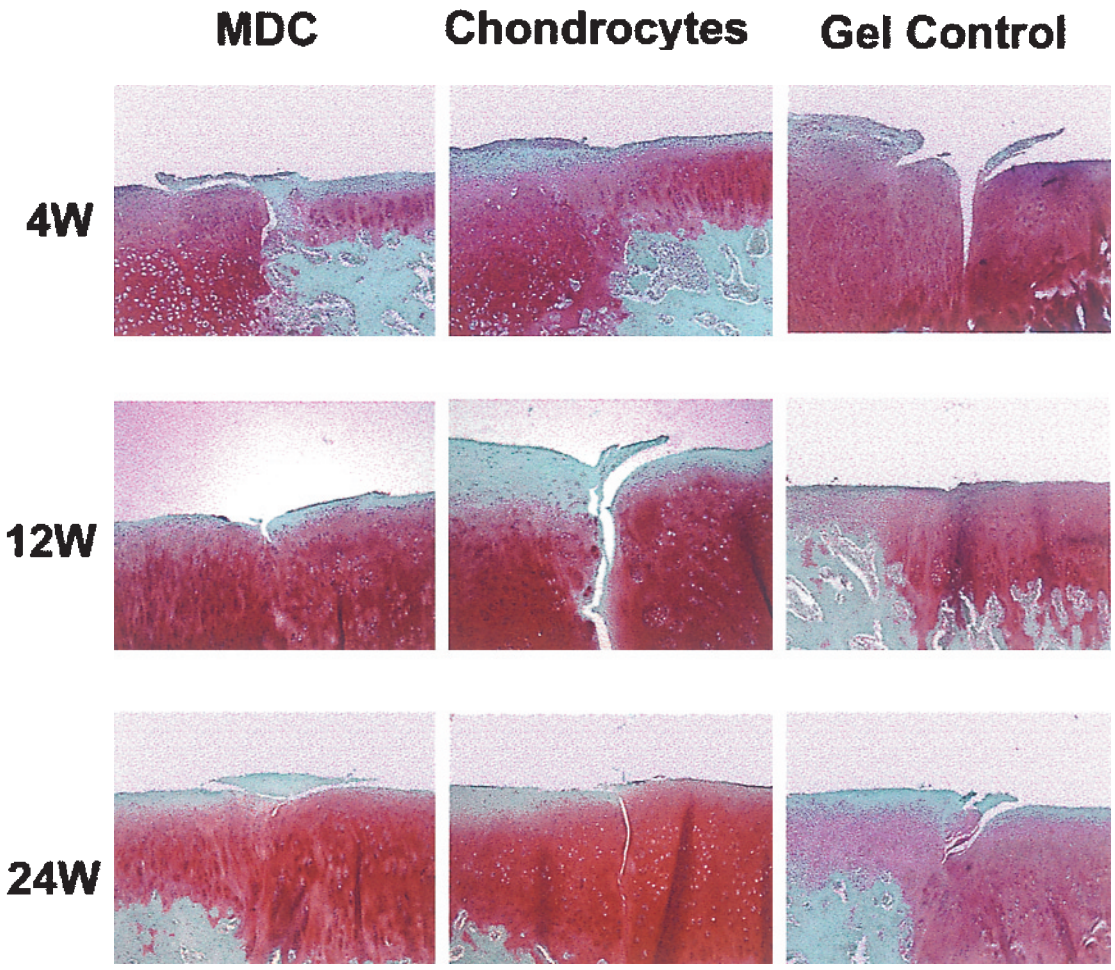

Figure 5. Histological findings at 4, 12, and 24 weeks after operation in each group. In each panel, left side is repaired tissue and right side is normal cartilage. At 24 weeks, in the MDC and chondrocyte groups, cells found in the regenerated tissues appear to maintain their typical chondrocyte-like morphology. The gel control group had no healing at 24 weeks postsurgery, while partial healing can be observed at 4 and 8 weeks postsurgery (original magnification ×100).

