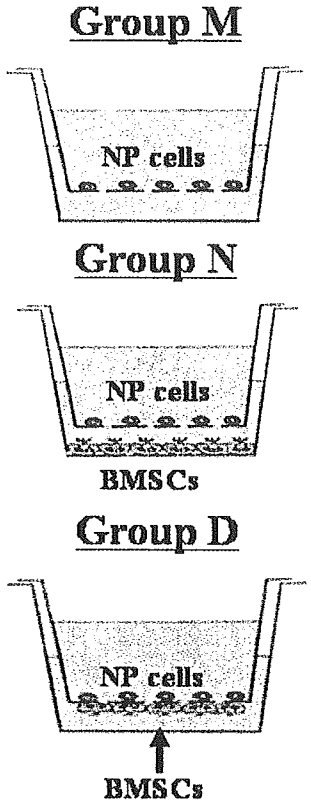

Figure 1. Diagrams of several culture systems used in the study. Group M: NP cells cultured monolayer (top). Group N: NP cells cultured in conventional coculture system with BMSCs having no cell-to-cell contact (middle). Group D: NP cells cultured in coculture system with BMSCs having direct cell-to-cell contact (bottom).

using a novel coculture system having direct cell-to-cell contact with autologous bone marrow-derived stromal cells (BMSCs) *in vitro* or not.

■ Materials and Methods

The current study was conducted under the protocol approved by the Animal Experimentation Committee of Tokai University School of Medicine.

Cell Isolation and Culture. To isolate BMSCs, bone marrow aspirates were taken from the iliac crest of 20 New Zealand white rabbits weighing an average of 1.5 kg while under inhalation anesthesia with 2.5% isoflurane (Abbot Laboratories, North Chicago, IL). Mononucleated cells were isolated with a density gradient (Nycoprep™; Axis-Shield, Oslo, Norway) centrifugation. These cells were then cultured with 25 cm^2 culture flask in Dullbecco Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) with 20% fetal bovine serum and penicillin (100 μg/mL), streptomycin (250 ng/mL), and amphotericin B (85 μg/mL) at 37 C in a humid atmosphere containing 5% CO_2. After 24 hours, nonadherent cells were discarded, and adherent cells were thoroughly incubated for 7 days. Next, the same rabbits were killed while under general anesthesia by intravenous injection of pentobarbital sodium (120 mg/kg) (Abbot Laboratories). Thoracolumbar spines including T5 to L7 levels were removed under aseptic conditions. Each disc was cut transversely, and NP was separated from the disc with a spatula. Obtained NP tissue was digested in a mixture of 0.4% pronase (Kakenkagaku, Tokyo, Japan) and 0.0125% collagenase P (Boehringer Mannheim, Mannheim, Germany) for 30 minutes. The digested cells were washed 3 times with DMEM, being collected by centrifugation at 1500 rpm. Six-well culture plate (Becton Dickinson, Franklin Lakes, NJ) and culture insert (Becton Dickinson) were used for coculture. The culture insert we used in the study consists of a polyethylene terephthalate track-etched membrane with 0.4 μm pores at the bottom, which prevent an exchange of cellular component. Diagrams of the culture systems used in this study are displayed in Figure 1. Nucleus pulposus cell cultured monolayer (Group M, Figure 1, top), conventional coculture system with no cell-to-cell contact with BMSCs (Group N, Figure 1, middle), and a new coculture system having direct cell-to-cell contact with BMSCs (Group D, Figure 1, bottom) were designed. The 1×10^4 NP cells and BMSCs were seeded in each group, and 7 sets of each group were made. In Group D, BMSCs were cultured on the reverse side of the membrane of the insert in DMEM with 10% fetal bovine serum. After 2 hours, culture insert was set in culture plate, and then NP cells were seeded on the front side of the membrane of the insert and cocultured in DMEM with 10% fetal bovine serum and penicillin (100 μg/mL), streptomycin (250 ng/mL), and amphotericin B (85 μg/mL) at 37 C in a humid atmosphere containing 5% CO_2. On day 4 of coculture, all groups were evaluated, because NP cells in Group D became confluent. The medium was exchanged once on the second day.

Evaluations. Evidence of direct cell-to-cell contact was evaluated in Group D. Cell proliferation, deoxyribonucleic acid (DNA) synthesis, proteoglycan (PG) synthesis, and cytokine expression analysis were evaluated for every groups.

Evidence of Direct Cell-to-Cell Contact. Specimens from Group D were processed for scanning electron microscopic (SEM) evaluation. Specimens were soaked in 0.1 mol/L phosphate buffer and fixed in 2.5% glutaraldehyde for 2 hours. Next, samples were fixed in 1% osmium solution for 1 hour and dehydrated in ascending concentrations of ethanol (50%, 70%, 80%, 90%, 95%, and 100%). Specimens were spatter-coated with gold and affixed to an adhesive interface for observation by SEM (JSM-840, Tokyo, Japan).

Measurement of Cell Proliferation. Cell proliferation was determined by WST-8 assay using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technology, Gaithersburg, MD).[11] A 96-well plate containing NP cells from each of the 3 groups was inoculated with 10 μL of prepackaged CCK-8 solution. They were incubated at 37 C in a humid atmosphere containing 5% CO_2 for 2 hours, and absorbance at 450 nm of the supernatant was measured spectrophotometrically. Cell counts were determined with a calibration curve.

Measurement of DNA Synthesis. The DNA synthesis was examined by uptake of [^{3}H]-thymidine.[12] Cultures were labeled with 74 kBq of [^{3}H]-thymidine per well for 2 hours. Cells were washed twice with phosphate buffered saline (PBS), and 2 mL of 10% trichloroacetic acid (TCA) was added to each well. Cultures were centrifuged (3000 rpm for 10 minutes), and supernatant (TCA) was removed. This procedure was repeated 5 times, and TCA-insoluble material was collected and dried

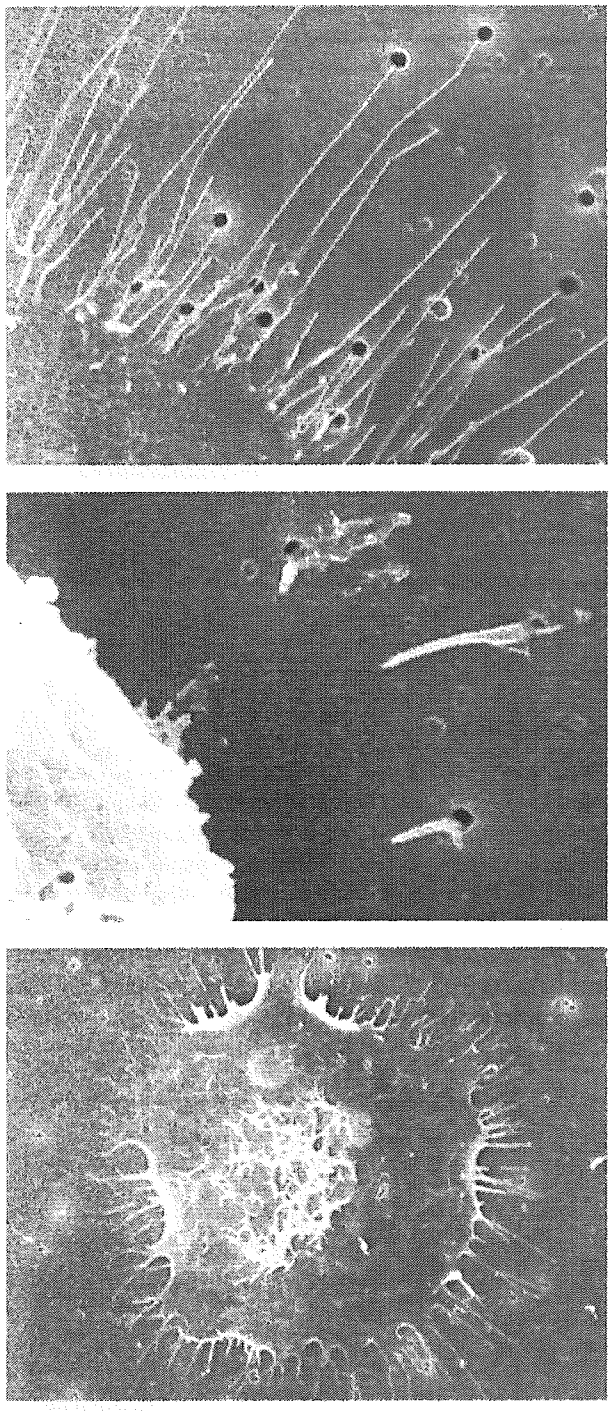

Figure 2. Electron microscopic view of the novel coculture system in Group D. Bone marrow-derived stromal cells on the reverse side of the membrane were adhering NP cells of the front side by extending cytoplasmic processus *via* pores of membrane (top). On the front side of the membrane, the cytoplasmic processus of BMSCs were extending toward NP cells through pores of membrane, and NP cells showed the longer processes and the faster wide spreading on the membrane (middle, bottom).

with 70% ethanol. The dried material was treated overnight with 1 mL of solvent (Solvable™; Packard, Meriden, CT) at 45 C and 10 mL of liquid scintillation cocktail (Atomlight™; Packard) was added for counting of emissions (Beckman LS4800, Fullerton, CA). Radioactivity, or disintegrations per minute (DPM), of these results was divided by number of cells counted by using the cell counting kit. The radioactivity of each sample was expressed as DPM per cell.

Measurement of Proteoglycan Synthesis. The incorporation of [^{35}S]-sulfate was used to measure PG synthesis. At the indicated times, cultures were labeled with 370 kBq of [^{35}S]-sulfate per well for 6 hours. Subsequent PBS washes, TCA treatment, drying, and scintillation counting were carried out with the same procedure as for [^{3}H]-thymidine uptake.

Protein Array Assay of Cytokine Expression. Ray Bio™ Cytokine Array (Ray Biotech, Norcross, GA) was used to evaluate cytokine expression of supernatants in every groups. To prepare media from 3 groups, cultures were performed in serum-free DMEM/F12 (DMEM: nutrient mixture Ham F-12; Gibco, Grand Island, NY). Cells were cultured for 5 days and supernatants were collected. Membranes immobilized with capture antibodies were blocked with 5% bovine serum albumin (BSA)/tris buffered saline (TBS) [0.01 M Tris HCl (pH 7.6), 0.15 M NaCl] for 1 hour. Membranes were then incubated with 1 mL of each media for 2 hours at room temperature. After extensive washing with TBS 0.1%/Tween 20 (3 times, 5 minutes each) and TBS (2 times, 5 minutes each) to remove unbound cytokines, membranes were incubated individually or collectively with biotin-conjugated streptavidin (2.5 pg/mL) for 1 hour at room temperature. Unbound materials were washed using TBS 0.01%/Tween 20 and TBS. Signals then were detected by an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Aylesbury, UK). The obtained expressions on tagged image file format (TIFF) images were quantified using CS analyzer 2.0 (Atto, Tokyo, Japan). Determining value of positive control as 1000, each value for transforming growth factor-beta (TGF-β), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) were quantified and evaluated.

Statistical Analysis. Statistical significance was determined based on $P < 0.01$. Comparison of group means between different cell cultures was determined using the Wilcoxon signed ranks test.

Results

Scanning Electron Microscopic Findings

In Group D, on day 4 of coculture, the BMSCs on the reverse side of the membrane were seen to adhere NP cells on the front by extending cytoplasmic processus through the 0.4 μm pores of membrane. Nucleus pulposus cells cultured on the front side of the membrane in Group D demonstrated longer processes and faster, wide spreading compared to NP cells cultured in other 2 groups (Figure 2).

