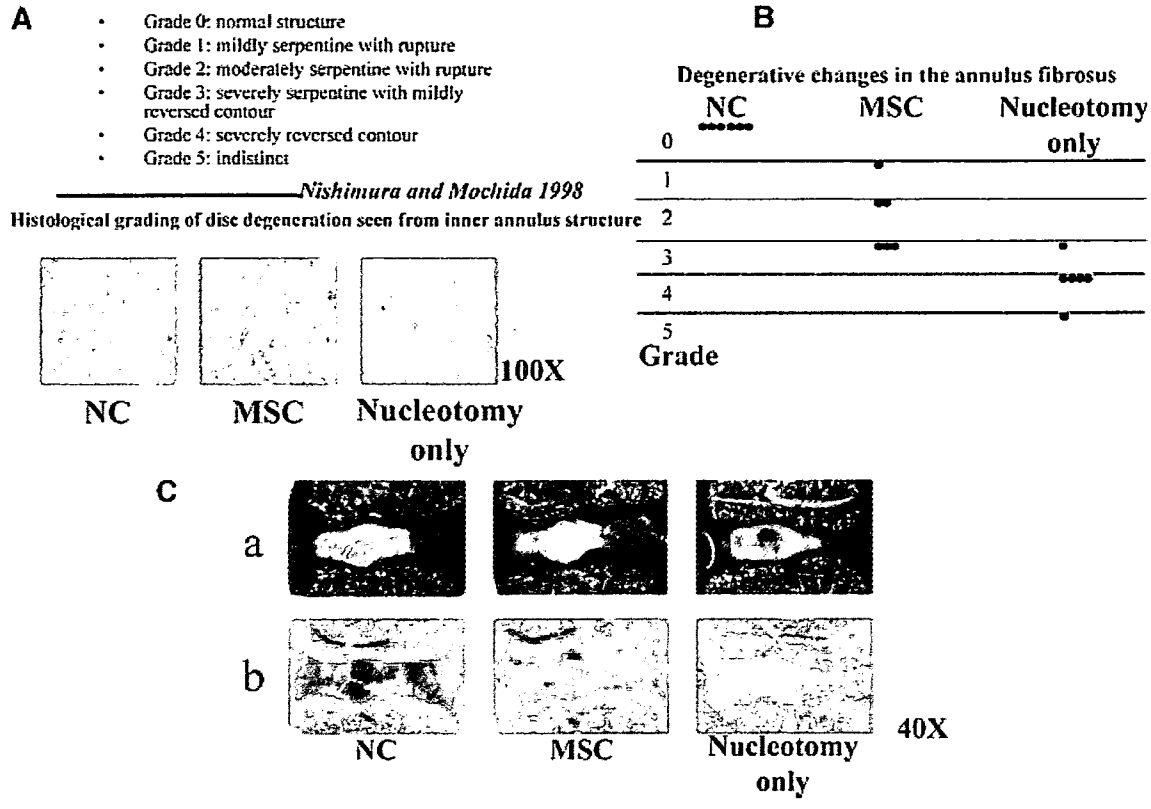

Figure 3. Histology. (A) Representative sections of the histology of disc degeneration seen from the inner annulus structure at 12 weeks after the first operation (original magnifcation ×100). Sections were stained with hematoxylin and eosin for evaluation. (B) The results of histological analysis using the disc degeneration grading system of Nishimura and Mochida,[50] which focuses on morphological changes in the annular structure. (Grade 0: normal structure; Grade 1: mildly serpentine with rupture; Grade 3 moderately serpentine with rupture; Grade 3: severely serpentine with mildly reversed contour; Grade 4: severely reversed contour; Grade 5: indistinct.) At 12 weeks after the first operation, discs from the nucleotomy-only group ($n = 6$ IVDs) had a severely reversed contour (Grade 4.0 ± 0.6), whereas the MSC-transplanted discs ($n = 6$; Grade: 2.3 ± 0.92) had preserved the inner annulus structure. (C) Hematoxylin and eosin (a) and Safranin-O staining (S-O) (b) were minimal in the nucleotomy-only group, while intense staining for S-O was seen in the MSC-transplanted group. These findings demonstrate that MSC transplantation therapy effectively suppressed a decrease in the proteoglycan (PG) content of degenerated discs (original mangifcation ×40).

percentage of GFP-positive MSCs in the NP region was $46.5 \pm 8.9\%$ at 8 weeks after MSC transplantation.

Results of RT-PCR Analysis

At 12 weeks after the first operation, semiquantification of the RT-PCR results demonstrated that FasL mRNA expression was significantly decreased in the nucleotomy-only group, in contrast to an increased expression of FasL mRNA in the MSC-transplanted group, compared to the NC and nucleotomy-only groups (NC: 0.85 ± 0.23; MSC: 1.02 ± 0.31; nucleotomy only: 0.74 ± 0.14, $p < 0.05$. β-actin value = 1.0) (Fig. 6). The results demonstrate that FasL mRNA was restored in the MSC-transplanted group when compared with the nucleotomy group.

DISCUSSION

Numerous studies using small animals indicate that MSC transplantation can decelerate the process of disc degeneration.[41–44] To study the clinical applications of MSC transplantation in humans, animal models of disc degeneration closer to human morphology must be used. One reason for this is that the IVD cells of rats or immature rabbits consist mainly of notochordal NP cells, as opposed to the phenotype of adult human NP cells.[45,46] Unfortunately, no animal model has been developed in which the process of disc degeneration in humans

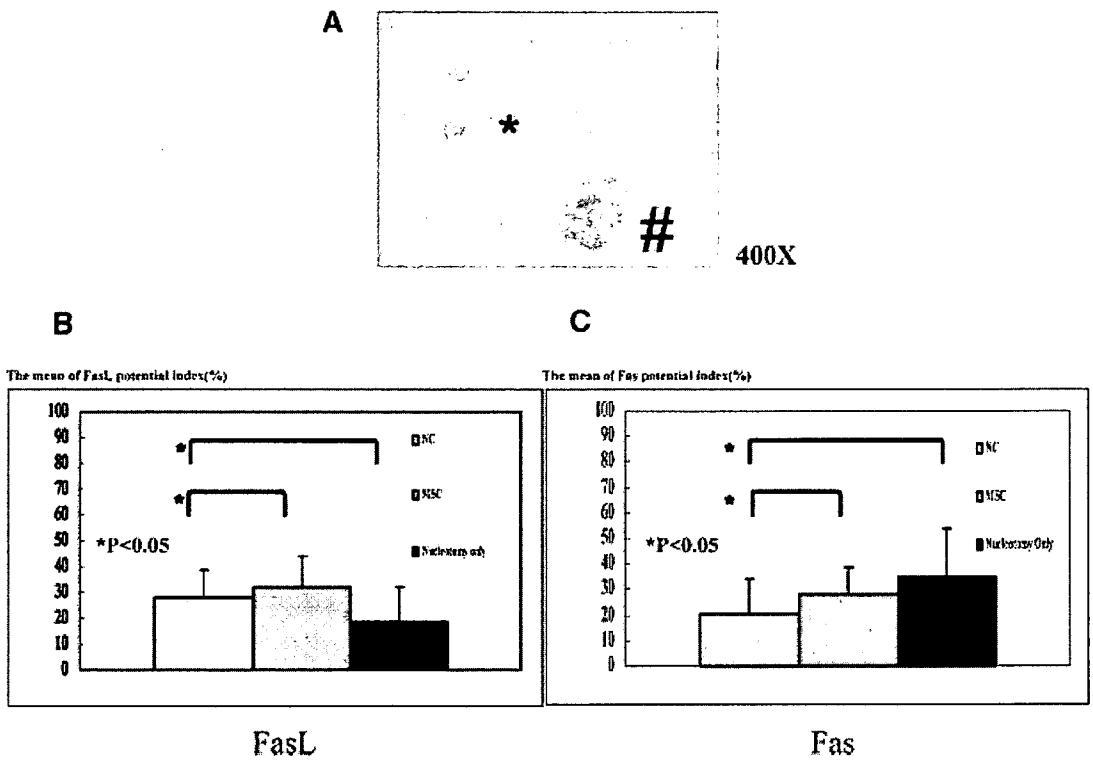

Figure 4. Immunohistochemical staining and analysis of Fas ligand (FasL) and Fas. (A) Immunohistochemical staining was used to evaluate the expression of FasL and Fas by disc cells from the nucleus pulposus (NP) region using the streptavidin–biotin method. FasL and Fas was expressed in the cytoplasm of nomal control group (NC) disc cells of the NP region. (The expression of the brown color indicates FasL and Fas-positive disc cells of the NP region. The expression of the blue color indicates FasL and Fas-negative disc cells of the NP region.) (Original magnifcation ×400.) (B) Immunohistochemical staining for FasL in canine IVDs. The proportion of FasL-positive cells in the NP region was significantly decreased in the nucleotomy-only group, compared to the NC group ($p < 0.05$). The proportion of FasL-positive cells in the NP region was restrained significantly in the MSC-transplanted group. (C) Immunohistochemical staining showing Fas in canine IVDs. Compared to the NC group, the proportion of Fas-positive cells in the NP region increased after induction of disc degeneration in the nucleotomy group, but that proportion was suppressed after MSC transplantation ($p < 0.05$). Significant differences among the groups are indicated by an asterisk ($^*p < 0.05$).

