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Human Wharton's Jelly Cells Activate Degenerative Nucleus Pulposus Cells *In Vitro*

Running Title: Cross-talk between Wharton's jelly cells and degenerative nucleus pulposus cells

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Abstract

In order to investigate the interaction between human Wharton's jelly cells (WJCs) and degenerative nucleus pulposus cells (NPCs), human WJCs were cocultured with degenerative NPCs with or without direct cell-cell contact. WJCs were isolated from the human umbilical cord and degenerative NPCs were isolated from the surgically obtained degenerative intervertebral disc tissue. The isolated WJCs positively expressed CD73, CD105, CD90, CD29, CD166 and human leukocyte antigen (HLA)-ABC, but negatively expressed CD34, CD45, and HLA-DR. After coculturing with 3 different WJCs:NPCs ratios for 7 days, the real-time polymerase chain reaction showed that the relative gene expression of NP-marker genes [aggrecan, type II collagen, and SRY-type HMG box-9 (SOX-9)] was significantly upgraded in all coculture groups (all *P* values < 0.05 compared with control groups). Coculture either with or without cell-cell contact significantly activated the expression of NP-maker genes than controls, but coculture with cell-cell contact yielded a higher gene expression than coculture without cell-cell contact. In coculturing with cell-cell contact and WJCs:NPCs of 25:75, the relative gene expression of aggrecan, type II collagen, SOX-9 for WJCs yielded the highest increase by 721-, 1507-, and 1463-folds, respectively (all $P < 0.05$ compared with WJCs control). In contrast, the highest relative gene expression of aggrecan, type II collagen, SOX-9 for NPCs was 112-, 84-, 109-folds, respectively, in coculture with cell-cell contact and in WJCs: NPCs of 75:25 (all $P < 0.05$ compared with NPCs control). In conclusion, the data indicated that coculturing human WJCs with degenerative NPCs induced the NP-like cell differentiation of WJCs and restored the biological status of degenerative NPCs and coculture WJCs and NPCs with direct cell-cell contact yielded more favorable gene expressions.

Keywords: Wharton's Jelly cells, nucleus pulposus cells, intervertebral disc degeneration, coculture

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Introduction

Degenerative disc disease is the natural consequence of intervertebral disc degeneration (IVDD). The low back pain from the degenerative disc disease is the most common cause of the limited activity for people younger than 45 years (1, 2). The biochemical process of IVDD is characterized with the nucleus pulposus fibrosis, the decrease of the type II collagen, and the reduction of the proteoglycan and water content (3-5).

Palliative pain management at the early stage and invasive operation at the late stage are the standard treatment strategies for IVDD (6). However, neither nonoperative nor operative approaches are capable of fully restoring the function of the degenerative intervertebral disc. Also, surgical intervention at the late stage is highly expensive as well as risky of infection and other complications. Thus, preventing the progression of IVDD should be considered at the very early stage. Mesenchymal stem cells (MSCs) possess the self-renewal ability, multilineage differentiation potential and immunomodulatory properties (7) and are recently explored in both basic research (8, 9) and clinical trials (7, 10) to treat IVDD. Vadala *et al.* reported that introducing stem cells to the nucleus pulposus (NP) effectively restored the molecular environment of the matrix, providing a possible option for the early intervention of IVDD (11). Currently, various types of tissue have been proposed as sources of MSCs, among which bone marrow and adipose tissue are most commonly explored in the IVDD-related studies (9, 11-14). However, MSCs derived from either bone marrow or adipose tissue are restricted in practice due to the painful invasive harvesting and the diminishing stemness properties (15-17).

Previous studies showed that MSCs derived from Wharton's jelly of the human umbilical cord (WJCs) hold a significantly greater cell proliferation (15), superior viability rates (14), and a better ability of differentiation than MSCs derived from other sources (17-19). Accordingly, WJCs might avoid the aforementioned disadvantages of MSCs derived from

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bone marrow or adipose tissue and function as a promising alternative for the stem cell augmented early intervention of IVDD (10, 15, 20-23). Our previous study indicated that WJCs, *in vitro* co-cultured with healthy nucleus pulposus cells (NPCs), were able to differentiate towards NP-like cells, as shown by the significantly increased gene expression of aggrecan, type II collagen, SRY-type HMG box-9 (SOX-9) (24). Likewise, WJCs, transplanted into the intervertebral discs of a canine disc degeneration model, efficiently achieved a significantly smaller reduction of the disc height, an improved T2-weighted signal intensity, and a better spinal segmental stability than control groups, indicating the potential of WJCs to restore the function of degenerative IVD (21). Although the in vivo data demonstrated the therapeutic effect of WJCs to treat disc degeneration, however, the *in vitro* evidence, to the best of our knowledge, is still required to explore the mechanism behind.

In the present study, to mimic the implantation of WJCs into the degenerative IVD, we cocultured WJCs with degenerative NPCs with or without direct cell-cell contact. We aimed to investigate the effect of WJCs on the function of degenerative NPCs and also the influence of degenerative NPCs on the differentiation of WJCs towards NP-like cells.

Materials and Methods

Experimental design

Human WJCs were isolated from the fresh human umbilical cords and human degenerative NPCs were separated from the degenerative IVD tissue. The surface makers of the isolated WJCs were analyzed by the flow cytometry and then cells were fluorescently labeled before seeding with degenerative NPCs. With or without a direct cell-cell contact, the WJCs were cocultured with NPCs in 3 cell ratios of 25:75, 50:50, and 75:25 (Table 1). The individually cultured WJCs and NPCs with a total cell number of 6.0×10^4 were used as controls. After culturing for 7 days, real-time polymerase chain reaction (PCR) was performed to evaluate the gene expression in each group. The relative gene

expression in the coculture groups was calculated using the gene expression of the house keeping gene GAPDH as references.