Cell Proliferation

Mean NP cell proliferation measured by WST-8 assay resulted in 3.97×10^4 cells in Group M, 21.7×10^4 cells in Group N, and 39.5×10^4 cells in Group D. Cell proliferation in Group N was approximately 5 times higher than that of Group M. Furthermore, cell prolifer-

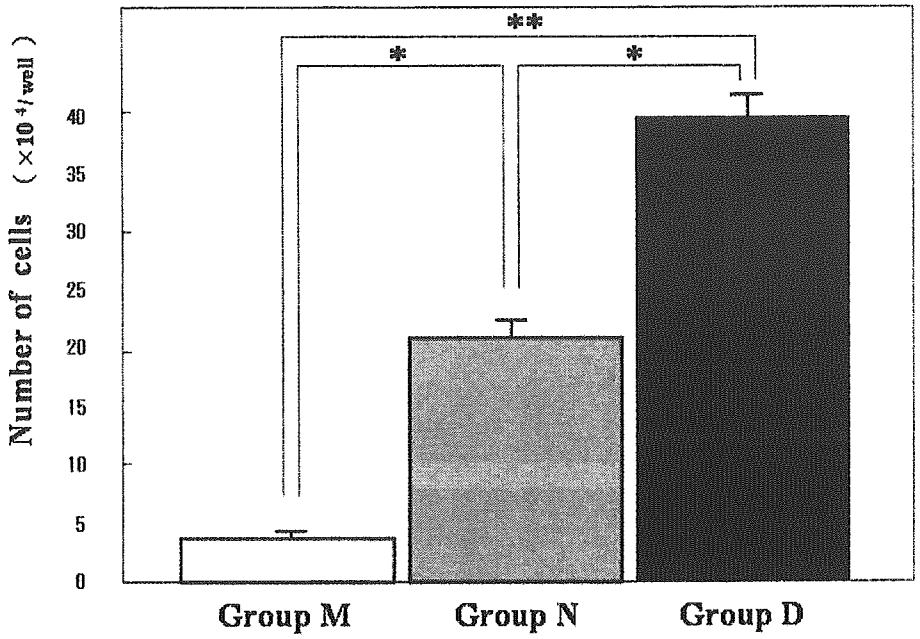

Figure 3. Effect of direct cell-to-cell contact coculture on cell proliferation. Upregulation of cell proliferation was accomplished in the order of in Group N ($P < 0.01$) and Group D ($P < 0.001$) compared to Group M. Furthermore, the differences between Group D and Group N ($P < 0.01$) were significant. Significant differences are indicated by $*P < 0.01$, and $**P < 0.001$. Data represent the mean ± SD (n = 20).

ation in Group D was 2 times higher than that of Group N. Results were significantly higher in Group N than that in Group M ($P < 0.01$), and in Group D than that in Group M ($P < 0.001$) or Group N ($P < 0.01$) (Figure 3).

DNA Synthesis

Mean NP cell uptake of [^{3}H]-thymidine was 5.03 ± 0.08 DPM per cell in Group M, 78.68 ± 0.85 DPM per cell in Group N, and 110.05 ± 1.55 DPM per cell in Group D. These results revealed that significant upregulation of DNA synthesis was accomplished in the order of in Group N ($P < 0.01$) and Group D ($P < 0.001$) compared to Group M. Furthermore, DNA synthesis in Group D was significantly more highly upregulated than that of Group N ($P < 0.01$) (Figure 4).

Proteoglycan Synthesis

Mean [^{35}S]-sulfate incorporation resulted in 3.90 ± 0.07 DPM per cell in Group M, 38.34 ± 0.58 DPM per cell in Group N, and 63.13 ± 0.78 DPM per cell in Group D. Proteoglycan synthesis in Group N was approximately 10 times higher than that of Group M. Furthermore, PG synthesis in Group D was 2 times higher than that of Group N. Proteoglycan synthesis was also significantly upregulated in the order of Group D ($P < 0.001$) and Group N ($P < 0.01$) compared to Group M (Figure 5).

Detection of Cytokines

Cytokine array assay results demonstrated significant increase of TGF-β1, IGF-1, EGF, and PDGF expressions in Group N and Group D. (In order of Group M, Group N, and Group D, TGF-β1: 19.94 ± 0.35, 63.22 ± 1.92, and 132.355 ± 1.36; IGF-1: 18.36 ± 0.39, 61.33 ± 0.64, and 129.81 ± 0.89; EGF: 4.06 ± 0.02, 38.20 ± 0.19, and 114.97 ± 2.05; PDGF: 10.69 ± 0.07, 51.41 ± 0.36, and 84.34 ± 1.41; $P < 0.01$) (Figure 6).

■ Discussion

Bone marrow-derived stromal cells make up approximately 0.125% of the total marrow cells and can be isolated from bone marrow by their tendency to adhere to tissue culture plastic.[13] Bone marrow-derived stromal cells have been known to express the capability of differentiating into osteoblasts, chondrocytes, adipocytes, skeletal muscle fibers, cardiomyocytes, hepatocytes, and epithelial cells.[14–18] From this unique ability of multilineage differentiation, there are vast growing numbers of

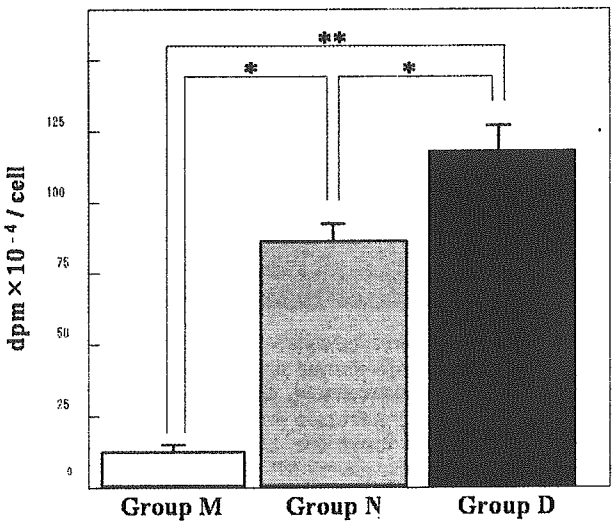

Figure 4. Effect of direct cell-to-cell contact coculture on DNA synthesis. The differences between Group N and Group M ($P < 0.01$) and between Group D and Group M ($P < 0.001$) were significant. Furthermore, DNA synthesis in Group D was significantly upregulated higher than that of Group N ($P < 0.01$).

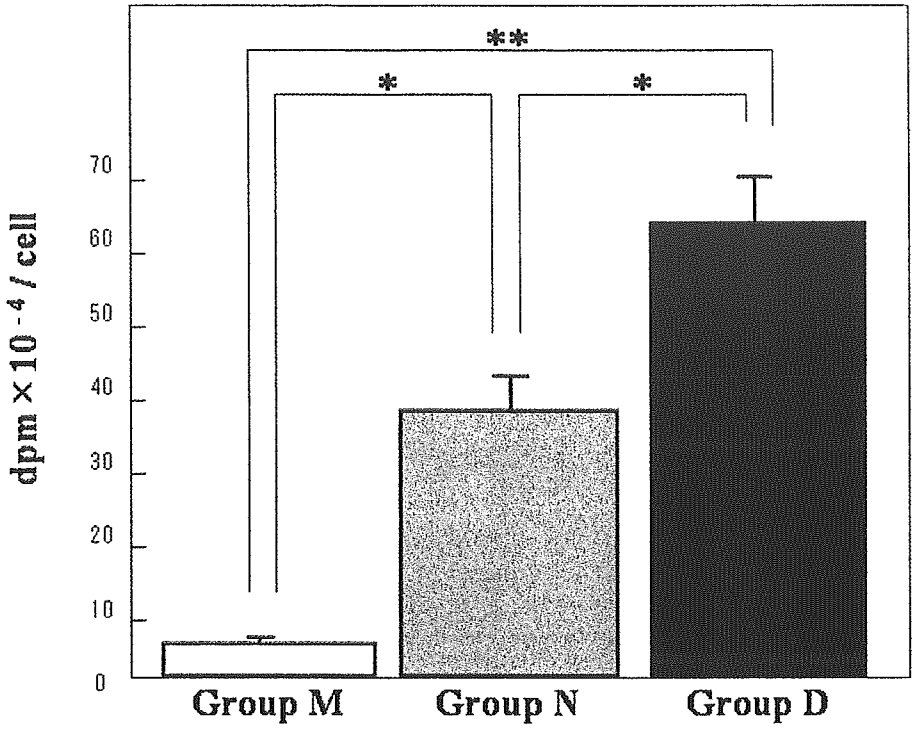

Figure 5. Effect of direct cell-to-cell contact coculture on PG synthesis. The differences between Group N and Group M ($P < 0.01$) and between Group D and Group M ($P < 0.001$) were significant. Furthermore, PG synthesis in Group D was significantly upregulated higher than that of Group N ($P < 0.01$).

reports focusing on the plasticity of BMSCs.[4,19] Yet, they possess another important nature, which is the maintenance function, serving as feeder or nursing cells for other cells. Recent studies report the significance of BMSCs functioning as feeder cells for hematopoietic progenitor cells, plasma cells, and hepatocytes.[20–24]

Therefore, the maintenance function by BMSCs to enhance the biologic and metabolic viability of NP cells was evaluated in the current study. In results, significant upregulation of NP cell proliferation, DNA synthesis, PG synthesis, and cytokine/growth factor expression was shown in conventional coculture system with BMSCs compared to monolayer culture of NP cells. The upregulation was also significant when comparing the data by coculture with AF cells, reported by ourselves.[25]

However, further upregulation of the biologic and metabolic viability of NP cells was desired in clinical application for the degenerative disc. Kawada *et al* had compared multiple coculture systems in expanding human umbilical cord hematopoietic progenitor cells and found that the direct cell-to-cell contact technique between BMSCs and hematopoietic progenitor cells achieved significant acceleration of cell proliferation, compared to conventional noncontact coculture.[26] They consider that direct cellular contact enhances cell signaling pathways and expression of specific adhesion molecules controlling proliferation, differentiation, and phenotypic expression of cells. Tsuji *et al* also reported that direct cell-to-cell contact through the specific adhesion molecules led not only the maintenance and differentiation of cells, but also enhanced production of cytokines by stromal cells.[27]

Therefore, the direct cell-to-cell contact coculture system was applied in the current study. The upregulation of all properties in the direct cell-to-cell contact coculture was significantly greater than the data in other culture systems. Additionally, the authors confirmed the significantly enhanced leading effects in inducing cytokines and growth factors from BMSCs by cytokine array assay. Transforming growth factor-β, IGF-1, EGF, and PDGF were significantly upregulated in the direct cell-to-cell contact coculture. Understandingly, Thompson *et al* already indicated that these growth factors were known to take part in proliferation and cellular metabolism of NP cells.[28] These results then support the importance of direct cell-to-cell contact in the coculture system.

Another advantage of the novel coculture system is the fact that enhancement of cell adhesion and proliferation dramatically accelerated in the earlier phase and NP cells reached full confluency as rapid as 4 days. Our previous studies showed that monolayer-cultured NP cells took approximately 16 to 17 days to reach to full confluent stage and conventional coculture took about 8 to 10 days (Figure 7). Based on all of the data presented here, it is strongly suggested that direct cell-to-cell contact plays an important role in upregulation of viability in NP cells *in vitro*.

Key Points

- The biologic and metabolic viabilities of NP cells by conventional coculture with BMSCs upregulated as compared to other culture systems.

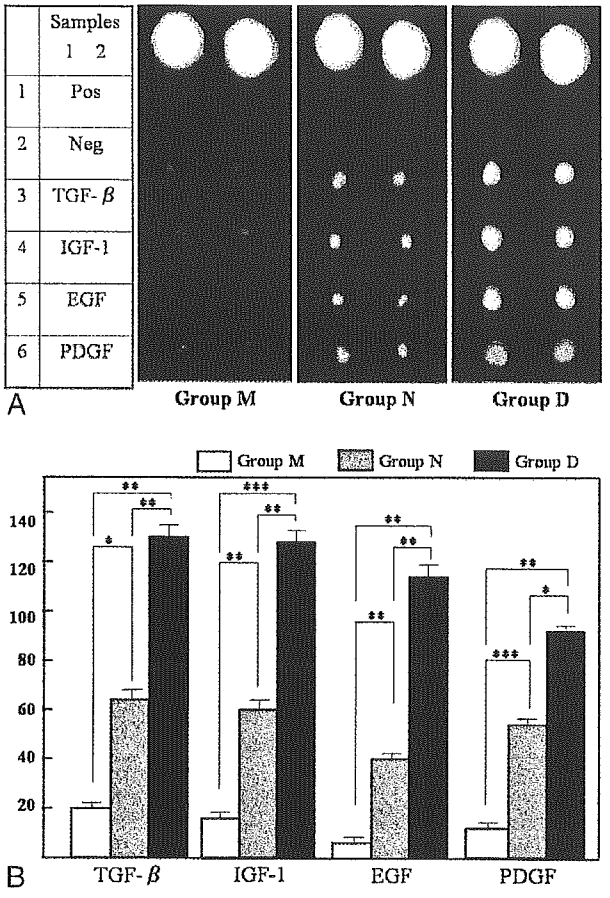

Figure 6. Coculture with direct cell-to-cell contact upregulated cytokine and growth factor expression. **A**, Template of the array. Array membranes were incubated with samples from each group. Pos = positive; Neg = negative. **B**, The obtained expressions were quantified. In each growth factor, the differences between Group M and Group N, Group N and Group D, and Group M and Group D were significant. Significant differences are indicated by $*P < 0.01$, $**P < 0.001$, and $***P < 0.001$. Data represent the mean ± SD (n = 6).

