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# Differentiation of Human Ligamentum Flavum Stem Cells Toward Nucleus Pulposus-Like Cells Induced by Coculture System and Hypoxia

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**Study Design.** Human ligamentum flavum (LF)-derived stem cells (LFSCs) and nucleus pulposus cells (NPCs) were cocultured under normoxia or hypoxia.

**Objective.** To isolate and identify human LFSCs and determine whether they can differentiate into NPCs when cocultured with NPCs under hypoxia.

**Summary of Background Data.** Mesenchymal stem cell (MSC)-based therapies have been proposed as a biological treatment for intervertebral disc degeneration. MSCs derived from various tissues are leading candidates for cell-based therapies, but such cells have not been reported in LF.

**Methods.** LF cells were isolated from patient samples and cultured using culture flasks coated with fibronectin, and their identity was confirmed using flow cytometry. The cells were induced to differentiate into osteoblasts, chondrocytes, and adipocytes, and their morphology, immunophenotype, cell proliferation capacity, cell cycle, and expression of stem cell-specific genes were compared with those of bone marrow-MSCs (BM-MSCs) derived from the same patients. NPCs and LFSCs were cocultured in 1- $\mu$ m-pore-size insert transwell-culture systems under hypoxia (2% O<sub>2</sub>) or normoxia. CD24 expression was measured by flow cytometry and confocal microscopy assay. On day 14, reverse transcription-polymerase chain reaction was used for comparing the expression of

chondrogenic genes (*Sox-9*, *collagen-II*, *aggrecan*) and novel marker genes (*KRT19*, *CA12*, *FOXF1*, *HIF-1 $\alpha$* ) between the 2 groups.

**Results.** LFSCs were obtained using the fibronectin differential-adhesion assay. The morphology of LFSCs was altered, and their immunophenotype, multilineage induction, cell proliferation capacity, cell cycle, and stem cell-specific gene expression were closely related—but not identical—to BM-MSCs, CD24 expression was highly significant in the differentiated LFSCs. RT/Real-time polymerase chain reaction revealed that compared with LFSCs grown under normoxia, hypoxia-treated LFSCs expressed higher levels of *Sox-9*, *collagen-II*, *aggrecan*, *KRT19*, *CA12*, and *HIF-1 $\alpha$*  genes except *FOXF1*.

**Conclusion.** Stem cells were identified in human LF, and LFSCs cocultured with NPCs were successfully differentiated into NP-like cells under hypoxia. This potentially provides new cell candidates for cell-based regenerative medicine and tissue engineering.

**Key words:** ligamentum flavum, bone marrow, mesenchymal stromal cells, nucleus pulposus cells, coculture, hypoxia, tissue engineering, intervertebral disc degenerative.

**Level of Evidence:** N/A

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Intervertebral disc (IVD) degeneration is closely related to low back pain,<sup>1</sup> which is experienced in around 40% of patients with back pain.<sup>2</sup> IVD degeneration involves a reduction in the function and number of viable cells in the disc, mostly through cellular senescence<sup>3</sup> and apoptosis,<sup>4,5</sup> and this is accompanied by diminished synthesis of extracellular matrix components such as collagens and proteoglycans and a reduction of the water content. Current IVD degeneration treatments mainly involve drug application and surgery, which is limited to relieve symptoms. However, in cell-based tissue engineering, the aim is to restore IVD structure and function. In clinical trials, pain was reduced in patients treated with disc-chondrocyte transplantation,<sup>6</sup> and in osteoarthritis treatment, implantation of autologous chondrocytes and matrix-induced autologous chondrocytes yielded favorable results.<sup>7</sup> Researchers aim to use a similar strategy in treating IVD degeneration and have previously

demonstrated the proliferative capacity and pluripotency of mesenchymal stem cells (MSCs), whose transplantation is considered a promising approach in developing new treatments of IVD degeneration. Several types of multipotent stem cells are present in adult tissues (such as in bone marrow, heart, brain, skeletal muscle, adipose tissues, synovial membrane, and menstrual blood<sup>8</sup>), and MSCs show similar characteristics such as multigenerational propagation, self-renewal, multilineage differentiation, and specific phenotypes. However, most MSCs are obtained with difficulty or invasively.

Current transforaminal endoscopic surgery and micro-endoscopic discectomy pass through ligamentum flavum (LF), which has been widely suggested to contain multipotent stem cells.<sup>9</sup> However, no study has identified a definitive stem/progenitor-cell population in LF. We tested whether stem cells present in LF (LF-derived stem cells; LFSCs) can be induced into nucleus pulposus cells (NPCs) *in vitro*. We isolated a rare cell population from human LF and showed that they exhibit several universal stem cell properties. Previously, methods such as hypoxia treatment, use of 3-dimensional scaffolds, and coculturing were employed to induce MSCs' differentiation into NP-like cells.<sup>10–13</sup> Here, we used hypoxia and a 3D coculture system to test whether LFSCs can differentiate into NP-like cells. Coculturing with NPCs potently induced chondrogenic differentiation, as shown by the upregulated expression of collagen-II, aggrecan, and Sox-9.<sup>14</sup> After differentiation, human NP-specific markers were also detected, such as CD24, cytokeratin-19 (KRT19), carbonic anhydrase XII (CA12), and forkhead box F1 (FOXF1)<sup>15</sup>; all these genes were upregulated when LFSCs were cocultured with NPCs. The use of autologous stem cells is regarded to be ethically appropriate, and we suggest that LFSCs could emerge as new seed cells for treating IVD degeneration.