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WJCs isolation and culture

With patient consent and approval by the Institutional Review Board (IRB) ethics committee, one fresh human umbilical cord was obtained and stored in the sterile D-Hanks' balanced salt solution at 4°C before the tissue processing for WJCs. Isolation and characterization of WJCs were performed using the previously standardized protocols (10, 25, 26). Firstly, the umbilical cord was transferred and carefully washed in 4°C phosphate-buffered saline (PBS) to remove the blood clot. Next, the umbilical cord was dissected to isolate the Wharton's jelly and cut into small fragments. The prepared fragments were treated with 0.2 mg/mL type II collagenase solution (Sigma, St Louis, USA) in serum-free medium containing 100 U/mL penicillin, 100mg/mL streptomycin, and 2.5 mg/mL amphotericin B antibiotics for 18 h at 37°C and then treated with 2.5% trypsin (Gibco, New York, USA) for 30 min at 37°C with agitation. Finally, the cells were washed and cultured in Dulbecco's modified Eagle's medium /F12 (DMEM/F12; Hyclone, South Logan, USA) supplemented with 20% fetal bovine serum (FBS) in 5% CO_2 at 37°C. The culture medium was replaced every 2 days. The cells were passaged when the confluence reached 80%-90%.

NPCs isolation and culture

With informed consent and institutional approval, degenerative IVD tissue was obtained during a lumbar fusion procedure of a 42-year-old patient diagnosed with spondylolisthesis at the levels of Lumbar 3 to Lumbar 4 (L3-L4), L4-L5, and L5 to Sacrum 1 (S1) (Fig. 1). The IVD of this patient was identified as the median degeneration and the grade III-IV according to the Pfirrmann grading system (27).

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Tissue processing of IVD and isolation of NPCs were performed as previously described (24, 28). Firstly, the cartilaginous endplate and the annulus fibrosus were carefully removed and the blood was washed away with PBS. The NP tissue were then digested in 0.4 mg/mL type II collagenase (Sigma, St Louis, USA) solution in the serum-free medium containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 2.5 mg/mL amphotericin B antibiotics for 5 h at 37°C. After digestion, the tissue debris was removed from the tissue/cell suspension using a 100 μm cell strainer. The cells were then pelleted by centrifugation at 400 g for 5 min and subsequently resuspended after removal of the supernatant. Next, the cells were cultured to confluence in a 25 cm^2 flask using DMEM/F12 supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2.5 mg/mL amphotericin B at 37°C in a humid atmosphere containing 5% CO_2. The culture medium was changed every 2–3 days.

Flow cytometry analysis of surface markers

The detection of surface markers was performed in the WJCs at passage 3. The cells were treated with the trypsin, suspended at concentration of 10^6/mL, and then washed twice using PBS. WJCs were labeled with antibodies to human leukocyte antigen (HLA)-DR, HLA-ABC, phycoerythrin (PE)-conjugated CD105, CD73, CD90, CD29, CD166, and fluorescein isothiocyanate (FITC)-conjugated CD34, CD45 (all from eBioscience, California, USA) for 30 min at 4°C. After the incubation and labeling, the cells were washed twice with centrifugations in order to remove the unbound antibodies. Afterwards, a flow cytometry analysis was performed using the EPICS XL MCL-4 (Beckman Coulter, California, USA).

Fluorescent labeling of WJCs

WJCs were trypsinized, counted, resuspended at a concentration of 10^6 in DMEM/F12, and washed twice with PBS. Then, 5, 6-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Sigma, St Louis, USA) was added and incubated for 30 min at 37°C. The reaction was stopped by adding FBS to a final concentration of 40% of the volume for 10 min. The cell suspension was then centrifuged at 400 g for 5 min, and the pellet was thereafter resuspended in DMEM/ F12 to a concentration suitable for seeding.

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Coculture of WJCs and NPCs

Both WJCs and NPCs at passage 3 were cultured together with or without direct cell-cell contact with 3 different WJCs:NPCs ratios (25:75, 50:50, and 75:25) (24). For coculture with direct cell-cell contact, CFSE-labeled WJCs and NPCs were mixed and seeded with different WJCs:NPCs ratios. For coculture without direct cell-cell contact, transwell six-well plates with polyethylene terephthalate (PET) track-etched tissue culture inserts (pore size 0.4 μm) were used. Particularly, CFSE-labeled WJCs were seeded on the base of the six-well plates, and NPCs were seeded on to the upper surface of the membrane of the tissue culture insert. In both coculture manners, the cells were maintained for 7 days in DMEM/F12 which supplemented with 10% FBS at 37 °C under a humidified atmosphere with 5% CO_2. The medium was changed every 2 days.

Separation of WJCs and NPCs cocultured with cell-cell contact

The cocultured cells were treated with trypsin, resuspended in DMEM/F12, and pelleted by centrifugation at 400 g for 5 min. The pellet was resuspended in 200 μl DMEM/F12 and passed through a 30μm sterile filter to remove the cell clumps. The samples were then sorted and analyzed using a MoFlo high-speed cell sorter (Dakocytomation, Carpinteria, USA) with an excitation wavelength of 490 nm and an emission wavelength of 530 nm. Generally, the cells fluorescing at 530 nm were selected as WJCs, and non-fluorescent cells were chosen as NP cells. The cells from each region were then resorted into a centrifuge tube containing PBS solution for the succeeding analysis of gene expression.