- Biologic and metabolic viability of nucleus pulposus cells were significantly upregulated by using a new coculture system having direct cell-to-cell contact.
- Existence of cell-to-cell contact between nucleus pulposus cells and bone marrow stromal cells induced an enhancement of growth factor secretion.
- This novel coculture system may provide NP cells with sufficient viability for clinical application of nucleus pulposus reinsertion.

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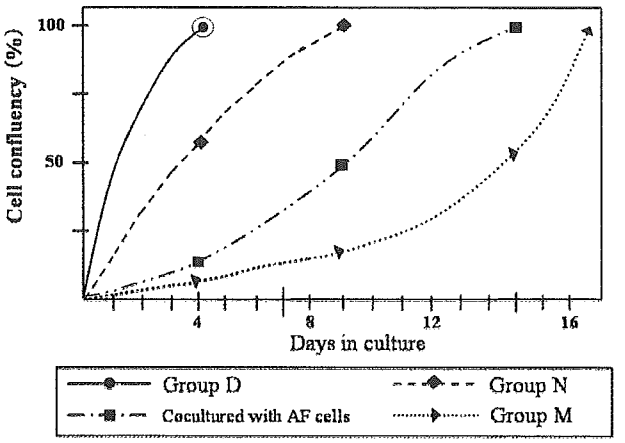

Figure 7. Growth curve in primary culture of NP cells using various culture systems. Cocultured NP cells having direct cell-to-cell contact (Group D) showed marked growth rate from early phase and reached to full confluency in 4 days.

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Immortalization of Human Nucleus Pulposus Cells by a Recombinant SV40 Adenovirus Vector

Establishment of a Novel Cell Line for the Study of Human Nucleus Pulposus Cells

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Study Design. Establishment and characterization of a *de novo* cell line derived from human nucleus pulposus cells using a recombinant simian virus 40 (SV40) adenovirus vector.

Objectives. To assess the feasibility of human nucleus pulposus cell line procurement and to evaluate the character of the resultant outcome to better understand the nature of human nucleus pulposus cells.

Summary of Background Data. Despite recent advances in disc cell biologic research, the fundamental nature of nucleus pulposus cells, especially in the context of human cell lines, is still not well understood. Therefore, a broad-based analysis of these cells is of significant necessity. Because of the limited amount of existing human cells, establishment of an immortal cell line would greatly facilitate resource supply.

Methods. After release of informed consent, tissue samples of nucleus pulposus were obtained from the lumbar intervertebral disc of a 19-year-old man undergoing anterior fusion for burst fracture. Samples with no apparent damage were selected and digested enzymatically for primary culture and then were infected with recombinant SV40 adenovirus vector (Ad/SV40). The infected cells were maintained in culture for more than 40 population doublings, after which they were considered immortalized. Next, confirmation of expression of T antigen was performed and resultant immortalized cell lines were designated and classified as human nucleus pulposus cell line derived from Ad/SV40 infection-1 (HNPSV-1). HNPSV-1 cells were characterized and compared with their mother cells under two designated culture conditions: monolayer and three-dimensional. Morphologic and immunocytochemical analyses were performed at various intervals. Cell proliferation, DNA synthesis, proteoglycan synthesis, gene expression profiling, and karyotypic analyses were also performed. Moreover, HNPSV-1 cells were injected into rabbit discs to assess the presence of tumorigenesis.

Results. Recombinant SV40 adenovirus vector infected nucleus pulposus cells with relatively high efficiency (90%> at multiplicity of infection 100). HNPSV-1 demonstrated marked prolongation of cell life with continuous cell doublings for over 5 months (60–100 cell population doublings). Despite significant increase in cell proliferation and DNA synthesis when compared with its mother cells, resultant cell lines expressed strikingly similar cell morphology and functional characteristics. Atypical karyotypes were noted; however, no apparent tumorigenesis was seen in rabbit discs 24 weeks after injection of HNPSV-1.

Conclusions. HNPSV-1 was successfully established using recombinant SV40 adenovirus vector. Results showed that human nucleus pulposus cells are capable of immortalization with maintenance of original cell characteristics. It is anticipated that these cells will be useful for *in vitro* studies of the biologic nature of human nucleus pulposus cells.

Key words: intervertebral disc, nucleus pulposus cells, immortalization, cell line. **Spine 2004;29:1515–1523**

Currently, there is a growing interest in investigating the nature of disc cells and finding ways at halting the irreversible process of intervertebral disc (IVD) degeneration.[1–3] Over the past decade, there have been numerous advances in our basic knowledge of the anatomy, biochemistry, and biomechanical features of disc cells. However, in comparison to research on cells of other skeletal organs, disc cell research and fundamental knowledge are certainly lacking.[1] Moreover, studies with human disc cells, especially focusing on nucleus pulposus (NP) cells, are seldom found in the literature. Perhaps some explanation for this dilemma in the study of human NP cells may be related to the inherent low NP cellularity combined with limited tissue sample resources.[2,4] Basic structural composition of disc cells is known to differ among species and undergo alteration with aging. However, further studies of human NP cells are needed to fully elucidate the fundamental details of cell function and structure.[5] Thus, development of new techniques that can both expand cell number and maintain cell phenotype is of critical importance to the field.[4]

One strategy to help define the biologic properties of cells is the establishment of an immortal cell line.[6] In this study, we introduced original-defective simian virus 40 (SV40) early gene into primarily cultured human NP cells by a recombinant SV40 adenovirus vector (Ad/

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SV40) and established an immortalized human NP cell line. The established cell line was named human nucleus pulposus cell line derived from Ad/SV40 infection-1 (HNPSV-1) and was compared to their mother cells (mNP cells) for possible changes in cell character. It is known that disc cells expand more rapidly when cultured in monolayer but lose their differentiated phenotype.[7,8] They can be redifferentiated in three-dimensional culture conditions.[9] Both types of culture methods were used in the study according to evaluation properties. HNPSV-1 and mNP cells underwent analysis in cellular morphology, immunocytochemistry, cell proliferation, DNA and proteoglycan (PG) synthesis, and gene expression profiling and karyotyping. Because cells immortalized with SV40 T antigen possess tumorigenesis capability,[6] HNPSV-1 cells were transplanted into rabbit IVDs. Paraffin sections of IVD specimens at 4, 8, 16, and 24 weeks after transplantation were prepared and evaluated for signs of tumor-associated changes.

Materials and Methods

All experimental protocols were approved by Institutional Review Board and Animal Experimentation Committee at the author's institution. Informed consent was obtained for all human tissue samples.

Preparation of Recombinant SV40 Adenovirus Vector. Recombinant adenovirus vector expressing SV40 early gene (Ad/SV40) used in the study was originally developed by Van Doren *et al*, where the origin-defective SV40 early gene with delta –E1/X cloned into the E1 region of the adenovirus.[6] Recombinant virus was grown in transformed human embryonic kidney cell lines, 293 cells by infecting the virus at the multiplicity of infection (MOI) at 0.1 plaque-forming units per cell in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Grand Island, NY), 2% fetal bovine serum. After a complete adenovirus-specific cytopathic effect was observed, the culture medium including the infected cells was frozen and thawed four times followed by centrifugation at 30,000 rpm for 15 minutes. The supernatant was used as virus stock and titration was performed (approximately 2×10^8 plaque-forming units/mL).

Primary Cultures of Human NP Cells. Tissue specimens of human NP cells were obtained from a 19-year-old man undergoing anterior fusion for burst fracture of the L2 vertebrae caused by a motorcycle accident. The patient was operated on approximately 8 hours after the accident, and only NP tissue showing no apparent gross damage was sampled. After careful debridement and washing, tissue specimens were minced with a scalpel and digested for 60 minutes at 37 C in DMEM containing 0.2% pronase (Calbiochem, La Jolla, CA), followed by 6 hours in 0.025% bacterial collagenase Type II (Sigma). The digest was filtered through a 75-μm cell-strainer (BD, Franklin Lakes, NJ) and cultured in 6-well culture plates (Primaria, BD) at a density of 1×10^5 cells/mL in DMEM, 10% fetal bovine serum (Gibco), 1% vol/vol penicillin and streptomycin in a 37 C, 5% CO_2 atmosphere. The medium was changed every 72 hours.

Infection of the Virus Vector. After two passages, half of the obtained cells were cultured in 12 30-mm culture dishes until reaching 80% confluency. These cells were then washed with phosphate-buffered saline (PBS, Gibco) and virus stock was added to each dish at MOI 1, 10, and 100. One set of negative control dishes was also prepared. They were incubated at 37 C, 5% CO_2 for 1 hour, washed with PBS, cultured in DMEM and 10% fetal bovine serum, and passaged. At every 10th passage, samples of infected cells were either cultured in monolayer or three-dimensionally in alginate beads as previously described[8,9] and carried out for evaluation.

Morphology and Vector Incorporation Analysis. Cellular morphology of the infected cells cultured in both monolayer and alginate beads were observed microscopically. The monolayer cultured cells were recovered by trypsinization and fixed in 4% paraformaldehyde at 4 C for 30 minutes and stained overnight with 4 C with mouse anti-SV40 T antigen antibody (Pab419; Oncogene, Boston, MA) at 1:100 dilution in PBS with 5% normal goat serum. Goat anti-mouse IgG Alexa fluor 594 (Molecular Probes, Eugene, OR) diluted at 1:50 in PBS was used as the second antibody. After PBS wash, the samples were analyzed using flow cytometry (FACSCalibur, BD) for quantification of positively stained viable cells. After confirming expression of T antigen, immortalized cells were designated as HNPSV-1.

Immunocytochemistry. Monolayer cultured mNP cells (passage = 1), mNP cells infected with Ad/SV40 (passage = 1), and established HNPSV-1 cells (passage = 40) were stained with mouse antibodies detecting chondroitin-4-sulfate, chondroitin-6-sulfate, and keratan sulfate (Seikagaku Co., Tokyo, Japan). These antibodies were diluted at 1:100–1:200 with PBS including 5% normal goat serum and stained overnight at 4 C. Horseradish peroxidase conjugated goat antimouse IgG was used as a second antibody and reacted with 0.02% 3'-diaminobenzidine containing 0.005% hydrogen peroxide. Hematoxylin was used as nuclear staining. For Type I and II collagen detection, mouse anti-Type I and II collagen (Daiichi Fine Chemicals, Toyama, Japan) was used with goat anti-mouse IgG Alexa fluor 588 (Molecular Probes) diluted at 1:200 in PBS as second antibody. In addition, HNPSV-1 cells were also stained with anti-SV40 T antigen antibody as described above. These specimens were counterstained with 40 mg/mL of 4',6-diamidino-2-phenylindole (Sigma) for immunofluorescent cytochemical evaluation. Furthermore, HNPSV-1 (passage = 40) cells were three-dimensionally cultured in alginate beads for 2 weeks and recovered using standardized techniques. To assess whether these cells possess redifferentiating ability to reproduce Type II collagen, alginate recovered cells were stained with Type I and II collagen antibodies, using protocol above.

Cell Proliferation. The effect of immortalization on cell proliferation was evaluated using WST-8 assay with a Cell Counting Kit (CCK-8; Dojindo Molecular Technology, Gaithersburg, MD) as previously described.[10] Briefly, HNPSV-1 (passage = 40) cells and mNP cells (passage = 1) were cultured in 60 mm monolayer culture dishes at 2.5×10^4 cells/mL with a total cell count of 1.0×10^5 cells per dish and evaluated at 1, 3, and 7 days after seeding. Next, cells were recovered by trypsinization and collected. They were then condensed into 96-well culture plates, and each sample was inoculated with 10 μL of CCK-8 solution. They were incubated at 37 C, 5% CO_2

for 2 hours, followed by spectrophotometric quantification using microplate reader with absorbance set at 450 nm. A calibration curve was made at the same time to obtain cell count data. All assays were repeated more than two times.