## MATERIALS AND METHODS

### Cell Culture

LF tissue (3–5 g) was obtained from patients who underwent transforaminal endoscopic surgery for lumbar degenerative disease (Table 1) (see Video, Supplemental Digital Content 1 .mp4, available at: <http://links.lww.com/BRS/A989>). Xinqiao Hospital's Institutional Review Board and Ethics Committee approved the study protocol. Written informed consent was obtained from all patients (see Supplemental Digital Content, Figure 1, available at: <http://links.lww.com/BRS/A990>, which shows the harvesting procedure). Magnetic resonance imaging/computed tomography and histological techniques were used to assess the LF state (see Supplemental Digital Content 2, Figure 2, available at: <http://links.lww.com/BRS/A991>). The LF-explant samples were cut into 1- to 2-mm<sup>3</sup> pieces and digested with 2% type-I collagenase (Sigma, St Louis, MO); counting the nucleated cells revealed that 1 g of tissue provided 2.5 to 5 × 10<sup>6</sup> cells and plated mononuclear cells at an initial density of 10,000 cells/cm<sup>2</sup> for obtaining a high yield. Cells were cultured in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) supplemented with 16.6% fetal bovine serum and 100 U/mL penicillin-streptomycin for 12 hours at 37°C in 5% CO<sub>2</sub>; the medium was changed twice weekly. Passage 3 was transferred to culture flasks coated with fibronectin (5  $\mu$ g/cm<sup>2</sup>) as described previously. LFSCs were positive for CD90, CD29, CD44, CD73, and CD105, and negative for STRO-1, CD34, CD45, and CD133, which were examined by an FACScan flow cytometer running CellQuest software (Beckman Coulter, Brea, CA) (see Supplemental Digital Content 3, Figure 3, available at: <http://links.lww.com/BRS/A992>) to measure the proliferation capacity and cell cycle of the LFSCs and bone marrow mesenchymal stromal cells (BM-MSCs), which was examined by Cell Counting Kit-8 and a FACSCalibur flow cytometer (BD

**TABLE 1. Details of the Patients Enrolled in This Study**

Case No.	Sex	Surgery	Disc Level	Age, yr	MRI-/CT-Evaluated Hypertrophy	Histological Morphology
1	F	TES	L3–L4	35	No	No
2	M	TES	L4–L5	38	No	No
3	M	MDS	L4–L5	41	No	No
4	M	TES	L4–L5	45	No	No
5	M	TES	L4–L5	69	No	No
6	M	TES	L5–S1	58	No	No
7	M	TES	L4–L5	54	No	No
8	F	MDS	L4–L5	28	No	No
9	M	TES	L4–L5	71	No	No
10	M	MDS	L4–L5	63	No	No

MRI indicates magnetic resonance imaging; CT, computed tomography; F, female; M, male; TES, transforaminal endoscopic surgery; MDS, microendoscopic discectomy surgery.

Biosciences, San Jose, CA) (see Supplemental Digital Content, Figures 4–5, available at <http://links.lww.com/BRS/A993> and <http://links.lww.com/BRS/A994>). The state of NP was assessed on the basis of magnetic resonance imaging, age, and disc level; the samples were obtained from patients undergoing corrective treatment of scoliosis. NP<sup>8</sup> and BM-MS<sup>16</sup> samples were isolated and grown as described previously and used at Passage 3.

### Testing LFSC Multipotency

#### Osteogenic Differentiation

Cells were incubated for 21 days in an osteogenic medium containing 10% fetal calf serum (FCS; Hyclone Laboratories, Inc., Logan, UT), 10 nmol/L dexamethasone, 10 mmol/L  $\beta$ -glycerophosphate, and 0.1 mmol/L l-ascorbic acid-2-phosphate; control cells were maintained in  $\alpha$ -MEM/10% FCS. Media were changed twice weekly. Mineral deposits were identified using alizarin-red staining.

#### Adipogenic Differentiation

Cells were incubated for 72 hours in an induction medium containing 10  $\mu$ g/mL insulin, 1  $\mu$ mol/L dexamethasone, 500  $\mu$ mol/L 3-isobutyl-1-methyl xanthine, and 100  $\mu$ mol/L indomethacin, and then for 24 hours in a maintenance medium (10  $\mu$ g/mL insulin in  $\alpha$ -MEM/10% FCS). This 96-hour treatment cycle was repeated 5 times and then cultures were incubated in the maintenance medium until 30 days. Control cells were maintained in DMEM/F12/10% FCS. Lipid droplets were stained with Oil Red O.

#### Chondrogenic Differentiation

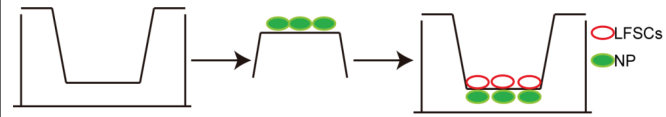
We used the micromass-culture method; to generate micromass cultures, we placed 20- $\mu$ L droplets of cell suspensions (in cell-culture medium;  $2.5 \times 10^5$  cells) in the center of multiwell-plate wells and after culturing for 2 hours under high humidity, we added a chondrogenic-differentiation basal medium (dexamethasone, ascorbate, ITS-Premix (Becton-Deckinson), sodium pyruvate, proline, and transforming growth factor (TGF)- $\beta$ 3) to the culture vessels and incubated them up to 21 days. The micromass cultures were fixed and stained with 1% Alcian blue.

