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Intervertebral Disc Cell-Mediated Mesenchymal Stem Cell Differentiation

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ABSTRACT

Low back pain is one of the largest health problems in the Western world today, and intervertebral disc degeneration has been identified as a main cause. Currently, treatments are symptomatic, but cell-based tissue engineering methods are realistic alternatives for tissue regeneration. However, the major problem for these strategies is the generation of a suitable population of cells. Adult bone marrow-derived mesenchymal stem cells (MSCs) are undifferentiated, multipotent cells that have the ability to differentiate into a number of cell types, including the chondrocyte-like cells found within the nucleus pulposus (NP) of the intervertebral disc; however, no method exists to differentiate these cells in an accessible monolayer environment. We have conducted coculture experiments to determine whether cells from the human NP can initiate the differentiation of human MSCs either with or without cell-cell contact. Fluorescent labeling of the stem cell population and high-speed cell sorting after coculture with cell-cell contact allowed examination of individual cell populations. Real-time quantitative polymerase chain reaction showed significant increases in NP marker genes in stem cells when cells were cocultured with contact for 7 days, and this change was regulated by cell ratio. No significant change in NP marker gene expression in either NP cells or stem cells was observed when cells were cultured without contact, regardless of cell ratio. Thus, we have shown that human NP and MSC coculture with contact is a viable method for generating a large population of differentiated cells that could be used in cell-based tissue engineering therapies for regeneration of the degenerate intervertebral disc. STEM CELLS *2006;24:707–716*

INTRODUCTION

Low back pain (LBP) is a condition that affects approximately 80% of the adult population at some point in their lives and has a massive impact on western economies due to both health care spending and loss of productivity [1]. Although the causes of LBP are thought to be multifactorial, in almost all cases there is evidence of intervertebral disc (IVD) degeneration, which makes curing disc degeneration one of the most important socioeconomic imperatives facing modern health care today.

The IVD is comprised of a central gelatinous nucleus pulposus (NP) surrounded by a more highly organized ring of predominantly type I collagen fibrils (the annulus fibrosus [AF]). Within the NP, rounded chondrocyte-like cells are embedded in a disorganized matrix of mainly type II collagen and aggrecan, supplemented with other proteoglycans, including versican [2, 3], and collagens, including type VI collagen. Aggrecan is a highly hydrophilic molecule. The amount of aggrecan in the NP is such that by imbibing water it generates sufficient swelling pressure to force even loaded vertebrae apart, a force resisted by the AF. Vertebral stability relies, in part, on the balance of these two opposing forces. During degeneration in the NP, there is enzymatic breakdown and decreased production of extracellular matrix components, in particular proteoglycans. This leads to loss of the hydrogel properties of the NP, including an increased fibrillar collagen content (particularly that of type I collagen), reduced swelling pressure, instability, and traumatic damage to the disc and surrounding structures [2, 4, 5]. This change in matrix composition is driven by an altered cell physiology. Our group has shown that this is probably caused by a relative increase in expression of the catabolic cytokine IL (interleukin)-1α [4] and the degradative enzymes MMP (matrix metalloproteinase)-1, -3, and -13 and ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs)-4 [6] as the severity of degeneration increases.

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Although there have been recent developments in therapies to treat IVD degeneration, such as those involving laser beam therapy or needle aspiration, the majority of preferred treatments involve either removal of tissue or fusion of the affected disc level; however, these treatments remove the symptoms rather than repair the tissue. Furthermore, they are often short-term solutions that frequently lead to complications due to alterations in the biomechanics of the spine. An ideal solution to managing disc degeneration would be to repair (or regenerate) the NP, producing a matrix with similar or improved biological and biomechanical properties compared with the original.

One such strategy is to use autologous cell implantation similar to that used in articular cartilage repair. As with articular cartilage, however, the IVD is relatively acellular, with only approximately 4000 cells per mm^3 in the NP [7], making the source of cells one of the main problems for any tissue repair strategy.

Recent studies using a number of species have suggested that reimplantation of extracted NP cells into the disc can retard degenerative changes [8–10]. However, the numbers of cells required for a successful treatment are more than can be reasonably acquired from a single disc. Furthermore, evidence suggests that removal of tissue from a degenerate disc can accelerate degeneration within that disc, whereas removal of cells from an adjacent normal disc could induce degeneration in that disc level [11]. In addition to this, cells from degenerate discs have an altered phenotype, showing increased senescence [12], increased expression of both catabolic cytokines and degradative enzymes [4, 6], and decreased expression of matrix components [2, 5].

One potential method to overcome these problems is to coculture NP cells with other cells types either to increase the number of NP-like cells or to increase the activity and matrix production rates of the NP cells. Aguiar et al. [13] cocultured canine and bovine NP cells with notochordal cells derived from adult canine discs and showed an increase in proteoglycan levels expressed by the NP cells. Although the NP is derived from the notochord during embryogenesis, the extent to which notochordal cells persist is species-dependant. Thus, whereas in certain breeds of dog, notochordal cells persist throughout life, in humans the NP is usually devoid of these cells by the age of 10 [14]. This lack of notochordal cells in adult human discs makes this strategy unworkable for treatment of human IVD degeneration.

We are examining a different strategy. As NP cells are essentially of the chondroid lineage [2], it should be possible to produce them from mesenchymal stem cells (MSCs). Previous studies have shown that these undifferentiated cells have a high proliferative capacity and have the ability to differentiate into a number of lineages, including both chondrocytes [15] and NP-like cells [16], although this study was performed using rat MSCs. This differentiation also required the use of a three-dimensional matrix such as alginate, although for use in a tissue engineering strategy differentiation in monolayer culture would be preferable. However, although studies have shown that adult human MSCs can be differentiated into chondrocytes for repair of articular cartilage defects [17, 18], the only data showing adult human MSC differentiation to NP-like cells were achieved using three-dimensional spheroid culture [19], which is not applicable to tissue engineering. A recent study by Yamamoto et al. [20] using rabbit NP cells and MSCs showed an increase in cell proliferation and proteoglycan synthesis during coculture where there was direct cell-cell contact. However, although the authors suggest that MSCs were stimulating NP cell proliferation and matrix production, the cells were not separated after coculture, and so the fundamental question of whether NP cells can induce MSC differentiation, or whether MSCs stimulate NP cells, has not been answered.