DNA Synthesis. Uptake of [^{3}H]-thymidine was measured to evaluate DNA synthesis of the immortalized cells. HNPSV-1 cells (passage = 40) cultured in monolayer for 7 days were labeled with 74 kBq of [^{3}H]-thymidine per well for 2 hours. Cells were washed twice with PBS, and 2 mL of 10% trichloroacetic acid (TCA) was added to each well. Cultures were centrifuged (3,000 rpm for 10 minutes), and supernatant (TCA) was removed. This procedure was repeated five times, and TCA-insoluble material was collected and dried with 70% ethanol. The dried material was treated overnight with 1 mL of solvent (Solvable, Packard, Meriden) at 45 C, and 10 mL of liquid scintillation cocktail (Atomlight, Packard, Meriden) was added for counting of emissions (Beckman LS4800, Fullerton, CA). Radioactivity (in disintegrations per minute [DPM]) of these results was divided by the number of cells counted using a cell counting kit. The radioactivity of each sample was expressed as DPM per cell. The results were compared with DNA synthesis of mNP (passage = 1) cells. ALL the isotope experiments were repeated more than two times.

PG Synthesis. Newly synthesized PG was measured by [^{35}S]-sulfate uptake to evaluate PG synthesis. HNPSV-1 cells (passage = 40) cultured in 6-well culture plates of alginate beads were labeled with 74 kBq of [^{3}H]-thymidine per well for 6 hours. Subsequent PBS wash, TCA treatment, drying, and scintillation counting were carried out using the same procedure as in the measurement of DNA synthesis.

Gene Expression Profiling. Gene expression profiling of genes that are known to interact with proliferation and metabolism of NP cells were assessed. The mRNA expression of these genes for mNP cells (passage = 1) and HNPSV-1 cells (passage = 40) was compared using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was extracted from HNPSV-1 and mNP cells cultured for 2 weeks in alginate beads using Isogen reagent (Nippon Gene, Tokyo, Japan). RNA samples were then reverse transcribed to cDNA using oligo dT primer and Multiscribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) followed by specific amplification of matrix specific genes and electrophoretic separation. Gene specific PCR primers were as follows:

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
5'-TCACCATCTTCCAGGAGCGA-3',
5'-CACAATGCCGAAGTGGTCGT-3';

Type I collagen
5'-TCTGCCCGTTGGGCTTATGA-3',
5'-CATTGCCTTTGATTGCTGGG-3';

Type II collagen
5'-ATGACAATCTGGCTCCCAAC -3',
5'-GAACCTGCTATTGCCCTCTG -3';

Aggrecan
5'-AGACAGTGACCTGGCCTGAC-3',
5'-TGGCCTCTCCAGTCTCATTC-3';

Versican
5'-CAGGAGTCGAGGCGCTGAT-3',
5'-GCAACCCAAAATGACTGAACG-3';

Insulin-like growth factor-1 (IGF-1)
5'-GGAGGGCGCCTCAGACA-3',
5'-ACATCTCCAGCCTCCTTAGATCAC-3'

Transforming growth factor-beta (TGF-β)
5'-CCGCGGGACTATCCACCT-3',
5'-ATGGCCTCGATGCGCTT-3'

Basic fibroblast growth factor (bFGF)
5'-AGTGGCTCATGCCCATATTC -3',
5'accttgacctctcagcctca-3'

Bone morphogenetic protein 2 (BMP2)
5'-TCAAGCCAAACACAAACAGC -3',
5'acgtctgaacaatggcatga-3'

Bone morphogenetic protein 4 (BMP4)
5'-TGATACCTGAGACGGGGAAG -3',
5'ccagactgaagccggtaaag-3'

The gels were scanned under UV light with a Densitograph system (Atto Biotechnologies inc., Tokyo, Japan) and band intensities were quantified densitometrically and normalized to GAPDH gene values using CS Analyzer (version, 2.01, Atto).

Karyotypic Analysis. Metaphase spreads were prepared from colchicine-arrested HNPSV-1 (passage = 40) grown in 60-mm culture dish using standard techniques.

Evaluation of Tumorigenicity. A total of 5×10^6 HNPSV-1 cells (passage = 40) were injected into degeneration-induced IVDs of New Zealand white rabbits as previously described.[11–13] In short, approximately 0.008 g of NP tissue was aspirated from L2 to L3, L3–L4, and L4–L5 discs under general anesthesia to induce degeneration. After 2 weeks, cells were suspended in 40 μL of DMEM and injected. Cyclosporin A (10 mg/kg body weight, Novartis AG, Basel, Switzerland) was subcutaneously administered for immunosuppression. At 4, 8, 16, and 24 weeks after injection, IVDs were harvested and made into paraffin sections. These sections were stained with hematoxylin and eosin and evaluated microscopically. Fluorescent immunohistochemistry of SV40 T antigen was also assessed to confirm survival of HNPSV-1 cells after injection.

Results

Primary Culture of human NP Cells

Gross findings of the obtained NP showed gelatinous tissue that could be distinguished from fibrous anulus tissue. NP cells were isolated and primarily cultured in monolayer expressed heterogeneous cellular constitution of widely spread cells with vacuoles, relatively small square-polygonal shaped cells, and relatively larger spindle-shaped fibroblast-like cells. These cells dedifferentiated into chondrocyte-like appearing cells after several passages in monolayer culture (Figure 1A, passage = 0).

Establishment of Human NP Cell Line

Primarily cultured NP cells infected with Ad/SV40 demonstrated accelerated proliferation forming proliferative colonies a few days after infection. These cells continued

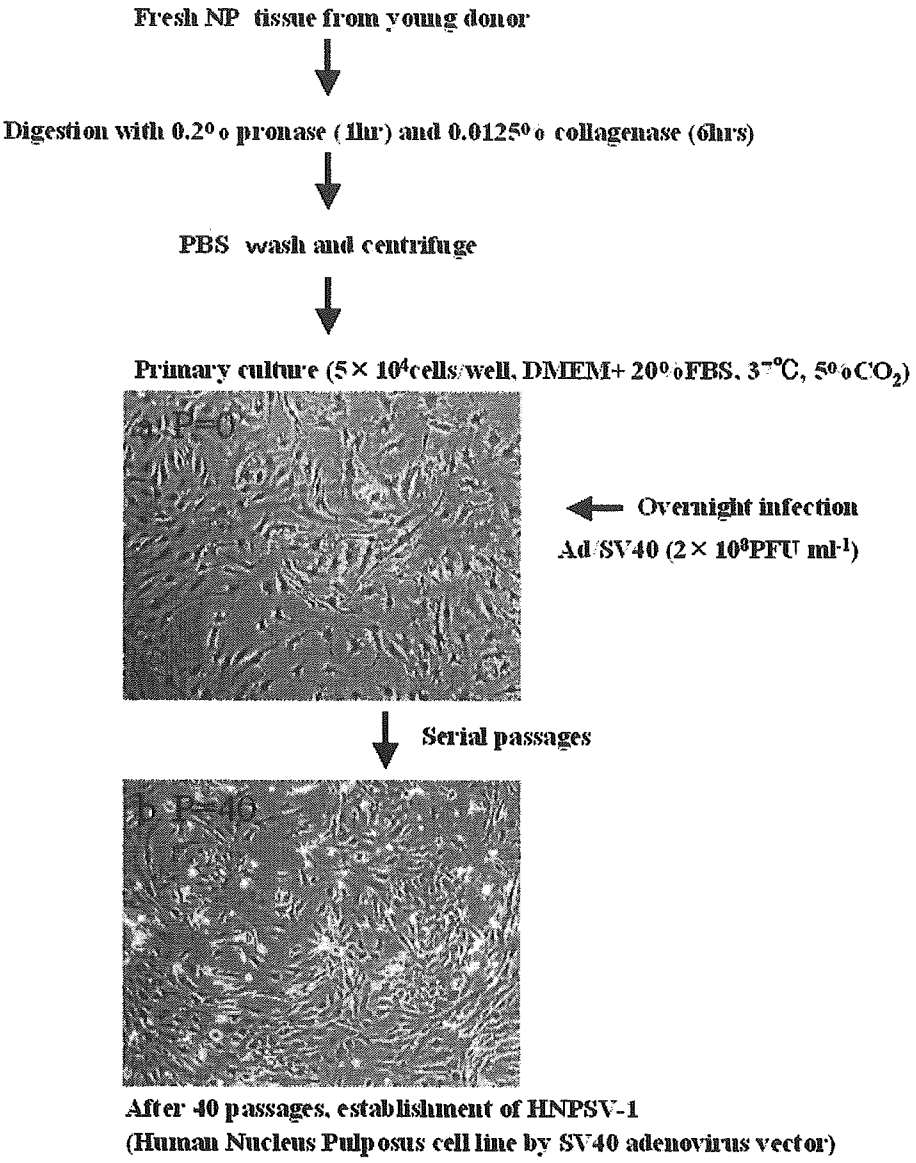

Figure 1. A schematic showing the process of cell line establishment. **A**: primarily cultured NP cells (passage = 0) with heterogeneous cellular composition. **B**: HNPSV-1 cells (passage = 40) demonstrate uniform chondrocyte-like appearing cells. Original magnifications, ×10.

to proliferate with more than 40 population doublings (approximately 20 passages) while mNP cells stopped proliferating after approximately 8 to 10 population doublings (4 or 5 passages). Infected cells that survived more than 40 doublings were considered immortalized and were designated as HNPSV-1 (Figure 1B, passage = 40). HNPSV-1 cells showed contact inhibition with gradual loss of proliferative activity between 60 and 100 doublings (approximately 5–6 months) and eventual cessation of growth (approximately 5–8 months).

Morphology and Vector Incorporation Data Analysis

Cellular morphology of HNPSV-1 cells under microscopy demonstrated uniform chondrocyte-like cell appearance (Figure 1B, passage = 40). These cells regained their spherical-shaped character when cultured in alginate beads and produced extracellular matrix molecules around the cells, resulting in colony formation (Figure 2). After several passages, these characteristics were compatible with mNP cell colony formation. Flow-cytometric analysis of infected cells showed that an average of 26 ± 8% of NP cells stained positive for SV40 T antigen when infected at MOI 1. At MOI 10, the positive percentage rose to 56 ± 7%; and at MOI 100, 90 ± 8% were detected positive (data not shown, n = 6 in each MOI).

Immunocytochemistry

Extracellular matrix components of NP cells shortly after infection with Ad/SV40 stained positively for Type II collagen, chondroitin-4-sulfate, chondroitin-6-sulfate, and keratan sulfate (Figure 3A). However, after several passages, these cells lost expression of Type II collagen, and instead, began to produce Type I collagen (Figure 3B). Fluorescent immunocytochemistry for SV40 T antigen showed that nearly all of the HNPSV-1 cells stained

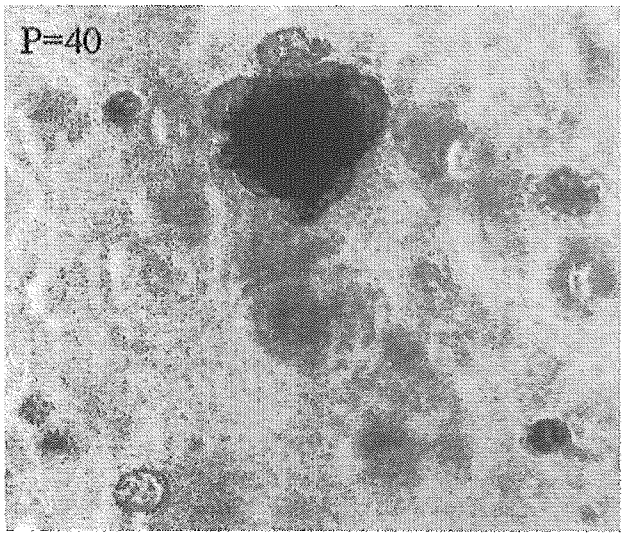

Figure 2. HNPSV-1 cells (passage = 40) demonstrate round morphology under three-dimensional culture in alginate. Colony formation with abundant cell-associated matrix is seen. Original magnifications, ×20.

positive (Figure 3C). HNPSV-1 cells recovered after 2 weeks of culture, and alginate beads showed reproduction of Type II collagen and decrease in Type I collagen.