#### LFSCs-NPCs Coculture

We used 6-well coculture plates consisting of 1- $\mu$ m-pore-size, high-pore-density, 3D polyethylene terephthalate track-etched tissue-culture inserts. Passage-3 LFSCs and NPCs were cocultured to allow direct contact between the cells, as described.<sup>17</sup> Briefly,  $5 \times 10^4$  NPCs were cultured on the reverse side of the membrane inserts for 12 hours, and after their adhesion, the inserts were turned upright and LFSCs at the same density were cultured on the front side (Table 2), respectively, for 14 days under 2%-O<sub>2</sub> or 20%-O<sub>2</sub> (normal) tension. We used hypoxia to induce LFSC differentiation because the native NP-tissue environment is hypoxic, and hypoxia represents a critical environmental factor that promotes MSC differentiation.<sup>10</sup> The control was non-cocultured LFSCs; Table 2 lists groups

**TABLE 2. Three Groups in This Research**

Group	Cells Density	O <sub>2</sub> Tension
Control	$5 \times 10^4$	20%
Coculture LF	$5 \times 10^4$	20%
Coculture LF	$5 \times 10^4$	2%



*LF indicates ligamentum flavum; LFSCs, ligamentum flavum–derived stem cells; NP, nucleus pulposus.*

used in this study. After coculture, LFSC morphology changes were examined. To denote a nucleus pulposus–like differentiation phenotype, the CD24-FITC (Santa Cruz Biotechnology, Inc., Dallas, TX)–labeled cells were analyzed using an FAC-Scan flow cytometer running CellQuest software (Beckman Coulter, Brea, CA). To detect CD24 were located in differentiated and undifferentiated LFSCs after 14 days and 21 days of coculture, which were methanol-fixed and incubated with CD24-FITC (mouse, 1:100, Santa Cruz Biotechnology, Inc., Dallas, TX) for 3 hours at 4°C, then washed, visualized with a Leica confocal microscope.

#### Real-Time/Reverse Transcription-Polymerase Chain Reaction Analysis

We used reverse transcription-polymerase chain reaction (RT-PCR) to evaluate the expression of differentiation-related genes (*ALP*, *OC*, *RUNX-2*; *PPAR-2*, *APP*, *LPL*; and *AGG*, *COL II*, *Sox-9*), and real-time PCR for testing stem cell-specific genes (*NANOG*, *Sox-2*, and *OCT-4*) and extracellular matrix or the characteristic NP markers (*HIF-1 $\alpha$* , *Sox-9*, *collagen-II*, *aggrecan*, *KRT19*, *FOXF1*, and *CA12*). After 2 weeks of coculture, LFSCs were isolated by trypsin from each group. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Concentration of total RNA was determined with a spectrophotometer (DU-500; Beckman, Fullerton, CA) at 260-nm wavelength. Two micrograms of total RNA were used to synthesize cDNA (MBI Fermentas, Sankt Leon-Rot, Germany). For RT-PCR, 1 mL of cDNA template was used for each reaction and sequences were amplified using Taq DNA polymerase. Differentiation related gene primers were designed and synthesized as shown in Table 3.  $\beta$ -actin was used to normalize the reactions. For Quantitative Reverse Transcription Polymerase Chain, Reaction Analysis Reactions (20  $\mu$ L) including 10  $\mu$ L of Sybr Green (BIO-RAD), 2  $\mu$ L of DNA, 1  $\mu$ L of each primer, and 6  $\mu$ L of sterile distilled water were performed in triplicates with C1000 Touch Thermal Cycler (BIO-RAD) for PCR amplification. Target gene expression was calculated by the  $2^{-\Delta\Delta C_t}$  method and normalized to  $\beta$ -actin and control. Differential expression was examined using 1-factor analysis of variance and was considered significantly different at *P* value of less than 0.05. The primers used are shown in Tables 4.

**TABLE 3. Primer Sequences for Reverse Transcription-Polymerase Chain Reaction Analysis**

Gene	Primer	Sequences	TA (°C)	Cycles
RUNX-2	Forward	ACGACAACCGCACCATGGT	59	30
	Reverse	CTGTAATCTGACTCTGTCTCT		
ALP	Forward	TGGAGCTTCAGA AGCTCAACACCA	59	28
	Reverse	ATCTCGTTGTCTGAGTACCAGTCC		
OC	Forward	ATGAGAGCCCTCACACTCCTC	59	30
	Reverse	GCCGTAGAAGCGCCGATAGGC		
PPAR-2	Forward	CGAGGGCGATCTTGACAGGAA	55	30
	Reverse	CAGGGGGGTGATGTGTTTGAAC		
APP	Forward	CTGTCCAAGTCCAACAGCAA	55	30
	Reverse	ACGTTGGCAGCTTTACGTCT		
LPL	Forward	TCCGCGTGATTGCAGAGAGAG	55	30
	Reverse	TGCTGCTTCTTTGGCTCTGACT		
AGG	Forward	TGAGGAGGGCTGGAACAAGTACC	57	28
	Reverse	GGAGGTGGTAATTGCAGGGAACA		
COL II	Forward	TTTCCCAGGTCAAGATGGTC	57	30
	Reverse	TCACCTGGTTTTCCACCTTC		
SOX-9	Forward	TGGCCGAGATGATCCTAAAAATAA	57	30
	Reverse	GCGCTTGGATAGGTCATGTTTGT		
$\beta$ -actin	Forward	GTGGGGCGCCCCAGGCACCA	55	45
	Reverse	CTTCCTTAATGTCACGCACGATTC		

### Statistical Analysis

Data are reported as means  $\pm$  SD. Histological and Immunofluorescence staining data are described qualitatively. Statistical analysis was performed using an analysis of variance, and the Fisher least significant difference test was used as a *post hoc* test; *P* value of less than 0.05 was considered statistically significant.

## RESULTS

### Characteristic of LFSCs

#### Cell Morphology

After selection using the fibronectin differential-adhesion assay, LFSCs appeared mostly uniform and spread out in culture flasks and remained monoplastic immediately after seeding (Figure 1A, B), and they formed colonies *in vitro* more efficiently than BM-MSCs did. We also examined the morphology of 7-day-cultured NPCs and BM-MSCs (Figure 1C, D).