Real-time PCR analysis of gene expression

Total RNA was extracted from the cocultured cells using the TRIzol reagent (Invitrogen, Carlsbad, USA) and used as a template for the cDNA synthesis (Invitrogen, Carlsbad, USA). After obtaining the cDNA by a reverse transcription, the relative gene expression of aggrecan, type II collagen, SOX-9, type I collagen, type VI collagen, and versican was determined by the real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

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was chosen as the reference housekeeping gene as previously suggested (21, 24, 29, 30). Sequences of primers for human aggrecan, type II collagen, SOX-9, type I collagen, type VI collagen, and versican genes were designed using the Primer Premier 6.0 software (PREMIER Biosoft, California, USA) as previously described (Table 2) (21). The real-time PCR analysis was performed with the Mini OpticonTM Detector System (Bio-Rad, California, USA) and the SYBR Green PCR kit (Takara Bio-technology, Dalian, China). A cycle threshold (Ct) value was obtained for each sample, and triplicate sample values were averaged. The $2^{-\Delta\Delta Ct}$ value was used to calculate the relative expression of each target gene (29, 30).

Statistical analysis

Statistical analysis were calculated with the Student t-test using the SPSS version 24.0 software (SPSS Inc., Chicago, IL, USA), and F-test was used to compare the variances. *P* value < 0.05 was considered to be statistically significant.

Results

Characterization of Wharton's Jelly cells

After isolation from the human umbilical cords, the *in vitro* WJCs displayed a fibroblast-like phenotype (Fig. 2A). Surface markers analysis showed that WJCs at passage 3 were positive for the MSC markers (CD73; CD105; CD90; CD29; CD166) and human leukocyte antigen (HLA)-ABC, but were negative for the hematopoietic stem cell markers (CD34; CD45) and HLA-DR (Fig. 3).

High-speed cell sorting of NPCs and CFSE-labeled WJCs

The MoFlo high-speed cell sorter was used for analysis and sorting of the cells after coculturing with cell-cell contact. Cell analysis was carried out with a gate placed on the forward *versus* 90-degree light scatter dot plot to reject the cell debris (Fig. 4). Cell mixture

of NPCs and CFSE-labeled WJCs were selectively separated with Gate R2 and Gate R3, respectively (Fig. 4B, D, F).

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Gene expression of WJCs after coculture

After coculturing of WJCs and NPCs with or without direct cell-cell contact for 7 days, the evaluation of the NP-related gene expressions detected a significant increase of aggrecan, type II collagen, SOX-9 (all $P < 0.05$ compared with WJCs control) (Fig. 5A, B). Compared with WJCs control, no significant change was found in the expression of type I collagen, type IV collagen, and versican. In coculturing of WJCs and NPCs with direct cell-cell contact, the expression of aggrecan was increased by 1507-, 207-, and 20-folds (all $P < 0.05$ compared with WJCs control) with WJCs:NPCs ratios of 25:75, 50:50, and 75:25, respectively. Similarly, the gene expression of type II collagen was upgraded by 1463-fold ($P < 0.05$) with the WJCs:NPCs ratio of 25:75, and was declined to less than 100 folds with WJCs:NPCs ratios of 50:50 and 75:25. Similarly, the gene expression of SOX-9 reached the highest increment (721-folds, $P < 0.05$ compared with WJCs control) when coculturing WJCs with NPCs in the ratio of 25:75 and with direct cell-cell contact. In coculturing WJCs and NPCs without cell-cell contact, the highest enhancement of gene expression was also seen with the WJCs:NPCs ratio of 25:75, as shown by 30-, 30-, and 33-folds of gene expression of aggrecan, type II collagen, and SOX-9, respectively (all $P < 0.05$ compared with WJCs control).

Gene expression of degenerative NPCs after coculture

The gene expression of the cocultured NPCs displayed a similar pattern as the cocultured WJCs (Fig. 5C, D). The expression of aggrecan, type II collagen and SOX-9 were significantly upgraded in all 3 cell ratios compared with NPCs control, while the difference of gene expression of type I collagen, type VI collagen, and versican did not reach the significance. Coculturing WJCs with NPCs in the ratio of 25:75 with cell-cell contact yielded a significantly upgraded gene expression of aggrecan, type II collagen and SOX-9 and (19-,

20-, and 17-folds, respectively; all $P < 0.05$ compared with NPCs control). Interestingly, the highest increase of expression of these three genes was observed in coculture with WJCs: NPCs of 75:25 as shown by 112-, 84-, 109-folds, respectively (all $P < 0.05$ compared with NPCs control). Similarly, in the coculturing of WJCs and NPCs without cell-cell contact, the highest increases of gene expression of aggrecan, type II collagen, and SOX-9 were observed in WJCs:NPCs of 75:25 with 54-, 60-, and 65-fold, respectively.

Discussion

In this study, with coculturing WJCs and degenerative NPCs with or without direct cell-cell contact, we demonstrated the positive effect of WJCs on degenerative NPCs. The data indicated that human WJCs could be induced to differentiate towards NP-like cells when coculturing with degenerative NPCs. Also, WJCs were able to restore the biological status of degenerative NPCs in the coculture system.

