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Impact of Direct Cell Co-Cultures on Human Adipose-Derived Stromal Cells and Nucleus Pulposus Cells

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ABSTRACT: Biologic and cellular treatment strategies aiming for curing intervertebral disc degeneration (IDD) have been proposed recently. Given the convenient availability and expansion potential, adipose-derived stromal cells (ADSCs) might be an ideal cell candidate. However, the interaction between ADSCs and nucleus pulposus (NP) cells still remains ambiguous, especially in direct co-cultures of the two types of cells. Nevertheless, NP markers in ADSCs after co-cultures were unidentified. Here, we addressed the interaction of human ADSCs and NP cells in a direct co-culture system for the first time. As a result, ADSCs could differentiate to the NP cell phenotype with a significant up-regulated expression of multiple genes and proteins in extracellular matrix (ECM) (*SOX9*, *COL2A1*, *ACAN*, and *COL6A2*), relative NP markers (*FOXF1*, *PAX1*, *CA12*, and *HBB*) and pertinent growth factors (*CDMP-1*, *TGF-β1*, *IGF-1*, and *CTGF*). Moreover, the gene expression of *COL2A1*, *ACAN*, and *COL6A2* of degenerate NP cells was also up-regulated. Collectively, these results suggest that direct co-cultures of ADSCs and NP cells may exert a reciprocal impact, that is, both stimulating ADSCs differentiation to the NP cell phenotype and inducing NP cells to regain functional phenotype. Accordingly, ADSCs might be a potential candidate in the development of cellular treatment strategies for IDD. © 2013 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 31:1804–1813, 2013

Keywords: direct co-culture; intervertebral disc degeneration; adipose-derived stem cells; nucleus pulposus

Intervertebral disc degeneration (IDD) is tightly linked with low back pain, which results in worldwide socioeconomic implications, wage losses, and consequently morbidity.[1] Current treatment options including conservative and surgical therapies aim for relieving symptoms rather than modifying the underlying pathological processes of IDD. Therefore, biologic and cellular treatment strategies aiming for curing disc degeneration have been attracting more and more attention of investigators these years.

Macroscopically, normal intervertebral disc consists of three sub-parts, the central nucleus pulposus (NP), the outer anulus fibrosus (AF) and the cartilage endplates connecting adjacent vertebras. In normal human disc, the central NP is made of abundant extracellular matrix (ECM) interspersed by a small number of NP cells that make up about 1% of the total volume.[2] As the disc degenerates, proteoglycan together with water content in NP decreases. NP becomes less gelatinous and more fibrous with the formation of cracks and fissures in AF, which eventually leads to IDD. The etiology of IDD is ascribed to numerous aspects, amongst which cell death is thought to play a crucial role.[3] NP cells are the vital machinery for the synthesis of functional ECM, the production of cytokines and the maintenance of relevant enzymes' activities. Cytology analysis of the disc has identified a variety of cellular alterations and provides gross information in molecular level. However, the therapeutic modalities are still in its infancy in terms of NP cell function restoration or translation.

Recently, the application of stem cells has shown a revolutionary opportunity upon cellar treatment strategies for IDD with the development of tissue engineering. Adult bone marrow-derived stromal cells (BMSCs) have been noted as a viable option for cell-based therapies for IDD.[4] Importantly, with the convenient availability and abundance in nature, adipose-derived stromal cells (ADSCs) gain intensive attention as a cell source in organ repairs.[5] As for disc regeneration, previous studies indicate that growth factors, specific culture conditions and soluble factors released by NP cells can guide ADSCs to differentiate into NP cell phenotype. Tapp et al.[6] noted that ADSCs could produce a type of proteoglycan and collagen I rich ECM following treatment with TGF-β in 3D cultures. Using transwell co-cultures, Lu et al.[7] found ADSCs could differentiate towards the NP cell-like phenotype in vitro. Furthermore, in their study of ADSCs implantation in a rabbit model, Chun et al.[8] found the injection of ADSCs into injured lumbar discs is an effective treatment modality for IDD by promoting cartilage regeneration. In addition, Minogue et al.[9] noted several novel NP markers

Abbreviations: ADSCs, adipose-derived stromal cells; AF, anulus fibrosus; BMSCs, bone marrow-derived stromal cells; CFDA, caboxyfluorescein diactetate; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IDD, intervertebral disc degeneration; MRI, magnetic resonance imaging; NP, nucleus pulposus; PBS, phosphate buffered saline; PE, phycoerythrin; TBST, tris-buffered saline and tween-20

Zhen Sun, Zhi-Heng Liu, Xu-Hong Zhao and Lu Sun contributed equally to this work.

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versus AF cells, and detected high level of gene expression of these markers in adult stem cells following cultured in type I collagen gels. However, the interaction networks between ADSCs and NP cells still remain unclear, especially with regard to direct co-cultures of the two kinds of cells. Nevertheless, the specific expression of NP cell markers on ADSCs after co-cultures was also unknown. So far, there have been no studies addressing the direct co-cultures of ADSCs and NP cells. Furthermore, as NP cells share a common phenotype with chondrocytes, little is known in the specific markers expression in ADSCs following co-cultures with NP cells. Accordingly, the objective of this study was to address the alterations of ADSCs and NP cells in a direct co-culture system. Moreover, specific NP markers in ADSCs were also studied following direct co-cultures.

MATERIALS AND METHODS

The Institutional Ethics Review Board of *Xijing Hospital* approved the study (No. 20111103-7). Each patient and volunteer signed the written informed consents. The same written informed consents were obtained from their relatives as for the cadaveric donors.

Human NP Cell Isolation and Cultures

The degeneration degree of the intervertebral discs was classified according to Pfirrmann's grading system. Grade IV discs from anterior interbody fusion are included in the degenerate group, and discs obtained from cadaveric donors are included in the normal group. All specimens were from the lumbar spine (L3/4–L5/S1). As for the normal group, grade I discs were obtained from cadavers. Magnetic resonance imaging (MRI) data in the records of the cadavers were collected. Both group samples were obtained within 2 h after surgery or autopsy. NP tissues were identified and separated using a stereotaxic microscope. Pfirrman grade V degenerated disc was excluded as dissection of NP from AF in this grade is difficult. The specimens were digested for 40 min in 0.2% pronase (Gibco-BRL, Carlsbad, CA), then washed with Hank balance salt solution and incubated in 0.25% type II collagenase (Gibco-BRL) at 37°C under gentle agitation. After 4 h, remaining tissue debris was removed through a 45-μm pore-size nylon mesh. Cells seeded in culture flasks cultured with DMEM/F12-based culture medium (containing 10% FBS, 1% P/S) in 5% CO_2 and 20% oxygen incubator at 37°C.