is perfectly replicated. This is partly because, in humans, the conditions of disc degeneration are varied and diverse, and multiple factors such as constitution, lifestyle habits, and occupation are involved in the etiology of the disorder; thus, the degree and conditions of disc degeneration are different in each case. In animal research, it is important to use the model that is most suitable to the objectives of the experiment. With this in mind, we chose a beagle model that has been developed by previous studies to examine the process of disc degeneration that occurs following nucleotomy (supplementary material). Among animal models that approximate the human condition, this model allows a consistent amount of nucleus tissue to be removed, thereby establishing reproducibility. Unfortunately, no animal model has been developed in which the process of disc degeneration in humans is perfectly replicated. We think that nucleus aspiration model is most suitable to the objectives of the experiment. However, one limitation of this nucleotomy model is the fact that it is an injury model that maynot truly represent human disc degeneration. There may be biological responses induced by the traumatic procedure. A preliminary study showed that excessive dissection and nucleotomy led to osteophyte formation and endplate injury in the vertebral body. Because a controlled technique was essential to obtain reproducible results, we used a hand-made stopper and performed the procedure under fluoroscopic imaging. Although it would be preferable to study a species that is bipedal and closer phylogenetically to human beings, to do this presents ethical and financial difficulties. There are

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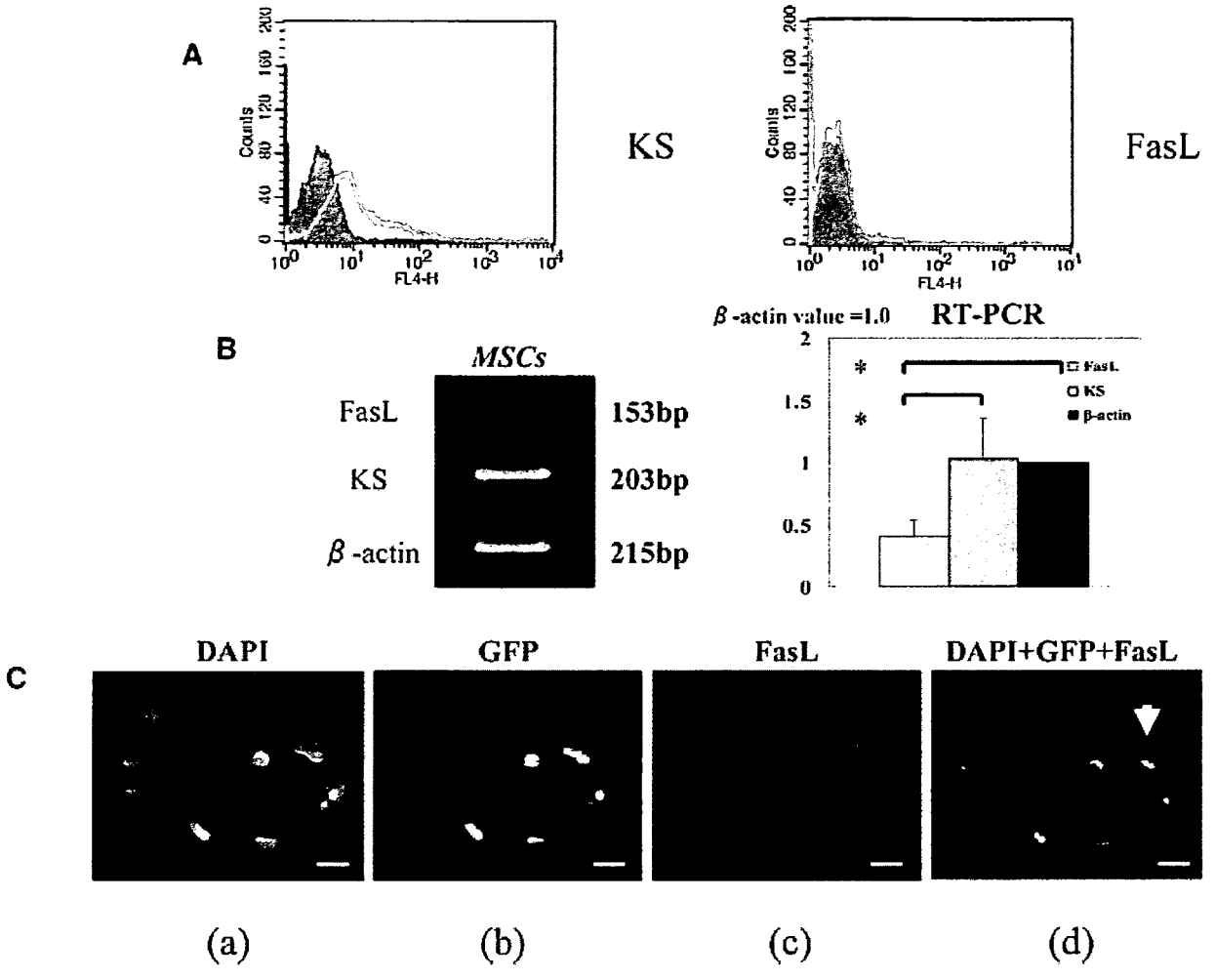

Figure 5. FACS analysis, RT-PCR analysis, and immunocytochemical triple-staining analysis. (A) FACS analysis of FasL expression in MSCs before transplantation. The results of the FACS analysis seen in the left panel shows that 30–40% of MSCs before transplantation express keratan sulfate (KS); however, as shown in the right panel, there was no expression of FasL. (B) RT-PCR analysis before transplantation showed expression of both FasL and KS mRNA in MSCs as normalized to β-actin, which was used as an internal control (FasL: 0.41 ± 0.12. KS: 1.03 ± 0.32. β-actin value = 1.0). Differences are indicated by an asterisk ($^*p < 0.05$). (C) Immunocytochemical triple-staining analysis using DAPI as a counterstain for nuclei. Sections were stained using fluorescence microscopy (a) DAPI, (b) GFP, (c) FasL, and (d) merged image of GFP, FasL, and DAPI. FasL showed a positive expression in both native NP cells and transplanted GFP-positive MSCs, as indicated by the arrow. Bar = 50 μm.

several reasons why we chose the beagle for our experimental model. The boundary between NP cells and AF cells is clear in the IVDs of beagles, and their IVDs have many chondrocyte-like cells that are more similar to those of adult humans.[47] Because they are a chondrodystrophic breed, beagles tend to suffer spontaneous disc degeneration or disc herniation.[48] In addition, beagles have been frequently used for disc degeneration studies, so there exists a body of literature to support further research employing them.[35]

To determine whether MSC transplantation can regenerate a degenerative disc, the %DHI and MRI (T2-weighted) images were used as the main parameters for clinical evaluation of disc degeneration. After MSC transplantation, the decline in the %DHI was controlled and in the MRI (T2-weighted) images, the luminance in the NP was less than normal, although remaining higher than that in the nucleotomy-only group. High signals in MRI (T2-weighed) images indirectly measure the water content of IVDs. From the results of this study, it is

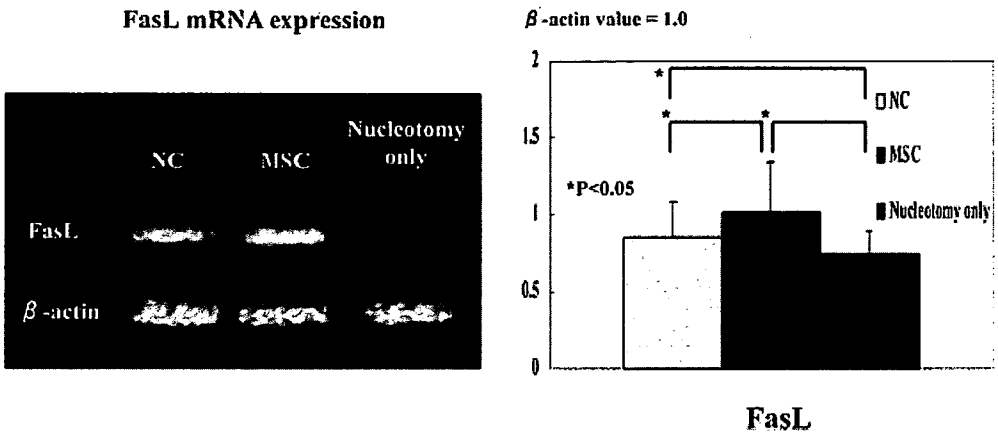

Figure 6. Expression of FasL genes by RT-PCR. In the MSC group, 4 weeks after nucleotomy, autologous MSCs were transplanted into the degenerated intervertebral discs (IVDs). The RT-PCR analysis after MSC transplantation at 8 weeks shows semiquantification of FasL mRNA expression in each group ($n = 6$ IVDs), normalized to β-actin, which served as an internal control. The results demonstrate that FasL mRNA was restored in the MSC-transplanted group compared with the nucleotomy group (β-actin value = 1.0). Differences among the groups are indicated by an asterisk ($^*p < 0.05$).

believed that MSC transplantation lessens the decrease in PG content, and thus, that also of the water content. The data therefore show MSC transplantation to be effective for the treatment of disc degeneration in this beagle disc degeneration model.

Several issues remain for future resolution, including the need for an animal model more closely mimicking human anatomy and physiology. Lacking this, the application of treatment data derived from animal models to the human condition remains problematical. There also remains a need to gather much more basic data on the types of disc degeneration and the optimal timing for MSC transplantation for maximal effectiveness. No specific marker for NP cells has as yet been developed. In this study, we did not investigate expression of the nonspecific markers of NP cells, such as type II collagen and aggrecan. We only focused on the expression of FasL to examine immune privilege in IVDs after MSC transplantation. We reported in our previous study that MSCs transplanted into a rabbit model of disc degeneration differentiated into type II collagen and aggrecan expressing cells.[22] The optimal number of MSCs needed for transplantation is also unknown. However, our preliminary analysis on comparing different number of cells in this particular model indicated that transplantation of approximately 1 million cells per IVD effectively led to regeneration of IVDs in canine model of disc degeneration, compared to 10 million cells per IVD and 100 thousand cells per IVD (data not shown). However, this does not mean that this number is the appropriate number to achieve similar data in humans, and further investigation is essential. Generally thought that the number of adult human NP cells is approximately 4 million/cm^3. Due to problems of breeding and research facility expenses, we could only follow up the animals for 12 weeks after the first operation. For clinical applications, longer term data to look for problems, such as ossification and tumorigenesis, subsequent to MSC transplantation are needed. Finally, a long-term study utilizing radiological, histologic, and biochemical analyses along with clinical results will be required to show more definitely that the transplantation of MSCs would be a useful clinical treatment for degenerating IVDs in humans.