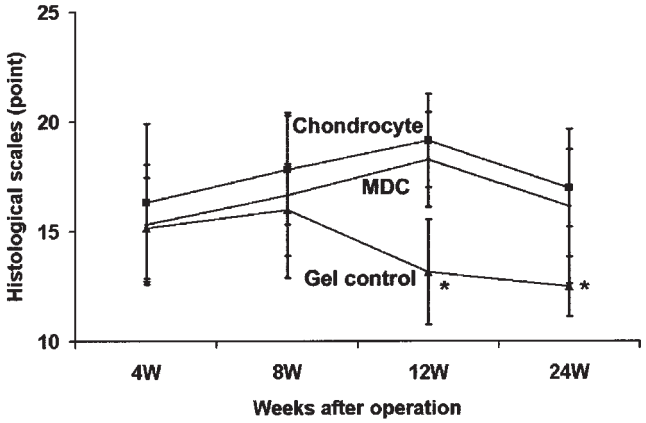

Figure 6. Chronological changes of the histological grading score. In the MDC and chondrocyte groups, histological scores improved until 12 weeks after transplantation. However, the scores in both groups decreased at 24 weeks. In the control group, although the scores at 4 and 8 weeks are similar to those in the MDC and chondrocyte groups, the scores deteriorate significantly at 12 and 24 weeks. *Significantly different from the MDC and chondrocyte groups.

In the MDC and chondrocyte groups, histological grading scores improved chronologically until 12 weeks after transplantation. The scores in both groups deteriorated between 12 and 24 weeks postsurgery. In the control group, although the scores at 4 and 8 weeks were similar to those in the MDC and chondrocyte groups, the scores deteriorated at 12 and 24 weeks after the operation. Statistical differences were observed between the transplanted cell groups (the MDC and chondrocyte groups) and the control gel group at 12 and 24 weeks after surgery.

Immunohistochemical analysis. Immunostaining for LacZ. LacZ positive cells were found in the repaired tissue at 2 and 4 weeks. Histochemical staining of the repaired tissues for LacZ in the MDC and chondrocyte groups at 2 and 4 weeks after transplantation is shown in Figure 7. At 2 weeks after transplantation, some cells in the tissue were stained blue, but distributions of LacZ positive cells were not uniform in both groups. At 4 weeks after transplantation, although the number