ADSCs Isolation and Verification

Human ADSCs were obtained from lipoaspirated fat tissue of the volunteers. After washed with phosphate buffered saline (PBS), fat samples were minced well in a sterile petridish and digested in 1 mg/ml collagenase type II (Sigma, St. Louis, MO) at 37°C under gentle agitation. Cells were passed through a sterile nylon mesh filter (70 μm pore size) (Falcon, Franklin Lakes, NJ) to remove undigested tissue. After centrifugation at 200*g* for 8 min, cells were harvested. The pellet was then resuspended and filtered through a 40 μm cell strainer to remove remaining tissue debris. After that, cells were counted and plated in plastic culture flasks (BD Biosciences, San Jose, CA). For verification of the cultured ADSCs, flow cytometry analysis was performed to identify positive and negative ADSCs markers. The cultured ADSCs were washed in PBS twice and incubated in blocking buffer for 30 min at 4°C. After being washed with PBS, the cells were incubated for 30 min at 4°C in dark environment with the fluorescein isothiocyanate (FITC)-conjugated antibodies or the phycoerythrin (PE)-conjugated antibodies: CD90/FITC, CD9/FITC, CD31/FITC, CD34/FITC, CD271/FITC, c-kit/FITC, VEGF/FITC, MAP-2/FITC, CD29/PE, CD45/PE, KDR/PE (BD Biosciences, Gibbstown, NJ). One percent of paraformaldehyde was used to fix the cells after staining. Isotype-identical antibodies (IgG) were used as controls. The samples were then analyzed by flow cytometry. It was noted that cell viability of each group was greater than 95.0%. Assessment of each sample was performed in three times.

Direct Co-Cultures of ADSCs and NP Cells and Subsequent Cell Sorting

Before direct co-cultures, cell-labeling of fluorescent was performed with ADSCs incubating in appropriate essential medium with a concentration of 10 μM 5, 6 caboxyfluorescein diactetate, succinimidyl ester (CFDA SE, Sigma) at 37°C in a dark environment for 30 min. Then excess media was added to halt the labeling reaction and cells were washed twice with PBS.

The direct co-culture system was established between ADSCs and NP cells in monolayer at a 50:50 ratio (6,000 cells/cm^2) in six-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ) in high-glucose (4,500 g/L) Dulbecco's modified Eagle's medium (DMEM-HG). The same ADSCs samples were cultured with both normal and degenerate NP cells, respectively. The control groups included sole CFDA-labeled ADSCs, sole normal and degenerate NP cells in the same density. Primarily cultured ADSCs and NP cells (passage 1) were used in this study. Demographic data were shown in Table 1. After 7 days, cells of co-culture groups were separated and harvested with flow sorter and analyzed along with the control groups. Briefly, following trypsinized and washed in PBS cells were filtered through a 40 μm filter to remove cell debris. Mixed cells were separate and analyze by flow cytometric after direct co-culture. Gates were set to exclude all dead cells, cell debris and cell clumps. Within the gated cell population, cells fluorescing at 518 nm were identified as CFDA-labeled ADSCs and nonfluorescent cells as unlabeled NP cells. During cell sorting, dead cells, cell debris and cell clumps were excluded in Region P1 (Fig. 2A). CFDA-positive cells (ADSCs) and CFDA-negative cells (NP cells) were chosen by different gates in fluorescence versus side scatter dot plot. Region P2 stand for CFDA-negative cells (NP cells) while the P2 were CFDA-positive cells (ADSCs) (Fig. 2B). The control groups were sorted by similar gating (Fig. 2C and D). Samples of both cell populations were reanalyzed by flow cytometry to check cell purity. This procedure was to verify whether there were contaminated CFDA-positive cells (ADSCs) in the CFDA-negative cell (NP cells) population and vice versa. As a result, each group of cell population had a purity of more than 98%.

Quantitative Real-Time PCR (qRT-PCR) Analysis of ADSCs and NP Cells

Total RNA in cells was isolated using TRIzol® Reagent (Ambion, Carlsbad, CA) according to the manufacturer's protocol. Reverse transcription to cDNA was performed using a High-Capacity cDNA Archive Kit (ABI, Foster City, CA). Predesigned primers were designed using OligoPerfect Designer Software (Invitrogen, Carlsbad, CA) and purchased from Sangon (Shanghai, China). RNA concentrations were

Table 1. Summary of Demographic Data Included in This Study

Patients No.	Age	Gender	Level	Degree[a]
Volunteers				
1	34	M		
2	38	M		
3	44	F		
4	47	F		
Degenerate group				
5	52	M	L45	IV
6	45	F	L45	IV
7	61	F	L45	IV
8	53	M	L45	IV
Normal group				
9	55	F	L45	I
10	43	M	L45	I
11	43	F	L45	I
12	47	M	L45	I

[a]Pfirrmann's grading system.

measured using a NanoDrop instrument (NanoDrop, Wilmington, DE). The levels of mRNA were normalized to GAPDH mRNA controls. All RT reactions, including GAPDH controls, were run in triplicate in a GeneAmp PCR 9700 Thermocycler (ABI). qRT-PCRs were performed on a StepOne Plus device (Applied Biosystems) with SYBR Premix Ex Taq kit (TaKaRa, Otsu, Shiga, Japan). The relative amounts of mRNA were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. The primers used in the study were shown in Table 2.