Evaluating the immune privilege of IVDs from FACS analysis, RT-PCR and immunohistochemical triple-staining data, we found FasL was expressed in MSCs before transplantation at the genetic level, but not as the protein. GFP-positive MSCs in the NP region expressed FasL protein when transplanted. These findings suggest the potential for MSCs to differentiate into cells expressing FasL protein. Therefore, transplanted MSCs might contribute directly to the recovery of immune privilege in degenerative IVDs. It is also possible that the FasL expression by the original NP cells increased due to the external stimulus from the transplanted MSCs. Transplanted MSCs may have, therefore, helped indirectly to regenerate the immune privilege in the degenerative IVDs. Because it is thought that that MSCs possess multilineage potential, depending on the environment, and FasL is expressed in tissue with immune

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privilege,[27,28] these results confirm that IVDs are actually a tissue with immune privilege. It has been reported that the ectopic expression of FasL induces the expression of various chemokines to produce inflammation.[49] In this study, an elevated level of FasL expression was detected by immunostaining and RT-PCR in the MSC-transplanted group, when compared to the NC group. Therefore, it is possible that MSC transplantation could produce undesirable inflammation in IVDs. However, because no increase in neutrophils and macrophages was seen in this experiment, we consider it more likely that the elevated FasL level results from FasL expression by differentiated transplanted MSCs in the IVD environment. A further study of the expression of inflammatory chemokines and FasL in MSC transplantation will be needed to elucidate this interaction. Because we found that FasL expression in IVD cells decreased with a greater degree of IVD degeneration, indicating reduced immune privilege in more seriously compromised IVDs, we speculated that cases involving advanced IVD degeneration may have better engraftment of transplant cells. This may be a factor in the clinical application of MSC transplantation therapy that determines the adaptation of the transplantation. An investigation of MSC transplantation efficiency based on differences in the degree of IVD degeneration is desirable in the future. However, with further research remaining regarding the function of FasL in general, it musut be warned that these results may not be the absolute evidence for immune privilege in the IVD environment. One other result drawn from immunohistochemistry is the fact that percentage of GFP positive cells in the NP region costaining with DAPI increases with time. Along with other data, we speculate that the GFP-positive MSCs survive and proliferate.

Although the current study demonstrated successful results in inhibiting disc degeneration, we can not identify the appropriate timing and type of disc degeneration that will be applicable for this therapy. We believe that biological treatments including growth factors, gene therapy, and cell transplantation all have difference in optimal time of administration. For example, growth factors would not be effective if there were not much cells left to react to these agents. The optimal timing that we believe to supplement cells in the IVD should be set between the early stage to the middle stage of IVD degeneration, where cells may be decreasing but nutrition status is still preserved.

In conclusion, in the beagle degenerative disc model, we found, for the first time, that MSC transplantation is partially effective in inhibiting disc degeneration, and may be responsible for maintaining immune privilege in IVDs. We believe that further study will determine whether transplanted MSCs are actually differentiated into NP cells as more information defining the phenotype of NP cells is revealed.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

A phenotypic comparison of intervertebral disc and articular cartilage cells in the rat

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Abstract The basic molecular characteristics of intervertebral disc cells are still poorly defined. This study compared the phenotypes of nucleus pulposus (NP), annulus fibrosus (AF) and articular cartilage (AC) cells using rat coccygeal discs and AC from both young and aged animals and a combination of microarray, real-time RT-PCR and immunohistochemistry. Microarray analysis identified 63 genes with at least a fivefold difference in fluorescence intensity between the NP and AF cells and 41 genes with a fivefold or greater difference comparing NP cells and articular chondrocytes. In young rats, the relative mRNA levels, assessed by real-time RT-PCR, of annexin A3, glypican 3 (*gpc3*), keratin 19 (*k19*) and pleiotrophin (*ptn*) were significantly higher in NP compared to AF and AC samples. Furthermore, vimentin (*vim*) mRNA was higher in NP versus AC, and expression levels of cartilage oligomeric matrix protein (*comp*) and matrix gla protein (*mgp*) were lower in NP versus AC. Higher NP levels of *comp* and *mgp* mRNA and higher AF levels of *gpc3*, *k19*, *mgp* and *ptn* mRNA were found in aged compared to young tissue. However, the large differences between NP and AC expression of *gpc3* and *k19* were obvious even in the aged animals. Furthermore, the differences in expression levels of *gpc3* and *k19* were also evident at the protein level, with intense immunostaining for both proteins in NP and non-existent immunoreaction in AF and AC. Future studies using different species are required to evaluate whether the expression of these molecules can be used to characterize NP cells and distinguish them from other chondrocyte-like cells.

Keywords Nucleus pulposus · Articular cartilage · Phenotype expression · Glypican 3 · Keratin 19

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Introduction

Low-back pain affects up to 80% of adults, with annual estimated direct and indirect costs upwards of $90 billion in the United States alone [25]. Although the aetiology of chronic back pain remains unknown and is likely to be multi-factorial, intervertebral disc degeneration appears to be a leading cause for chronic axial low-back pain [26, 40].

A healthy intervertebral disc consists of three distinct regions the central nucleus pulposus (NP), the outer annulus fibrosus (AF) and the inferior and superior cartilage endplates. The NP is a highly hydrated gel-like matrix composed of negatively charged aggregating proteoglycans, randomly organized collagen fibres and radially oriented elastin fibres [5, 9, 18, 32, 50]. The surrounding AF consists of a series of concentric lamellae of predominately type-I collagen fibres [19, 28]. Throughout growth and skeletal maturation, the boundary between annulus and nucleus becomes less obvious, with the nucleus generally becoming more fibrotic and less gel-like with age [4, 27, 30]. The most significant biochemical change to

occur at the onset of disc degeneration is a loss of proteoglycan [1, 27], and a subsequent decrease in the load-bearing capability of the disc—as the proteoglycan content decreases, the osmotic pressure of the disc falls and the disc is less able to maintain hydration under load [1, 27].

The cells of the AF, particularly in the outer region, appear "fibroblast-like", with an elongated and thin morphology aligned parallel to the collagen fibres and primary synthesis of type-I collagen. By comparison, the cells of the mature human NP are commonly characterized as "chondrocyte-like"—spherical cells synthesizing primarily type-II collagen and aggrecan. This traditional characterization of AF cells as "fibroblast-like" and NP cells as "chondrocyte-like", however, appears to be an over-simplification. It has recently been suggested that cells of the AF could be chondrocytic at a different stage of differentiation than cartilage cells [35]. From the tissue-level perspective, the structure of the proteoglycans differs [6] and the proteoglycan : collagen ratio is higher in NP compared to articular cartilage (AC) [31]. Functionally, such differences are manifested as distinctly different mechanical properties—whereas cartilage behaves largely like a viscoelastic solid, NP can behave both as a fluid and as a viscoelastic solid under different loading conditions [17].

As an added complication, in young individuals and in adults of certain species there is a second population of cells in the NP, the so-called "notochordal cells", presumed remnants of the embryonic tissue that guided formation of the spine and the NP [47, 48]. Based on the concurrent timing of age-related changes and the disappearance of notochordal cells in humans [48] and the prevalence of disc degeneration in chondrodystrophoid canines (no notochordal cells in adult animals) versus non-chondrodystrophic canines (notochordal cells present in adults), a possible connection between loss of notochordal cells and disc degeneration has been surmised.

One of the fundamental issues in understanding disc degeneration and regeneration is to comprehend the cellular biology of the intervertebral disc. Despite the high prevalence of degenerative disc disease (DDD) and the debilitating consequences, the answer to such a basic question as to what defines a disc cell remains surprisingly puzzling. Furthermore, if a stem cell-based tissue engineering approach is envisaged for NP regeneration, it is crucial to know whether stem cell derived "chondrocyte-like" cells are more similar to AC or NP cells.

Thus, the current study was performed to identify a set of genes that could potentially be used to characterize a NP cell. Since—as stated above—the phenotype expression of NP cells and articular chondrocytes are very similar and essentially the same markers, such as type-II collagen, aggrecan, or Sox-9 are used to identify these two cell types, we focused on genes that may distinguish NP from AC cells. We used rat coccygeal discs and AC from both young and aged animals and a combination of microarray, real-time RT-PCR and immunohistochemistry techniques to compare NP, AF and AC cells.

Materials and methods

Microarray analysis

A total of 16 Wistar rats (14–16-weeks old) were used for tissue harvest for the microarray analysis. AC was harvested from the femoral heads, trochlear grooves, femoral condyles and tibial plateaus; AF and NP were harvested from coccygeal discs (8–10 discs/rat). Cells were enzymatically isolated from their respective tissues using sequential pronase (Roche, Basel, Switzerland) and type 2 collagenase (Worthington Biochemical, Lakewood, NJ) digestion with DNase II (Sigma, St. Louis, MO) to prevent cell clumping (AC: 0.2% pronase, 1 h and 0.1% collagenase, overnight; AF: 0.2% pronase + 0.004% DNase, 1 h and 0.1% collagenase + 0.004% DNase, overnight; NP: 0.2% pronase + 0.004% DNase, 1 h and 0.04% collagenase + 0.004% DNase, 8 h). After enzymatic isolation, cells were lysed in TRI Reagent supplemented with 0.5% polyacryl carrier (both Molecular Research Center, Cincinnati, OH), and RNA was cleaned using a modified TRIspin method [37]. Briefly, 1-bromo-3-chloro-propane (0.1 ml/1.0 ml of TRI; Sigma) was added to the cell lysate, and the RNA in the resulting aqueous layer was cleaned by column purification with the GenElute Mammalian Total RNA Kit (Sigma) according to the manufacturer's specifications. RNA was eluted in 50 µl of RNase-free water and treated with DNase I (Sigma) to remove genomic DNA. RNA was precipitated (7 mM NaCl and 70% EtOH, 3 h at –80°C) and dissolved at a concentration of 1 µg/µl.

