

Potential of Human Nucleus Pulposus-Like Cells Derived From Umbilical Cord to Treat Degenerative Disc Disease

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BACKGROUND: Degenerative disc disease (DDD) is a common spinal disorder that manifests with neck and lower back pain caused by the degeneration of intervertebral discs (IVDs). Currently, there is no treatment to cure this debilitating ailment.

OBJECTIVE: To investigate the potential of nucleus pulposus (NP)-like cells (NPCs) derived from human umbilical cord mesenchymal stem cells (MSCs) to restore degenerated IVDs using a rabbit DDD model.

METHODS: NPCs differentiated from MSCs were characterized using quantitative real-time reverse transcription polymerase chain reaction and immunocytochemical analysis. MSCs and NPCs were labeled with fluorescent dye, PKH26, and transplanted into degenerated IVDs of a rabbit model of DDD (n = 9 each). Magnetic resonance imaging of the IVDs was performed before and after IVD degeneration, and following cell transplantation. IVDs were extracted 8 wk post-transplantation and analyzed by various biochemical, immunohistological, and molecular techniques.

RESULTS: NPC derivatives of MSCs expressed known NP-specific genes, *SOX9*, *ACAN*, *COL2*, *FOXF1*, and *KRT19*. Transplanted cells survived, dispersed, and integrated into the degenerated IVDs. IVDs augmented with NPCs showed significant improvement in the histology, cellularity, sulfated glycosaminoglycan and water contents of the NP. In addition, expression of human genes, *SOX9*, *ACAN*, *COL2*, *FOXF1*, *KRT19*, *PAX6*, *CA12*, and *COMP*, as well as proteins, *SOX9*, *ACAN*, *COL2*, and *FOXF1*, suggest NP biosynthesis due to transplantation of NPCs. Based on these results, a molecular mechanism for NP regeneration was proposed.

CONCLUSION: The findings of this study demonstrating feasibility and efficacy of NPCs to regenerate NP should spur interest for clinical studies to treat DDD using cell therapy.

KEY WORDS: Cell therapy, Human umbilical cord mesenchymal stem cells, Intervertebral disc, Nucleus pulposus, Rabbit model, TGFB pathway, Tissue engineering

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The intervertebral disc (IVD) is a fibrocartilaginous tissue that contributes to spine flexibility. Genetic-, aging-, and trauma-associated effects on IVD organization, structure,

and function have been implicated in chronic lower back pain.¹ The complex progressive disorder collectively referred to as degenerative disc disease (DDD) affects approximately 60% to 80% of people in the western countries.^{2,3} DDD causes substantial economic and societal burden in North America and Europe.⁴ As a result, there is a great deal of interest in elucidating the DDD process and relieving the significant societal burden associated with chronic back pain.

The IVD is composed of hydrated and gelatinous nucleus pulposus (NP), surrounding annulus fibrosus, and cartilaginous endplates.⁵ The extracellular matrix (ECM) of NP is composed of water, proteoglycans, and collagen

ABBREVIATIONS: **CM**, conditioned medium; **DAPI**, 4,6-diamidino-2-phenylindole; **DDD**, degenerative disc disease; **DM**, differentiation medium; **DMEM**, dulbecco's modified eagle's medium; **ECM**, extracellular matrix; **GM**, growth medium; **H&E**, hematoxylin and eosin; **IVD**, intervertebral disc; **MRI**, magnetic resonance imaging; **MSC**, mesenchymal stem cell; **NP**, nucleus pulposus; **NPC**, nucleus pulposus-like cell; **PAS**, periodic acid-schiff; **SEM**, standard error of the mean; **sGAG**, sulfated glycosaminoglycan; **qRT-PCR**, quantitative real-time reverse transcription polymerase chain reaction

that act as a shock absorber and maintain the mechanical load bearing capacity of the IVD.⁶ ECM is produced by NP cells derived from notochord precursors/progenitors, their number are decreased during the second decade of human life, thus limiting the potential to repair damaged NP.⁷ Degenerative changes in NP are characterized by the loss of cellularity and matrix integrity.⁸⁻¹¹ IVD degeneration is irreversible and can manifest clinically as chronic neck and lower back pain.¹²

There is no cure for DDD, and current treatments only address the symptoms rather than the cause of the disease.¹³ Patients generally seek relief using analgesics and/or physiotherapy.¹⁴ In severe cases, surgical procedures such as discectomy and spinal fusion are performed to mitigate pain. However, these interventions are insufficient to restore normal disc function and can further reduce the flexibility of the spine.¹³ Therefore, tissue regeneration to restore the biological and mechanical function of NP is more desirable for the treatment of symptomatic degenerated IVDs.¹⁵⁻¹⁷