The present study aimed to investigate the effect of human MSC and NP cell coculture on the differentiation of MSCs to NP-like cells in monolayer culture. Cells were cocultured with and without cell-cell contact and at a variety of different ratios to investigate whether these factors affected cell differentiation. MSCs were labeled with a cell-permanent inert green-fluorescent dye, and this allowed NP cells and MSCs to be separated by high-speed cell sorting subsequent to the coculture with cell-cell contact. Finally, gene expression of the transcription factor *SOX-9*, which is involved in differentiation of MSCs to chondrocyte-like NP cells, the proteoglycans aggrecan and versican, and the collagens types I, II, and VI, was measured by real-time polymerase chain reaction (PCR) to examine the differentiation states of both the MSC and NP cells.

Materials and Methods

NP Cell Source and Culture

Normal cadaveric IVD disc tissue (which showed no evidence of degeneration based on the system described by Sive et al. [2]) was removed from disc level L4-L5 of an 18-year-old male. Informed consent from relatives and local Ethical Committee approval was obtained for the use of the sample for research. NP and AF tissue were separated and digested in a 0.4% collagenase solution in serum-free medium containing antibiotics. The digested tissue/cell suspension was passed through a 100-μm cell strainer to remove tissue debris, and cells were then pelleted by centrifugation at 400*g* for 5 minutes. The supernatant was removed, and cells were resuspended and cultured to confluence in a 75-cm^2 flask with high-glucose (4500 g/l) Dulbecco's modified Eagle's medium (DMEM-HG), supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 100 μg/ml sodium pyruvate, and 2 mM L-glutamine at 37°C in a humid atmosphere containing 5% CO_2, with medium changed every 3 days.

MSC Source and Culture

To obtain human mesenchymal stromal stem cells, bone marrow was obtained from proximal femur samples removed during hip replacement surgery on a 62-year-old male. The patient gave informed consent, and local Ethical Committee approval was obtained for the use of the sample for research. Mononucleated cells were isolated using a Histopaque-1077 (Sigma, St. Louis, http://www.sigmaaldrich.com) density gradient method [21]. These cells were cultured in a 75-cm^2 flask with Minimum Essential Medium, α-modification (α-MEM), supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 2 mM L-glutamine at 37°C in a humid atmosphere containing 5% CO_2. After 7 days, nonadherent cells were discarded and adherent

cells were cultured to confluence, with medium changed every 3 days.

Fluorescent Labeling of MSCs

At confluence, MSCs were trypsinised, counted, and resuspended at a concentration of 1×10^6 in α-MEM. A sufficient number of cells for the experiment were incubated for 30 minutes at 37°C with a final concentration of 10 μM 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFDA; Sigma). After incubation, the reaction was stopped by adding an excess of medium, centrifuging at 400*g* for 5 minutes, and resuspending in DMEM-HG to a concentration suitable for seeding.

Coculture of MSCs and NP Cells

All cocultures were conducted in triplicate in 24-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) and using 0.4-μm pore size, high pore density, polyethylene terephthalate (PET) track-etched tissue culture inserts (Becton, Dickinson and Company) where appropriate. CFDA-labeled MSCs and unlabeled NP cell control wells were prepared using 6×10^3 cells per cm^2. For coculture wells without contact, CFDA-labeled MSCs were seeded on tissue culture plastic, whereas NP cells were seeded on the upper surface of the membrane of the tissue culture inserts. For cocultures with direct cell-cell contact, both NP cells and CFDA-labeled MSCs were seeded in the base of the appropriate wells of a 24-well plate. Cells were seeded at ratios of 75:25% NP cells/MSCs, 50:50% NP cells/MSCs, and 25:75% NP cells/MSCs. The actual number of cells seeded in each well is summarized in Table 1. Cocultured cells were maintained for 7 days in DMEM-HG at 37°C and 5% CO_2 in a humidified atmosphere with medium being changed every 2 days.

Table 1. The number of cells seeded into wells of a 24-well plate

	NP cell concentration	MSC concentration
NP control	1.2×10^4	
MSC control		1.2×10^4
NP/MSC 75:25% without contact	1.2×10^4	4×10^3
NP/MSC 50:50% without contact	1.2×10^4	1.2×10^4
NP/MSC 25:75% without contact	4×10^3	1.2×10^4
NP/MSC 75:25% with contact	9×10^3	3×10^3
NP/MSC 50:50% with contact	6×10^3	6×10^3
NP/MSC 25:75% with contact	3×10^3	9×10^3

For samples without contact, NP cells were seeded into the upper surface of the membrane of a tissue culture insert, whereas all other cells were seeded on the base of the 24-well plates.
Abbreviations: MSC, mesenchymal stem cell; NP, nucleus pulposus.

Separation of MSCs and NP Cells Cocultured with Contact Using High-Speed Cell Sorting

Cocultured cells were trypsinised, resuspended in DMEM-HG, and pelleted by centrifugation at 400*g* for 5 minutes. The pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) and passed through a 30-μm sterile filter (Partec, Münster, Germany, http://www.partec.com) to remove cell clumps. The samples were then analyzed and sorted using a MoFlo high-speed cell sorter (Dako, Glostrup, Denmark, http://www.dako.com) and Summit software (Dako). The cells were passed through a 488-nm argon ion laser, and cells fluorescing at 530 nm selected as MSCs, non-fluorescent cells selected as NP cells, and particles of incorrect forward and 90 degree light scatter rejected as debris. Cells from each region were sorted into 14-ml polypropylene (BD Falcon; BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) tubes containing 12 ml of DMEM-HG and then pelleted and lysed in 1 ml of TRIzol.