Western Blotting

Separated ADSCs following co-cultured with degenerate NP cells were prepared in MOPS buffer on ice for 30 min. Debris of the cells was removed by centrifugation for 20 min at 4°C. The protein concentration was measured by the BCA assay (Sigma). Following electrophoresized in 10% Bis-Tris gel, equal amounts of 15 μg proteins were transfered to PVDF membrane (0.45 mm). Then samples were blocked overnight with 3% skim milk TBST. Subsequently, the membranes were incubated for 12 h at 4°C with following primary antibodies: mouse monoclonal anti-SOX9 antibody (Abcam, Cambridge, MA); mouse monoclonal anti-collagen II antibody (Abcam); mouse monoclonal anti-aggrecan antibody(Abcam); mouse monoclonal anti-collagen VI antibody(Abcam) and

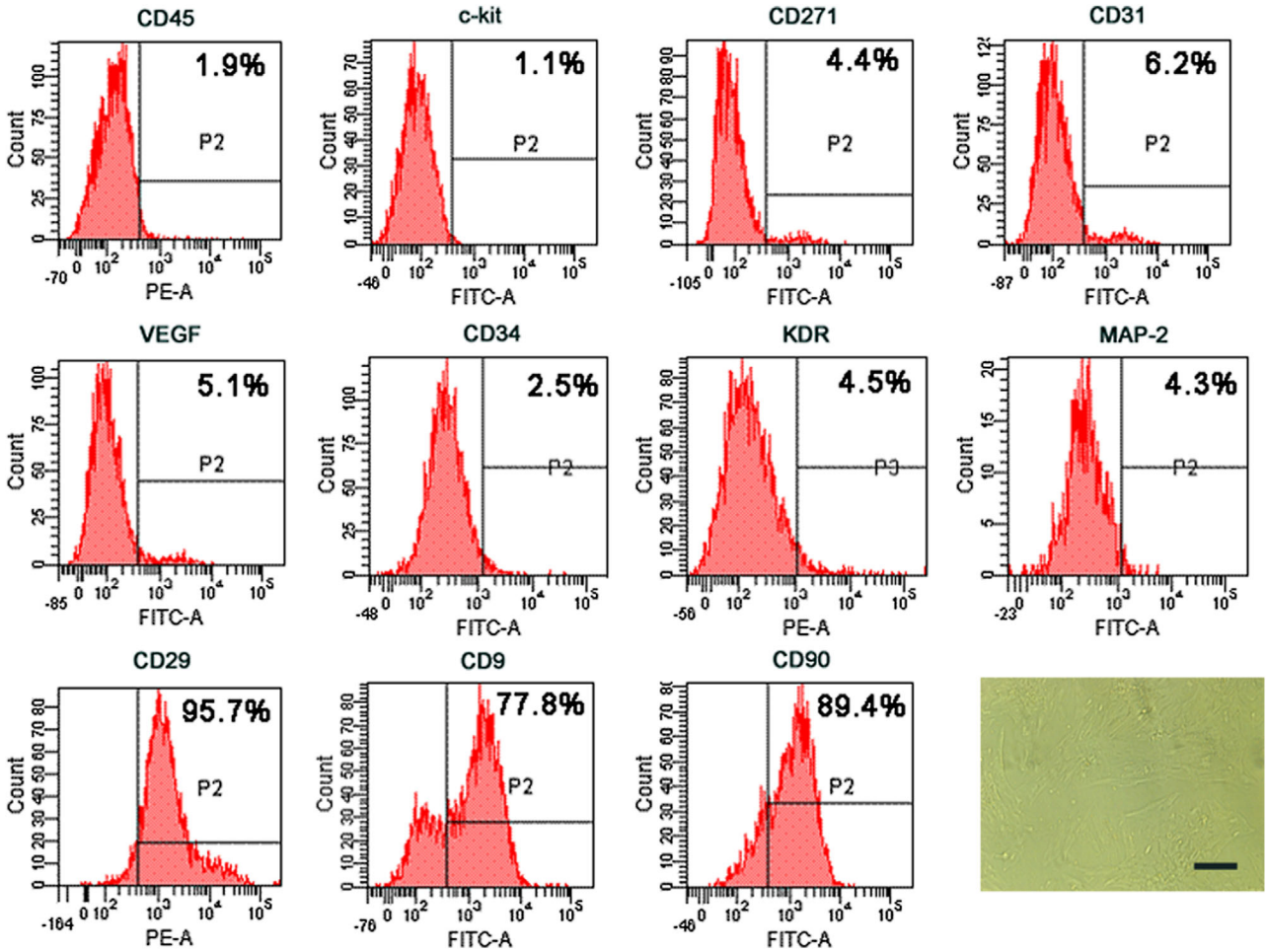

Figure 1. Flow cytometry (FCM) analysis of human adipose-derived stromal cells (ADSCs). FCM results of ADSCs on day 10 after cell cultures. It is notable that CD29+, CD9+, and CD90+ cells were highly expressed in the population of total stem cells. Spindle-shaped morphological feature of the ADSCs were shown in the right lower corner. Scale bar = 50 μm. The percentage shown represents the mean values for three experiments.

mouse monoclonal antibody specific to β-actin (Sigma) as control. Antibody labeling was identified using anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, Boston, MA), Membranes were treated with ECL Plus according to the manufacturer's instructions (Amersham Pharmacia Biotech, Umea, Sweden). Images were analyzed by densitometry from Scion Image. The relative amount of immunoreactive protein in each sample was quantitated by densitometry (AMBIS Radioanalytic and Visual Imaging System, Ambis, San Diego, CA).

Statistical Analysis

Statistical significance was determined using the Student's *t* test. The SPSS statistical package (SPSS, Chicago, IL) for statistical analysis was used. A p value <0.05 was considered significant.

RESULTS

Isolated ADSCs exhibited high level staining of positive ADSCs markers, including CD90, CD9, and CD29. The expression of CD90, CD29 was approximately 90% of the total cell population, and CD9 displayed nearly 80%. In contrast, there was only a small proportion with negative ADSCs markers (Fig. 1).

Direct Co-Cultures and Cell Sorting

CFDA has been shown not to affect cell function, phenotype, or proliferation rate including NP cells and stem cells.[10] Native morphological appearance of cells was maintained and no changes were observed in cell shape microscopically after 7 days co-cultures of ADSCs and NP cells. Each group of cell population had a purity of more than 98%. Cell sorting data were shown (Fig. 2).