Human umbilical cord is a promising source of MSCs with unique advantages (15, 31, 32). Diverse components of the umbilical cord MSCs could be used to isolate MSCs, including the amnion, the subamnion, the perivascular zone, and the Wharton's jelly (15, 33). Previous studies have proved Wharton's jelly, compared with other compartments of the umbilical cord, to be the best source of MSCs for the clinical application (15). Also, isolation of MSCs from the Wharton's jelly is less time-consuming and labor-intensive than isolation from the subamnion and the perivascular zone and yields more MSCs efficiently with less risk of cross-contamination (34, 35). Besides, WJCs also hold a superior ability of chondrogenic differentiation than other components of the umbilical cord, indicating a possible potential to differentiate towards the chondrocyte-like NPCs (15).

The most important finding is that WJCs cocultured with degenerative NPCs are able to differentiate towards NP-like cells. Our data indicated that degenerative NPCs were induced to express significantly more aggrecan, type II collagen, and SOX-9 when

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coculturing with WJCs with or without cell-cell contact. These data also confirm the findings of a great deal of the previous works in this field (36-40). The possible mechanism of such reversed degeneration is the enhanced production of extracellular matrix by directly adopting and/or maintaining the disc-like phenotypes themselves to ameliorate the effects of disc degeneration (41, 42) or in-directly by yielding cytokines and cellular factors to revert the degenerative NPCs to behave towards healthy NP cells (43).

Previous study found that MSCs could survive in *ex vivo* IVD tissue for 15 days after injection, and to reactivate the resident degenerative NPCs also play an important role in the stem cell therapy (6). With the coculture circumstance, we also demonstrated that the resident degenerative NPCs were reactivated by the cocultured WJCs, as showed by the significantly upgraded expression of aggrecan, type II collagen and SOX-9 in NPCs. Therefore, WJCs, as valuable cell resources of stem cell therapy of IVDD, are not only able to reactivate the degenerative NPCs but also differentiate towards NP-like cells by themselves.

Changes in disc extracellular matrix play important roles to increase the cellular degeneration and alter the function of the degenerative disc (44). In this study, the upregulated gene expression of aggrecan, type II collagen and SOX-9 indicates a significant favorable interplay between the cocultured WJCs and degenerative NPCs on the chondrogenesis of the degenerative disc. NPCs are considered as chondrocyte-like cells as the normal NP tissue and the hyaline cartilage share the similar macromolecules in the extracellular matrix (24). Aggrecan and type II collagen are essential to keep the osmotic pressure and maintain the mechanical function of the nucleus pulposus (3-5, 14). Increase of aggrecan and type II collagen of degenerative NPCs can enhance the effect of the implanted WJCs and facilitate the water absorption of impaired NP tissue to partially regain the biomechanical function. SOX-9 is a crucial regulator of the type II collagen synthesis and chondrogenesis (43, 45), which also plays important roles in the chondrocyte differentiation and maturation of NP cells (46).

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Le Visage C *et al.* cocultured degenerative NPCs with bone marrow derived MSCs and failed to attribute the increased growth factors to either NPCs or MSCs (39). However, in the current study we separated WJCs and NPCs using the high-speed cell sorting and it allowed for an individual evaluation and in-depth analysis of the effect of coculture on each type of cells. The data indicated that coculture of WJCs and NPCs with cell-cell contact yielded the higher gene expressions than coculture without cell contact, however both coculture systems significantly activated the matrix-associated gene expression than controls. Our findings are consistent with previous studies in which coculture without cell-cell contact showed similar but weaker effect as coculture with cell-cell contact (47-49). Moreover, the trophic effects of MSCs were accomplished by secreting growth factors and cytokines into their vicinity (38, 50). In the present study, such paracrine interactions between WJCs and degenerative NPCs were observed and further debilitated by the introduction of a membrane with 0.4 μm pores, possibly implying that implantation of MSCs might be more effective than introduction of growth factors or cytokines only to treat IVDD.

Three coculture ratios of WJCs:NPCs were tested in this study and the gene expression levels varied significantly. In agreement with our previous study, the current study also proved that WJCs:NPCs at 25:75 was optimal for stimulating the differentiation of WJCs towards NP-like cells (24). However, degenerative NPCs showed the lowest gene expression level at the ratio of WJCs:NPCs of 25:75, and exhibited a uptrend when more WJCs were presented. The possible explanation of such data might be the complex inter-cellular metabolic exchange with different cell ratios, as both the gene expression and the succeeding cell fate of the cocultured WJCs and NPCs were meticulously regulated by the local concentration of the metabolites secreted in such coculture systems (51, 52). Therefore, in-depth comparisons between the refined cell proportions are required to ensure the optimum effect of such coculture and further guide the possible clinical application.

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This study holds some limitations. First, the impact of CFSE labeling on WJCs was not tested in the current study, as previous studies showed that CFSE labelling caused no significant influence on the gene expression of WJCs (24, 36). Additionally, only one donor was included in this study to avoid the heterogeneity among different subjects, such as age, gender and degenerative severity. And how these factors affect the cross-talk between WJCs and NPCs would be one direction of our future work. Furthermore, the data were evaluated after coculture for only 7 days, and the results of gene expressions may alter over time. Moreover, the specific stem cells for the IVD treatment need to sustain under the high *in vivo* intra-disc pressure and the chemical microenvironment if injected with certain biomaterials, therefore the sophisticated *in vivo* effects between WJCs and NPCs are still warranted to be explored.