Real-Time PCR Analysis of Gene Expression

Control cells were rinsed in PBS, and cells were lysed in TRIzol (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). For cells cocultured without contact, tissue culture inserts were removed to clean wells, cells were rinsed in PBS, and TRIzol was added to both the original wells (MSCs) and the inserts (NP cells) to lyse the cells.

Total RNA was then extracted according to manufacturer instructions. Briefly, chloroform was added to each sample, and sample tubes were centrifuged to enable phase separation. RNA was precipitated by addition of isopropanol to the aqueous phase, followed by centrifugation. Precipitated RNA pellets were washed in 75% ethanol and then resuspended in distilled RNAse-free water.

cDNA was prepared from RNA using Superscript II RT (Invitrogen). RNA ($<$1 μg) was mixed with random prime hexamers (250 ng) and dNTPs (500 μM each dNTP) and then incubated at 65°C for 5 minutes. Tubes were cooled on ice, and then 5× first strand buffer, 0.1 M dithiothreitol (DTT), 40 U RNAseOUT RNAse-inhibitor (Invitrogen), and 200 U reverse transcriptase were added, giving a final volume of 20 μl. Samples were then incubated at 42°C for 90 minutes and finally heated to 70°C for 15 minutes.

Gene expression was analyzed by real-time PCR using an ABI Prism 7000 sequence detection system (Applied BioSystems, Foster City, CA, http://www.appliedbiosystems.com). Primer/probe sets of housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *18S* were predesigned and purchased from Applied BioSystems. Primers for *SOX-9*; collagen types I, II and VI; proteoglycans aggrecan and versican; and elastin were designed using the Applied BioSystems Primer Express 2 software or derived from Martin et al. [22] (Table 2).

Reactions were carried out in duplicate in 96-well plates in a final volume of 25 μl. For *GAPDH* and *18S*, the reaction mix contained 12.5 μl mastermix (Applied BioSystems), 9.25 μl RNAse-free water, 1.25 μl primer/probe mix, and 2 μl cDNA. For target genes, the reaction mix contained 12.5 μl SYBR green mastermix (Applied BioSystems), 5.5 μl water, 2.5 μl forward primer (900 nM), 2.5 μl reverse primer (900 nM), and 2 μl cDNA.

Table 2. Real-time polymerase chain reaction primer details

Primer	Forward and reverse primer sequences 5′→3′	Amplicon size (bp)	GenBank accession no.
SOX-9	GACTTCCGCGACGTGGAC CAGTACCTGCCGCCCAAC	99	Z46629
Type I collagen (COL1A1)	CAGCCGCTTCACCTACAGC TTTTGTATTCAATCACTGTCTTGCC	83	[a]
Type II collagen (COL2A1)	GGCAATAGCAGGTTCACGTACA CGATAACAGTCTTGCCCCACTT	79	[a]
Type VI collagen (COL6A2)	GACGCTGTTCTCCGACCT GGTCTGGGCACACGATCT	89	BT007617
Aggrecan	TCGAGGACAGCGAGGCC TCGAGGGTGTAGCGTGTAGAGA	85	[a]
Versican	TGGAATGATGTTCCCTGCAA AAGGTCTTGGCATTTTCTACAACAG	98	[a]
Elastin	CAGGTGTAGGTGGAGCTTTTGC GGCAGTTTCCCTGTGGTGTAG	142	M36860

Accession number given for primers designed in-house using Primer Express 2.0 software (Applied BioSystems).
[a] Primers taken from [22].
Abbreviation: bp, base pairs.

The PCR reaction consisted of an initial enzyme activation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A cycle threshold (Ct) value was obtained for each sample, and duplicate sample values were averaged. The $2^{-\Delta\Delta Ct}$ method was then used to calculate relative expression of each target gene [23]. Briefly, mean Ct value of target genes in each sample was normalized to its averaged housekeeping gene Ct value (the average of the mean *18S* and mean *GAPDH* values [24]) to give a ΔCt value. This was then normalized to control samples (ΔΔCt), which in all cases was MSC or NP cells cultured alone, and finally the $2^{-\Delta\Delta Ct}$ value obtained.

Coculture of MSCs with Human Dermal Fibroblasts

To ensure that the changes seen in MSC and NP gene expression after coculture with contact were caused by the NP cells, MSCs were cultured in contact with human dermal fibroblasts (HDFs). The same experimental setup and cell ratios were used as for the NP-MSC coculture. CFDA-labeled MSCs were separated from HDFs using MoFlo high-speed cell sorting as described earlier. Cells were lysed in TRIzol, and RNA was extracted and reverse-transcribed to cDNA. This was then used in real-time PCR for housekeeping genes *GAPDH* and *18S* as well as collagen types I and II, *SOX-9*, aggrecan, and elastin as described earlier.

Statistical Analysis

Statistical significance was determined using the Student's t test, in which $p < .05$ was considered significant.

Results

High-Speed Cell Sorting of NP Cells and CFDA-Labeled MSCs

The MoFlo high-speed cell sorter was used for analysis and sorting of the cells after coculture. Cell analysis was carried out, and a gate was placed on the forward versus 90 degree light scatter dotplot to reject all cell debris.

As the CFDA molecule is divided evenly between daughter cells with each cell division, it was important to isolate cells that were distinctly positive or negative after culture (Fig. 1). Therefore the region 1 (R1) gate was placed over a small percentage of "negative" NP cells to ensure no contamination with MSCs that had undergone several divisions. Similarly, the region 2 (R2) gate only covered cells that were definitely positive. After 7 days, the HDF-MSC control coculture samples were sorted using the same method, with unlabeled HDFs gated on region 2 and labeled MSCs gated on region 3 (Fig. 2). A sample of each cell population was reanalyzed on the MoFlo high-speed cell sorter to check cell purity. This demonstrated whether there were contaminating labeled cells (MSCs) in the unlabeled cell (NP or HDF) population and vice versa, and it was established that each population had a purity of more than 97% (i.e., insignificant numbers of contaminating cells).