Several studies have attempted to use cell therapy for the regeneration of IVD with encouraging but limited success.^{10,18-23} However, in order to determine the best mode of regenerative therapy, an in depth understanding of the IVD microenvironment is required. Thus, the matrix and cell composition of the disc as well as the importance of intrinsic and extrinsic factors on the mechanical and biological function of the IVD needs to be examined. Some reports describe the potential of autologous, allogeneic, and xenogeneic chondrogenic cells and mesenchymal stem cells (MSCs) to regenerate IVDs using animal models.^{18,21,23-32} This study evaluated for the first time the potential of NP-like cells (NPCs) derived from human umbilical cord MSCs to restore physicochemical, histological, and molecular properties of degenerated IVDs. These results could provide a rationale and stimulus for clinical trials to treat DDD using NPCs.

METHODS

Maintenance and Culturing of MSCs

Human umbilical cord MSCs were isolated as previously reported^{33,34} under approved protocols (HIC# 2012-101 and IRB# 400244). MSCs were cultured and maintained in growth medium (GM) containing Dulbecco's Modified Eagle's Medium (DMEM) with 4500 mg/L glucose and 2 mM L-glutamine (Life technologies, Carlsbad, California), supplemented with 10% fetal bovine serum (FBS) (Aiken Biologicals, Nash, Texas), and antibiotics (0.1% gentamicin, 0.2% streptomycin, and 0.12% penicillin; Sigma Aldrich, St. Louis, Missouri).

Differentiation of MSCs into NP-Like Cells

MSCs were differentiated into NPCs using NP conditioned medium (CM) from rabbit NP cells³⁵ or differentiation medium (DM) composed of a cocktail of growth factors (20 ng TGF β 1, 20 ng BMP7, 20 ng GDF5, 10 ng insulin, 100 nM dexamethasone, and 100 μ M ascorbic acid).

Flow Cytometry

Flow cytometric analysis for the expression of cell surface markers (CD90-FITC/CD73-APC, CD44-FITC/CD105-APC and CD29-APC), and cell size was determined on FACS Canto II using Diva Software (Becton Dickinson, Franklin Lakes, New Jersey).

Animal Studies

Animal experiments were approved by the Animal Care Committee (ACC#AL-13-06) and Institutional Biosafety Committee (IBC#2858). Skeletally mature female New Zealand white rabbits (n = 27), aged 6 to 12 mo, weighing 3.2 to 3.5 kg, were used in this study. Experimental design consisted of 3 randomly assigned groups of 9 animals each recipient of sham (media only), NPCs, or MSCs.

Magnetic resonance imaging Evaluation

Magnetic resonance imaging (MRI) evaluations of rabbits were performed before and 2 wk after puncture to confirm the degeneration as well as 6 wk after transplantation. Rabbits were tranquilized using ketamine (40 mg/kg) and xylazine (3 mg/kg). A localizing midsagittal T2-weighted image was obtained to view the L1-2 through L5-6 using a Philips 3T Ingenia (Philips Healthcare, Best, The Netherlands). Three-mm-thick midsagittal sections were taken using T2-weighted imaging sequences to evaluate signal characteristics within the IVDs. IVD degeneration and regeneration was determined by loss or gain of signal intensity and/or loss of IVD height on the T2-weighted sequence of the disc, respectively. Quantification of IVD height was determined by measuring the distance between the upper and lower vertebral bodies in the sagittal T2-weighted MRI images at the L2-3, L3-4, and L4-5 as previously published.³⁶ NP content was analyzed by measuring the mean signal intensity using Philips DICOM viewer version R3.0 SP3 (Philips Healthcare).

Transplantation of Labeled MSCs and NPCs Into the Rabbit IVD

Rabbit DDD model was developed via needle puncture as previously published.²¹ Needle punctures were performed on the 2 levels of the lumbar IVD segments at L2-3 and L4-5 to induce IVD degeneration. After 2 wk, 1×10^6 cells labeled with PKH26 dye (Sigma-Aldrich), or DMEM alone (sham) in 20 μ l volume were injected into the IVD at L4-5 through a Hamilton syringe with a 22-gauge needle guided by antero-posterior and lateral fluoroscopy. L2-3 served as degenerated and L3-4 served as the healthy IVDs, and the animals were followed up to 8 wk after cell injections.