In conclusion, coculturing human WJCs with degenerative NPCs induced the NP-like cell differentiation of WJCs and was able to restore the biological status of degenerative NPCs. Coculture WJCs and NPCs with direct cell-cell contact yielded more favorable gene expressions. These data might reveal the key early effect of WJCs on the degenerative NPCs, possibly providing the prospective evidence for the further investigation of WJCs in the treatment of intervertebral disc degeneration.

Disclosure Statement

There are no competing financial interests.

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TABLE 1. The TREATMENT GROUPS AND THE AMOUNT OF WJCS AND DEGENERATIVE NPCS SEEDED IN THE TWO COCULTURE SYSTEM

	WJCs	Degenerative NPCs
Control groups		
WJC control	6.0×10^4	
Degenerative NPCs control		6.0×10^4
Cell-cell contact groups		
WJCs:NPCs 25:75	1.5×10^4	4.5×10^4
WJCs:NPCs 50:50	3.0×10^4	3.0×10^4
WJCs:NPCs 75:25	4.5×10^4	1.5×10^4
No cell-cell contact groups		
WJCs:NPCs 25:75	2.0×10^4	6.0×10^4
WJCs:NPCs 50:50	6.0×10^4	6.0×10^4
WJCs:NPCs 75:25	6.0×10^4	2.0×10^4

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TABLE 2. SEQUENCES OF PRIMERS USED IN THE GENE ANALYSIS

Gene	Primer Sequence (5'→3')
GAPDH	F: GAAGGTCGGAGTCAACGG
	R: GGA AGA TGG TGA TGG GAT T
Aggrecan	F: GCAGGACCAGACTGTCAGATAC
	R: TCCAGGCGTGTGATGAAGAAC
Type II collagen	F: GGCAATAGCAGGTTCACGTACA
	R: CGATAACAGTCTTGCCCCACTT
SOX-9	F: GACTTCCGCGACGTGGAC
	R: CAGTACCTGCCGCCCAAC
Type I collagen	F: CAGCCGCTTCACCTACAGC
	R: TTTTGTATTCAATCACTGTCTTGCC
Type VI collagen	F: GACGCTGTTCTCCGACCT
	R: GGTCTGGGCACACGATCT
Versican	F: TGGAATGATGTTCCCTGCAA
	R: AAGGTCTTGGCATTTTCTACAACAG

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Figure legends

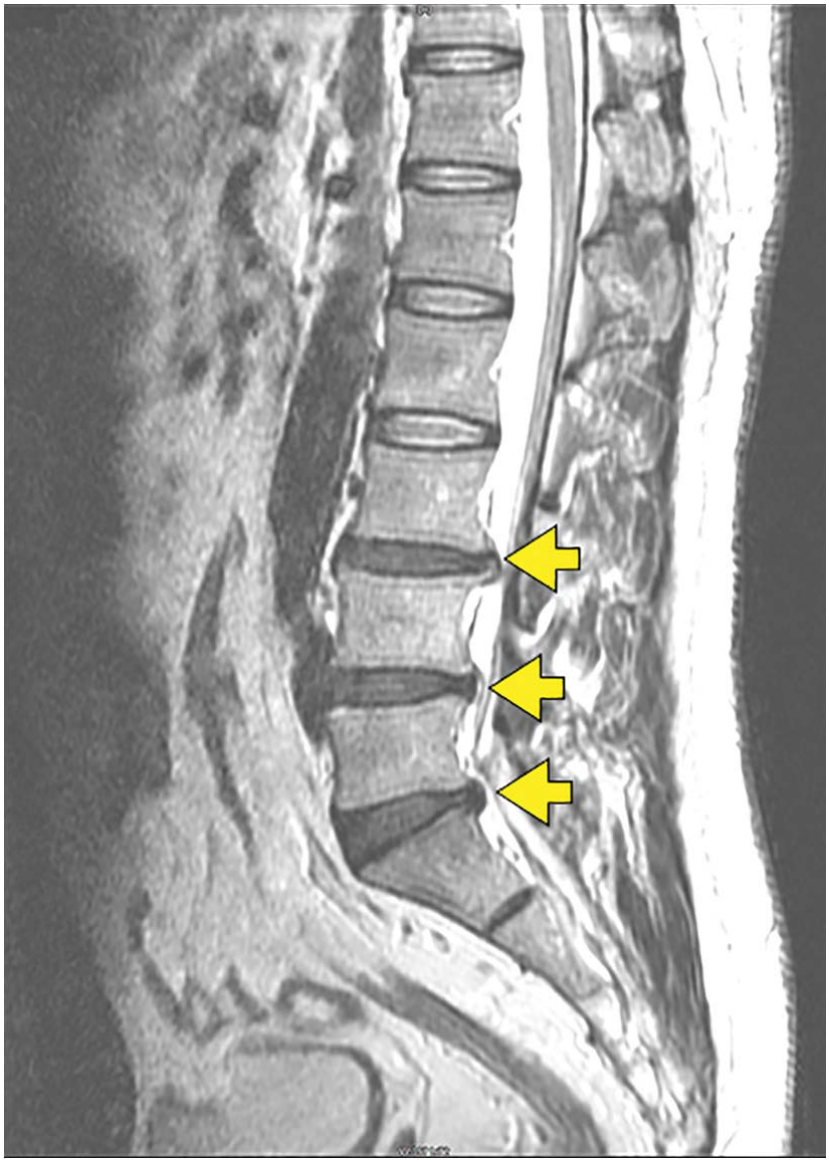

FIG. 1 T2-weighted MRI image of a 42-year-old male patient with lumbar spondylosis. The degeneration (Pfirrmann grade IV) occurred at the levels of L3-4, L4-5, and L5-S1. Of note, the degenerated disc tissue is extruded posteriorly to compress the spinal cord (yellow arrows).