Table 2. Human Oligonucleotide Primers Used for Real-Time Quantitative Polymerase Chain Reaction Analysis

Gene Symbol	Forward Primer	Reverse Primer
SOX9	GACTTCCG CGACGTGGAC	GTTGGGCGGCA GGTACTG
COL2A1	GGCAATA GCAGGTTCAC GTACA	CGATA ACAGTCTTGCC CCACTT
ACAN	TCGAGGACAG CGAGGCC	TCGAGGGTG-TAGCGTGTAGAGA
COL6A2	GACGCTGTTCTC CGACCT	GGTCTGGGCACA CGATCT
CDMP-1	CTGTGCGAGTTC CCATTGC	TGGAGTTCA TCAGGGTCTGGAT
TGF-β1	CGCGCATCCT AGACCCTTT	CTGTGGCAGGTC GGAGAGA
IGF-1	AGCCTGTCCACCCT TGAGAA	CCCTGGAGCC AC AGAGC AT
CTGF	CCCTGCATCTTCGG TGGTA	GGCACGTGCACTG GTACTTG
GAPDH	GCACCGTCAAGGC TGAGAAC	GGATCTCGCTCCT GGAAGATG

Analysis of Gene Expression in ADSCs After Direct Co-Cultures With NP Cells

The expression of the mRNAs of classic markers, including *SOX9*, *COL2A1*, *ACAN* and *COL6A2* of ADSCs significantly increased following direct co-cultures with either degenerative or normal NP cells. No significant difference was observed between normal and degenerate NP cells in inducing ADSCs differentiate into NP phenotype (Fig. 3). In addition, as novel NP markers, *FOXF1*, *PAX1*, *CA12*, and *HBB* in ADSCs significantly increased in mRNA expression following direct co-cultures with degenerate NP cells. There was also significant increase in ADSCs of *FOXF1*, *PAX1*, *CA12*, and *HBB* gene expression after co-cultures with normal NP cells (Fig. 4).

Meanwhile, the gene expression of growth factors, including *CDMP-1*, *TGF-β1*, *IGF-1* and *CTGF*, in ADSCs increased significantly after direct co-cultures with normal NP cells. The gene expression of *CDMP-1*, *IGF-1*, and *CTGF* increased significantly after co-cultures with degenerate NP cells. No statistical difference was observed between ADSCs co-cultured with degenerate or normal NP cells (Fig. 5). To ensure that CFDA labeling had no effect on ADSCs phenotype, ADSCs labeled with CFDA were studied after 7 days culture and showed no significant change (Fig. 6).

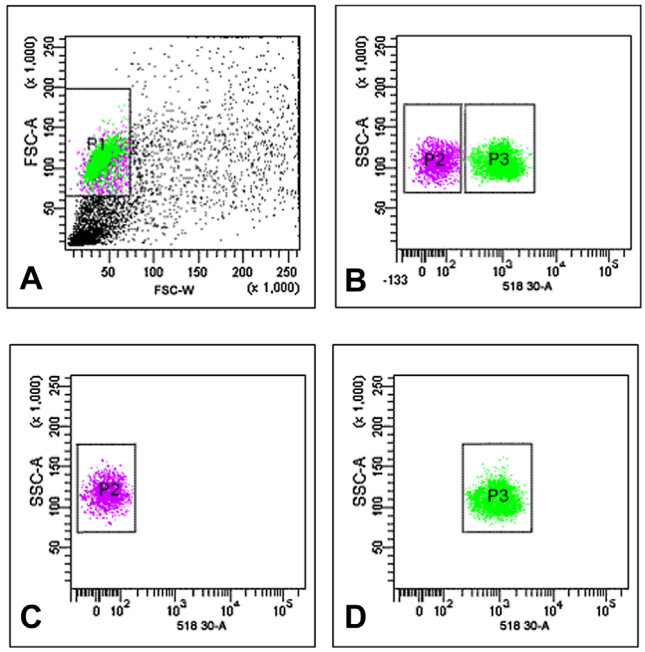

Figure 2. Representative cell sorting data for the separation of carboxyfluorescein diacetate (CFDA)-labeled adipose-derived stromal cells (ADSCs) and unlabeled nucleus pulposus cells (NP cells) following direct co-cultures. (A) 2D dotplot for co-cultured cells. Gate P1 was placed around single live cells. (B) 2D dotplot showed CFDA-labeled ADSCs in gate P3. Unlabeled NP cells were in gate P2. (C) NP cells without co-cultures were sorted in gate P2 as controls. (D) CFDA-labeled ADSCs without co-cultures were sorted in gate P3 as controls.