RNA was sent to MWG, Ebersberg, Germany, for Hybridization Plus Service. This service included T7 amplification of the RNA, Cy3 or Cy5 fluorescent labelling of the RNA, co-hybridization (NP/AF and NP/AC) to the MWG Rat 10K array, array imaging, and analysis. A total of four microarrays were prepared: NP/AF and NP/AC co-hybridizations, each with dye-swap to exclude dye bias. The relative expression of each gene in NP versus AF and NP versus AC was expressed as a ratio of fluorescence intensities.

Real-time RT-PCR

The expression levels of seven genes [annexin A3 (*anx3*), cartilage oligomeric matrix protein (*comp*), glypican 3 (*gpc3*), keratin 19 (*k19*), matrix gla protein precursor

(*mgp*), pleiotrophin heparin binding factor (*ptn*) and vimentin (*vim*)] were further evaluated using real-time RT-PCR. In addition to confirming the findings of the microarray analysis for the isolated rat cells, the effects of enzymatic cell isolation on gene expression was evaluated by comparing expression profiles in RNA extracted from isolated cells to RNA extracted directly from tissue. Furthermore, real-time PCR was used to compare the levels of these genes in young (same age as cell source; $n = 3$ isolations separate tissue pools) and old rat tissue (~2-years old; $n = 6$ animals). For RNA extraction from tissues, tissue was first powderized under liquid nitrogen prior to the addition of the TRI Reagent, and RNA was isolated as described above.

Reverse transcription of total RNA to cDNA was performed using the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the primers and probes listed in Table 1 and TaqMan Universal PCR MasterMix (Applied Biosystems) under standard thermal conditions (10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C; AB 7500, Applied Biosystems). Human 18S rRNA (Applied Biosystems) was used as the endogenous control and data were analysed using the Relative Quantification Method with C_t values from isolated AC cells as the arbitrarily chosen experimental calibrator (ABI Prism User's Bulletin, Applied Biosystems).

Immunohistochemistry

Coccygeal discs and femoral condyles were dissected from 16-week-old rats, fixed in 4% paraformaldehyde, decalcified, dehydrated, frozen in OCT compound and cryosectioned. Sections were treated with 0.3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidase and then washed with phosphate-buffered saline (PBS). Then they were blocked with 2% normal serum for 30 min at room temperature and incubated with the primary antibody at 4°C for 18–24 h. Mouse monoclonal anti-cytokeratin 19 antibody (Progen Biotechnik GmbH, Heidelberg, Germany) and rabbit anti-*gpc3* antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were applied at concentrations of 12.5 and 8 μg/ml, respectively, in 1% BSA/PBS. Negative controls consisted of respective isotype matched irrelevant antibodies (rabbit or mouse IgG). Sections were washed and then treated with biotinylated anti-mouse or anti-rabbit IgG (Vector Laboratories, Burlingame, CA), respectively, for 45 min at room temperature. Slides were then processed using the Vectastain ABC Kit (Vector Laboratories), developed with 3,3′-diaminobenzidine (DAB) substrate (Vector Laboratories) and counterstained with hematoxylin.

Table 1 GenBank reference of the genes evaluated, and oligonucleotide primers and probes used for real-time RT-PCR

Gene (Abbr.)	Accession number	fw primer (5′–3′)	rv primer (5′–3′)	Probe (5′–3′)	Normalized fluorescence	
					AC/NP	AF/NP
Keratin 19 (k19)	X81449.1	cca gga ccg acc tgg aga t	act aat ttc ctc ctc gtg gtt ctt c	cct gaa gga gga gct ggc cta cct ga	15.48	10.94
Glypican 3 (gpc3)	NM_012774.1	ggc cct gag cca gtg gtt	ttt acc ctt ggg cac aga cat	caa act gaa gca cat taa cca gct cct gag a	12.93	10.87
Pleiotrophin heparin binding factor (ptn)	NM_017066.1	ctc aga gat gta aga tcc ctt gca	caa gcc tgg aac tgg tat ttg c	ctg gaa gaa gca gtt tgg agc tga g	9.88	10.80
Vimentin (vim)	NM_031140.1	gca ccc tgc agt cat tca ga	gca agg att cca ctt tac gtt ca	agg atg ttg aca atg cgt ctc tgg cac g	7.87	*2.97*
Annexin A3 (anx3)	NM_012823.1	gtg gaa gag acg aaa gcc tga a	atc cgt gcc cca ttt ttt ct	tgg cca aaa aag atg ccc aga ccc	5.90	5.68
Cartilage oligomeric matrix protein (comp)	NM_012834.1	gtt tcc cgg acg aga agc tt	atc ctc ctg ccc tga att gg	agc gcc agt gcc gca agg ac	0.11	0.11
Matrix gla protein precursor (mgp)	NM_012862.1	gct ccc tct ggc cat cct	ttc cat gct ttc gtg aga ttc ata	tgg ccg tgg cag ccc tgt g	0.06	*0.24*

Statistical analysis

Statistical significance for RT-PCR data was determined using Kruskal–Wallis non-parametric analysis with Mann–Whitney *U* post-hoc testing. Significance was set at $P < 0.05$.

Results

Microarray

To identify genes that could be used to uniquely distinguish NP cells from AF cells and articular chondrocytes, a comparative microarray analysis of 10,368 genes was performed. NP/AF and NP/AC comparisons were made by co-hybridizing the RNA from the two pairings onto separate arrays. Since the purpose of this study was to identify genes that could be used to characterize a given cell type, genes that had normalized fluorescence intensities <5 for *both* cell types on an array were excluded from further analysis. Furthermore, since we were looking for genes that could be used to distinguish between the different cell types, we further narrowed our search to genes with relative intensity differences of at least five, rather than the more commonly used factor of two.

From the NP/AC co-hybridization, 19 genes were identified that had a fluorescent intensity ratio of at least five, and 22 genes with a ratio <0.2 (i.e. AC/NP ratio >5) (Table 2). Three of these genes had ratios of ten or higher and four had ratios of 0.1 or lower. Interestingly, among the list of genes showing substantially higher expression in articular chondrocytes compared to NP cells was *procol2a1* (NP/AC = 0.14), the gene coding for the α1 chain of type-II collagen, which is the predominant collagen in the NP.

From the NP/AF co-hybridization arrays, 27 genes were identified with fluorescence ratios of at least five, and 36 genes with ratios below 0.2 (Table 3). Three of these genes had ratios of ten or higher and ten genes had ratios of 0.1 or lower.

Keratin 19 and *gpc3* had ratios >10 in both the NP/AC and NP/AF comparisons (Table 4). *ptn* also showed large differences between NP/AF and NP/AC (11.1- and 9.9-fold, respectively). *vim* also showed a large difference between NP and AC cells (NP/AC = 7.7), but not between NP and AF cells (NP/AF = 2.9). These four genes were chosen for further analysis by real-time RT-PCR as potential "markers" for NP cells. Additionally, to obtain a better understanding of the relative sensitivities between microarray analysis and RT-PCR, *anx3* was chosen for evaluation by RT-PCR; the ratios of both NP/AC and NP/AF were at least five, but still had a relatively low intensity in the NP (normalized fluorescence intensity = 5.4).

In an effort to also identify genes that could potentially be used to determine that a cell is *not* a NP cell, two genes which were highly expressed in articular chondrocytes and had AC/NP ratios near ten were also analysed by RT-PCR—*comp* and *mgp* (9.3- and 15.8-fold lower intensity in NP versus AC, respectively).

Real-time RT-PCR

The differences in *anx3*, *comp*, *gpc3*, *k19*, *mgp*, *ptn* and *vim* expression were confirmed by real-time RT-PCR using RNA extracted from isolated cells in the same manner as for the microarray hybridization (Fig. 1; cells). Relative mRNA levels of *anx3*, *gpc3*, *k19* and *ptn* were significantly higher in NP samples compared to AF and AC samples ($P < 0.05$). Additionally, *vim* mRNA levels were higher in NP samples compared to AC, and *comp* and *mgp* levels were lower in NP compared to AC ($P < 0.05$). It should be noted that the magnitude of difference in gene expression levels as measured by RT-PCR was much greater than shown by microarray fluorescence intensities. For example, whereas there was a 13–14-fold difference in intensities for *k19* and *gpc3* (NP versus AC) on the microarrays, there was a 100–1,000-fold difference by RT-PCR.

The RT-PCR data also confirmed that there were no significant differences in the relative gene expression measured from RNA extracted from tissue compared to RNA extracted from isolated cells (Fig. 1). There were, however, significant differences in gene expression among young and aged rats (Fig. 2). Compared to levels measured in young tissue, aged tissue had higher NP levels of *comp* and *mgp* mRNA, and higher AF levels of *gpc3*, *k19*, *mgp* and *ptn* mRNA. The elevated *comp* expression of aged NP samples was similar to the level measured for AC (young rat tissue), but the elevated *mgp* expression in the aged NP remained nearly tenfold lower than in AC. The age-related changes in AF expression of *gpc3*, *k19* and *ptn* resulted in statistically similar expression levels of these genes in the NP and AF for aged rats.