#### Multipotency of Putative LFSCs

Adipogenic differentiation occurred was confirmed on the basis of the presence of Oil Red O–stained intracellular lipid vacuoles (Figure 2a A); but they were not detected in the growth medium (Figure 2a D). Osteogenic differentiation

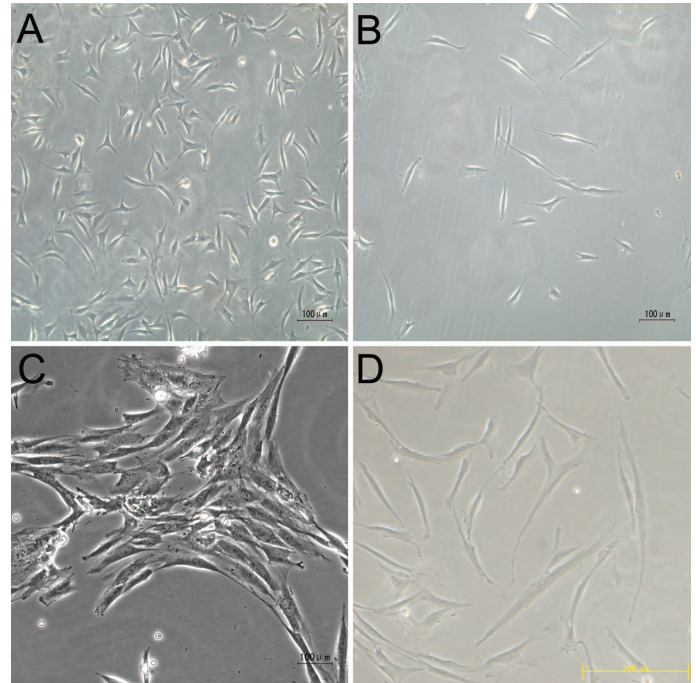
was verified on the basis of the deposition of an alizarin-red-positive mineralized matrix at 3 weeks after induction (Figure 2a B); this was not observed in control cells (Figure 2a E). Chondrogenic differentiation was confirmed on the basis of Alcian blue staining, which revealed glycosaminoglycan deposition (Figure 2a C), and in the growth culture, no cells stained positively for Alcian blue (Figure 2a F).

#### PCR for Differentiation- and Stem Cell–Related Gene Expression

RT-PCR results showed that all induction-related genes (*ALP*, *OC*, *RUNX-2*; *PPAR-2*, *APP*, *LPL*; and *AGG*, *COL II*, *Sox-9*) were expressed at higher levels in the 3 induction groups than in the control group (Figure 2b A–C). Compared with BM-MSCs, LFSCs expressed higher levels of *NANOG*, *Sox-2*, and *OCT-4* (Figure 3). The results were confirmed using real-time RT-PCR. LFSCs and BM-MSCs did not express significantly different levels of *NANOG* and *Sox2* (2-tailed,  $t = 0.37$ ,  $P = 0.68$ , and  $t = 1.83$ ,  $P = 0.22$ , respectively); however, the *OCT4* mRNA level was higher in LFSCs than in BM-MSCs (2-tailed,  $t = 12.8$ ,  $P = 0.004$ ). In LFSCs, *sox2*, *NANOG*, and *OCT4* mRNAs were measured to be (mean  $\pm$  SD)  $300.40 \pm 71, 125 \pm 226$ , and  $83 \pm 6.9$  fg/mg, respectively, and in BM-MSCs, these were  $298.45 \pm 61, 118 \pm 7$ , and  $42.5 \pm 1.8$  fg/mg, respectively.

### Genes Expression After Coculture

To explore the Expression of CD24 during differentiation of Human LFSCs, we examined its expression pattern by confocal microscopy assay and flow cytometry. Regarding the confocal microscopy assay, the results revealed that there were a large number of cells that expressed CD24 in the differentiated LFSCs. This indicated that LFSCs have some potential to differentiate into cells sharing features with the phenotype of the healthy NP cell. Levels of CD24 expression were consistent with the immunostaining pattern, compared with undifferentiated group (Figure 4A), which was highly significant ( $P < 0.001$ ) (Figure 4B). The CD24 expression of undifferentiated group (45.9%, Figure 4C) was lower than differentiated (82.8%, Figure 4D). For quantitative analysis in differentiated LFSCs and undifferentiated, the former CD24 level was higher than the latter after 14 days and 21 days of coculture (Figure 4E) ( $P < 0.05$ ). PCR analysis of NP-marker



**Figure 1.** Morphology of cells derived from ligamentum flavum, nucleus pulposus, and bone marrow. Morphology of ligamentum flavum-derived cells was selected by fibronectin differential adhesion assay after immediately seeded (Figure 3B) and before selected (Figure 3A). Morphology of nucleus pulposus (Figure 3C) and bone marrow-mesenchymal stem cells (Figure 3D). A–C Bar = 100  $\mu\text{m}$ , D Bar = 200  $\mu\text{m}$ .

expression in differentiated LFSCs showed that after 14 days of coculture, the expression of the following genes was significantly increased relative to control ( $P < 0.05$ ) in both normoxic- and hypoxic-group cells (Figure 5): *KRT19* (A), *CA12* (C), *ACAN* (D), *COL2A1* (E), *Sox-9* (F), and *HIF-1 $\alpha$*  (G). However, under hypoxia, *FOXF1* (B) was not upregulated.