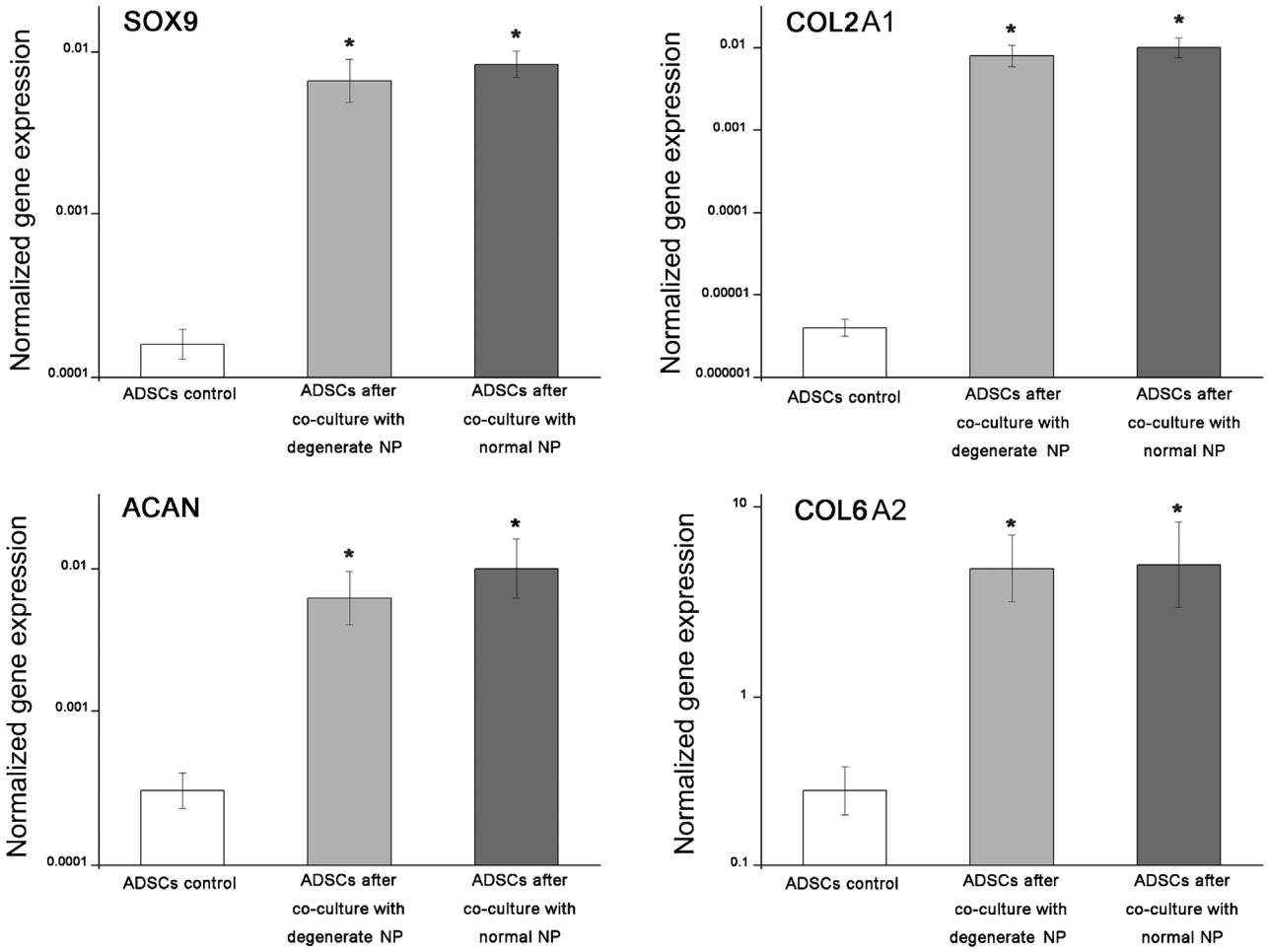

Figure 3. Relative ECM gene expression in adipose-derived stromal cells (ADSCs) after 7 days of direct co-cultures with nucleus pulposus cells (NP cells). Data were averaged from four different individual samples and performed in triplicate. Error bars represent SEM. $^{*}p < 0.05$.

Analysis of Gene Expression in Degenerate NP Cells After Direct Co-Cultures With ADSCs

Following direct co-cultures with ADSCs, degenerate NP cells showed an increase in ECM expression, including *CLO2A1*, *ACAN*, and *COL6A2* in gene expression, which indicated with a trophic effect of ADSCs on degenerate NP cells (Fig. 7).

Western Blotting Results

ADSCs significantly increased the expression of *SOX9*, *COL2*, *ACAN*, and *COL6* after direct co-cultured with degenerate NP cells. Quantitative examination revealed that the average protein/β-actin ratios in ADSCs after co-culture were 192.3%, 137.8%, 146.1%, and 132.8% of the controls ($p < 0.05$). These results were almost consistent with the findings of qRT-PCR analysis (Fig. 8).

DISCUSSION

Accumulating evidence has shown that stem cells have the potential for biologic cell-based treatment strategies for IDD.[11,12] Due to the altered phenotype and viability, as well as the difficulty in collection, NP cells might not be an ideal candidate for the biologic treatment for IDD. At this point, stem cells have been the mainstay for the biologic therapies candidates. Whereas BMSCs have been a viable option to increase the number of NP-like cells capable of producing functional and appropriate ECM, ADSCs are receiving more and more attention. Compared with BMSCs, ADSCs are more extremely abundant, easer to obtain with minimal invasiveness, and can be cultured to reach satisfactory densities for autologous transplantation without ethical issues. Meanwhile, studies have shown indirect co-cultures of ADSCs and NP cells could guide ADSCs to differentiate into NP phenotype. In contrast, co-cultures of BMSCs and NP cells might not have such significant changes, which indicate that ADSCs are more capable of differentiating than BMSCs. In addition, Lin et al.[13] found ADSCs possess a higher proliferation potential and greater ECM expression underwent differentiation compared with BMSCs. These distinct advantages make ADSCs especially promising for clinical application in the treatment for IDD.