Immunohistochemistry

Immunohistochemical analysis was performed to identify and localize the protein expression of *k19* and *gpc3*, whose mRNA expression levels were strikingly different between NP and AC cells in both young and aged rats.

In general, the NP of young rat discs appeared inhomogeneous with a central area consisting of single cells including large vacuoles and of acellular regions, whereas the peripheral part contained groups of condensed cells and an extended extracellular matrix. Since the acellular

Table 2 Genes with NP/AC fluorescence intensity rates of >5 or <0.2 in the NP/AC co-hybridization arrays

Gene	Accession number	Normalized fluorescence		
		AC	NP	NP/AC
Keratin 19	*X81449.1*	***5.10***	***79.71***	***15.48***
Avian sarcoma virus 17 v-jun oncogene homologue	*NM_021835.1*	*2.84*	*41.10*	*14.11*
Glypican 3	*NM_012774.1*	***2.64***	***34.58***	***12.93***
Pleiotrophin heparin binding factor	NM_017066.1	**2.60**	**27.94**	9.88
Vimentin	NM_031140.1	**31.02**	**263.39**	7.87
Serum-inducible kinase	NM_031821.1	0.78	5.79	7.39
Sialoprotein osteopontin	NM_012881.1	14.00	104.39	7.00
Lysosomal trafficking regulator	NM_053518.1	2.77	18.70	6.87
Ige binding protein	NM_031832.1	63.85	431.35	6.73
Glutathione s-transferase	J03752.1	1.36	10.62	6.61
Annexin a3	NM_012823.1	**0.94**	**5.43**	5.90
Carbonic anhydrase iii ec 4.2.1.1	NM_019292.1	30.74	196.36	5.85
c-fos protein aa 1-380	X06769.1	27.42	158.33	5.61
Striated muscle alpha tropomyosin aa 81-284	NM_057208.1	0.88	5.47	5.54
Class i beta-tubulin	AB011679.1	8.30	44.32	5.28
Precursor of alpha-1-protease inhibitor	NM_022519.1	3.26	18.84	5.23
Cytokeratin 8 polypeptide	M63482.1	15.78	83.64	5.21
Basigin ox47 antigen	NM_012783.1	6.18	31.13	5.12
Hypothetical protein smap31—homo sapiens	RATTUS01096	6.41	33.03	5.07
Testostrone-repressed prostate message 2	NM_053021.1	62.80	12.91	0.19
Arachidonate 5-lipoxygenase activating protein	NM_017260.1	14.15	4.14	0.18
Collagen alpha 1 type x col10a1	AJ131848.1	8.48	2.02	0.17
Early quiescence protein-1	RATTUS00790	5.79	1.00	0.17
Nuclear protein 1	NM_053611.1	67.14	10.66	0.16
Chondroadherin	NM_019164.1	46.23	7.93	0.16
Connective tissue growth factor	NM_022266.1	286.93	44.82	0.16
o-acetyltransferase milk fat glolbule membrane protein	NM_012811.1	79.71	12.32	0.16
Procollagen ii alpha 1 col2a1	NM_012929.1	819.25	134.84	0.14
Scrapie responsive protein 1	NM_033499.1	12.38	1.69	0.14
3-*o*-sulphotransferase	AF177430.1	11.03	1.41	0.13
Protease nexin	M17784.1	33.30	4.24	0.13
Collagen alpha 2 type v	AJ224880.1	5.54	0.67	0.12
Integrin-binding sialoprotein bone sialoprotein ii	NM_012587.1	13.06	1.50	0.11
smhs1 protein	NM_080399.1	20.81	3.03	0.11
Organic cation transporter	NM_022270.1	7.86	0.81	0.11
Cartilage oligomeric matrix protein	NM_012834.1	**269.93**	**29.00**	0.11
Myocilin	NM_030865.1	13.03	1.41	0.10
Zinc finger protein 6	*U78117.1*	*31.55*	*3.36*	*0.08*
Matrix gla protein precursor	*NM_012862.1*	***252.20***	***15.95***	***0.06***
Megakaryocyte stimulating factor precursor	*RATTUS02946*	*52.08*	*3.05*	*0.05*
60 kDa secreted protein transin aa 1-475	*X02601.1*	*64.63*	*3.21*	*0.05*

Italicized text indicate a ratio of >10. Genes analyzed by RT-PCR are marked in bold-face type

regions and the vacuoles did not exhibit any immunoreactivity, the central part generally appeared less stained than the peripheral areas.

Consistent with the gene expression data, positive immunostaining for *k19* was noted in the NP of young rat discs (Fig. 3a). The staining was localized intracellularly

Table 3 Genes with NP/AF fluorescence intensity rates of >5 or <0.2 in the NP/AF co-hybridization arrays

Gene	Accession number	Normalized fluorescence		
		AF	NP	NP/AF
Glypican 3	*NM_012774.1*	***2.35***	***28.91***	***10.94***
Keratin 19	*X81449.1*	***6.81***	***79.89***	***10.87***
Pleiotrophin heparin binding factor	*NM_017066.1*	***2.46***	***27.34***	***10.80***
Avian sarcoma virus 17 v-jun oncogene homologue	NM_021835.1	2.15	19.40	8.76
DNA-damage-inducible transcript 1	NM_024127.1	2.16	18.05	8.51
Solute carrier family 12, member 2	NM_031798.1	2.35	19.04	8.39
Cytokeratin-18	U67992.1	9.04	80.81	8.17
Keratin 18	X81448.1	2.03	17.29	7.85
Cytokeratin 8 polypeptide	M63482.1	13.39	90.33	6.81
Sprague_dawley trembllq9jme5 fragment	RATTUS02626	0.75	4.96	6.44
Lysosomal trafficking regulator lyst	NM_053518.1	2.49	16.46	6.44
Hypothetical protein smap3	RATTUS01096	5.18	32.75	6.39
Carbonic anhydrase iii	NM_019292.1	36.35	231.32	5.89
Nerve growth factor-induced protein	NM_012551.1	1.20	7.16	5.89
Striated muscle alpha tropomyosin aa 81-284	NM_057208.1	0.77	4.62	5.88
Protein tyrosine phosphatase, non-receptor type 16	NM_053769.1	16.84	98.14	5.75
Annexin a3	NM_012823.1	**0.91**	**5.47**	**5.68**
Metallothionein I	M11794.1	20.66	114.91	5.63
Ninjurin 2	NM_021595.1	0.85	4.73	5.50
Apolipoprotein e	S76779.1	7.39	39.92	5.43
Brain alpha-tropomyosin tmbr-1; 2; 3	M18135.1	0.75	4.07	5.43
Serum-inducible kinase	NM_031821.1	0.86	5.20	5.32
Transgelin	NM_031549.1	1.65	8.51	5.20
Metallothionein 2	M11794.1	25.26	129.84	5.16
c-fos protein aa 1-380	X06769.1	20.25	112.10	5.10
jun b proto-oncogene	NM_021836.1	5.27	26.85	5.09
Histone h4 precursor aa -1 to 102	NM_022686.1	0.77	3.88	5.06
nov protein	NM_030868.1	16.00	3.26	0.20
o-acetyltransferase milk fat glolbule membrane protein	NM_012811.1	47.56	9.07	0.19
Precursor cystatin c c-terminal fragment	X16957.1	82.32	16.63	0.19
Kelch family protein nd1-l—mus musculus	RATTUS00028	7.51	1.37	0.18
Lipopolysaccharide binding protein	NM_017208.1	21.52	3.80	0.18
Megakaryocyte stimulating factor precursor	RATTUS02946	7.13	1.23	0.18
4-hydroxyphenylpyruvic acid dioxygenase	NM_017233.1	3.90	0.67	0.18
Thrombospondin-4	X89963.1	5.78	1.01	0.17
Alpha-1 type i collagen colia1	M27208.1	435.43	75.35	0.17
Osteonectin	NM_012656.1	288.15	49.35	0.17
Chondroadherin	NM_019164.1	73.75	12.86	0.16
Protease nexin	M17784.1	5.05	0.78	0.16
Alcohol dehydrogenase 3	NM_019286.1	25.44	3.85	0.15
Fatty acid elongase 2	AB071986.1	8.76	1.61	0.14
Unknown	AF093569.1	5.36	0.76	0.14
Clone: 1300008g01—mus musculus	RATTUS01267	23.78	4.96	0.14
Early quiescence protein-1	RATTUS00790	7.17	0.98	0.14
Myocilin	NM_030865.1	8.48	1.19	0.13
Gelsolin protein—mus musculus	RATTUS02839	25.03	4.58	0.13
mhc class ib antigen rt1.m5	AF055667.1	16.27	2.23	0.12

Table 3 continued

Gene	Accession number	Normalized fluorescence		
		AF	NP	NP/AF
Contrapsin-like protease inhibitor related protein	NM_031531.1	57.44	6.84	0.12
Osteoglycin precursor	RATTUS01982	10.50	1.22	0.12
smhs1 protein	NM_080399.1	22.94	3.22	0.11
Testostrone-repressed prostate message 2	NM_053021.1	223.91	24.62	0.11
Cartilage oligomeric matrix protein	NM_012834.1	**217.03**	**24.29**	**0.11**
Ryudocan/syndecan 2	NM_013082.1	16.46	1.84	0.11
Death effector domain-containing protein deft	*NM_031800.1*	*11.94*	*1.18*	*0.10*
3-o-sulphotransferase	*AF177430.1*	*23.24*	*2.25*	*0.10*
pro1 collagen type iii; alpha 1	*X70369.1*	*76.53*	*7.29*	*0.09*
Precursor truncated-cadherin cadherin-13	*RATTUS02522*	*17.15*	*1.48*	*0.09*
Dermatan sulphate proteoglycan-ii decorin	*Z12298.1*	*163.16*	*13.91*	*0.09*
Versican v3 isoform precursor	*AY007691.1*	*24.31*	*2.16*	*0.08*
Collagen alpha 2 type v	*AJ224880.1*	*19.23*	*1.33*	*0.07*
60 kDa secreted protein transin aa 1-475	*X02601.1*	*21.36*	*1.41*	*0.06*
Zinc finger protein 6	*U78117.1*	*34.55*	*2.98*	*0.06*
Procollagen, type i, alpha 2	*NM_053356.1*	*677.00*	*35.20*	*0.05*

Italicized text indicate a ratio of >10. Genes analyzed by RT-PCR are marked in bold-face type

throughout all the NP cells. In contrast, no *k19* immunostaining was identified in AF cells. Furthermore, articular chondrocytes were also immunonegative for *k19* (Fig. 3b).