Effect of CFDA Labeling on MSC Gene Expression

To ensure that CFDA labeling had no effect on MSC gene expression or phenotype, the expression of *SOX-9*, types I and II collagen, aggrecan, and elastin genes were measured by real-time PCR. Type II collagen was virtually undetectable (being seen in only five of 12 wells; data not shown), and the remaining genes showed no significant change in expression (Fig. 3).

Gene Expression after NP and MSC Coculture Without Contact

We examined whether coculture of human NP cells and MSCs affected the differentiation state of either cell population and whether this differentiation was affected by either cell ratio or cell-cell contact. We used real-time PCR to study changes in gene expression between control and coculture samples and expressed this as a relative gene expression normalized to housekeeping genes and control cell samples.

Where cells were cocultured without contact, there were only minor changes in the genes studied (Fig. 4). Although a number of these changes were significant, none of the increases or decreases was larger than 10-fold over controls. It was also evident from the data that cell ratio had no effect on gene expression.

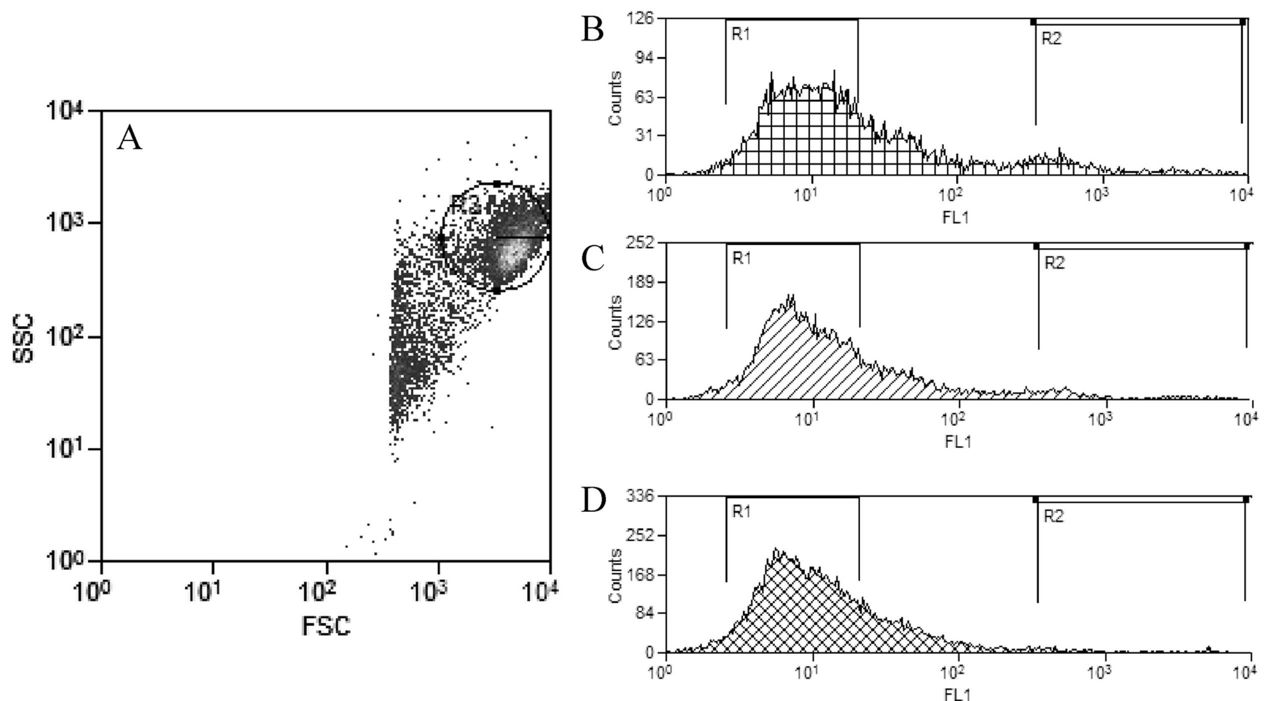

Figure 1. MoFlo data showing parameters for separation of CFDA-labeled MSCs and unlabeled NP cells following coculture with cell-cell contact. **(A):** MoFlo dotplot showing the cultured mesenchymal stem cells (MSCs) and nucleus pulposus (NP) cells after 7 days in culture. **(B–D):** Histograms showing the cultured MSCs and NP cells after 7 days in culture. R3 gate was placed on the FSC versus SSC dotplot to eliminate cellular debris from the histograms. Histograms show regions of unlabeled NP cells (R1) and labeled MSCs (R2) that were sorted. Histogram **(B)** is 75:25% MSC/NP, **(C)** is 50:50% MSC/NP, and **(D)** is 25:75% MSC/NP. Abbreviations: R, region; FSC, forward scatter; SSC, side scatter.