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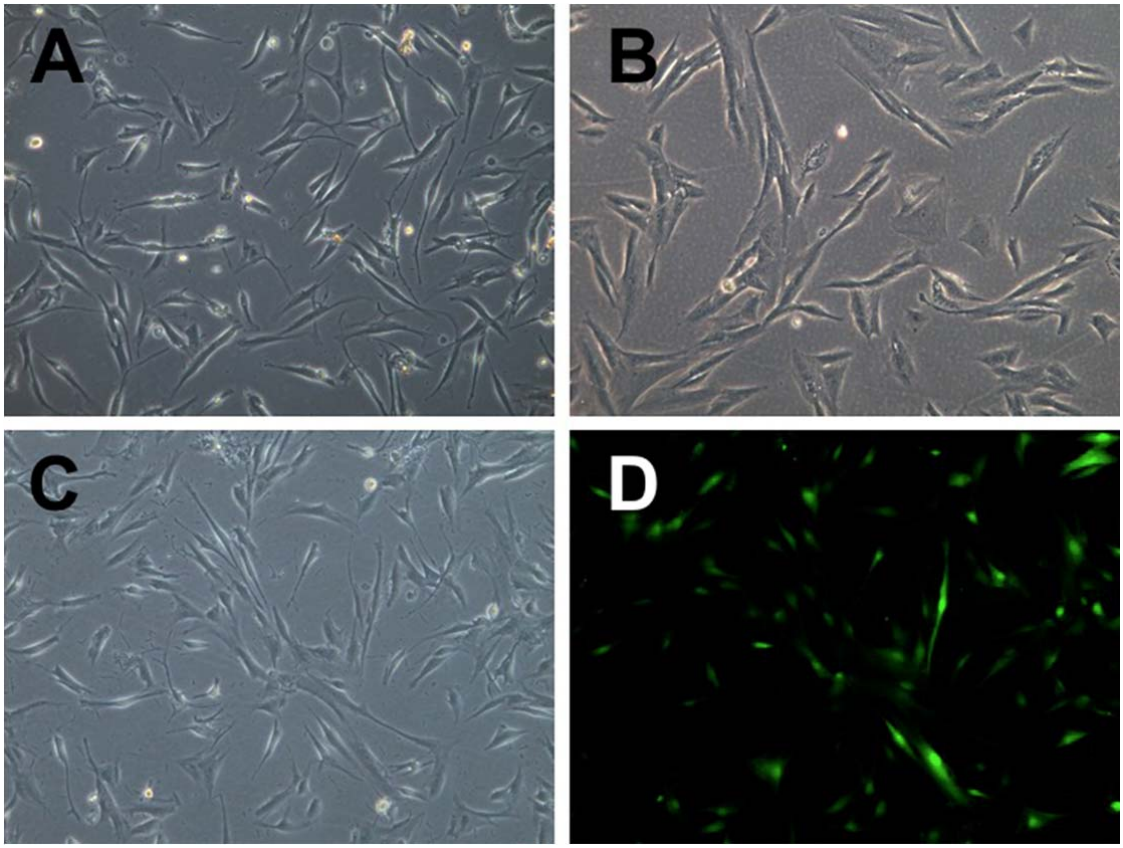

FIG. 2 Morphological observation (×100) of WJCs and degenerative NPCs. (**A**) Degenerative NPCs at 7 days in culture. (**B**) WJCs at 3 days in culture attached to the flask and displayed the fibroblast-like morphological characteristics, which was similar to mesenchymal stem cells derived from bone marrow or adipose tissue. (**C**) The mixed degenerative NPCs and WJCs at 7 days in coculture could not be distinguished from each other under the bright-field microscope. (**D**) Fluorescence microscopic image of (**C**) indicated an evenly distribution of WJCs labeled by CFSE with the green fluorescence.