Cell Proliferation

Cell proliferation analysis of mNP cells and HNPSV-1 cells showed significant increases in cell number for HNPSV-1 cells *versus* mNP cells after 7 days of culture (Figure 4). Cell number counted 1 day after culture showed an average of $3.2 \pm 0.8 \times 10^5$ cells with mNP cells compared to $5.4 \pm 0.7 \times 10^5$ with HNPSV-1 cells (168.75% increase; $P > 0.01$). Assay results after 3 days of culture showed $10.2 \pm 1.8 \times 10^5$ cells with mNP and $15.3 \pm 1.4 \times 10^5$ cells with HNPSV-1 (150.0% increase; $P > 0.01$). The final cell number obtained after 7 days in culture was $14.7 \pm 1.2 \times 10^5$ cells with mNP and $33.4 \pm 1.8 \times 10^5$ cells with HNPSV-1 demonstrating a significant difference (227.2% increase, $P < 0.001$).

DNA Synthesis

The results of [^{3}H]-thymidine incorporation also showed marked upregulation with HNPSV-1 compared to mNP. The mean [^{3}H]-thymidine incorporated into DNA of HNPSV-1 cells was approximately 10 times higher than that incorporated into DNA of mNP cells (mNP cells: $3.9 \pm 0.7 \times 10^{-4}$ dpm/cell, HNPSV-1 cells: $32.6 \pm 1.1 \times 10^{-4}$ dpm/cell; $P < 0.0001$, Figure 5).

PG Synthesis

Despite significant upregulation seen in DNA synthesis, results of PG synthesis quantified by uptake of [^{35}S]-sulfate did not show a significant difference. The mean [^{35}S]-sulfate uptake for mNP cells was $15.6 \pm 1.3 \times 10^{-4}$ dpm/cell compared to $18.5 \pm 1.5 \times 10^{-4}$ dpm/cell for HNPSV-1 cells ($P > 0.01$, Figure 5).

Gene Expression Profiling

Results of RT-PCR for all the genes evaluated showed that HNPSV-1 cells expressed identical patterns with mNP cells in 7 of 9 genes evaluated under the same culture conditions. A significant difference was found in genes responsible for proliferation of NP cells. The mRNA expressions levels of IGF-1 and TGF-β genes in HNPSV-1 cells were upregulated 2 to 3 times more than that of mNP cells ($P < 0.001$, Figure 6).

Karyotypic Analysis

Analysis of HNPSV-1 (passage = 40) showed that of 16 cells examined, 6 cells had normal karyotypes (46,XY), 8 cells had near-diploid (range 39–44, XY), and 2 cells had near-tetraploid (89, XXY).

Tumorigenicity

HNPSV-1 cells injected into rabbit IVDs failed to produce tumors in any of the rabbit discs, at least until 24 weeks postinjection. Histologic evaluation of the paraffin sections revealed that in some discs loss of NP cells was restored by injection of HNPSV-1 cells. Immunohistochemistry of these sections using an anti-SV40 T antigen antibody and a fluorescent second antibody confirmed that cells reconstituting the NP were indeed HNPSV-1 cells. No apparent immunologic response was noted (Figure 7).

Discussion

The primary function of the NP (and ultimately the major function of the disc as well) is to preserve water content of the intervertebral disc, thus providing safe shock/weightbearing absorbance during axial loading and range of motion activity. Since disc degeneration is a primary cause of low back pain in humans, studies of the human disc cells are important. Previous reports claim that the viability of NP cells is responsible for maintaining intrinsic disc structure.[14,15] The NP cells are a heterogeneous group of cells that compose the NP tissue. A major drawback in the studies of NP cells is in the difficulty in procuring primary culture because of the inherent low cell count number usually totaling approximately 4,000 cells/mm^3 as compared with 9,000 cells/mm^3 in anulus fibrosus and 14,000 cells/mm^3 in articular cartilage.[16] In humans, cellular composition changes of the NP occur early in life, mostly by the second decade, followed by a gradual transition of cell type with notochordal cells replaced by chondrocyte-like cells.[5,16] Many reports have suggested that this change in cellular composition is the actual beginning the process of IVD degeneration associated with aging.[16–19] Therefore, in performing appropriate research protocols for NP cell research, obtaining fresh and fully viable tissue samples from young donors without major disc degeneration is considered an important factor. However, the impracticability of obtaining young human NP tissues with minimal degeneration is a major limiting factor. From experience in our laboratory, primary cultures of human NP cells typically produce fewer than 5×10^5 cells depend-

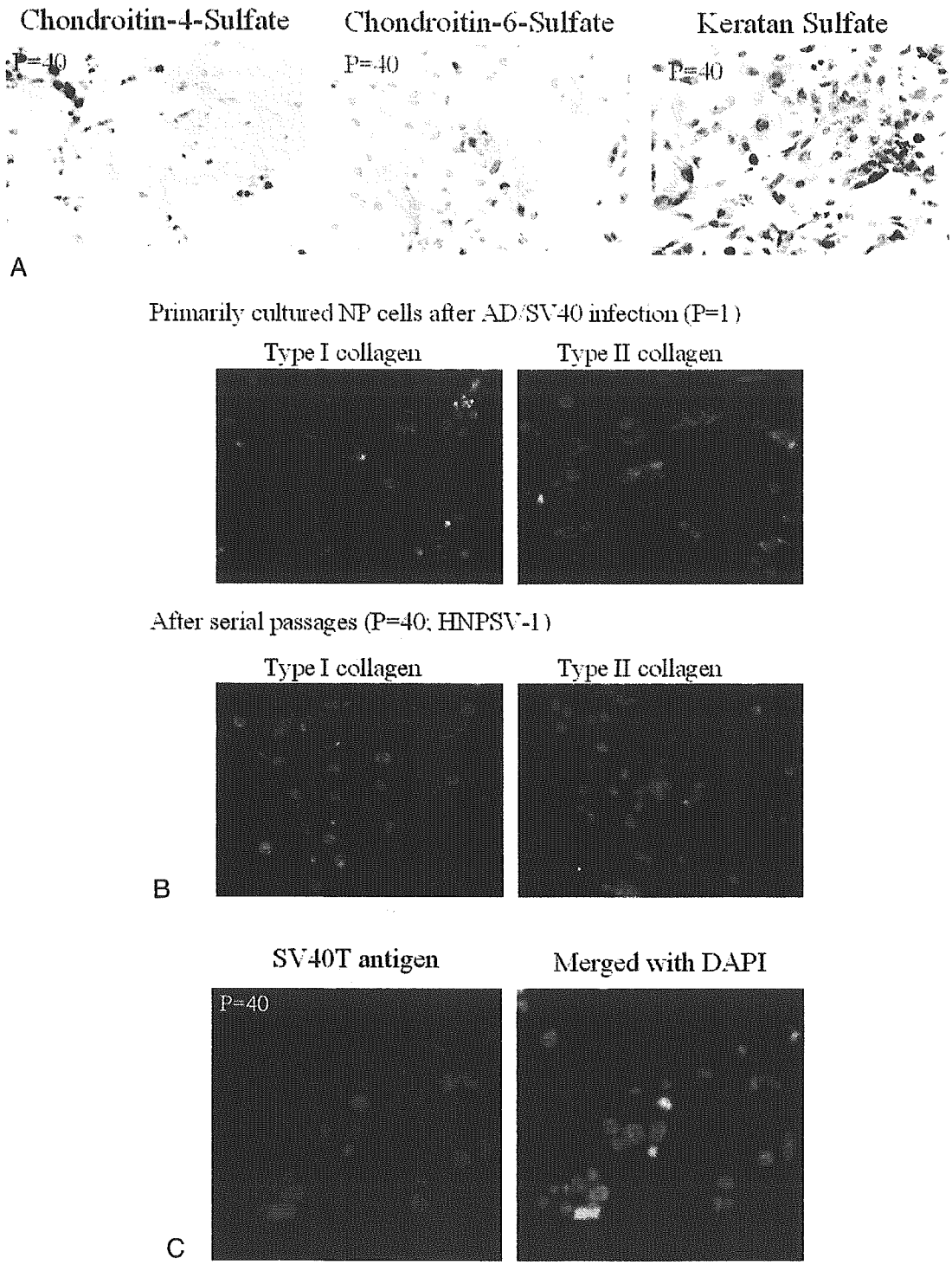

Figure 3. **A**: Immunocytochemistry results showing production of matrix molecules consistent with mNP cells in HNPSV-1 cells (passage = 40). **B**: Turnover of collagen production is seen between primarily cultured NP cells after Ad/SV40 infection (passage = 1) and HNPSV-1 cells (passage = 40). Production of Type II collagen is seen in passage = 1 cells but not in passage = 40 cells. Type I collagen is not detected in passage = 1 cells but is detected in passage = 40 cells. **C**: SV40 antigen is stained in nearly all of the HNPSV-1 cells (passage = 40). Nuclear staining is confirmed by DAPI. Original magnifications, ×20.

ing on the amount of tissue samples. Such primary cultures are capable of about 5 to 6 passages and never survive more than 13 passages, making it difficult for usage in broad-based studies.

To investigate the fundamental characteristics of human NP cells and to use these findings in eventual development of a successful treatment for disc degeneration, we have established an immortalized human NP cell line

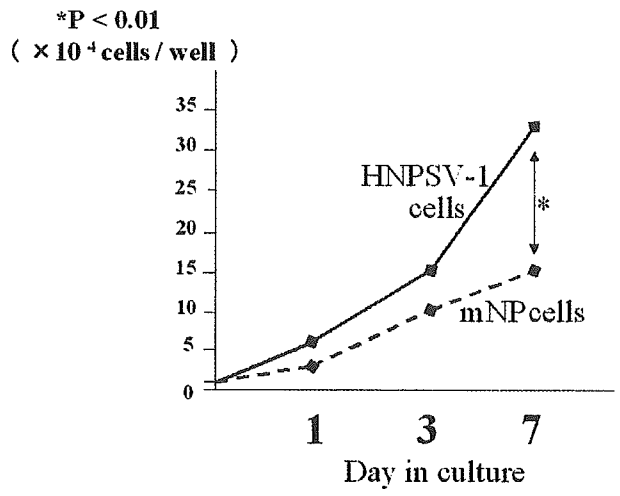

Figure 4. Evaluation of cell proliferation by using WST-8 assay showing significant upregulation in HNPSV-1 cells (passage = 40) *versus* mNP (passage = 1) after 7 days of monolayer culture.

using Ad/SV40 infected cell lines. Successful immortalization of human cell lines that were previously difficult to obtain *in vitro* has provided researchers with a chance to more fully explore the underlying feature of these cells.[20,21] However, there has been no reported study in the literature describing successful use of cell lines derived from human NP cells. Although the use of origin-defective SV40 has become one of the most important techniques in immortalization of cells, the low rate of DNA transfection in human cells has proven to be a significant drawback.[6] In the current study, we used a recombinant SV40 adenovirus vector, a novel virus vector that enables immortalization or transformation of human cells with a resultant high efficiency outcome. A variety of human cell lines established by infection of this virus vector have been reported, including derivations from fibroblasts, keratinocytes, and esophageal epithelial cells.[6,20,21] Cell lines immortalized by this virus vector do not produce free SV40 DNA; consequently, the effect is often transient. This transient effect is another significant advantage of this virus vector, accounting for reduced tumorigenesis ability.

A previous study by Moon *et al* has shown that exogenous genes can be successfully and efficiently cloned into human disc cells using adenovirus vector.[22] In our study, flow-cytometric analysis of the infected NP cells demonstrated that more than 90% of primarily cultured human NP cells were infected with recombinant SV40 adenovirus vector at designated MOIs. Infection resulted in establishment of a veritable HNPSV-1 cell line as demonstrated by marked prolongation of cell life, thereby permitting these cells to be defined as immortalized, as evidenced by their ability to grow at rates 4 to 10 times that of population doublings compared with their mother cells. The fact that HNPSV-1 cells were initially determined immortalized but later developed senescence after 60 to 100 cell population doublings demonstrated that the effect of Ad/SV40 was indeed transient. These results proved that immortalized cell lines could be successfully established from human NP cells by Ad/SV40 infection.