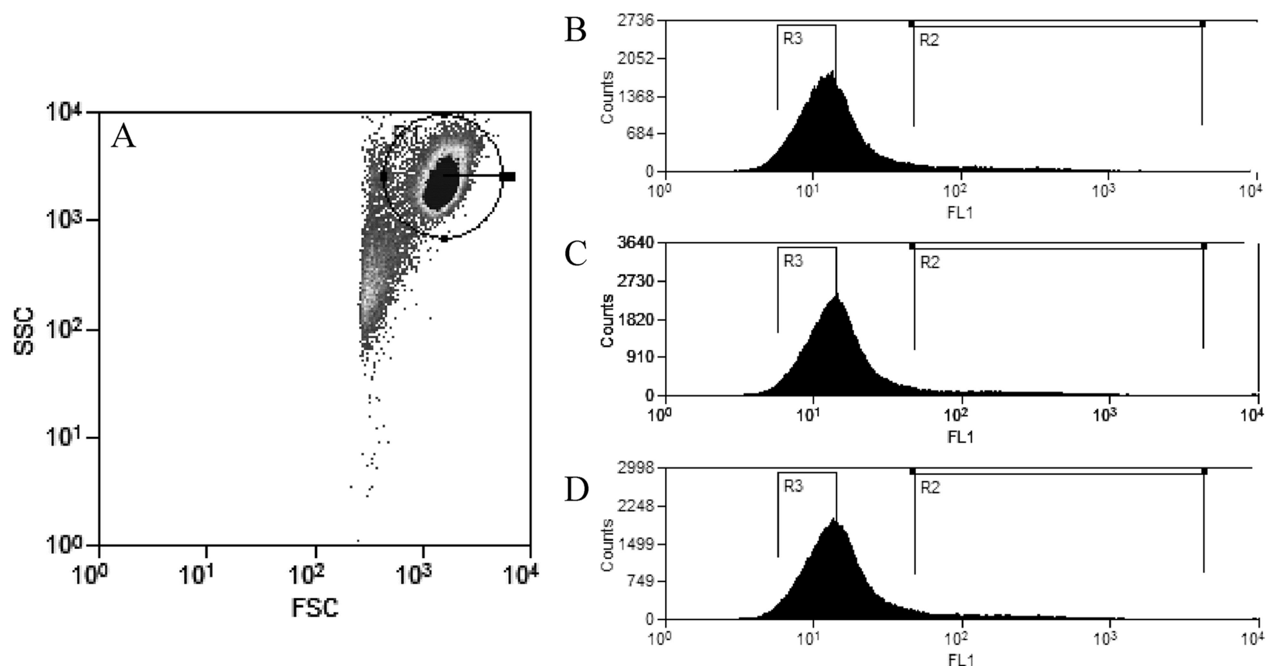

Figure 2. MoFlo data showing parameters for separation of CFDA-labeled MSCs and unlabeled HDF cells following coculture with cell-cell contact. **(A):** MoFlo dotplot showing the cultured mesenchymal stem cells (MSCs) and human dermal fibroblast (HDFs) after 7 days in culture. **(B–D):** Histograms showing the cultured MSCs and HDFs after 7 days in culture. R1 gate was placed on the FSC versus SSC dotplot to eliminate cellular debris from the histograms. Histograms show regions of labeled (R2) MSCs and unlabeled nucleus pulposus (NP) cells (R3) that were sorted. Histogram **(B)** is 75:25% MSC/NP, **(C)** is 50:50% MSC/NP, and **(D)** is 25:75% MSC/NP. Abbreviations: FSC, forward scatter; R, region; SSC, side scatter.

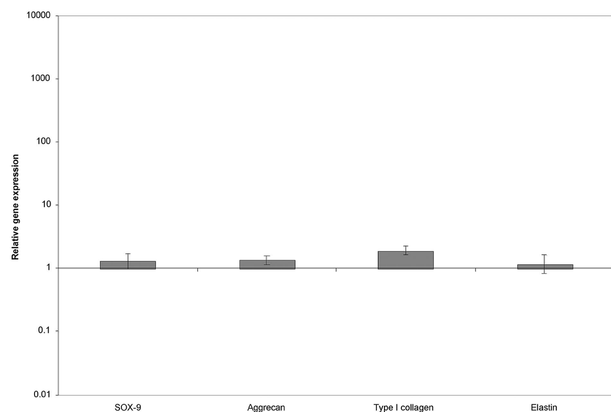

Figure 3. Relative expression of *SOX-9*, aggrecan, type I collagen, and elastin mRNA by 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFDA)-labeled and -unlabeled mesenchymal stem cell (MSCs) after 7 days in culture. Gene expression in CFDA-labeled cells normalized to average of housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*] and *18S*) and unlabeled cells. *, statistical significance ($p < .05$) from control cells.

Gene Expression after NP and MSC Coculture with Contact

Cells cultured with contact were separated by high-speed cell sorting. Where cells were cultured with contact, increases in a number of matrix marker genes were seen. After 7 days in coculture with contact *SOX-9*, mRNA was significantly ($p <$ .05) increased in MSCs in all three cell ratios (Fig. 4A), with the largest increase being seen at a ratio of 75:25% NP/MSC (700-fold over controls compared with 63-fold and 51-fold in 50:50% NP/MSC and 25:75% NP/MSC, respectively). Although *SOX-9* mRNA levels in NP cells were raised significantly, these increases were below 10-fold over controls.

Type II collagen expression after 7 days in coculture with contact (Fig. 4B) was increased significantly in MSCs at all ratios, with the largest increase of almost 6000-fold again being seen at a ratio of 75:25% NP/MSC. A significant increase was seen in type II collagen expression in NP cells at the same ratio; however, there was a small but not significant increase in expression in the 50:50% sample and a significant decrease in the 25:75% NP/MSC sample. Although after 7 days MSCs at all cell ratios with contact showed significant increases in expression of type I collagen, the increases were relatively low, being below a 10-fold increase in every case (Fig. 4C).

Type VI collagen showed statistically significant increases in expression in MSCs at all ratios, with the largest increase being seen with the most NP cells present (75:25% NP/MSC) (Fig. 4D). These increases were small, with the largest increase being only fivefold over controls. NP cells showed significant increases at 75 and 50% NP ratios, although again these were very small increases (both 1.6-fold).

Examination of the changes in expression of the proteoglycans aggrecan and versican showed very different results. Although MSCs showed large and significant increases in aggrecan expression (Fig. 4E), there was no significant change in versican mRNA expression (Fig. 4F). Aggrecan expression in MSCs showed the largest increase in samples with the highest number of NP cells (5876-fold) and decreased as NP cell number decreased (485-fold at 50% and 11-fold at 25%). NP cells, however, showed small but significant increases in aggrecan at 75 and 50% NP, but a decrease at 25% NP, although again there was no significant change in expression of versican.