### DISCUSSION

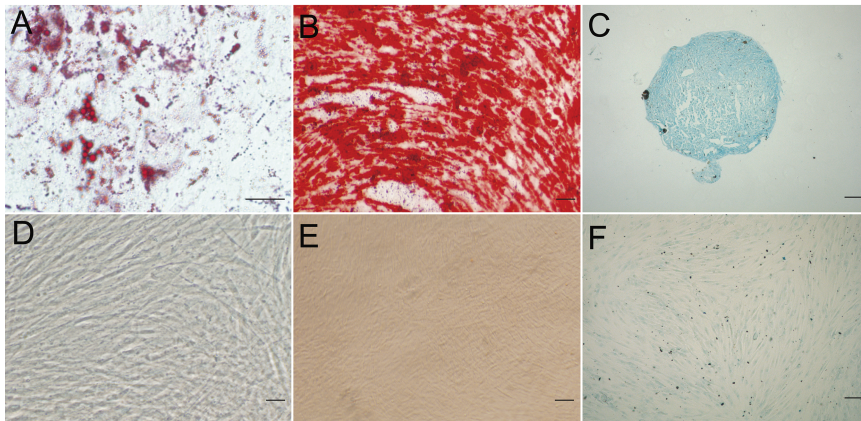
The tissue-resident adult stem/progenitor cells are unique compared with other embryonic/umbilical cord/fetal/placental-derived stem cell sources in that they are enriched in a readily accessible anatomical location. Bone marrow-derived stromal cells constitute 0.125% of the total marrow cells. However, we identified a large LFSC subpopulation and isolated these cells from LF. LF tissue is an abundant, accessible, and replenishable source of adult stem cells. LFSCs coexpress MSC markers such as CD90 and CD105, which were detected in 2.17% to 3.25% of the total cell mass. We found that 1 to 3 g of tissue is required for expanding the isolated cells. The use of *ex vivo*-expanded adult stem/progenitor cells and their differentiated progenies offers great promise for cell-replacement therapies with multiple applications in humans. Moreover, the chondrogenesis induction observed highlights the robustness of the described biological phenomenon and suggests that alternative cell sources, which can be harvested under minimally invasive conditions, might be considered for clinical implementation of the described paradigms.

**TABLE 4. Primer Sequences for Reverse Transcription-Polymerase Chain Reaction Analysis**

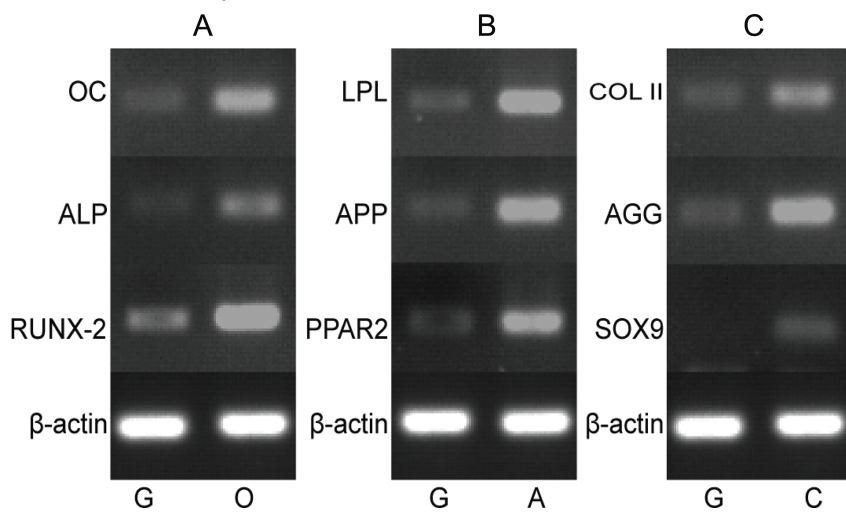
Gene	Primer	Sequences (5'–3')
ACAN	Forward	TGAGGAGGGCTGGAACAAGTACC
	Reverse	GGAGGTGGTAATTGCAGGGAACA
COL2A1	Forward	TTTCCCAGGTCAAGATGGTC
	Reverse	TCACCTGGTTTTCCACCTTC
SOX-9	Forward	TGGCCGAGATGATCCTAAAAATAA
	Reverse	GCGCTTGGATAGGTCATGTTTGT
KRT19	Forward	AACGGCGAGCTAGAGGTGA
	Reverse	GGATGGTCGTGTAGTAGTGCC
CA12	Forward	CGTGCTCTGCTGGTGATCT
	Reverse	AGTCCACTTGAACCGTTCCT
FOXF1	Forward	TCCCTGGAGCAGCCGTATC
	Reverse	TGGGCGACTGCGAGTGATA
HIF-1 $\alpha$	Forward	CCCTACCCAACGAAAAATTAC
	Reverse	GGGACTATTAGGTGAAC
SOX-2	Forward	CCCCCTGTGGTTACCTCTTC
	Reverse	GGCCGCTCTGGTAGTGCTG
NANOG	Forward	ACCCCGTTTACTGTGTTAGC
	Reverse	GACGGCAGCCAAGGTTATTTAA
OCT4	Forward	GGCAAGCGATCAAGCAGCGAC
	Reverse	GGGAAAGGGACCGAGGAGTAC
$\beta$ -actin	Forward	GTGGGGCGCCCCAGGCACCA
	Reverse	CTTCTTAATGTCACGCACGATTTTC

ACAN indicates aggrecan; COL2A1, collagen II  $\alpha$  1; KRT19, cytokeratin-19; CA12, carbonic anhydrase XII; FoxF1, forkhead box F1.