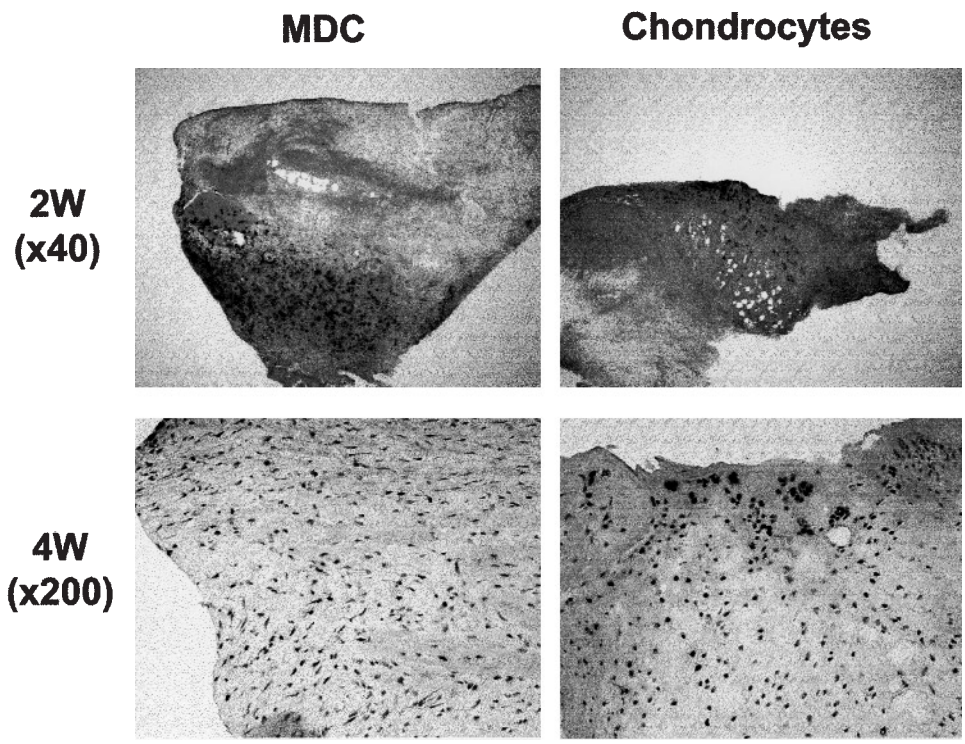

Figure 7. LacZ staining of the repaired tissues at 2 and 4 weeks after transplantation. At 2 weeks, some LacZ-expressing cells can be found in the repaired tissue, but the distributions of cells are not uniform in the 2 groups. At 4 weeks, although the LacZ positive cells in the repaired tissue decreased dramatically in both groups, we can still observe LacZ positive cells in the repaired tissue. In contrast, we cannot detect any LacZ positive cells in the repaired tissue at later time points postsurgery (original magnification ×40 and ×200).

of LacZ positive cells in the repaired tissue decreased dramatically in both groups, we could still observe LacZ positive cells. After 4 weeks, we could detect no LacZ positive cells in the repaired tissue.

Collagen typing of the repaired cartilage. SDS-PAGE for collagen typing of repaired tissues revealed that the repaired cartilage was composed of a mixture of type I and type II collagen in every group. However, in the MDC and chondrocyte groups, the tissues were almost entirely composed of type II collagen as indicated by the substantial amount of CB 10 band and a small amount of CB 3,5, whereas in the control group, it was almost entirely composed of type I collagen as indicated by the apparent amount of CB 3,5 band and a small amount of CB 10 band (Figure 8). The densitometric scanning of CB 3,5 and CB 10 bands revealed that the percentages of type II collagen and type I collagen were 67.3 ± 10.0% and 32.8 ± 10.0% in the MDC group, 69.3 ± 8.6% and 30.8 ± 8.6% in the chondrocyte group, and 36.3 ± 8.1% and 63.8 ± 8.1% in the control group, respectively.

DISCUSSION

We evaluated the feasibility of *ex vivo* gene therapy for repair of full thickness articular cartilage defects using retrovirally transduced cells. We compared 2 different cell populations, muscle derived cells (MDC) and chondrocytes. Our first step was to examine chronological cell proliferation and the length of transgene expression in collagen gels *in vitro*, and compare the results between the MDC and chondrocytes. We found that retrovirally transduced MDC showed significantly better proliferation in collagen gels than chondrocytes *in vitro*. After 4 weeks of culturing, the cell number in the MDC group was about 1.5 times higher than in the chondrocytes, a statistically significant difference between the 2 groups. More important, both MDC and chondrocytes could express the LacZ reporter gene at high levels throughout the culture periods up to 4 weeks. Although the percentages of LacZ positive cells decreased chronologically, both cell types were more than 75% LacZ positive even after 4 weeks of culturing periods. In *in vivo* experiments in which retrovirally transduced MDC and chondrocytes embedded in collagen gels were transplanted into full thickness articular cartilage defects of rabbits, transgene expression was detected in the repaired tissues only at 2 and 4 weeks after transplantation. At later time points, the LacZ positive cells could not be found in the repaired tissue. Although transgene expression of MDC was detected only at early time points after transplantation, there is a possibility that MDC, as well as chondrocytes, can be used as a gene delivery vehicle for cartilage repair. As for the cell prolifera-