Gene Expression after HDF and MSC Coculture with Contact

HDFs and MSCs were cocultured with contact for 7 days, and changes in expression of a number of NP and HDF marker genes were measured by real-time PCR. At a ratio of 75:25% HDF/MSC, both cell types showed small (2.8-fold in HDF and 2.9-fold in MSCs) but significant increases in *SOX-9* (Fig. 5A), whereas there was also a significant increase in *SOX-9* expression (6.3-fold) in MSCs at a ratio of 75:25% NP/MSC. Type II collagen could not be reproducibly detected in any of the samples (data not shown), and although detectable, aggrecan did not show any significant changes (Fig. 5B). Similarly, there was very little change in expression of type I collagen, with only MSCs at a ratio of 75% showing a small but significant decrease (Fig. 5C). The largest changes were seen in the expression of elastin, with both HDFs and MSCs showing significant increases in expression after 7 days in coculture (Fig. 5D).

DISCUSSION

Tissue engineering or cell-based therapies for repair of the degenerate IVD require a large number of cells (e.g., 25×10^6 cells/ml [25]) with an NP-like phenotype that can be implanted into the NP of the diseased disc to produce a new matrix. As the IVD contains relatively few cells (4000 cells per mm^3 in the normal adult NP [7]) and since these cells cannot be removed without damaging the resident disc tissue, the main problem for these strategies is the generation of a usable cell population. Studies have shown that multipotent, bone marrow-derived MSCs from both humans and rats can differentiate into cells with an NP-like phenotype [16, 19] and that these cells may be useful for generating a large number of NP-like cells that can be used in therapeutic treatments of IVD degeneration. Although autologous MSCs would be the ideal choice, recent studies have shown that MSCs lack HLA class II receptors [26] and that human recipients receiving MSCs from sibling donors do not exhibit an immunogenic response [27], suggesting that allogeneic MSCs could be used.

Although a number of different methodologies can be employed to induce and maintain a differentiated phenotype in MSCs, these usually involve addition of growth factors or culture in a three-dimensional environment that makes subsequent usage difficult [16, 19]. The current study aimed to elucidate the effects that coculture had on the differentiation state of both human NP cells and MSCs and whether this gene expression was modulated by culture with or without cell-cell contact or the alteration of cell ratio.

Our findings suggest that rather than inducing matrix production by NP cells, as suggested by Yamamoto and co-workers [20], the NP cells induce differentiation and matrix component gene expression by MSCs. We were able to study this by using a novel fluorescent cell-labeling coculture method, combined with high-speed cell sorting to isolate both NP cells and MSCs after coculture with cell-cell contact.

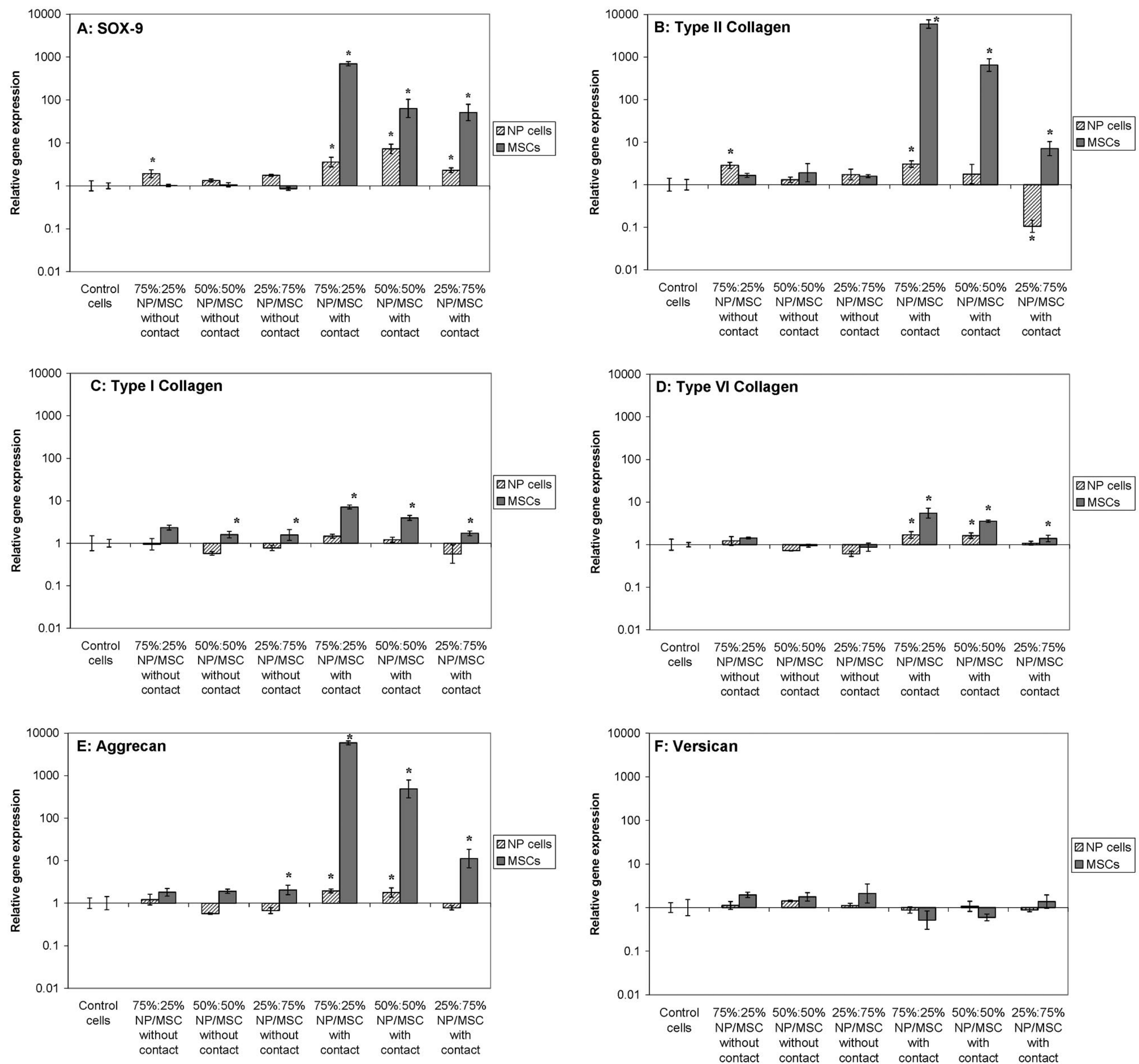

Figure 4. Relative gene expression in NP cells and MSCs after coculture with or without contact for 7 days. Expression normalized to average of housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*] and *18S*) and control cells cultured alone (level expressed as 1). **(A):** *SOX-9* mRNA expression. **(B):** Type II collagen. **(C):** Type I collagen. **(D):** Type VI collagen. **(E):** Aggrecan. **(F):** Versican. *, statistical significance ($p < .05$) from control cells. Abbreviations: MSC, mesenchymal stem cell; NP, nucleus pulposus.