Nucleus pulposus tissue also demonstrated a strong intracellular immunoreaction for *gpc3* (Fig. 4a). *Gpc3* staining was identified in a majority of the cells throughout the NP, but was most intense towards the periphery, in particular towards the border to the AF. No immunoreactivity for *gpc3* was found in AF and AC cells (Fig. 4b).

Control sections probed with irrelevant immunoglobulins did not show any staining for neither disc nor cartilage specimens.

Discussion

No genes were identified that were clear "on/off" markers to distinguish NP cells from AC or AF cells. There were, however, a total of 63 genes with at least a fivefold difference in fluorescence intensity between the NP and AF cells compared to 41 genes with a fivefold or greater difference comparing NP cells and articular chondrocytes.

In order to increase the purity of the RNA extracted for microarray hybridization, cells were first isolated from their extracellular matrix by enzymatic digestion. It may be expected that such a process can change gene expression levels, thus it was important to confirm the findings from the microarray with real-time RT-PCR using RNA extracted both from isolated cells and directly from the tissue. For the genes examined by RT-PCR (*annexin A3*, *comp*, *gpc3*, *k19*, *mgp*, *ptn* and *vim*), there was no significant difference in the gene expression for cell or tissue isolation. The possibility remains, however, that true differences in gene expression that would be seen with RNA extracted directly from tissue were not detected because the cell isolation procedure neutralized such differences. In addition, our results were based on one single microarray analysis. Therefore the present study cannot be regarded as comprehensive or conclusive over the phenotypical differences between the different cell types; it rather reports on the identification of a number of genes that are differently expressed in the cells/tissues analysed.

In choosing the subset of genes for RT-PCR analysis, relative levels of gene expression (very high or very low-fluorescence ratios on the microarray), individual fluorescence intensities and reported functions/classifications of the genes were considered. The relative levels of individual fluorescence intensities were used to eliminate genes which were expressed at very low levels in all three cell types since the purpose was to find a set of genes that could be used to identify a given cell type. Additionally, although *procol2a1* met the criteria of at least a fivefold difference (AC > NP), it was highly expressed in both NP and AC cells; thus, while its expression is one of the criteria currently used to characterize a cell as an NP cell, it may not be used to distinguish an NP from an AC cell. Finally, it was desired to have a set of genes that could potentially lend insight into development/differentiation of the NP cell

Table 4 Genes from microarray showing at least a fivefold higher or lower intensity ratio for BOTH NP/AF and NP/AC comparisons

Gene	Accession number	Normalized fluorescence					
		AC	NP	NP/AC	AF	NP	NP/AF
Keratin 19	X81449.1	*5.10*	*79.71*	*15.48*	*6.81*	*79.89*	*10.87*
Avian sarcoma virus 17 v-jun oncogene homologue	NM_021835.1	*2.84*	*41.10*	*14.11*	2.15	19.40	8.76
Glypican 3	NM_012774.1	***2.64***	***34.58***	***12.93***	***2.35***	***28.91***	***10.94***
Pleiotrophin heparin binding factor	NM_017066.1	**2.60**	**27.94**	**9.88**	***2.46***	***27.34***	***10.80***
Serum-inducible kinase	NM_031821.1	0.78	5.79	7.39	0.86	5.20	5.32
Lysosomal trafficking regulator	NM_053518.1	2.77	18.70	6.87	2.49	16.46	6.44
Annexin a3	NM_012823.1	**0.94**	**5.43**	**5.90**	**0.91**	**5.47**	**5.68**
Carbonic anhydrase iii	NM_019292.1	30.74	196.36	5.85	36.35	231.32	5.89
c-fos protein aa 1-380	X06769.1	27.42	158.33	5.61	20.25	112.10	5.10
Striated muscle alpha tropomyosin aa 81-284	NM_057208.1	0.88	5.47	5.54	0.77	4.62	5.88
Cytokeratin 8 polypeptide	M63482.1	15.78	83.64	5.21	13.39	90.33	6.81
Hypothetical protein smap3	RATTUS01096	6.41	33.03	5.07	5.18	32.75	6.39
Testostrone-repressed prostate message 2	NM_053021.1	62.80	12.91	0.19	223.91	24.62	0.11
Early quiescence protein-1	RATTUS00790	5.79	1.00	0.17	7.17	0.98	0.14
Chondroadherin	NM_019164.1	46.23	7.93	0.16	73.75	12.86	0.16
o-acetyltransferase milk fat glolbule membrane protein	NM_012811.1	79.71	12.32	0.16	47.56	9.07	0.19
3-*o*-sulphotransferase	AF177430.1	11.03	1.41	0.13	*23.24*	*2.25*	*0.10*
Protease nexin	M17784.1	33.30	4.24	0.13	5.05	0.78	0.16
Collagen alpha 2 type v	AJ224880.1	5.54	0.67	0.12	*19.23*	*1.33*	*0.07*
Smhs1 protein	NM_080399.1	20.81	3.03	0.11	22.94	3.22	0.11
Cartilage oligomeric matrix protein	NM_012834.1	**269.93**	**29.00**	**0.11**	**217.03**	**24.29**	**0.11**
Myocilin	NM_030865.1	13.03	1.41	0.10	8.48	1.19	0.13
Zinc finger protein 6	U78117.1	*31.55*	*3.36*	*0.08*	*34.55*	*2.98*	*0.06*
Megakaryocyte stimulating factor precursor	RATTUS02946	*52.08*	*3.05*	*0.05*	7.13	1.23	0.18
60 kDa secreted protein transin aa 1-475	X02601.1	*64.63*	*3.21*	*0.05*	*21.36*	*1.41*	*0.06*

Italized text indicate a ratio of >10. Genes analyzed by RT-PCR are marked in bold-face type

population and/or functional differences among NP and AC or AF cells (see Table 5 for a summary of gene functions and their relevance to cartilage and disc cells).

Although it was not a primary objective of this study to identify a gene that could be used to characterize aging of the intervertebral disc, we had anticipated substantial age-related changes in the rat NP based on reports that the cell population of the rat NP changes from a predominately notochordal cell type to a more mature ("chondrocyte-like") cell type around 12 months of age [43]. For the

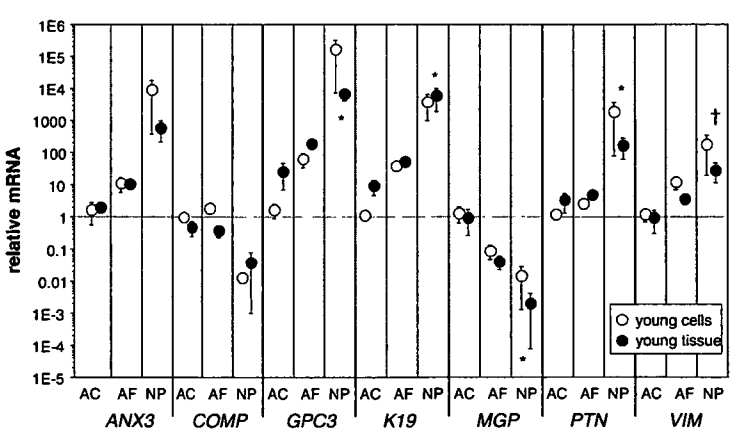

Fig. 1 Relative mRNA expression in tissue (*filled circles*) or isolated cells (*open circles*) of annulus fibrosus (*AF*), nucleus pulposus (*NP*) and articular cartilage (*AC*) of 14–16-week-old rats. Data were normalized to the expression levels of AC cells. No significant differences were seen between cell and tissue extracts for any genes. *$P < 0.05$ compared to AC and AF; †$P < 0.05$ compared to AC

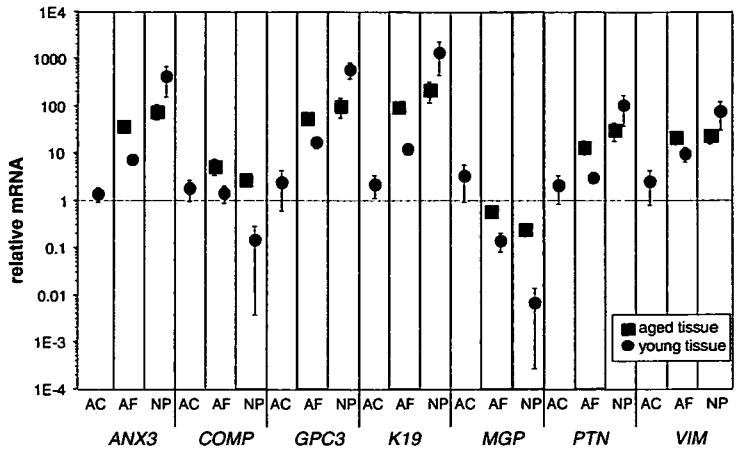

Fig. 2 Relative mRNA expression in annulus fibrosus (*AF*), nucleus pulposus (*NP*) and articular cartilage (*AC*) tissue of young (14–16 weeks; *circles*) and old (2 years; *squares*) rats. Data were normalized to the expression levels of young AC cells. *Shaded* groupings reflect a significant difference ($P < 0.05$) between aged tissue levels of mRNA relative to young tissue