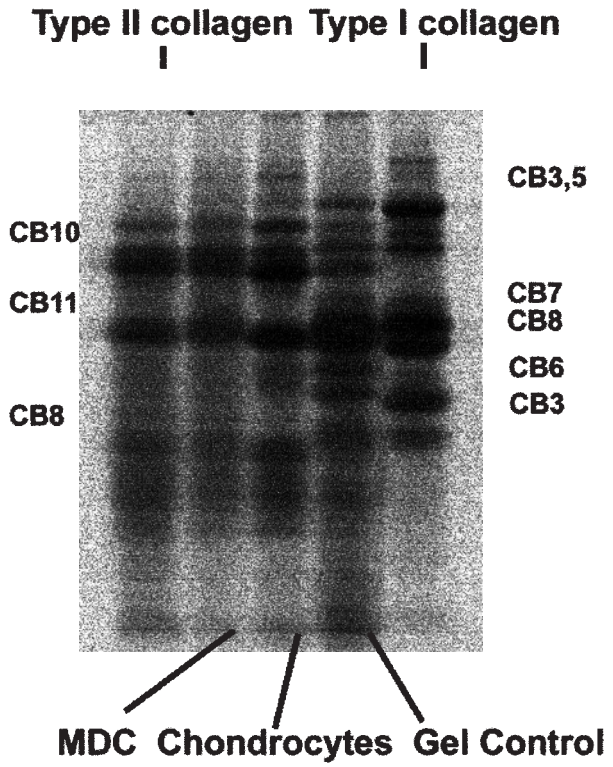

Figure 8. Representative SDS-PAGE result for collagen typing of repaired tissues at 24 weeks after operation. It can be seen that the repaired tissues are composed of a mixture of type I and type II collagen in all groups. However, in the MDC and chondrocyte group, the repaired tissues are mainly composed of type II collagen, whereas they are mainly composed of type I collagen in the gel control group.

tion in collagen gels, it was proved that MDC had an advantage over chondrocytes. For the clinical application of autologous chondrocyte transplantation in which chondrocytes are harvested from the patients, one of the major problems is that only a small number of cells can be derived from articular cartilage biopsy, as cartilage slices can only be harvested from the nonarticulating area of the knee joint, which contains very little cartilage. Therefore, many researchers have focused on the enhancement of chondrocyte proliferation using growth factors or hyaluronic acid[29,30]. There are some advantages to using MDC as a gene delivery vehicle for such application. Muscle biopsy is less invasive than chondrocyte harvesting even under arthroscopy, and it can be done in outpatient clinics without special instruments. Since skeletal muscle represents up to 40–50% of our total body weight, we can harvest adequate amounts of muscle tissue and they can regenerate after injury created from the biopsy. The presence of satellite cells that proliferate and regenerate myofibers after muscle injuries supports the muscle repair after trauma[31]. Although the amount of skeletal muscle tissue necessary for such an application is difficult to determine at this point, it has been reported that an open biopsy of 200 mg of the quadriceps femoris is enough to produce millions of myogenic cells[32]. This open biopsy procedure was not harmful to the donor[32]. Therefore, MDC are good cell candidates for gene delivery vehicle to enhance cartilage repair.

However, one concern about these cells gene delivery vehicles shown in this study is that we could detect transgene expression of MDC and chondrocytes in the repaired tissues only up to 4 weeks after transplantation. These short term expressions of transgene in the transplanted cells are consistent with other studies[33,34]. Although the optimal duration of growth factor expression for cartilage repair *in vivo* is still under investigation, 4 weeks of transgene expression may be too short to generate mature repaired tissue. Until sustained transgene expression can be achieved, application and utility of this method may be limited.

The cause of this rapid loss of transgene expression is still unclear. Kang, *et al* suggest that rapid loss of gene expression may reflect (1) loss of gene expression, (2) dilution of the transplanted cells by host bone marrow cells migrated into the defect, (3) death of allogeneic cells due to a cytotoxic immune response, (4) death resulting from the trauma of cell culture and transplantation, or (5) programmed cell death[33]. Our data do not clarify the apparent causes of rapid loss of transgene expression. Further studies using autologous and allogeneic cells to evaluate the immune response and/or apoptosis are required.