The initial primary culture of mNP cells obtained from a young donor in the current study showed evidence of heterogeneous cell composition. Yet, with multiple passages, these cells dedifferentiated into chondrocyte-like appearing cells. It has been noted that NP cells in adult humans are chondrocyte-like cells that originally migrated from the inner anulus or the cartilage endplate.[5,8,17,23–25] However, this finding is not fully substantiated, and more investigation is needed to confirm the precise pattern of origin in these chondrocyte-like cells because only small numbers of studies deriving results based on purely histologic foundations have been reported for human NP. However, despite the lack of direct proof in the literature, we hypothesize that chondrocyte-like cells are isolated originally from either the inner anulus or cartilage endplate.

It is known that immortalization of normal cells may possibly alter normal cell function and character.[26] We therefore examined the effect of immortalization on function and character of human NP cells. Results from cell proliferation and DNA synthesis studies demonstrated a significant upregulating effect after immortalization. This effect can in part be explained at the gene

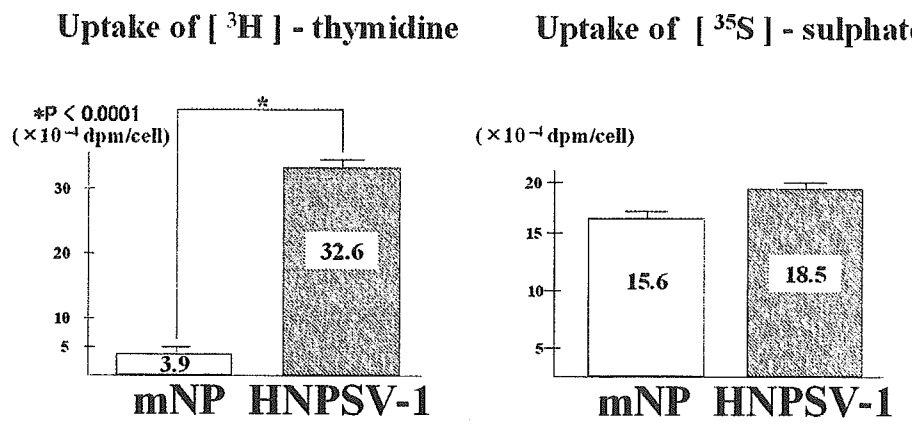

Figure 5. **Left**: Significant upregulation of DNA synthesis in HNPSV-1 cells (passage = 40) *versus* mNP (passage = 1), monolayer culture. **Right**: PG synthesis was not affected by immortalization, three-dimensional culture.

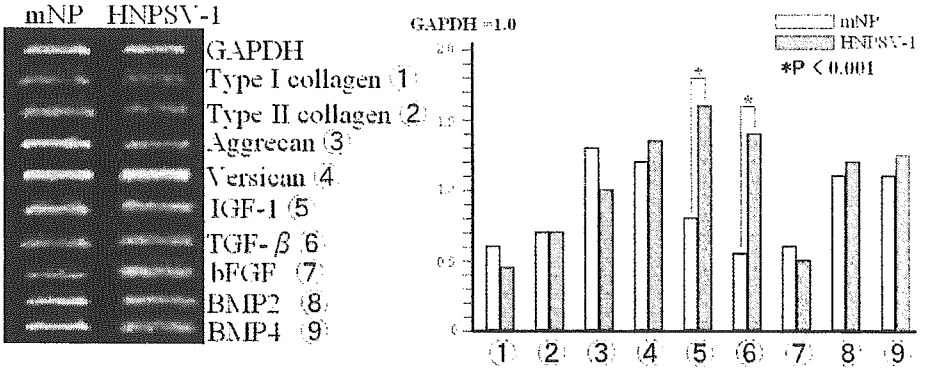

Figure 6. Gene expression profiling by RT-PCR. The majority of the genes expressed similar patterns. IGF-1 and TGF-β mRNA levels were significantly enhanced, suggesting association with upregulation of cell proliferation and DNA synthesis, compared between HNPSV-1 cells (passage = 40) and mNP cells (passage = 1), three-dimensional culture.

level, based on the fact that mRNA expressions for IGF-1 and TGF-β genes, growth factors that are known to stimulate the cell viability of NP cells,[24,27,28] were also shown to be upregulated during immortalization.

Despite the upregulation effects of immortalization on cell growth and proliferation, production of extracellular matrix molecules was less affected. Disc cells are known to produce high levels of large proteoglycan and collagen molecules, primarily Type II.[7,24,25] However, similar to chondrocytes, these disc cells rapidly modulate their phenotype after serial passages resulting in a decrease in production of large proteoglycan and Type II collagen molecules and contrary upregulation of Type I collagen. This phenotypic change in monolayer cultured NP cells was to be observed in our study, in which primarily cultured NP cells and cells from second passage after Ad/SV40 infection produced Type II collagen. However, after several passages, these cells produced Type I collagen instead of Type II collagen. Immunocytochemical analysis of HNPSV-1 showed a dominant production of Type I collagen over Type II collagen using monolayer culture. However, after culture in a three-dimensional environment, redifferentiation into Type II collagen dominant production was observed. Furthermore, neither PG synthesis nor mRNA expression results of matrix components showed a significant difference between HNPSV-1 cells and mNP cells. These results suggest that immortalization of human NP cells by infection with Ad/SV40 accounts mostly for cell proliferation.

A major disadvantage of SV40 mediated transformation is the resultant cancer-associated changes.[6,20,21] However, we did not detect any apparent cancer-associated change in established cell lines. This might have been related to the fact that, as mentioned, the effect of adenovirus vector infection is often transient and, thus, we suspect that HNPSV-1 regained senescence after serial passages. No apparent loss of contact inhibition or cellular piling with unlimited density growth was noted. Moreover, tumorigenesis expression was ineffective at least up to 24 weeks when injected in rabbit IVDs. Previous reports on the establishment of cell lines using immortalization methods note karyotype changes after immortalization.[29,30] This is often explained as an unavoidable phenomenon. The karyotypic analysis of HNPSV-1 cells also demonstrated various atypical karyotypes. Despite this fact, many researchers have insisted that immortalized cell line investigations are essential to defining the fundamental nature of uncommon cells.

Currently, many researchers worldwide are attempting to develop an ultimate treatment methodology for IVD degeneration.[1–3] More than a few reports are found claiming that deterioration of NP cells is the initial trigger for IVD degeneration.[11–15,18,19] Thus, many of the recent studies aimed at decelerating or reversing the irreversible process of IVD degeneration focus on investigating the process of NP cell function and restoration.[11–13,17,18,22,31,32] However, as mentioned before, very little is understood

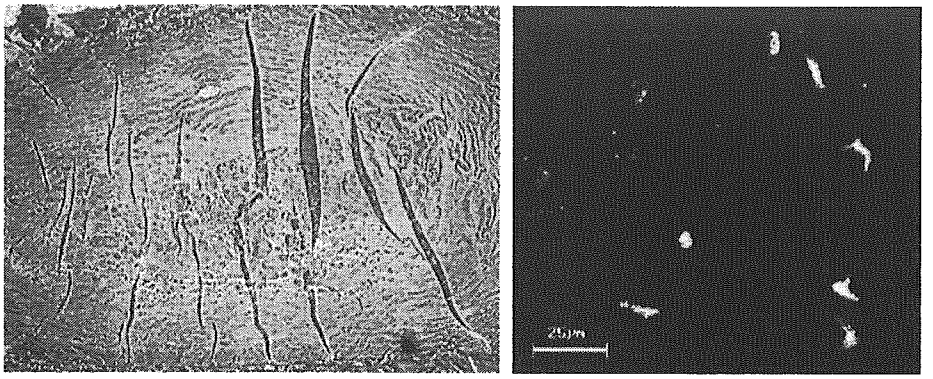

Figure 7. **Left**: Histology of degeneration-induced IVD with HNPSV-1 cells injected at 24 weeks after injection. No apparent tumorigenesis is seen. Original magnification, ×10. **Right**: Survival of HNPSV-1 cells after injection confirmed by staining with anti-SV40 T antigen.

regarding the nature of human NP cells. For instance, no ultimate specific marker has been reported for NP cells, making it difficult to distinguish these cells from anulus fibrosus cells or chondrocytes. Much remains to be learned concerning human NP cells, especially the association between NP cells and the process of IVD degeneration. The final goal in such studies is the development of an efficient application of techniques for treatment of IVD degeneration. Availability of an immortalized line of human NP cells capable of maintaining some differentiated properties or possessing the ability to reexpress some of these properties in designated culture conditions should be of great value in helping to define the biologic function and precise character of human NP cells.

■ Key Points

- Human nucleus pulposus cells were successfully immortalized *via* infection of a recombinant SV40 adenovirus vector, establishing a human nucleus pulposus cell line, HNPSV-1.
- HNPSV-1 gained significant upregulation capabilities for cell proliferation and prolongation of cell life with the retention of most of its original characteristics.
- HNPSV-1 showed evidence of some atypical karyotypes but were not tumorigenic when injected into rabbit intervertebral discs.
- These cells should be useful in further understanding the nature of human nucleus pulposus cells *in vitro*.

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Differentiation of Mesenchymal Stem Cells Transplanted to a Rabbit Degenerative Disc Model

Potential and Limitations for Stem Cell Therapy in Disc Regeneration

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Study Design. An *in vivo* study to assess the differentiation status of mesenchymal stem cells (MSCs) transplanted to the nucleus pulposus of degenerative discs in a rabbit model.

Objectives. To evaluate the fate of MSCs transplanted to the nucleus pulposus of degenerative discs in a rabbit and to determine whether they are a suitable alternative for cell transplantation therapy for disc degeneration.

Summary of Background Data. Although MSCs have been proposed as candidate donor cells for transplantation to treat intervertebral disc degeneration, their differentiation after transplantation has not been adequately investigated.

Methods. Autologous MSCs, labeled with green fluorescent protein, were transplanted into mature rabbits. Consecutive counts of transplanted MSCs in the nucleus area were performed for 48 weeks after transplantation. Differentiation of transplanted cells was determined by immunohistochemical analysis. The proteoglycan content of discs was measured quantitatively using a dimethylmethylene blue assay, and mRNA expression of Type I and II collagen, aggrecan and versican was measured semi-quantitatively using reverse transcription polymerase chain reaction.

Results. Many cells that were positive for green fluorescent protein were observed in the nucleus pulposus of cell-transplanted rabbit discs 2 weeks after transplantation. Their number increased significantly by 48 weeks. Some GFP-positive cells were positive for cell-associated matrix molecules, such as Type II collagen, keratan sulfate, chondroitin sulfate, aggrecan, and the nucleus pulposus phenotypic markers, hypoxia inducible factor 1 alpha, glutamine transporter 1, and matrix metalloproteinase 2. MSCs did not show significant expression of these molecules before transplantation. Biochemical and gene expression analyses showed significant restoration of total proteoglycan content and matrix-related genes compared with nontransplanted discs.

Conclusions. MSCs transplanted to degenerative discs in rabbits proliferated and differentiated into cells expressing some of the major phenotypic characteristics of nucleus pulposus cells, suggesting that these MSCs may have undergone site-dependent differentiation. Further studies are needed to evaluate their functional role.

Key words: intervertebral disc, mesenchymal stem cells, stem cell therapy, disc degeneration, disc regeneration. **Spine 2005;30:2379–2387**

Low back pain is second most frequent reason for visits to physicians.[1] Degeneration of the intervertebral disc accounts for more than 20% of low back pain and hence is a leading source of healthcare costs, lost wages, and patient morbidity.[2–4] Curative treatment for intervertebral disc degeneration is a major challenge. This is because of the nonregenerative nature of the cells that form the intervertebral disc, nucleus pulposus (NP), and anulus fibrosus (AF) cells. Thus, development and application of a treatment for repair of disc degeneration are of great therapeutic significance. Although recent experimental studies have proposed the use of a variety of new techniques, a method that has been sufficiently validated for broad clinical application is still lacking.