Histological Analysis

IVDs were harvested following intravenous overdose of phenobarbitone sodium (120 mg/kg, Sigma-Aldrich) euthanasia. IVDs were fixed in 10% buffered formalin overnight, decalcified using 11% formic acid, cryoprotected in 20% sucrose, and transferred to OCT for cryosectioning. Ten μ m sections were stained with periodic acid-schiff (PAS), alcian blue, and hematoxylin and eosin (H&E) to evaluate the presence of glycoproteins, proteoglycan content, and cellular architecture, respectively.

Immunocytochemical Analysis

Cells and sections fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.5% triton X-100 (Sigma-

TABLE 1. List of Primary and Secondary Antibodies Used in Immunohistochemical Staining

Antibody	Primary	Secondary
ACAN	Rabbit polyclonal	Anti-rabbit-FITC
COL2	Goat polyclonal	Anti-goat-FITC
FOXF1	Goat polyclonal	Anti-goat-FITC
SMAD2	Rabbit polyclonal	Anti-rabbit-FITC
SOX9	Goat polyclonal	Anti-goat-FITC
TGF β R2	Rabbit polyclonal	Anti-rabbit-FITC
TUBB	Goat polyclonal	Anti-goat-FITC

Aldrich), blocked with 2% bovine serum albumin (Sigma-Aldrich) for 1 h. Samples were then incubated in primary antibody (1:100) for 2 h, washed with Phosphate-buffered saline (PBS), followed by treatment with secondary antibody (1:200) at 37°C for 1 h, and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were captured using a confocal microscope (NIKON Instruments Inc, Melville, New York). Nonimmune rabbit immunoglobulin was used as the negative control in all the immunostaining experiments on the rabbit specimens. Antibodies used are listed in Table 1.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

Transcriptional analysis was performed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Cellular RNA was isolated using GeneJet RNA purification kit (Thermo Fisher Scientific, Waltham, Massachusetts). IVD tissue was homogenized in Trizol reagent to isolate RNA. Isolated RNA was used to synthesize cDNA using iScript kit (BioRad, Hercules, California). qRT-PCR was carried out using SsoAdvanced SYBR Green Supermix and CFX96 Real-Time PCR System (BioRad). All reactions were prepared in triplicate and normalized to reference genes, *GAPDH*, and *ACTIN*. The primer sets used for qRT-PCR are listed in Table 2.

Statistical Analysis

Data were presented as the mean \pm standard error of the mean (SEM) of triplicates. Results with a *P*-value $\leq .05$ were considered statistically significant. Homogeneity of variance was determined with an F-test. All analyses were performed on log transformed data, using 1-way Analysis of variance (ANOVA) test and analyzed for equal variance using SPSS version 11.5 (SPSS, IBM Inc, Armonk, New York).