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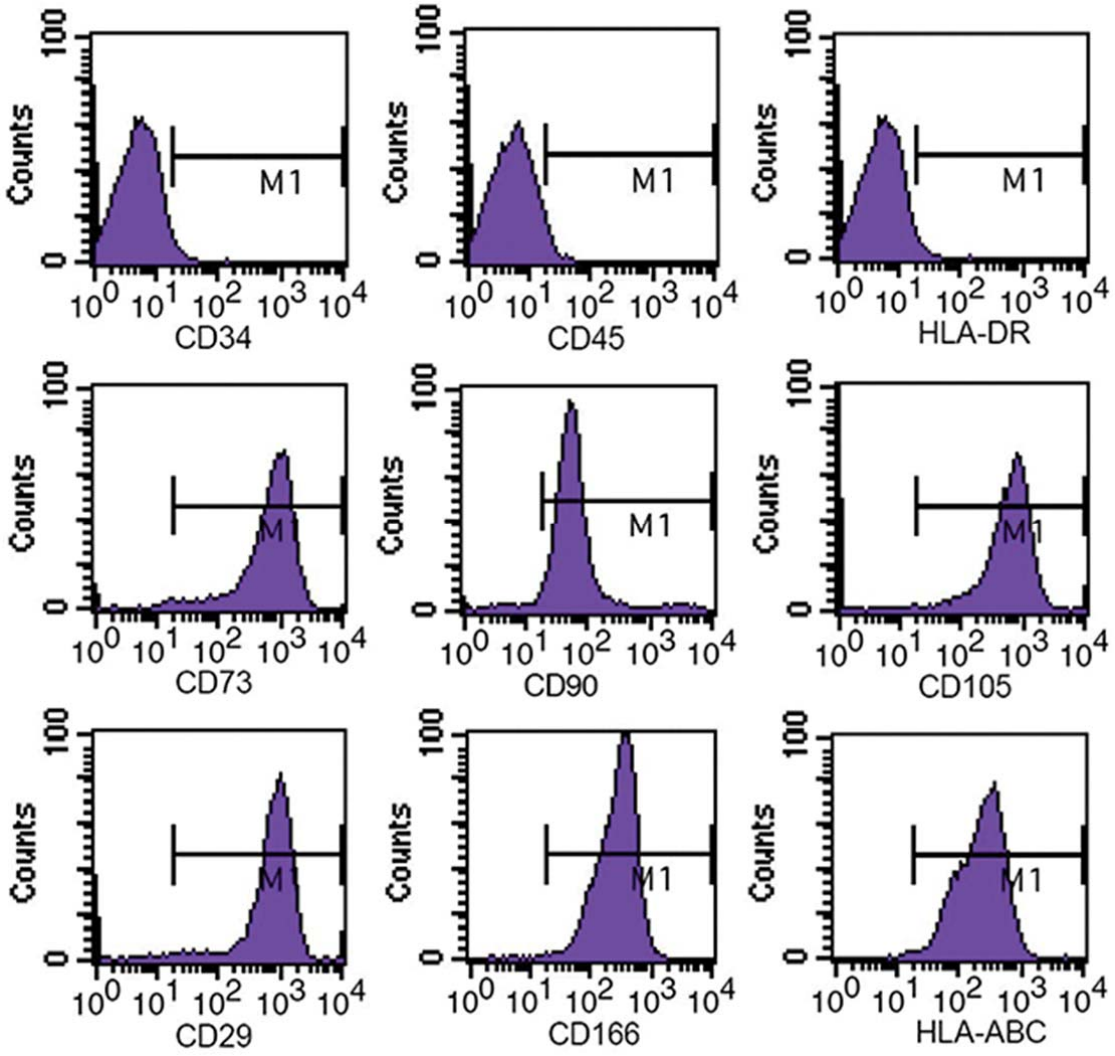

FIG. 3 Flow cytometry analyses of WJCs at passage 3. WJCs positively expressed CD73, CD105, CD90, CD29, CD166, and human leukocyte antigen (HLA)-ABC, while negatively expressed CD34, CD45, and HLA-DR.

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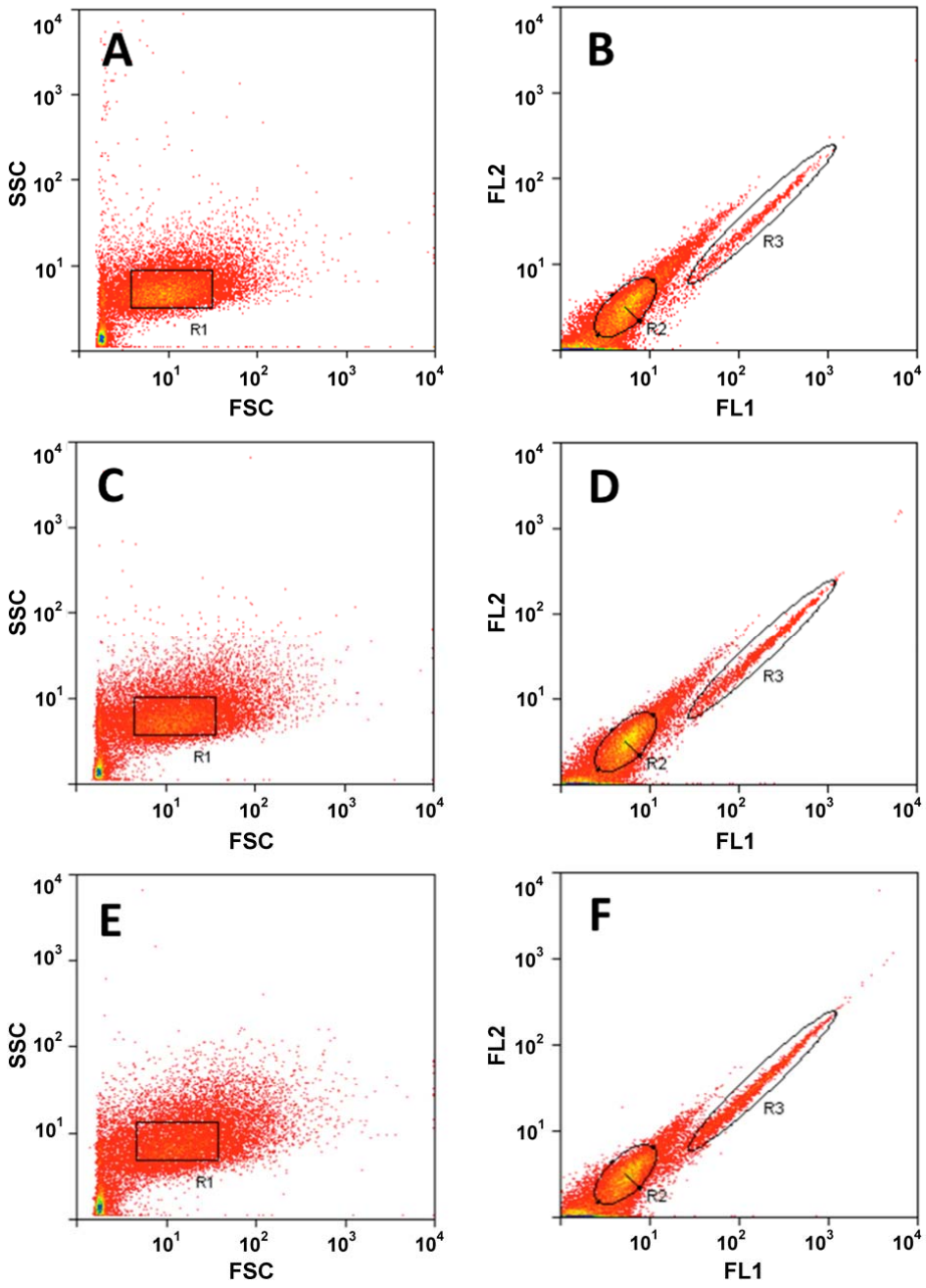