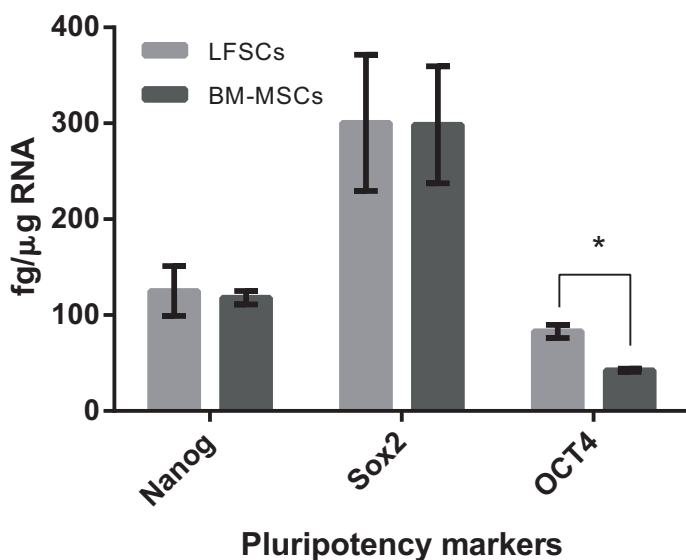
a multidifferentiation potential



b The mRNA expression of differentiation

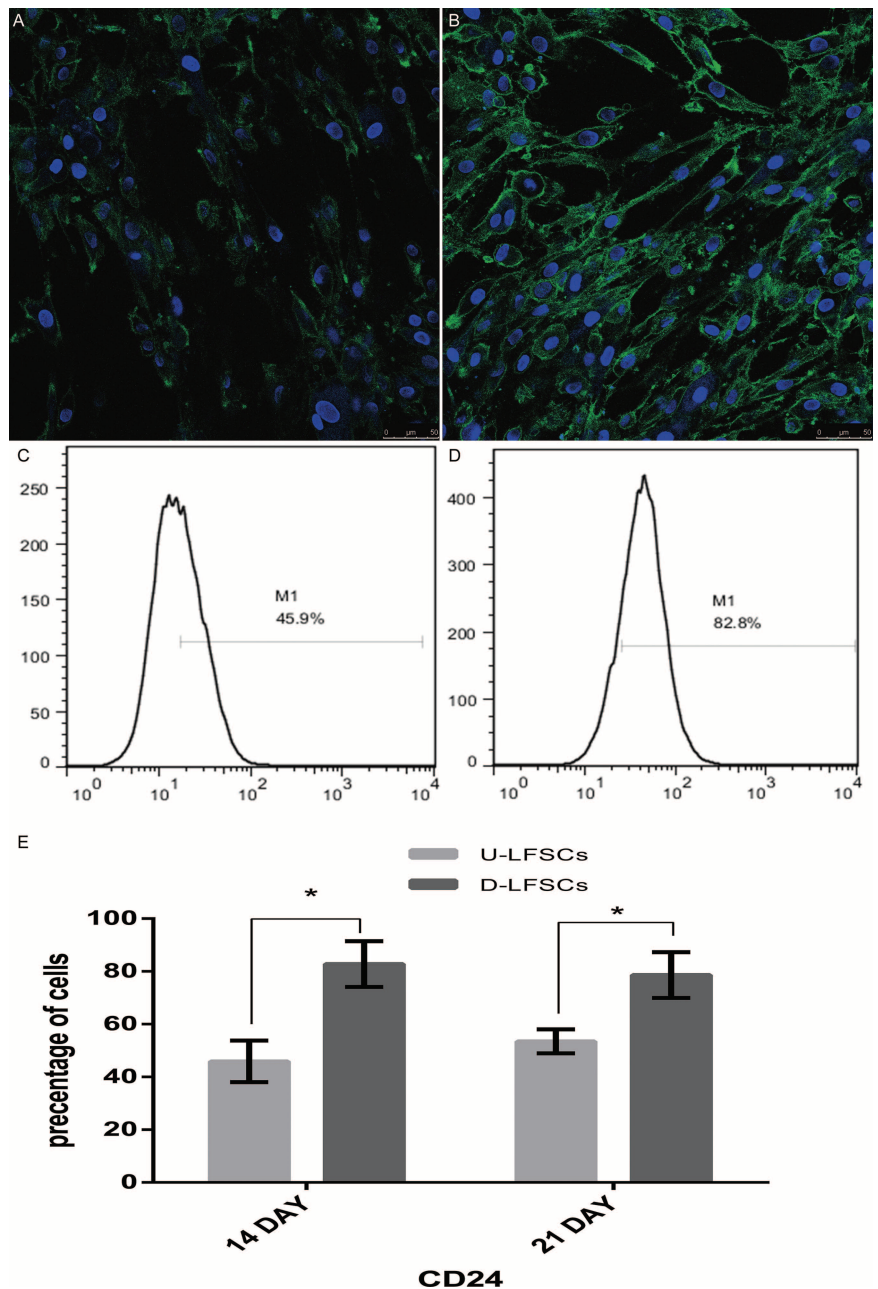


**Figure 2.** a *In vitro* osteogenic, adipogenic, and chondrogenic assay of ligamentum flavum-derived progenitor cells from case 10. Cells cultured in adipogenic medium formed intracellular lipid droplets that were stained with Oil Red O (A), and no adipocytes could be found in the noninduction growth medium (D). Cells cultured in osteogenic medium produced an alizarin red positive matrix (B) and no positive staining in the growth medium (E). Pellets cultured in chondrogenic induction medium (C) and the non-induction growth group (F) stained with Alcian blue. Differentiation-related genes were markedly upregulated under induction medium compared with the noninduction growth group. G, O, A, and C indicates the growth, osteogenic, adipogenic, and chondrogenic induction medium, respectively (Figure 2b).



**Figure 3.** Real-time reverse transcription-polymerase chain reaction for sex-determining region Y-related HMG box 2 (*Sox2*), Nanog, and POU5F1 stem cell genes (*OCT4*, *NANOG*, and *SOX-2*), which were expressed both in BM-MSCs and LFSCs from case 10. BM-MSCs indicates bone marrow-mesenchymal stem cells; LFSCs, ligamentum flavum-derived stem cells. \* $P < 0.05$  compared with BM-MSCs.