TABLE 2. List of Primer Sequences Used in qRT-PCR

Gene	Primer sequence		Product length
	Forward (5'-3')	Reverse (5'-3')	
hSOX9	AGCGAACGCACATCAAGAC	CTGTAGGCGATCTGTTGGGG	85
hACAN	AGCCTGCGCTCCAATGACT	GGAACACGATGCCTTACC	103
hCOL2	CTCGTGGCAGAGATGGAGAA	CACCAGGTTACCAGGATTG	252
hFOXF1	AAGCCGCCCTATTCCCTACATC	GCGCTTGGTGGGTGAAC	63
hKRT19	GCCACTACTACAGACCATCC	CAAACCTGGTTCGGAAGTCAT	126
hCOMP	AAGGACAGTGATGGCGATGG	TGTCCGAGAGTCTGATGT	154
hCA12	TGGAATCAGAATTGGAATCAC	GACACAGCAACACACAT	169
hPAX6	CTTTGCTTGGGAAATCCGAG	AGCCAGGTTGCGAAGAACTC	103
hTGF β 1	GACACCAACTATTGCTTCAG	CAGGCTCAAATGTAGGG	156
hSMURF1	GTCCAGAAGCTGAAAGTCCCTCAGA	CACGGAATTCACCATCAGCC	166
hSMURF2	CCTCGGCTGTCTGCTAACT	CGCTTGACTTGGGACTGT	128
hMEKK1	ATCTCTCAAACACAGCGCA	TGCGTGTGTTGAGGAGAT	200
hSMAD2	TGACCTGAGAACCGAATGGC	GAGAATCCAACAGCCTCCC	119
hSMAD3	TAGACTTGGGATGGGAGGG	GGCGCTCATCGATTTTCCC	123
hSMAD4	GGTAGCTGGAGAGGAAGGGA	TCAATCCAAGCCCGTGATC	84
hSMAD5	CTTCCACCAGCCCAACAACAC	TAGGCAGGAGGCGTATCAG	151
hERK1	TCAACACCACCTGCGACCTT	GCGTAGCCACATACTCCGTC	102
hERK2	GTTCCAAATGCTGACTCAA	CTCGGTCGTAATACTGCTCC	126
hTGF β R1	GTGACAGATGGGCTCTGCTT	GCAATGGTAAACCAGTAGTTGGA	211
hTGF β R2	AGTTTCTGTTTCCCCGCGAG	TCAGTGGATGGGCGATCCTAT	491
hBMP2	CGCTGTCTTAGCGTTGCT	GGGGTGGGTCTCTGTTTCCAG	191
hBMP4	CTGAGTATCTAGCTTGTCTCCC	TCAGCATTCGGTTACCAGGA	310
hBMPR2	CTTTACTGAGAATTTCCACCTCCTG	GCCAAAGCAATGATTATTGTCTCATC	90
hFOXC2	CGCCTAAGGACCTGGTGAAG	GGAAGCGGTCCATGATGA	123
hNODAL	TGTTGGGGAGGAGTTTCATC	GCACAACAAGTGAAGGGAC	93
hGDF5	ATCAGCATCTTCTTATTGACTCT	ACACGACTCCACGACCAT	75
hGDF10	TGCCTAAGATCGTTCTCCAT	CCCAAGGGAGTTCATCTTATCG	119
hGAPDH	ACAACCTTGGTATCGTGAAGG	GCCATCACGCCACAGTTTC	101
hACTIN	AATCTGGCACCACACCTTCTAC	ATAGCACAGCCTGGATAGCAAC	170

RESULTS

Differentiation of MSCs into NP-like Cells

The results depicted in Figure 1A showed that MSCs differentiated in CM had an elongated morphology, whereas DM cultured cells were large and flat (Figure 1A). The differentiated cells stained with alcian blue indicating the presence of glycoproteins in the ECM (Figure 1B). The fact that matrix protein expression was higher in cells differentiated in DM suggest that it is more inductive than CM. DM-differentiated cells expressed chondrogenic-specific genes, *SOX9*, *ACAN*, and *COL2*, as well as NP-specific markers, *FOXF1*, *PAX6*, *CA12*, and *COMP*, with poorly expressed *KRT19* (Figure 1C). They also expressed proteins, SOX9, ACAN, COL2, and FOXF1 (Figures 1D and 1E). The differentiation efficiency of MSCs into NPCs was 36% to 45% as determined by a decrease in the percentage of cells positively expressing MSC-specific surface markers (Figures 1F and 1G). These cells also had larger cell size than MSCs (Figure 1H). Based on the ability of MSC derivatives to produce ECM and express NP-specific markers, they were denoted NPCs.

Transplantation of NP-like Cells in the Degenerated IVDs

NPCs labeled with PKH26 with >95% labeling efficiency (Figure 2A) and >95% cell viability (Figure 2B) were transplanted into the degenerated IVDs, L4-5, using a Hamilton syringe into the central NP region of the IVDs. IVDs displayed degeneration 2 wk after puncture, and a progressive loss of disc height was confirmed by MRI analysis 6 wk post-transplantation (Figure 2C). Disc height improved in NPC-transplanted IVDs by 25% and 38% ($P < .05$) as compared to the degenerated and sham IVDs, respectively. NPC-treated IVDs had only 12% less disc height when compared with healthy IVDs (Figure 2D). In addition, there was a significant increase in NP T2-weighted MRI signal intensity of NPC-treated IVDs compared to the degenerated disc and sham (Figures 2C and 2E).

Physicochemical Evaluation of IVDs

The physical content of the NP was significantly reduced in degenerated and sham IVDs as compared to healthy discs. In fact, degenerated and sham IVDs were calcified and displayed partial loss of NP. Degenerated IVDs that received NPCs showed significant improvement in NP content with concomitant increase in sulfated glycosaminoglycan (sGAG) and water content when compared with sham IVDs (Figures 3A-3C). After 8 wk, sGAG decreased 38% and 53% in degenerated and sham IVDs, respectively. The sGAG content in the NPC-treated IVDs improved to 91% compared to sham and was similar to healthy IVDs (Figure 3B). Likewise, water content of NP in transplanted IVDs was not significantly different from healthy IVDs (Figure 3C).