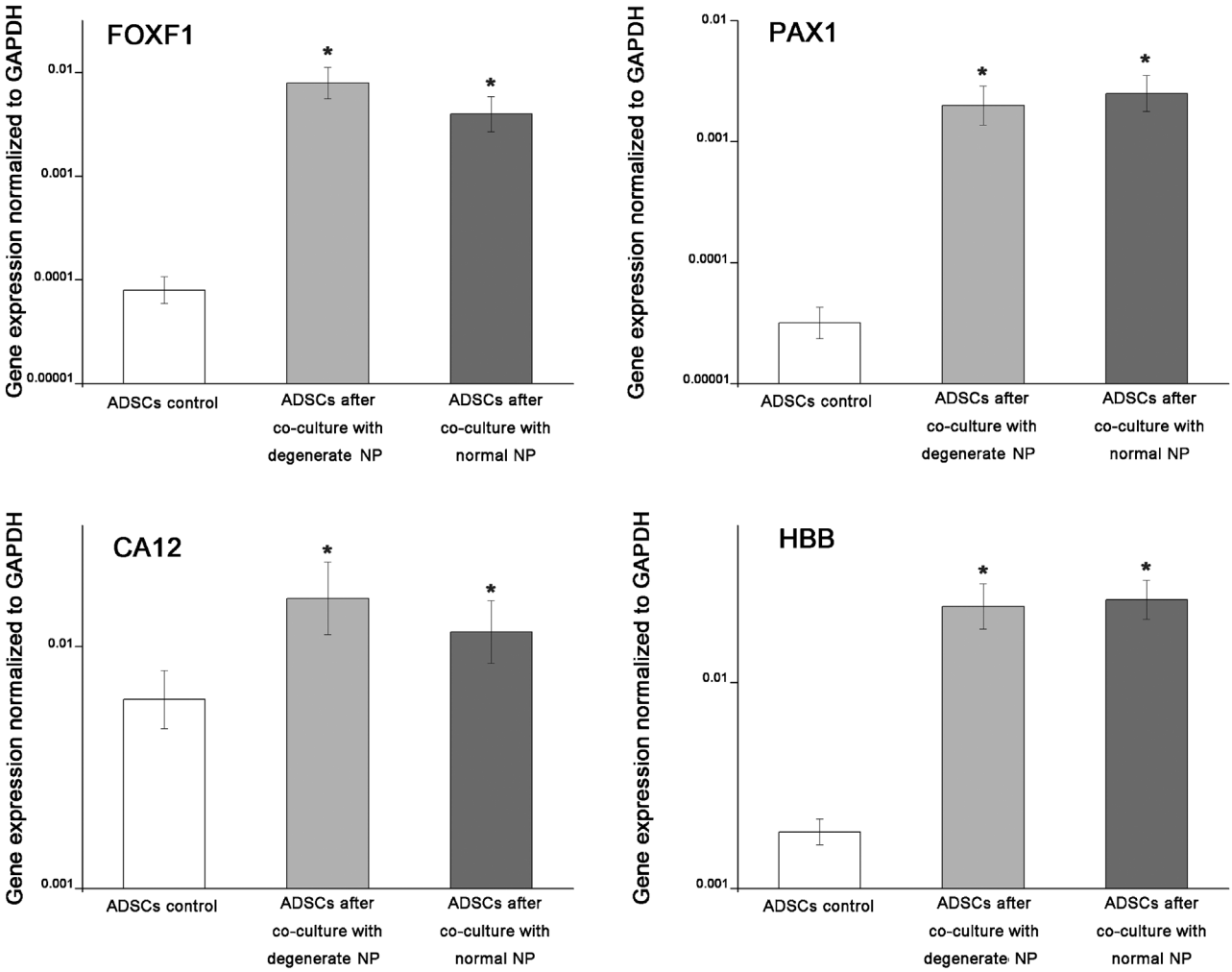

Figure 4. Relative gene expression of NP markers in adipose-derived stromal cells (ADSCs) after 7 days of direct co-culture with nucleus pulposus cells (NP cells). Data were averaged from four different individual samples and performed in triplicate. Error bars represent SEM. $^{*}p < 0.05$.

Until now, several lines of evidence have demonstrated that ADSCs could differentiate into NP cell phenotype under the stimulation of growth factors, specific culture conditions and soluble factors released by NP cells.[14,15] ADSCs contribute to chondrogenesis in co-cultures with human articular chondrocytes.[16] However, there is a paucity of information in the literature pertaining to the reciprocal effects of NP cells and ADSCs especially with regard to direct co-cultures. Considering these, we proposed that direct co-cultures of ADSCs and NP cells might guide ADSCs to differentiate NP cells phenotype and regain the function of degenerate NP cells.

To our knowledge, the study is the first addressing the interaction in a direct co-culture system between human ADSCs and NP cells in vitro. Here, we found that ADSCs can be guided to differentiate into NP-like cells after direct co-cultures with normal and degenerate NP cells in vitro. Moreover, a reciprocal impact of ADSCs on degenerate NP cells was found. Besides, some NP markers were also shown an increased expression in ADSCs after co-cultures.

To classify cellular differentiation of ADSCs following direct co-cultures, we examined the expression of *SOX9*, *COL2A1*, *ACAN*, and *COL6A2* as classic NP cell markers that are closely associated with functional ECM.[17] *SOX9* is one of the most commonly used markers in the studies of stem cell differentiation to chondrocyte-like cells, especially NP cells. Yang et al.[18] found that *SOX9* could facilitate differentiation of ADSCs into a chondrocyte-like phenotype in vitro. Type II collagen is highly expressed in NP and the loss of its expression is a classic pathological hallmark of IDD. Moreover, type VI collagen and aggrecan express abundantly in NP but in small quantities in ADSCs. As for clinical cases, it might be more practical of degenerate NP cells' function to induce ADSCs differentiate into NP cell phenotype. We extend our focus to the protein levels of *SOX9*, *COL2*, *ACAN*, and *COL6* in ADSCs co-cultured with degenerate NP cells