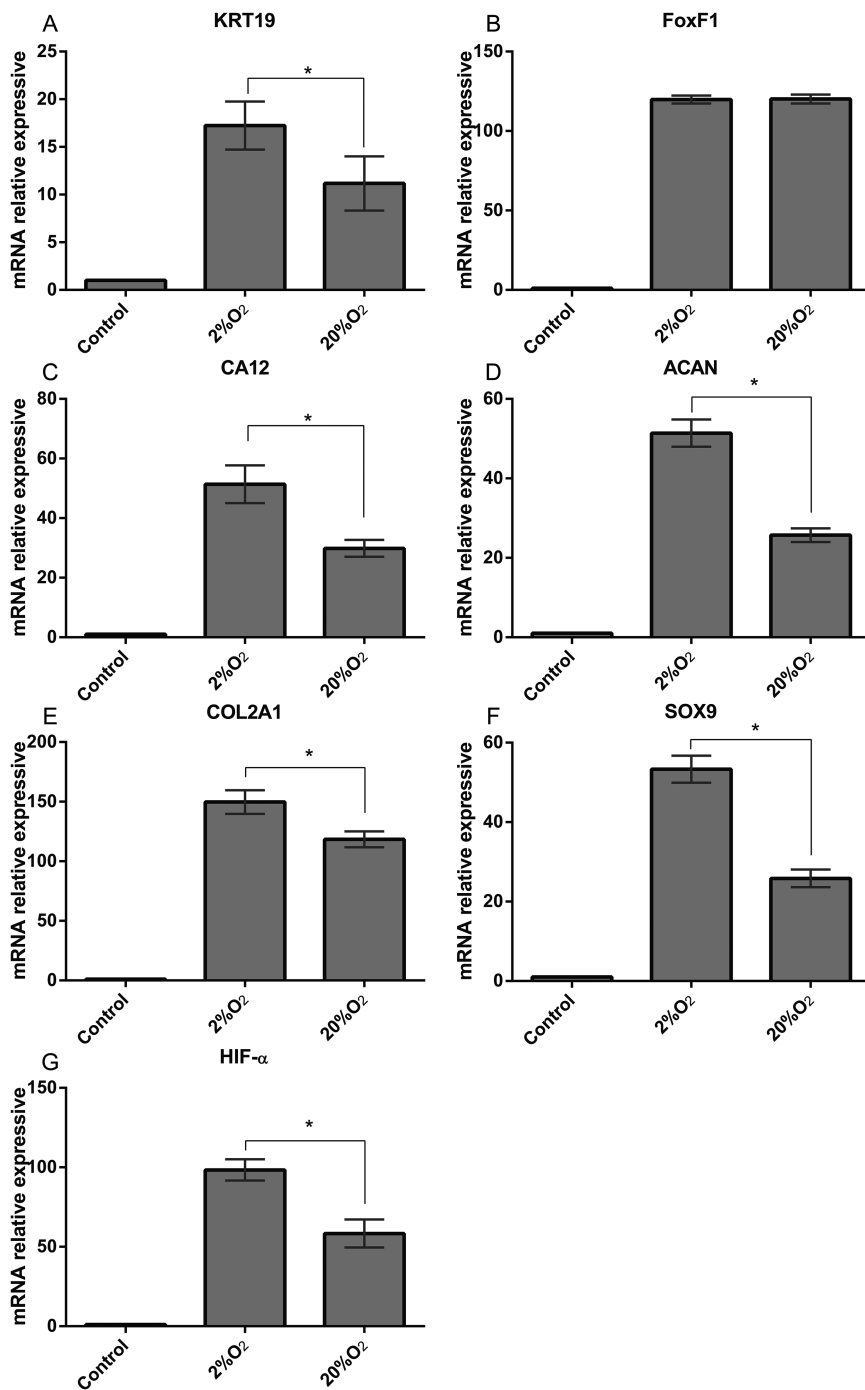
Because LFSCs have been poorly characterized and their specific markers remain unidentified, we sought to identify stem cells in human LF and evaluate their potential to differentiate into NP-like cells *in vitro*. Using the fibronectin differential-adhesion assay, we obtained homogeneous LFSCs, our results provided evidence that LSCs on FN induced a higher rate of migration than on a glass surface. Migration of cells is facilitated by the adhesive interaction of cells with the substrate, which is determined by specific integrin-ligand binding affinity, level of ligands, generation of forces at binding sites. as demonstrated using flow cytometry, the proliferation rate, cell cycle, and RT-PCR. Recently, primary fibroblast-like cells derived from diverse human tissues were shown to be capable of differentiating into at least 1 mesenchymal lineage.<sup>18</sup> Although LFSCs and BM-MSCs expressed many of the same markers, the expression patterns were not identical. LFSCs highly expressed CD90, CD29, CD44, CD73, and CD105 but not the BM-MSC-marker *stro-1*; although not mentioned in the International Society for Cellular Therapy's (ISCT) criteria as a suitable marker to identify multipotent cells,<sup>19</sup> *stro-1* might serve a selective negative marker for LFSCs. LFSCs could also differentiate into osteoblasts, adipocytes, and chondrocytes. To further confirmed the unique phenotype of the



**Figure 4.** CD24 are expressed in the undifferentiated LFSCs (A). Differentiated LFSCs express high levels of CD24 (B). Scale bars represent 50  $\mu$ m. Flow cytometric analysis of CD24 in undifferentiated (C) or differentiated LFSCs (D), the former cells indicate that subpopulation is approximately 45% and the latter is up to 82%. Differences in the expression of CD24<sup>+</sup> were calculated after 14 days and 21 days of coculture (E). All experiments were performed in triplicate and data are shown as mean  $\pm$  SD. Data in inset show statistical significance at  $*P < 0.05$ . LFSCs indicate ligamentum flavum–derived stems.

isolated LFSCs. We also used Immunocytochemistry staining. Specifically, all LFSCs expressed highly expressed  $\alpha$ -SMA, type I collagen, and fibronectin, but not expressed type II collagen (Supplemental Digital Content, Figure 6 available at: <http://links.lww.com/BRS/A995>). LFSCs and BM-MSCs expressed the same stem cell–related genes (*OCT-4*, *NANOG*, and *Sox-2*), and *OCT-4* amplifications were significantly detected in LFSCs. These genes are required for efficient self-renewal of embryonic stem cells, Tai *et al*<sup>20</sup> have shown that *OCT-4* is expressed in several adult human stem cells. The induction-related genes were markedly upregulated in the respective induction groups. We have also compared the morphology, proliferation potential, and cell cycle of LFSCs with those of BM-MSCs from the same subjects. These data suggest that LFSCs are closely related—but not identical—to BM-MSCs.