The immunogenicity of the scaffolds can also be responsible for the transient transgene expression observed in our study. Type I collagen gels used in this study may be immunogenic because the telopeptide regions that harbor the antigenic determinants on the peptide chains of type I collagen were not deleted. There is novel collagen gel commercially available in which telopeptides are deleted[7,35]. This type I collagen gel is less immunogenic and has already been used for wrinkle treatment in plastic surgery and dermatology. In 2000, Uchio, *et al* evaluated the human chondrocyte proliferation and matrix synthesis cultured in low immunogenic collagen gels, and showed the potential of this collagen gel as a carrier for chondrocyte transplantation[35]. It is also necessary to perform the experiment by combining autologous cells and this type of collagen gel to reduce immune response. This combination is closer to actual clinical application and may give us a better understanding of the fate of the grafted cells.

This study also demonstrated that the repaired tissues in the MDC and chondrocyte groups were similarly better than the gel control group macroscopically and histologically. Although the histological scores for the MDC, chondrocyte, and control groups were not statistically different at the early time periods of 4 and 8 weeks, the scores in the MDC and chondrocyte groups were statistically better than the control group at 12 and 24 weeks after transplantation. Moreover, collagen typing of repaired tissue using electrophoresis of CNBr peptides revealed that the repaired tissues in the MDC group were mainly composed of type II collagen, as in the chondro-

cyte group, whereas in the control group tissue was mainly composed of type I collagen. Despite the scarcity and early disappearance of the grafted cells in the repaired tissue in early time periods after transplantation, the cartilage healing was better in the MDC and chondrocyte groups than in the control group at 12 and 24 weeks after transplantation. Therefore, grafted MDC played an important role at the early time periods of the healing process.

Several studies suggest the presence of pluripotent stem cells in skeletal muscle that can differentiate into other lineages[23-25], potentially into chondrogenic lineage. Thus there is a possibility that some purified MDC could differentiate into chondrogenic cells and contribute as a cell source for cartilage healing. Cell isolation from human skeletal muscle has been described[32,36,37]. With the preplating technique for myogenic cell purification, previously described by Rando and Blau[26] and Qu, *et al*[27], we isolated different populations of MDC that contained different ratios of desmin (myogenic-specific marker) positive and negative cells. We have also reported that the percentage of muscle derived stem cells increases after 5 days of preplating[23,25]. Pluripotent MDC express myogenic markers and stem cell markers, such as Bcl-2, CD34, FLK-1, and Sca-1, but differ from mesenchymal and hematopoietic stem cells[23,25]. It has been found that 95% of highly purified myogenic cells are desmin positive and coexpress stem cell markers such as CD34 and Bcl-2 markers[23,25]. Thus grafted MDC for cartilage defects may contain some of these stem cells and consequently have the potential to differentiate into chondrogenic lineage and contribute as a cell source for the repaired tissues. However, because the identification and characterization of these pluripotent cells from skeletal muscle are still under investigation, further studies are required to determine whether the MDC used in this study indeed contain stem cells.

Chondrocytes have been extensively studied and are a natural and logical cell source for cartilage repair. As shown in this study, MDC are also a good candidate as a gene delivery vehicle and a cell source for cartilage repair. With MDC based *ex vivo* gene therapy, we can potentially deliver appropriate therapeutic genes to the articular cartilage defects. As a gene delivery vehicle, the genetically engineered MDC can produce growth factors that stimulate chondrogenic differentiation of progenitor cells from bone marrow. For example, the genetically modified cells can express therapeutic genes (such as IGF-1, TGF-ß, and BMP2) that promote cartilage healing. Moreover, if MDC themselves can differentiate into chondrogenic lineage, they can also participate as a cell source for cartilage repair and can also be enhanced by the growth factors they produce in an autocrine fashion.

Genetically modified muscle derived cells embedded in collagen gels were used for full thickness articular cartilage defects, demonstrating the feasibility of MDC based *ex vivo* gene therapy for cartilage repair.

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