The Effect of Cell-Cell Contact

Yamamoto et al. [20] and others have suggested that coculture with cell-cell contact induces growth factor expression, in particular transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), that is not seen in coculture without contact, and Thompson et al. [28] showed that these growth factors are responsible for matrix production by NP cells. Our work using coculture with and without contact suggests that cell-cell contact itself is required for MSC differentiation and matrix production by NP cells and MSCs, since coculture without contact did not show any significant changes in matrix gene expression by either NP cells or MSCs. This differs from other studies using different cell types cocultured with MSCs where no cell-cell contact has been shown to have an effect, although not to the same extent as when cells were cultured with cell-cell contact [29]. Our results may therefore be due to the specific cell type (i.e., the NP cell) used for coculture.

Cells cultured with cell-cell contact showed large increases in gene expression of *SOX-9* and a number of matrix molecules, in particular aggrecan and type II collagen, indicating that direct cell-cell contact is essential for MSC differentiation to NP cells. The changes in cell proliferation and gene expression profiles of cells cocultured with cell-cell contact demonstrated in both this study and that of Yamamoto et al. [20] could also be due to expression of a number of cell adhesion molecules or changes to

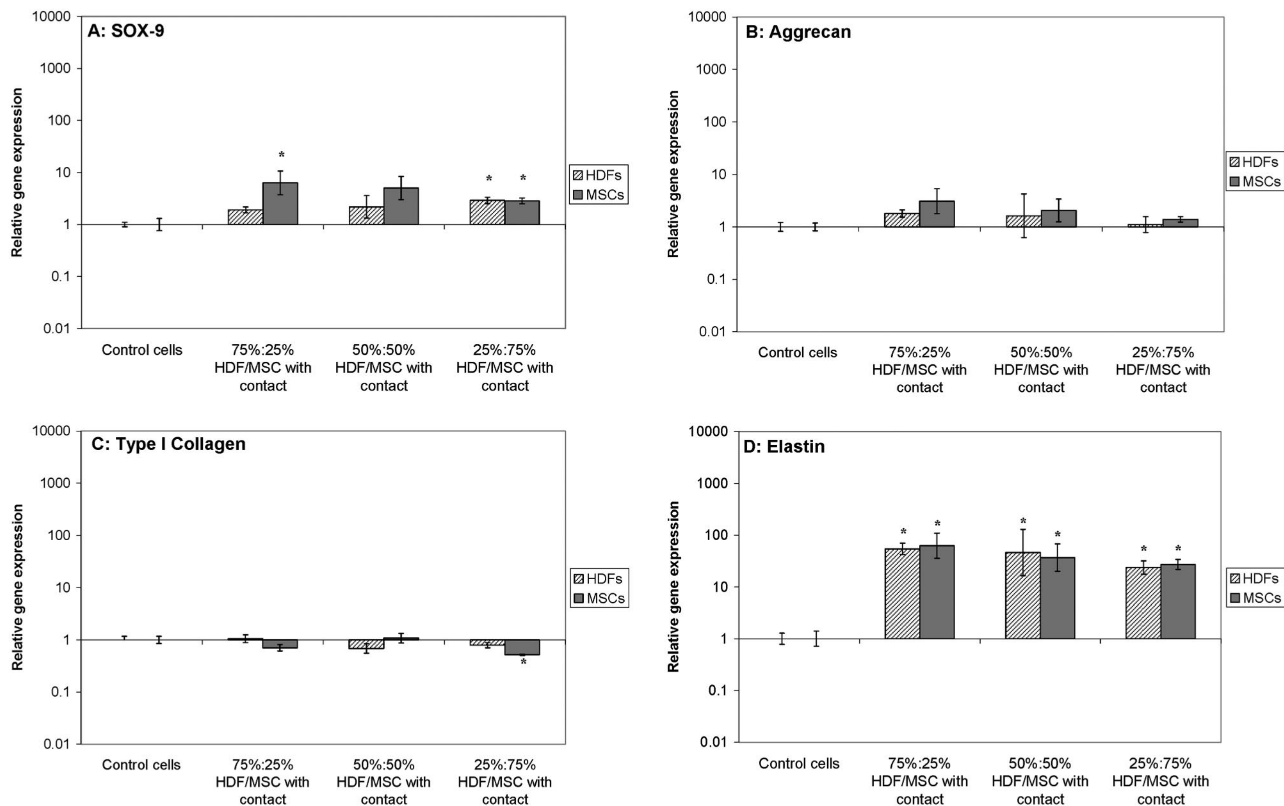

Figure 5. Relative gene expression in HDFs and MSCs after coculture with or without contact for 7 days. Expression normalized to average of housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*] and *18S*) and control cells cultured alone (level expressed as 1). **(A):** *SOX-9* mRNA expression. **(B):** Aggrecan. **(C):** Type I collagen. **(D):** Elastin. ∗, statistical significance ($p < .05$) from control cells. Abbreviations: HDF, human dermal fibroblast; MSC, mesenchymal stem cell.

cell signaling pathways, and further studies are needed to clarify this.