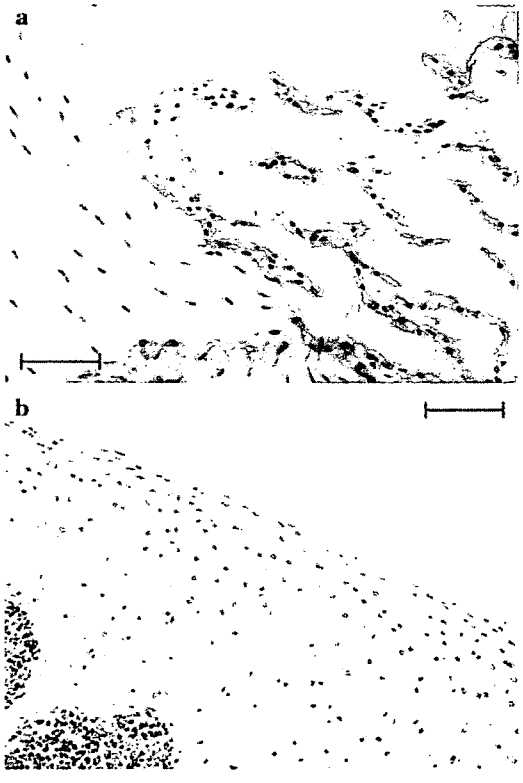

Fig. 3 Immunolocalization of keratin 19 in rat **a** coccygeal disc and **b** articular cartilage, assessed using DAB as the chromogen. **a** Intense intracellular *k19* staining was observed in the NP, which was virtually absent in the AF. **b** Articular chondrocytes were immunonegative for *k19*. *Scale bars* = 0.1 mm

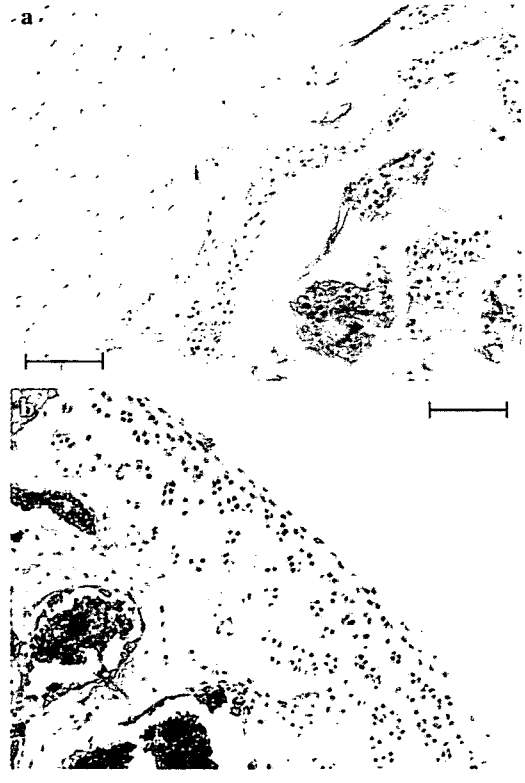

Fig. 4 Immunolocalization of glypican 3 in rat **a** coccygeal disc and **b** articular cartilage, assessed using DAB as the chromogen. **a** Intense intracellular *gpc3* staining was observed in the NP, while AF was immunonegative for *gpc3*. **b** Articular chondrocytes showed no *gpc3* immunostaining. *Scale bars* = 0.1 mm

genes chosen for RT-PCR analysis in the current study, however, the only statistically significant age-related changes in the NP were with *comp* and *mgp* expression; with *comp* expression in the aged NP cells increasing to near the level measured in young rat AC.

In contrast to the few age-related changes in the NP, *gpc3*, *k19*, *mgp* and *ptn* expression changed with age in the AF. In all cases, gene expression shifted towards levels measured in the NP in the older tissue samples. It is unclear from this study whether this shift is due to a true shift in the AF cell or due to partial inclusion of NP tissue in the AF samples. The NP/AF boundary is less distinct in the older animals and since it was anticipated that NP cells would be more similar to AC cells, tissue was harvested as to err on

Table 5 Genes chosen for validation using real-time RT-PCR

Abbr.	Alternate name	Description/function	References
k19	Cytokeratin-19	Intermediate filament typically co-expressed with *vim*; expression reported in epidermal stem cells, notochord cells, various tissues during embryonic development	[44, 49]
gpc3		Cell-surface heparin sulphate proteoglycan; inhibits cell proliferation and regulates cell survival; modulates activity of several growth factors; expression reported in selective embryonic mesoderm and human fetal cartilage	[29, 34, 42, 51]
ptn	Osteoblast stimulating factor-1	Heparin-binding multi-functional protein; can up-regulate proteoglycan synthesis and osteoblast proliferation; expression reported in mesoderm and neuroectoderm during embryonic development, and fetal, juvenile, and osteoarthritic cartilage	[36, 45, 46]
vim		Intermediate filament typically co-expressed with *k19;* expression reported in AF cells, large ("notochordal") and small ("chondrocytic") cells of the NP, and notochord cells; expression also documented in cartilage	[3, 7, 21, 22]
anx3		Ca^{2+} and phospholipid binding protein; influences membrane organization and traffic; regulates Ca^{2+} currents across membranes and/or intracellular Ca^{2+} concentrations	[12]
comp		Extracellular matrix glycoprotein that can cross-link type-II collagen; found abundantly in articular cartilage; also identified in NP and AF	[8, 20, 39]
mgp		Vitamin-K dependent protein found in bone and cartilage; expression up-regulated in endochondral bone formation, pathological calcification of cartilage, and immature and periosteal hypertrophic chondrocytes during fracture healing	[2, 15, 16, 23, 24, 33]

the side of including NP tissue with AF rather than including AF tissue with NP.

Immunohistochemical studies basically confirmed the gene expression data at the protein level. The strong *gpc3* reaction of the NP cells, which was absent in AF and AC, suggests that *gpc3* expression may be an explicit feature of NP cells. High levels of *gpc3* have been found in most tissues during mammalian development [34], whereas in the adult, *gpc3* expression is strongly limited [42]. Supporting our observation, Pellegrini et al. reported that the *gpc3* labelling of developing mouse vertebrae remained, after ossification, only in the NP [34]. *Gpc3* is a highly versatile proteoglycan that has been shown to regulate various growth factors, depending on the tissue, the developmental stage, and its membrane-bound or secreted form [10]. Since *gpc3* has also been shown to induce apoptosis in certain cell-types [13], one could speculate that this proteoglycan may contribute to the change in cell populations within the NP. On the other hand, *gpc3* mRNA was still highly expressed in the NP of aged rats, suggesting that NP might be one of the rare tissues that retain *gpc3* expression.

The large differences in *k19* mRNA levels between NP and AC cells were also reflected by the *k19* immunoreactivity of the respective tissues. *K19* has previously been associated with notochord and chordoma [14, 44]. Thus, this protein might be expected to be present primarily in notochordal cells of the NP. Nevertheless, the majority of the NP cells stained *k19* positive. This is, however, consistent with the finding that *k19* mRNA was expressed at high levels also in NP of old rats that are expected to be devoid of notochordal cells. Notably, several of the molecules found to be highly expressed in NP, namely *k19*, *gpc* and *ptn*, play an important role in tissue development and differentiation. This reflects, in addition to the variable occurrence of notochordal cells also on a molecular basis, that in certain species the NP may continue to express developmental molecules after birth and possibly throughout life.

Recently, Risbud et al., suggested that hypoxia inducible factor 1α (HIF-1α) could be used to distinguish NP cells and articular chondrocytes [38]. NP cells uniquely expressed functionally active HIF-1α protein under normoxic conditions and only modestly increased their HIF-1α

transcriptional activity in a low-oxygen environment. In contrast, AF cells, chondrocytes and osteoblasts showed low-level expression of HIF-1α under normoxia, with significant protein induction at hypoxia. Confirming reports that HIF-1α mRNA is consistently expressed in most cell types and at best modestly induced by hypoxia ([41]; Risbud MV, personal communication) there were no significant differences in HIF-1α gene expression levels between the cell types analysed by microarray. Thus, while HIF-1α protein expression can be considered as specific for NP cells, its mRNA expression is irrelevant as a phenotypic NP marker.

A recently published study, which also employed microarray analysis of RNA extracted from rat coccygeal discs, identified CD24 as a potential marker for NP cells [11]. In the current microarray, the NP : AC intensity ratio for CD24 was 2.3, supporting their findings that CD24 is more highly expressed in the NP compared to AC, but not meeting our stricter criteria of an intensity ratio >5 to warrant further investigation. It is possible, however, that enzymatic isolation of the cells from their extracellular matrix affects the expression of cell surface proteins, such as CD24. Due to the potential utility of using a cell surface marker such as CD24 for cell sorting and/or immunolocalization, CD24 remains an interesting candidate and warrants further investigation.

Conclusions

The present study evaluated a set of genes that are distinctly higher expressed in rat NP compared to AC cells. In view of a stem cell-based tissue engineering approach for NP regeneration, expression of these molecules could become instrumental in monitoring and, eventually, triggering stem cell differentiation towards IVD cells. However, future studies using different animal models in which notochordal cells are not present after birth are necessary to confirm whether the expression of *gpc3* and *k19* can be used to distinguish a mature NP cell from an articular chondrocyte, especially with respect to the human situation. In particular, it will be essential to evaluate whether these genes can generally be attributed to NP cells or whether they depend on the presence of notochordal cells. In addition to the limitation of the rat model due to the ambiguity of the notochordal cells, the functions of the rat coccygeal disc in comparison to the functions of the human IVD need to be considered in the interpretation of the findings of this study. Nonetheless, the rat was an appropriate model for this study due to the need to have an abundant supply of healthy disc tissue, the commercial availability of a rat microarray and the common use of the rat model to study the etiology of disc diseases.