Many researchers worldwide are seeking biologic ways to repair degenerated intervertebral discs.[5,6] Gene therapy, growth factor injections, cell-based tissue engineering, and cell therapy have been proposed as major candidates.[7–14] Cell transplantation is a new therapy that is based on the supplementation of matrix-producing cells because a decrease of matrix components, primarily proteoglycan and collagen, is the major initial trigger for disc degeneration. Several animal studies have been conducted using notochordal nucleus pulposus cells, autologous disc chondrocytes (nucleus pulposus cells and anulus fibrosus cells), elastic and costal cartilage, and mesenchymal stem cells (MSCs).[15–23] The results of these animal model pilot studies are promising, and imply that it is possible to slow the process of degeneration or regenerate tissue. However, investigations that are more detailed are needed to validate animal models, to define the effective time window for treatment in cases of progressive intervertebral disc degeneration, and to identify appropriate donor cell types.

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To achieve the most effective tissue regeneration, it is desirable to restore the normal cellular constituents of tissues. Therefore, when disc degeneration is induced by evacuation of nucleus pulposus cells and matrix, a therapy that supplies original nucleus pulposus cells would be appropriate. However, access to healthy nucleus pulposus cells, especially autologous cells, is very limited in clinical settings. Although the phenotypic character of nucleus pulposus cells is still not defined, it has been established that they share the main characteristics of chondrocytes, the so-called chondrocyte-like cells, in adult humans.[24–26] The question that arises is whether any kind of chondrocyte can be used to treat intervertebral disc regeneration. Considering that the final forms of tissues that contain chondrocytes differ widely (*e.g.*, nucleus pulposus, anulus fibrosus, articular cartilage, auricular cartilage), the use of differentiated chondrocytes from tissues different to those of the transplant tissue is of questionable efficacy. Therefore, the use of progenitor cells that can differentiate into nucleus pulposus chondrocyte-like cells may be of great value. As chondrocytes originate from the mesenchyme, there is a good chance that autologous MSC transplantation may become an applicable treatment if MSCs can be differentiated into cells expressing a nucleus pulposus cell phenotype. Risbud *et al* have shown that MSCs that are exposed to hypoxic conditions *in vitro*, as occurs in the nucleus pulposus region of the intervertebral disc, can show differentiation toward nucleus pulposus cells with up regulation of hypoxia-responsive genes.[27] However, the differentiation of MSC has not been sufficiently addressed *in vivo*.

Advances in stem cell biology have shown that differentiation of MSCs depends primarily on the environment in which they are placed.[28,29] In this study, we focused on the questions of whether MSC exhibit site-dependent differentiation when transplanted to the nucleus pulposus region of intervertebral discs in a classic rabbit disc-degeneration model, and whether the transplantation affects the cellular and matrix components of MSC-transplanted discs.

■ Materials and Methods

The Institutional Review Board and Animal Experimentation Committee of the author's institution approved all experimental protocols.

Isolation and Labeling of Autologous MSC. MSCs were isolated from rabbit bone marrow by gradient isolation of mononuclear cells and cell attachment to tissue culture plastic as described previously.[21,28] In short, marrow collected by aspiration from the iliac crest was carefully poured over 20 mL of Nycoprep 1.077 Animal (Axis-Shield PoC AS, Oslo, Norway) and centrifuged using 600*g* for 30 minutes. Mononucleated cells were recovered from the middle layer, washed three times with phosphate-buffered saline (PBS), and primarily cultured. Cells that adhered to culture plastic formed colonies that were expanded in monolayer culture were recovered and had been designated as MSCs. MSCs progressing to transplantation were infected with pMSCV-eGFP, retrovirus vector expressing green fluorescent protein (GFP) gene under the control of long-terminal repeat at multiplicity of infection 20 as described before.[30,31] Vector incorporation data were analyzed by quantitative analysis of the infected cells, and only GFP-positive MSCs were obtained by cell sorter (FACS Vantage, Becton Dickinson, MA).

Immunocytochemistry of MSCs Before Transplantation. To evaluate the phenotypic character of MSCs before transplantation, passage 2 MSCs were seeded on culture slides and cultured for 5 days. After confirmation of cell expansion, cells on the slides were fixed in 4% paraformaldehyde-PBS for 1hour at 4 C and washed 3 times in PBS. MSCs were stained with following primary antibodies (using following dilutions) after blocking with 5% bovine serum albumin (BSA)–PBS (5% BSA, 0.02%Tween20, 0.02%NaN_3 in PBS) for 30 minutes: keratan sulfate ($mIgG_{2b}$, 1:200; Chemicon, Temecula, CA), chondroitin-4-sulfate ($mIgG_1$, 1:200; Chemicon), Type I collagen (mIgG1, 1:100; Daiichi Finechemicals, Toyama, Japan), and Type II collagen ($mIgG_2$, 1:200; Daiichi). Antimouse Alexa 598 (chicken, 1:400; Molecular Probes, Eugene, OR) was used as second antibody.

Degenerative Disc Model. Forty-eight female New Zealand white rabbits (average weight, 3 kg) were divided into three equal-sized groups: a normal control (NC) group, a degenerative disc (DG) group, and an MSC-transplanted (MSC) group. To induce disc degeneration, nucleus pulposus aspiration was performed under inhalation anesthesia in the degenerative disc and MSC-transplanted groups: the nucleus pulposus from the L2–L3, L3–L4, and L4–L5 intervertebral discs were aspirated through an anterolateral approach using a 21-gauge needle and a 10-mL syringe, as described by Okuma *et al.*[19] Aspirated disc fragments were examined under a dissecting microscope to confirm that only nucleus pulposus was aspirated. The weight of nucleus pulposus inside the needle ranged from 5 to 8 mg wet weight.

Transplantation of MSC. Cultured MSCs at passage 2 were embedded in atelocollagen and transplanted into degenerative L2–L3, L3–L4, and L4–L5 discs in the anesthetized MSC transplantation group rabbits 2 weeks after the first operation. Atelocollagen gel (CELLGEN, Lot; 334060, Koken, Tokyo, Japan), medium solution (0.02 mL), in which the MSCs were embedded at a density of 1×10^6 cells/mL, was injected with careful manipulation into each disc through a 27-gauge insulin microinjector.

Tissue Preparation. At 2, 4, 8, 16, 24, and 48 weeks after transplantation, six rabbits, including two degenerative disc model rabbits and two normal controls, were killed by intravenous injection of high-dose pentobarbital sodium (120 mg/kg, Abbott). The L2–L3, L3–L4, and L4–L5 discs were excised immediately in continuity with their cranial and caudal vertebral bodies. These columnar tissues were removed, muscles and nerves were cut off, and the vertebral body–disc–vertebral body units were separated. The units were then fixed in 10% neutral buffered formalin solution (Wako), decalcified in Plank–Rychlo solution (Decalcifying Solution A; Wako), soaked in 20% sucrose-PBS, and processed individually in Tissue Tek O.C.T. Compound (Sakura Finetechnical Co., Ltd.,

Tokyo, Japan) and rapidly frozen. Discs were sectioned with cryotome in axial direction to make 6-μm-thick sections.

Evaluation

Immunohistochemical Double-Staining. Immunohistochemistry was performed using sections obtained from MSC-transplanted rabbit discs 48 weeks after transplantation. Before staining with designated primary antibodies, sections were blocked with 5%bovine serum albumin (BSA)-PBS (5% BSA, 0.02% Tween20, 0.02% NaN_3 in PBS) for 30 minutes at room temperature. Primary antibodies used were directed against the following antigens (using following dilutions): keratan sulfate ($mIgG_{2b}$, 1:200; Chemicon); chondroitin-4-sulfate ($mIgG_1$, 1:200; Chemicon); chondroitin-6-sulfate (mIgG1, 1:200; Chemicon); Type I collagen (mIgG1, 1:100; Daiichi Finechemicals); Type II collagen ($mIgG_2$, 1:100; Daiichi); hypoxia-inducible factor-1-alpha (HIF-1α, mIgG1, 1:200; R&D systems, Minneapolis, MN); glutamine transporter-1 (GLUT-1, sheep, 1:200; FabGennix Inc. Shreveport, LA); matrix metalloproteinase-2 (MMP-2, goat, 1:200; Santa Cruz Biotechnology Inc.); and GFP (chicken, 1:2000; Chemicon). Species-specific secondary antibodies were used for double labeling: Alexa 488 and 594 (1:200; Molecular Probes). Tissues on the slides were mounted with VECTASHIELD Mounting medium with 4′, 6′-diamino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). For staining of proteoglycan epitopes, sections were digested for 1 hour with chondroitinase ABC (Sigma) before staining.

Adipogenesis and Osteogenesis in MSC-Transplanted Discs. Oil red O and Von Kossa staining for identification of lipid and calcium deposition was also performed using standard techniques and evaluated to assess any possible adipogenesis or osteogenesis in the MSC-transplanted discs. Hematoxylin was used for counterstaining.

Microscopic Observation and Analysis. The sections were examined under confocal laser scanning microscope (Carl Zeiss, Germany) or fluorescent microscope (Olympus, Japan) with control slides lacking primary or secondary antibodies. Cells were determined by nuclear staining with DAPI and detected using an UV-1A filter (excitation wavelengths 365 nm, emission wavelengths 420 nm). Next, of the DAPI stained cells, GFP-positive MSCs were detected using FITC filter (excitation wavelengths 465 nm, emission wavelengths 505 nm) and Type I and II collagen, proteoglycan epitopes, HIF-1α, GLUT-1, and MMP-2-positive cells were detected using G-1A filter (excitation wavelengths 546 nm, emission wavelengths 575 nm). Observations were performed by randomly taking 5 points in the nucleus region under 20× magnifications. To obtain an equalized data, a total of 20 axial sections, 10 sections vertically in the sagittal direction above and below the very center of the disc, were analyzed. The sections obtained from the center of the disc were easily identified since the center portion of the disc in the outer anulus was marked before sectioning. Only the cells in view stained with DAPI presenting clear visible nucleus were counted. These cells costained with GFP, detecting transplanted MSCs, were consecutively counted and analyzed. A percentage of DAPI- and GFP-positive cells over all DAPI-positive cells in the 20× magnification view in the nucleus region was calculated and compared in sections obtained from MSC-transplanted rabbits killed 2, 4, 8, 16, 24 and 48 weeks post-MSC transplantation. Determination of whether transplanted MSCs expressed Type I and II collagen, proteoglycan epitopes, HIF-1α, GLUT-1 and MMP-2 were then analyzed. The observations were carried out by the first author and then confirmed objectively by a different histologist. Agreement between the two observers was 92.4%.

Biochemical Analysis. Total intervertebral disc tissues from disc in each group after 48 weeks after transplantation were detached from upper and lower endplates with careful manipulation using a dissecting microscope. These discs were minced and digested for 18 hours at 55 C in papain solution (200 μg/mL in 50 mmol/L EDTA, 5 mmol/L L-cysteine). The DNA content of the tissues was evaluated by taking an aliquot of the digest and by adding 100 μL of Hoechst 33258 dye solution (1 μg/mL, pentahydrate, Molecular Probes). Two hours later, the emission spectrum of the mixture was determined for an excitation at 365 nm by measuring fluorescence emission of 460 nm using plate reader (FL500, Bio-Tek). The standard curve was determined using known concentration of calf thymus DNA (Sigma). Proteoglycan content was measured by mixing 75 μL of the digest with 25 μL of 2.88 mol/L GuHCl solution and 200 μL of dimethylmethylene blue reagent in 96-well plate, and immediately the absorbance at 530 nm and 595 nm was measured by plate reader (SPECTRA MAX250, Molecular Devices). Purified bovine nasal septum-D1 proteoglycan (Sigma) was used as a standard, and the ratio of 530 nm/595 nm was calculated. Data were obtained as micrograms of GAG per microgram of DNA and compared among groups by calculating percent control with regarding averaged data of normal control group discs as a control. Assays were performed more than three times.

Changes in Matrix-Related Gene Expressions Detected by RT-PCR. From intervertebral discs in each group, total RNAs were extracted 24 weeks after transplantation using Isogen reagent and its suggested protocols (Nippon Gene, Tokyo, Japan). RNA samples were then reverse-transcribed to cDNA using oligo dT primers and Multiscribe Reverse Transcriptase (Applied Biosystem, Foster City, CA), followed by specific amplification of matrix specific genes and electrophoretic separation. Gene specific PCR primers were as follows shown in Table 1. The gels were scanned under UV light with a Densitograph system (Atto Biotechnologies Inc., Tokyo, Japan), and band intensities were semi-quantified densitometrically and normalized to GAPDH gene values using a CS Analyzer (version, 2.01, Atto). Experiment was repeated three times.