FIG. 4 CFSE-labelled WJCs and degenerative NPCs were separated by the high-speed MoFlo cell sorter after 7 days of coculture. Two-dimensional dot-plots showing the soring of WJCs and NPCs cocultured with the ratios of 25:75 (**A, B**), 50:50 (**C, D**), and 75:25 (**E, F**). Gate R1 indicates the single live cells, Gate R2 implies the degenerative NPCs without CFSE label, and Gate R3 shows the CFSE-labeled WJCs. FL, fluorescence. SSC, side scatter. FSC, forward scatter.

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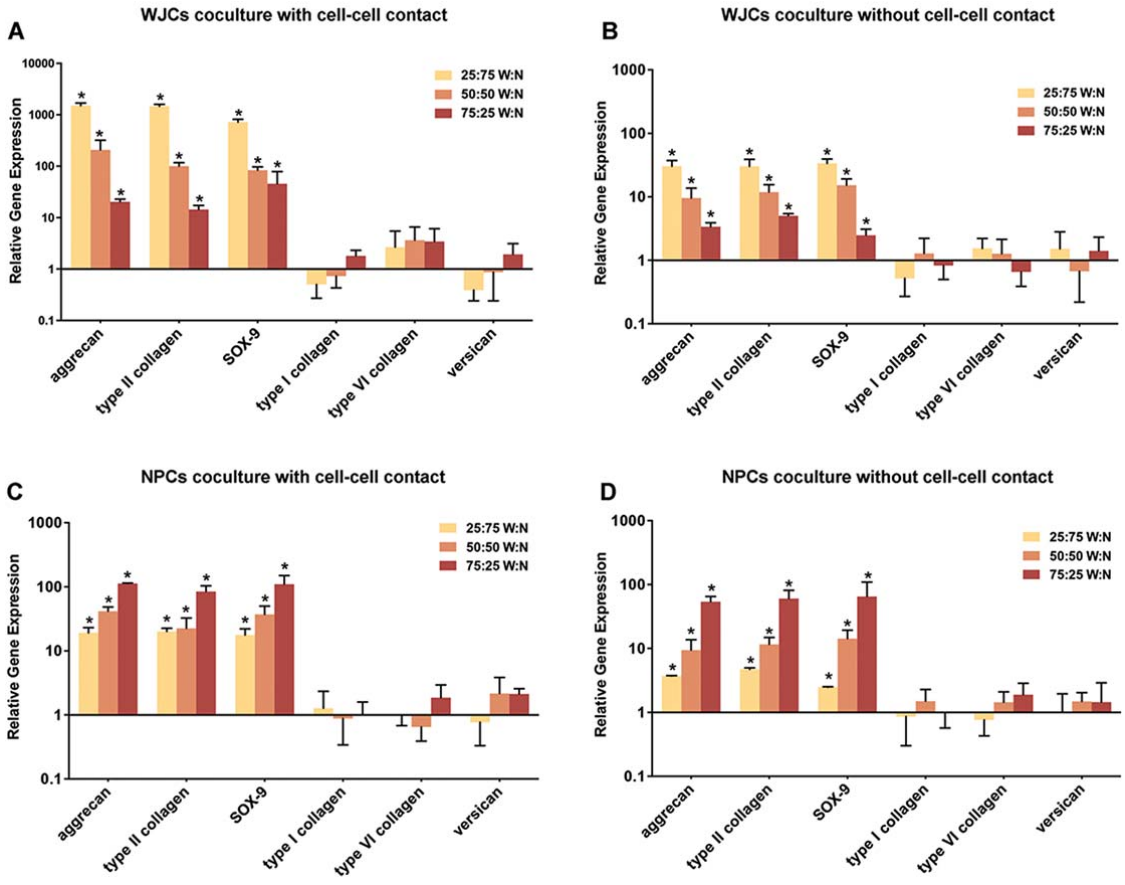

FIG. 5 Relative gene expression of aggrecan, type II collagen, SOX-9, type I collagen, type VI collagen, versican of WJCs and NPCs after coculture with or without cell-cell contact. The relative gene expression was calculated using the gene expression of the housekeeping gene GAPDH as reference. Relative gene expression of WJCs after coculture with (**A)** and without (**B**) cell-cell contact. Relative gene expression of degenerative NPCs after coculture with (**C**) and without (**D**) cell-cell contact. W, WJCs. N, degenerative NPCs. $^{*}P < 0.05$ compared with individual cultured WJCs or NPCs.