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Feasibility of Using a Human Nucleus Pulposus Cell Line as a Cell Source in Cell Transplantation Therapy for Intervertebral Disc Degeneration

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Study Design. Assessment of the potential use of an immortalized human nucleus pulposus cell line as an alternative cell source in cell transplantation therapy for intervertebral disc degeneration.

Objectives. To evaluate the effect of transplanting the human nucleus pulposus cell line into a disc degeneration model in rabbits and to define whether it is capable of becoming an alternative cell source for cell transplantation therapy for disc degeneration.

Summary of Background Data. Interest in cell transplantation therapy for disc degeneration has been growing for several years, and a range of different cell types have been examined as possible donor cells. In addition, the establishment of a novel cell line that possesses some of the major characteristics of a normal human nucleus pulposus cells has been reported.

Methods. Human nucleus pulposus cell line was established, and cells were transplanted into a rabbit disc degeneration model. At 4, 8, and 24 weeks after transplantation, inhibition of intervertebral disc degeneration was assessed by examining the disc height, macroscopic appearance, histologic findings, and immunohistochemistry. In addition, aggrecan, versican, and Type II collagen gene expression in the nucleus pulposus were measured semiquantitatively at the mRNA level. Furthermore, the survival of transplanted cells was examined using immunohistochemistry for Simian Virus 40 T antigen, and the presence of graft-*versus*-host reaction was assessed by immunohistochemistry for CD4 and CD58.

Results. The disc height was significantly greater in the transplanted group than in the degenerative group's disc from 4 weeks' posttransplantation. Macroscopically, the nucleus pulposus was absent and there was loss of disc height in the degenerative group at 24 weeks after transplantation, whereas the nucleus pulposus was preserved in the transplanted group. Histologic examination showed that the structure of the inner anulus fibrosus was significantly preserved in the transplanted group, and the boundary between the nucleus and anulus could be clearly visualized. Expression of mRNAs of the nucleus pulposus matrix, aggrecan, and Type II collagen was significantly greater in the transplanted group than in the degenerative group. This indicates that transplantation of human nucleus pulposus cell line helped to preserve the matrix of the nucleus pulposus. Thus, transplantation of a human nucleus pulposus cell line was shown to delay disc degeneration in this rabbit model.

Conclusion. The human nucleus pulposus cell line may become an alternative cell source for cell transplantation therapy of intervertebral disc degeneration.

Key words: intervertebral disc degeneration, human nucleus pulposus cell line, cell transplantation therapy.
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Low back pain is extremely common, experienced at some stage by three fourths of the world's population. Low back pain is alleviated within 3 months in 90% of sufferers, but sometimes becomes chronic and associated with pain in the lower limbs, causing long-term impairment of the quality of life.

The causes of low back pain are multiple, including myofascial disease, nerve root compression, and intervertebral joint instability. In recent years, much attention has been focused on intervertebral disc (IVD) degeneration. The main causes of IVD degeneration are unknown, but the process is thought to involve loss of cells and decreased function due to apoptosis, leading to a decreased disc matrix, which is composed of proteoglycans and collagen.[1] Destruction of the IVD follows, with associated loss of function.

Surgical procedures such as herniotomy and vertebral fusion are the most widely used and effective current treatments for IVD herniation and spondylolisthesis, conditions that can arise from IVD degeneration. However, these procedures sacrifice the function of the IVD and increase the mechanical load on the adjacent discs. Therefore, such treatments cannot be considered curative.

In recent years, methods of inhibiting the progression of IVD degeneration have been under development. New treatments that are being considered include injection of growth factor into the IVD,[2–7] induction of genes using viral vectors,[2] and transplantation of cells or tissues.[8–15]

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However, the effects of growth factor are only temporary, and there are ethical problems with gene transfer.[16] Accordingly, our group decided to investigate cell transplantation. In particular, we have been investigating cell transplantation therapy using nucleus pulposus (NP) cells for tissue regeneration.[10–12]

The IVD is an avascular, low-pH, low-oxygen environment that is extremely inhospitable to cells.[17] The NP has a low cell density compared with other tissues[18] and is a site where proliferation is difficult; it contains different types of cells, such as notochordal and chondrocyte-like cells, depending on the subject's age. Therefore, it is very difficult to culture NP cells, and it is both technically and ethically difficult to obtain healthy human NP cells, as most IVDs resected at operation have a degenerate NP. Therefore, the development of cell transplantation therapy using healthy NP cells is extremely problematic. To assist in the investigation of nucleus pulposus cells, we introduced the simian virus (SV) 40 T antigen into human NP cells using a recombinant adenovirus vector, and succeeded in establishing an immortal human nucleus pulposus cell line (human nucleus pulposus SV40: HNPSV).[19] This novel cell line retains its original characteristics but can be amplified in large amounts without becoming tumorigenic.

This article presents the results of our study of the use of this cell line as a source for cell transplantation therapy in a rabbit IVD degeneration model. Inhibition of IVD degeneration was assessed by examining the disc height, macroscopic appearance, histologic findings, and immunohistochemistry. The expression of mRNA for genes involved in IVD degeneration (NP–aggrecan, versican,[20] and Type II collagen) was measured semiquantitatively. The survival of transplanted cells was examined using SV40 T antigen immunostaining, and the presence of a graft-*versus*-host reaction was assessed by immunostaining for CD4 and CD58, markers for lymphocytes and monocytes. Finally, the transplanted nucleus pulposus cells were monitored for a maximum of 24 weeks to assess tumorigenicity.

■ Materials and Methods

The Institutional Review Board and Animal Experimentation Committee of the author's institution approved all experimental protocols. Informed consent was obtained for human tissue sampling.

Establishment of HNPSV-5

Isolation of Human NP. Human NP cells were obtained from a 19-year-old woman undergoing anterior fusion for a burst fracture of the L3 vertebra caused by a traffic accident. The patient was operated on about 10 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 40 minutes at 37 C in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Grand Island, NY) containing 0.27% pronase (Calbiochem, La Jolla, CA), followed by 3 hours of digestion 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 a six-well culture plate (Primaria BD) at a density of 1×10^5 cells/mL in DMEM with 10% fetal bovine serum (Gibco), 1% vol/vol penicillin and streptomycin at 37 C, in a 5% CO_2 atmosphere. The culture medium was changed every 72 hours.

Transvection With Recombinant SV40 Adenovirus Vector. Based on the method of Sakai *et al*,[19] the cultured NP cells were infected with recombinant SV40 adenovirus vector. Briefly, after the second passage, the cells were cultured in 10-mm culture dishes until reaching 80% confluence. The cells were then washed with phosphate-buffered saline (PBS, Gibco) and virus stock was added to each dish at multiplicity of infection 10. One set of negative control dishes was also prepared. Cells were incubated at 37 C, 5% CO_2, for 1 hour, washed in PBS, and recultured in DMEM and 10% fetal bovine serum. After five passages, a proportion of the cells was sampled and SV40 T expression was confirmed using immunocytochemical staining with a mouse anti-SV40 T antigen antibody (Pab419; Oncogene, Boston, MA) and goat anti-mouse IgG Alexa fluor 594 (Molecular Probes, Eugene, OR). Overall, 82.4% of the cells infected with Ad-SV40T stained positive for SV40 T antigen when analyzed by flow-cytometry. These cells were designated as human NP cells (HNPSV-5).

Degenerative Disc Model. Forty-four female New Zealand white rabbits (average weight, 3 kg) were divided into three groups: a normal control (NC) group; 14 rabbits (12 rabbits; 4 rabbits killed at 6, 10, and 26 weeks after the first operation were used for paraffin sectioning and 2 rabbits killed at 26 weeks after first surgery for RT-PCR analysis), a degenerative discs (DG) group; 14 rabbits (12 rabbits; 4 rabbits killed at 6, 10, and 26 weeks after the first operation were used for paraffin sectioning and 2 rabbits for RT-PCR), and a HNPSV-5 transplanted (Tx) group; 16 rabbits (12 rabbits; 2 rabbits killed at 6 and 26 weeks after first surgery for frozen sectioning and 2 rabbits killed at 26 weeks after first surgery for RT-PCR analysis), 3 discs obtained from L2–L3, L3–L4, and L4–L5 were used from each rabbits. To induce disc degeneration, nucleus aspiration was performed under inhalation anesthesia in the DG and Tx groups: the NP from the L2–L3, L3–L4, and L4–L5 IVDs were aspirated through an anterolateral approach using a 21-gauge needle and a 10-mL syringe, as described.[11,12] Aspirated disc fragments were examined under a dissecting microscope to confirm that only NP was aspirated. The weight of NP inside the needle ranged from 0.005 to 0.008 g.

Transplantation of HNPSV-5. Cultured HNPSV-5 cells embedded in atelocollagen were transplanted into degenerative L2–L3, L3–L4, and L4–L5 discs in the anesthetized HNPSV-5 transplantation group rabbits 2 weeks after the first operation. Injection site in the center of the outer anulus was observed directly without magnification and needle of a 27-gauge microinjector was inserted carefully. Changes in the pressure when the needle passes through the anulus was detected and confirmation with the depth marker on the needle that ensures that the needle tip is in the nucleus, atelocollagen gel (CELLGEN, Lot; 334060, Koken, Tokyo, Japan)–medium solution (0.02 mL, pink in color), in which the HNPSV-5 cells were embedded at a density of 1×10^6 cells/mL, was injected. Careful manipulation was performed to assure minimal leakage occurred.