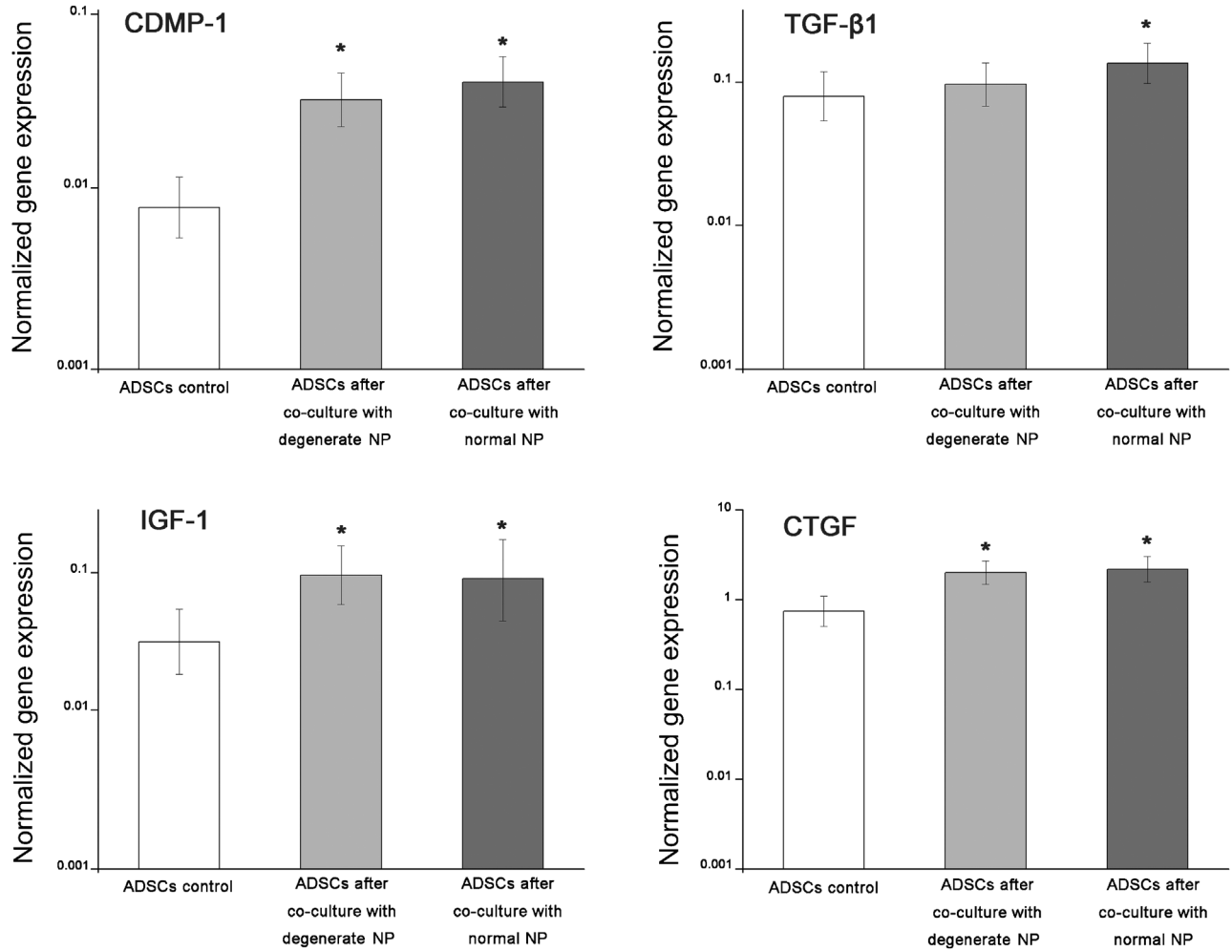

Figure 5. Relative gene expression of growth factors in adipose-derived stromal cells (ADSCs) after 7 days of direct co-culture with nucleus pulposus cells (NP cells). Data were averaged from four different individual samples and performed in triplicate. Error bars represent SEM. $^{*}p < 0.05$.

and found an increased expression of these ECM functional proteins. These results support the qRT-PCR analysis. Taken together, our findings suggest that ADSCs could be guided to differentiate into NP cell phenotype.

Although the exact phenotypes of human NP cells remain largely unknown, several studies have attempted to detect specific NP cell markers.[9,19,20] In particular, Minogue et al.[9] found *FOXF1*, *PAX1* and *CA12*, *HBB* positively expressed in NP cells versus AF cells by microarray analysis. Importantly, we found that the gene expression of *FOXF1*, *PAX1*, *CA12* and *HBB* as NP cell specific markers increase in ADSCs after direct co-cultures with NP cells. From this point, it is remarkable that ADSCs could be guided to differentiate to NP-like phenotype rather than other types of cells. This directed differentiation implied that these markers could be used to characterize NP-specific differentiation and ADSCs might be a potential option for the treatment strategies for IDD.

The increase in the gene expression of growth factors in ADSCs suggests that ADSCs exert a trophic effect following direct co-cultures with NP cells. Indeed, studies demonstrate that stem cells could secret growth factors and cytokines following induced differentiation.[21,22] Yamamoto et al. found that cell–cell contact between BMSCs and NP cells induced the secretion of growth factors including *TGF-β1*, *IGF-1*, *EGF*, and *PDGF* into the medium.[12] However, there have been no studies addressing the secretion of growth factors by ADSCs. Despite we did not address the secretion of growth factors; we found that NP cells notably improve the mRNA expression of *CDMP-1*, *TGF-β1*, *IGF-1* and *CTGF* in ADSCs following direct co-cultures. Recently, many pieces of evidence have shown the impact of growth factors on stem cells differentiation to a chondrogenic lineage and on the anabolic modulation of NP cells.[23,24] *CDMP-1* and *TGF-β1* could enhance chondrogenesis in adenovirus-transduced BMSCs.[25] Stimulation of disc cells with recombinant CDMP-1 resulted in an increase of proteoglycan and collagen synthesis.[26] In addition, growth factors have a close relationship with functional ECM secretion in NP cells.[27] For degenerate NP