We sought to test whether LFSCs can be used to overcome a shortage of seed cells required for disc regeneration. To date, studies have applied methods commonly used to induce chondrogenesis and have reported that 2%-O<sub>2</sub> tension promoted the differentiation of human BM-MSCs into NP-like cells.<sup>21</sup> Here, we used the transwell system, which facilitates close cell-cell contact and is a widely accepted 3D cultivation system; in this system, hypoxia induced LFSC differentiation into NP-like cells that synthesized an extracellular matrix similar to that synthesized by NPCs. In these transwells, 2 types of cells are adhered to 2 sides of porous membranes, and this coculture method enables direct contact between the 2 types of cells through extended cytoplasmic processes. Recently, the Spine Research Interest Group made the recommendation of the following healthy NP phenotypic markers:



**Figure 5.** Quantitative reverse transcription-polymerase chain reaction analysis of special mRNA expression in stem cells derived from ligamentum flavum (LFSCs) after coculture. Expression of (A) *KRT19*, (B) *FOXF1*, (C) *CA12*, (D) *ACAN*, (E) *COL2A1*, (F) *SOX-9*, and (G) *HIF-1α* was normalized to  $\beta$ -actin and control. Values are the mean  $\pm$  SD.  $n = 10$ . \* $P < 0.05$ .

stabilized expression of HIF-1 $\alpha$ , GLUT-1, aggrecan/collagen II ratio greater than 20, Shh, Brachyury, KRT18/19, CA12, and CD24.<sup>22</sup> In our study, We predicted that LFSCs have the potential to differentiate into cells sharing features with populations of NP progenitor cells or immature NP morphology that are CD24 robustly positive. This indicated that LFSCs have some potential to differentiate into cells sharing features with the phenotype of the healthy NP cell. We also examined NP-specific markers HIF-1 $\alpha$ , Sox-9, collagen-II, aggrecan, KRT19, FOXF1, and CA12. HIF-1 $\alpha$  is a crucial mediator of the adaptive response of cells to hypoxia, which is essential for the growth and survival of growth-plate chondrocytes *in*

*in vivo*,<sup>23</sup> but HIF-1 $\alpha$  is also robustly expressed under normoxic culture conditions.<sup>24</sup> Sox-9 is expressed in all chondrocyte progenitors and chondrocytes,<sup>25</sup> and this is required for chondrocyte differentiation, cartilage formation, and expression of chondrocyte-marker genes such as *Col2a* and *aggrecan*.<sup>26</sup> The collagen-II gene is an early and abundant chondrocyte-differentiation marker. Keratins comprise the largest subfamily of intermediate-filament proteins and provide structural support to the nucleus and tensile strength to the cell.<sup>27</sup> Lee *et al*<sup>28</sup> identified *KRT19* as an NP-marker gene that is highly differentially expressed, and Sakai *et al*<sup>29</sup> performed comparative microarray analysis on *KRT19*. *FOXF1* has been poorly



studied, but recent findings have revealed its nonrandom association with birth defects such as spinal malformations and vertebral fusion.<sup>30</sup> Recently, Dahia *et al*<sup>31</sup> reported that hypoxia induced Sonic hedgehog signaling, which is required for IVD differentiation, but we found that *FOXF1* expression did not differ between hypoxia and normoxia groups. CA12 is potentially related to NPCs' adaptation to their harsh niche;<sup>32</sup> its identification in hypoxic NPCs might reflect the use of a similar homeostatic mechanism under hypoxia.<sup>33,34</sup> CA12 also maintains acid-base equilibrium by transporting CO<sub>2</sub> from cells residing in acidic environments<sup>35</sup>; thus, acid-base physiology could play a crucial role in NP-like cell survival and function in the IVD. Although no complete set of markers has yet been established for NP cells, findings of this study suggest that LFSCs may be induced to differentiate into NP-like cells as demonstrated by the presence of multiple NP-associated markers.

In summary, LFSCs are favorable cell-therapy candidates because they can be accessed readily, they proliferate rapidly, and obtaining them causes limited donor-site morbidity. Here, we identified certain LFSC-specific markers and demonstrated that LFSCs were successfully differentiated into NP-like cells, which produced an extracellular matrix similar to that of NPCs and expressed characteristic NP markers. Moreover, hypoxia enhanced the expression of these NP markers in differentiated LFSCs. Furthermore, using an injectable hydrogel to deliver cells to the injured disc might minimize the surgical risk of transplantation. To fully harness LFSCs' potential for IVD regeneration, an appropriate vehicle is required to facilitate effective LFSC delivery into the disc and additionally provide an optimized environment for tissue regeneration once the cells are *in situ*, and LFSCs can be expanded *in vitro* in sufficient quantities for tissue engineering. Although expanding LFSCs is less straightforward than originally expected, several approaches have been used for this purpose, including the development of specialized media, coculture with other cell types, identification and use of growth factors that exert proliferative effects on LFSCs, and culture on 3D scaffolds within bioreactors that simulate the *in vivo* environment. The optimal material used for IVD treatment must be injectable, nonantigenic, nonmigratory, volume-stable, and safe for human use.

## ➤ Key Points

- ❑ This study identifies the presence of stem cells in the human ligamentum flavum.
- ❑ Ligamentum flavum–derived stem cell have some specific markers, such as negative of STRO-1, highly expressed  $\alpha$ -SMA, type I collagen, and fibronectin. (Supplemental Digital Content, Figure 6, available at:)
- ❑ Real-time PCR shows that the LFSCs upregulate the expression of Sox-9, type II collagen, aggrecan, KRT19, CA12, and HIF-1 $\alpha$  but not *FOXF1*.

- ❑ LFSCs were successfully differentiated into NP-like cells, which could provide new cell candidates for cell-based regenerative medicine and tissue engineering.
- ❑ Ligamentum flavum is thought to be better candidate for cell therapy because of its ease of access, limited donor-site morbidity, and high proliferation rate.

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