Statistical Analysis. Statistical analyses of the data were carried out using a nonpaired Welch's *t* test with significance as-

Table 1. Primers Used for RT-PCR Analysis

Gene	Sequence
GAPDH	5′-TCACCATCTTCCAGGAGCGA-3′
	5′-CACAATGCCGAAGTGGTCGT-3′
Type I collagen	5′-TCTGCCCGTTGGGCTTATGA-3′
	5′-CATTGCCTTTGATTGCTGGG-3′
Type II collagen	5′-ATGACAATCTGGCTCCCAAC-3′
	5′-GAACCTGCTATTGCCCTCTG-3′
Aggrecan	5′-AGACAGTGACCTGGCCTGAC-3′
	5′-TGGCCTCTCCAGTCTCATTC-3′
Versican	5′-CAGGAGTCGAGGCGCTGAT-3′
	5′-GCAACCCAAAATGACTGAACG-3′

sumed at $P < 0.05$. Error bars were set to represent standard deviation (SD).

Results

Vector Incorporation Analysis and Percentage of GFP-Positive Cells After Cell Sorting

Vector incorporation analysis showed that 36% ± 15% of MSCs were adequately infected by the vector. After sell sorting, 98% ± 2% of the obtained MSCs stained positively for GFP.

Immunocytochemistry of MSCs Before Transplantation

Immunocytochemistry for proteoglycan epitopes and collagens demonstrated that obtained GFP-positive MSCs stained very poorly for keratan sulfate, chondroitin-4-sulfate, and Type II collagen, whereas some of the GFP-positive MSCs stained strongly positive for Type I collagen (Figure 1).

Observations of MSCs After Transplantation

GFP-positive cells were detected in the nucleus pulposus region from sections obtained 2 weeks after transplantation in most of the MSC-transplanted rabbit intervertebral discs. The percentage of GFP-positive MSCs rose from 15% ± 8% at 2 weeks after transplantation up to as much as 55% ± 7% at 48 weeks (Figure 2). Results of immunohistochemical double-staining demonstrated that no GFP-positive cells were detected in disc specimens obtained from normal control group or degenerative disc group (Figure 3a). On the other hand, both GFP-negative cells detecting native nucleus pulposus cells and GFP-positive cells detecting MSCs were found in MSC-transplanted group discs. As with the native nucleus pulposus cells, some of the GFP-positive MSCs in the nucleus pulposus region of MSC-transplanted rabbit discs costained together with keratan sulfate (72% ± 15%), chondroitin-4-sulfate (34% ± 5%), chondroitin-6-sulfate (24% ± 8%), and Type II collagen (35% ± 7%) (Figure 3b–e). However, most of the GFP-positive MSCs did not stain positively for Type I collagen (3% ± 8%) (Figure 3f). Regarding nucleus pulposus phenotype markers, confocal laser scanning microscopic analysis showed that GFP-positive MSCs costained with HIF-1 alpha (75% ± 14%), GLUT-1 (61% ± 11%), and MMP-2 (48% ± 11%) (Figure 4).

No apparent depositions of lipid or calcium were noted in the nucleus of MSC-transplanted discs showing absence of adipogenesis and osteogenesis (Figure 5).

Result of Biochemical Analysis

Result of biochemical analysis showed that the proteoglycan content of MSC-transplanted disc was similar to normal control group discs without cell transplantation, whereas the proteoglycan content of degenerative disc group discs showed a significant decrease (MSC, 83% ± 13% control; DG, 52% ± 16% control; Figure 6).

Result of RT-PCR Analysis

Semi-quantification of RT-PCR results demonstrated that in degenerative disc group discs, Type II collagen and aggrecan mRNA expression markedly decreased and, in contrast, Type I collagen mRNA increased compared with normal control group discs after induction of degeneration. On the other hand, in MSC-transplanted

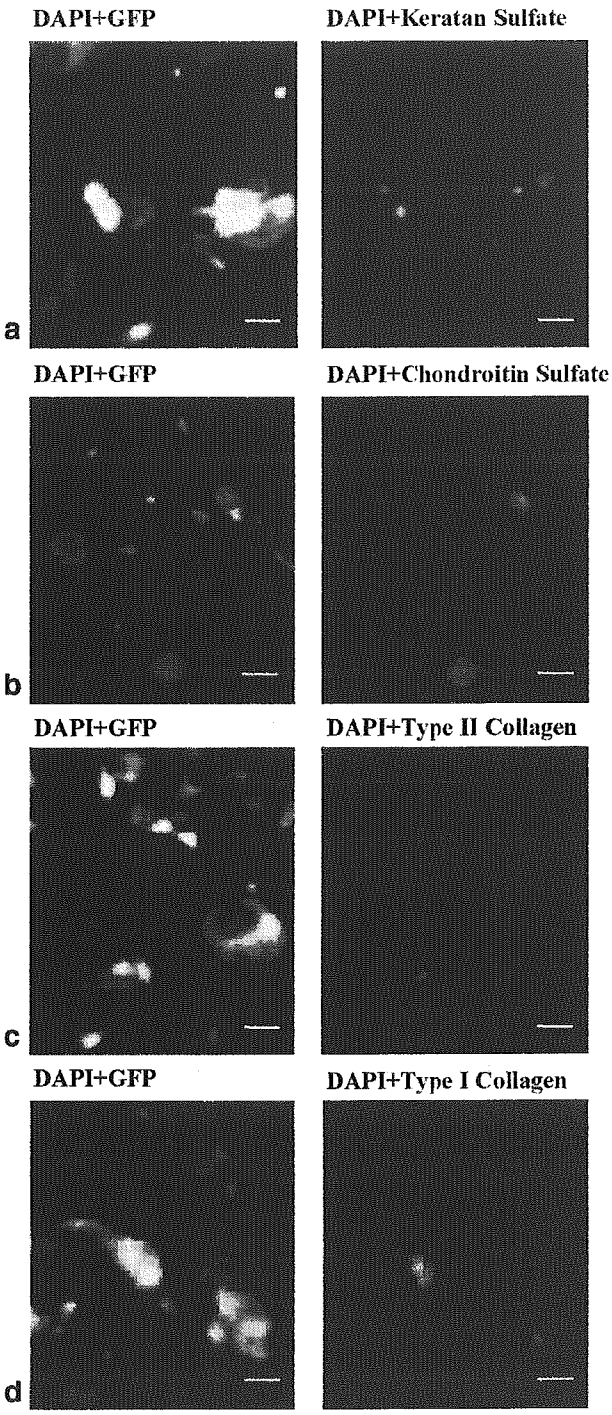

Figure 1. Immunocytochemistry results of MSCs before transplantation. Image on the left in each panel shows that MSCs express GFP and image on the right shows expression patterns for cell-associated matrix: **a**, no expression detected for keratan sulfate; **b**, very small amounts detected for chondroitin-4-sulfate; **c**, no expression for Type II collagen; **d**, positive expression of Type I collagen. Bars = 10 μm.

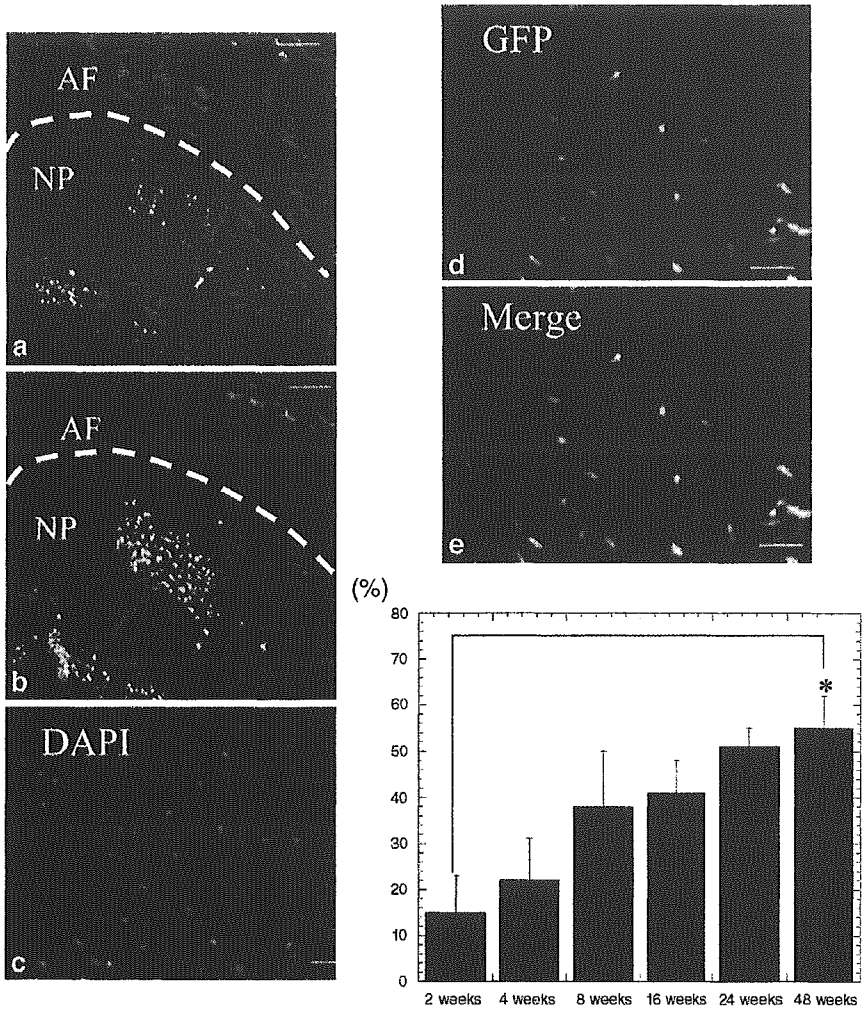

Figure 2. MSCs transplanted to nucleus pulposus area demonstrate time-dependent increase: **a**, 2 weeks; **b**, 16 weeks. Actual calculation of the cells in the nucleus region was performed using the ×20 magnification image with counting DAPI + GFP-positive/all DAPI-positive cells. **c**, DAPI. **d**, GFP. **e**, Merge. **f**, Significant rise in percentage of GFP-positive cells was detected in the nucleus region of MSC-transplanted disc 48 weeks after transplantation. Bars = 500 μm (**a**,**b**), 50 μm (**c**,**d**,**e**).

discs, recovery of Type II collagen and aggrecan mRNA expression was apparent with decrease in Type I collagen mRNA suggesting an antidegenerative effect of MSC transplantation (Figure 7).

Discussion

Autologous MSC transplantation has become a major treatment option for various diseases.[32–35] Previously reported animal studies of MSC transplantation to the intervertebral disc have shown that MSCs can survive and proliferate in intervertebral discs that show delayed degenerative changes by histologic grading, immunohistochemistry, disc height, and the T2 MRI signal.[21–23,36] However, it is not clear whether these effects are mediated by functional cellular supplementation originating from transplanted MSC. Therefore, it is essential to determine whether MSCs that are transplanted to the nucleus pulposus region of intervertebral disc undergo differentiation in concert with the environmental stimulation that results from restoration of cellular and matrix components.

Although the precise character of nucleus pulposus cells is still undefined, most of these cells are clearly chondrocyte-like in human adults. Despite having many features in common with chondrocytes, they are known to differ in expression of hypoxia-responsive proteins *in vivo* and *in vitro*. Rajpurohit *et al* have shown that the up-regulated expression of HIF-1, GLUT-1, and MMP-2 that is observed in nucleus pulposus cells *in vivo* can be used as a phenotypic marker to distinguish these cells from those of neighboring tissues.[37]

Immunohistochemical analysis has shown that GFP-positive MSCs can be detected in the nucleus pulposus region of MSCs transplanted discs from 2 weeks post-transplantation. That these cells increased proportionally over time suggests that some of the transplanted cells survived and had proliferated. These results are compatible with a study reported by Crevenstine *et al* using MSC implantation in rat-tail intervertebral discs.[23]

Immunocytochemical analysis of MSCs before transplantation showed that they expressed a different phenotype than nucleus pulposus cells *in vivo*. They showed strong expression of Type I collagen, in contrast to a very low staining intensity for Type II collagens and proteoglycan epitopes. Double-staining with GFP and cell-associated matrix molecules in the transplanted MSCs