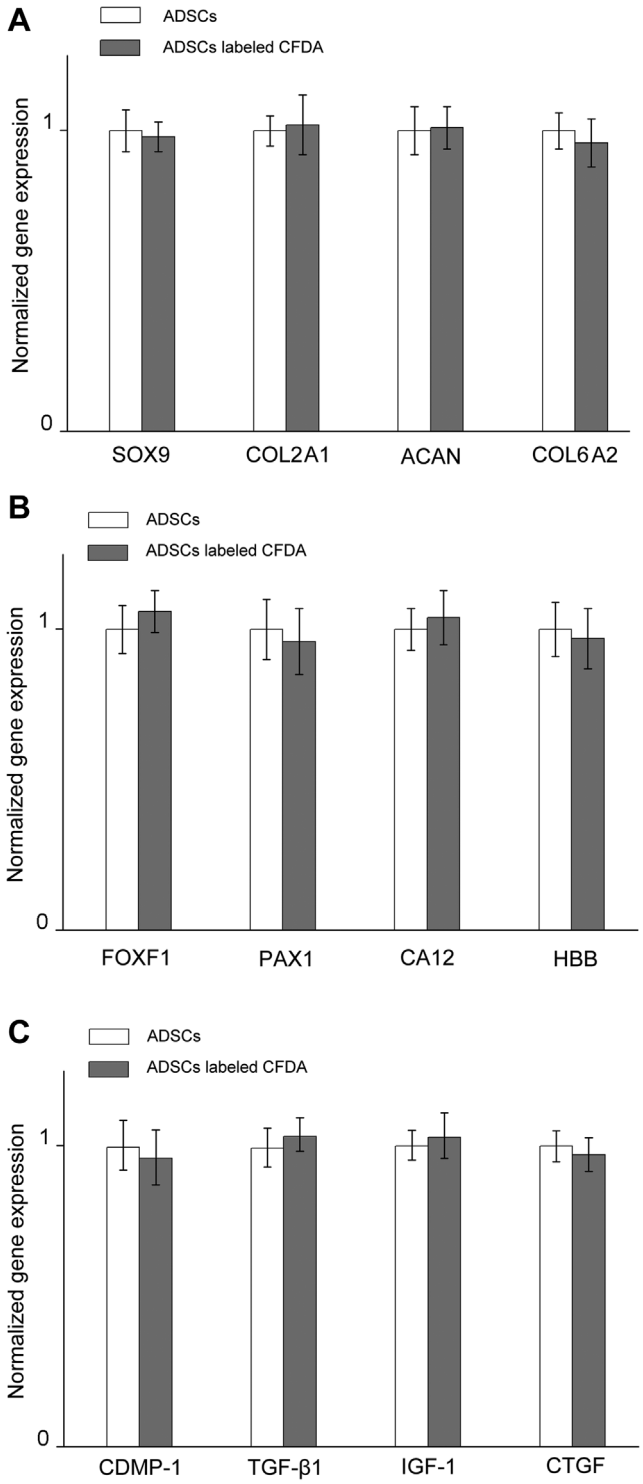

Figure 6. Gene expression of carboxyfluorescein diacetate (CFDA)-labeled adipose-derived stromal cells (ADSCs) were quantified and normalized to ADSCs without CFDA labeling, the value of which was set at 1.0. Data were performed in triplicate. Error bars represent SEM. (A) ECM genes, (B) NP markers, (C) growth factors.

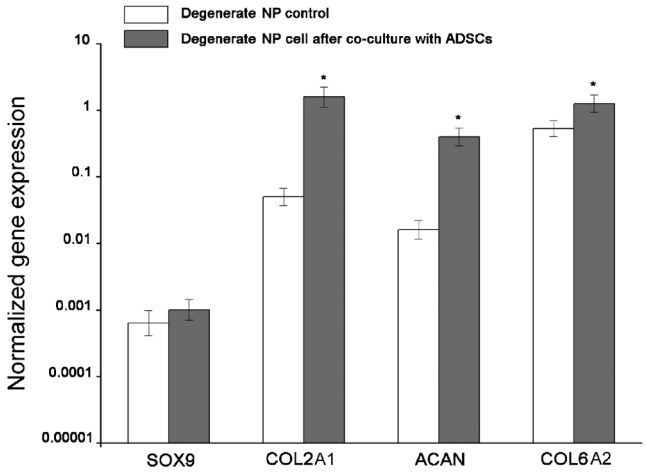

Figure 7. Relative gene expression in degenerate nucleus pulposus cells (NP cells) after 7 days of direct co-cultures with adipose-derived stromal cells (ADSCs). Data were averaged from four different individual samples and performed in triplicate. Error bars represent SEM. $^{*}p < 0.05$.

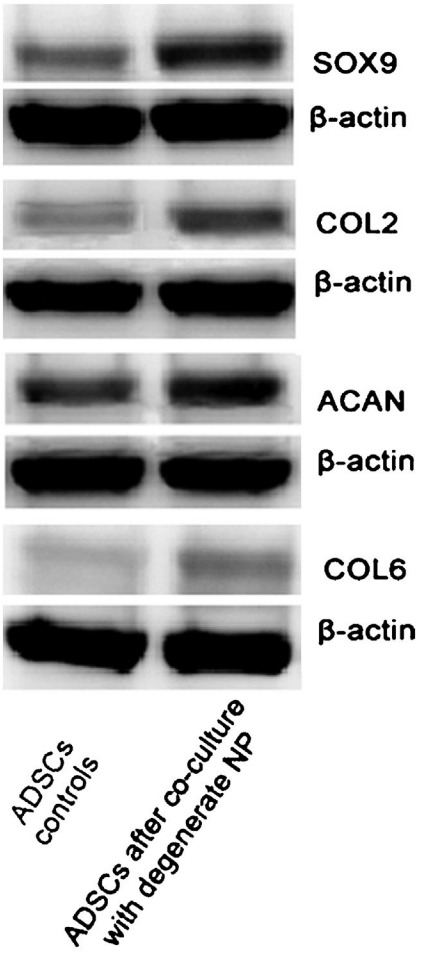

Figure 8. Protein expressions of ECM proteins in adipose-derived stromal cells (ADSCs) following direct co-cultured with degenerate NP cells. Western blot analysis of *SOX9*, *COL2*, *ACAN*, and *COL6* protein levels in ADSCs after direct co-cultured with degenerate NP cells. Shown were representative blots from six independent experiments with similar results. β-Actin served as loading control.

cells after co-cultures, ECM gene expression increases as well. This finding implies that during co-cultures degenerate NP cells might regain normal phenotypes which are capable of maintaining disc function to some degree. In the direct co-culture system, ADSCs may behave in a trophic manner on degenerate NP cells.

There are several limitations to our present study. First, it is known that NP cells lose their phenotype in monolayer culturs. Therefore, we used primarily cultured NP cells (passage 1) to minimize the phenotype alternation. Second, the histology analysis was not examined following co-cultures. This was due to the poor cell status after sorting, which prevented us from obtaining appropriate results. However, western blotting showed an increased expression of ECM in ADSCs following co-cultures. In addition, potential cell materials exchange might influence the cell sorting outcome. In fact, bi-directional exchange of membrane components has been observed in the study of Strassburg et al.,[28] who found a high exchange of membrane-bound substances between BMSCs and NP cells after cultures. In the case of ADSCs, this effect might exist and cause the ADSCs to attain a NP-like phenotype, rather than through intrinsic cell differentiation. However, we detected an up-regulation of NP markers in gene level, which might be less affected by membrane-bound substances exchange. Moreover, as the studied markers are mostly secreted proteins rather than membrane-bounded, membrane exchange might not contribute to their expression. Further studies are needed to classify the molecular mechanisms in the differentiation of ADSCs to NP cells, especially in terms of membrane exchange.

In conclusion, this study provides evidence for the interaction of human ADSCs and NP cells in a direct co-culture system for the first time. Expression of ECM, NP markers and pertinent growth factors increases in ADSCs following co-cultures. Meanwhile, degenerate NP cells could regain toward normal phenotypes co-cultured with ADSCs. Interestingly, it is notable that there is no significant difference between normal and degenerate NP cells in inducing ADSCs differentiate into NP phenotype. Accordingly, ADSCs might be a potential candidate in the development of cellular treatment strategies for IDD.

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