Our results have shown that the differentiation pathway of MSCs is dependant on the cell type with which they are cocultured. In our study, control experiments, in which MSCs were cultured with HDFs with cell-cell contact, showed increases in elastin that were not seen after MSC coculture with NP cells (data not shown). This is important because elastin is a key component of dermal tissue and has been shown to be expressed by cultured dermal fibroblast [30]. There were small, yet significant increases in *SOX-9* mRNA expression, which could possibly be due to release of factors by the HDFs that affect *SOX-9* expression, such as members of the fibroblast growth factor (FGF) family [31]. FGFs have been shown to stimulate *SOX-9* expression in both chondrocytes and MSCs [32], and this could account for the increased expression of *SOX-9* during MSC coculture with HDFs. There was, however, no increase in either type II collagen or aggrecan, which showed the largest increases in MSCs after coculture with NP cells, suggesting that this increase in *SOX-9* mRNA expression is not causing differentiation of either the MSCs or HDFs to chondrocyte-like cells.

These findings are consistent with those of Ball et al. [33], who demonstrated that coculture of MSCs and vascular endothelial cells with cell-cell contact produced a different effect on MSC differentiation than did coculture of MSCs with HDFs. It is therefore clear that the cells with which MSCs are cocultured determine their fate and that coculture of MSCs with NP cells with cell-cell contact produces a population of differentiated NP-like cells from the stem cells.

Gene Expression and the Effect of Cell Ratio

Most previous coculture studies have used only a 50:50% cell ratio, and although these studies have shown that coculture affects cell phenotype, no studies have investigated whether this ratio is the optimum for directing differentiation. It is for this reason that we investigated a range of cell ratios. Importantly, we have shown that in most cases a ratio of 75:25% NP cells/MSCs was the optimal ratio for stimulating MSC differentiation, possibly due to the increase in cellular signals received by the MSCs from the greater NP cell population. This was evidenced by the relative increases in *SOX-9*, type II collagen, and aggrecan gene expression after 7 days, particularly at a ratio of 75:25% NP cells/MSCs. Whereas type I and VI collagen showed only minor, yet still significant, changes in expression, results suggest that type I collagen was constitutively expressed at high levels by both NP cells and MSCs in monolayer culture. Type VI collagen has been shown to account for as much as 5% of the IVD tissue dry weight (compared with only approximately 1% of the dry weight of articular cartilage [34]). Type VI collagen is involved in proteoglycan complex interactions, which combined with its abundance in the NP of the disc (approximately 20% of total collagen content [34]) makes it a potentially important collagen in the proteoglycan-rich NP. Our studies have shown that coculture leads to signif-

icant increases in expression by both NP cells and MSCs, which should be a key factor in production of a integrated tissue-engineered matrix.

Preliminary real-time PCR data using primers specific for the type IX collagen isoforms (data not shown) showed low levels of expression of the short-form of type IX collagen in NP cells rather than the long-form, although this expression was unchanged by coculture. MSCs did not show detectable levels of either form in control samples or samples cocultured without contact. However, after coculture with NP cells with cell-cell contact, MSCs did show detectable levels of the short-form, but not the long-form. As NP cells have previously been shown to express only the short-form of type IX collagen, whereas articular chondrocytes express the long-form [35], this molecule may be a usable marker of an NP-specific phenotype, rather than a chondrocytic phenotype.

One of the largest and potentially most important changes was the increase in expression of aggrecan. A recent study comparing proteoglycan and collagen ratios in NP tissue and cartilage showed a 27:1 ratio of proteoglycan/collagen in the NP, but only a 2:1 ratio in cartilage, demonstrating the importance of proteoglycans, in particular aggrecan, in the structure and function of the IVD [36]. Therefore the increase in aggrecan, particularly in MSCs after 7 days in coculture with cell-cell contact with NP cells, suggests that these cells have differentiated to cells with an NP-like phenotype and could potentially be used in therapeutic strategies.

The Application of CFDA Labeling and High-Speed Cell Sorting

MSCs were labeled with CFDA to allow separation of MSCs and NP cells after coculture with direct cell-cell contact. This inert, non-fluorescent molecule is cell-permeable and once within a cell is cleaved by intracellular esterases to a cell-permanent green fluorescent dye that binds covalently to intracellular proteins. This dye is shared evenly between daughter cells and has been used extensively to track cell division in a number of cell types, including NP cells and stem cells, and has been shown not to affect cell phenotype, function, or proliferation rate [37–41]. Results comparing CFDA-labeled and -unlabeled MSCs showed no significant change in gene expression after culture for 7 days, suggesting that CFDA is a safe and reliable molecule for labeling cells.

High-speed cell sorting allows each population of cocultured cells to be examined individually. The MoFlo high-speed cell sorter has the theoretical ability to identify, classify, and sort individual cells at rate of up to 100,000 cells per second at above 95% purity, allowing exact subpopulations to be identified and separated [42, 43]. This means that CFDA-labeled MSCs can be separated from unlabeled NP cells with high purity and specificity and that these cells can be used for further culture or application in tissue engineering therapies to treat IVD degeneration.

Conclusion

We have shown that coculture of NP cells and MSCs causes MSC differentiation to an NP-like phenotype only if cells have cell-cell contact and that the optimum cell ratio for differentiation is 75:25% NP/MSC. We have also shown that this differentiation can be achieved within 7 days, and the unique cell labeling method we have developed also allows subpopulations of cells to be purified and used in subsequent applications. Furthermore, the effects of NP cells on MSCs suggest that if partially or completely differentiated MSCs were implanted into the disc, the surrounding cells and matrix molecules would prompt retention of phenotype and production of an NP-like matrix by the implanted cells that could restore disc height function, and future in vivo studies could elucidate this.

Although only one cell population has been used, our results suggest that this novel methodology has major implications for the future of cell-based, tissue engineering strategies for treatment of the degenerate IVD, because it allows rapid production of NP-like cells from MSCs without the dangers of removing IVD tissue.

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Disclosures

The authors indicate no potential conflicts of interest.

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