Postharvested Histology of Rabbit IVDs

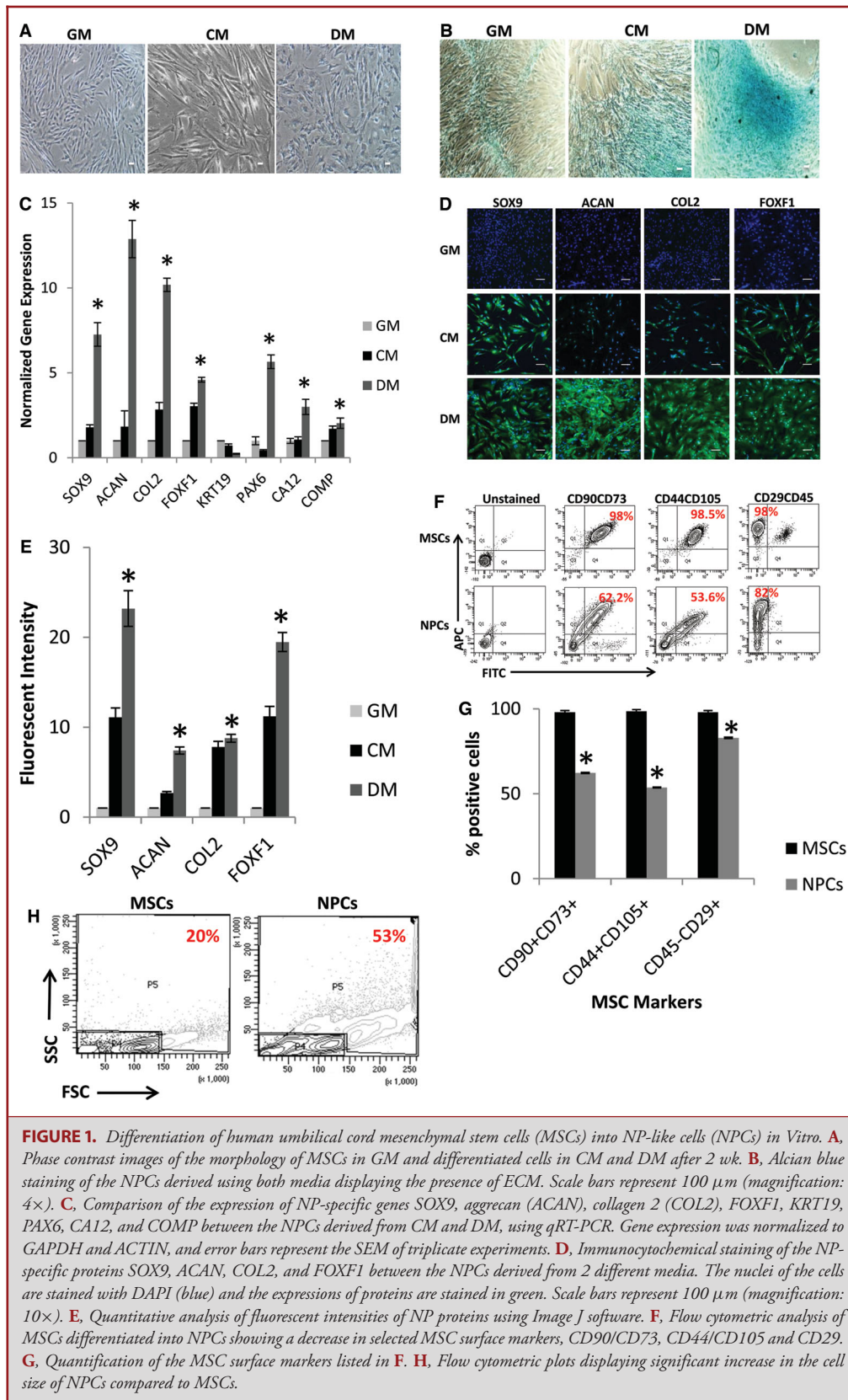
Histological analysis of IVDs was performed to determine the presence of ECM proteins as well as cellular and

structural integrity. PAS staining indicated fissured morphology with less ECM content in the NP of the degenerated compared to the healthy IVDs (Figure 3D). In contrast, ECM content was improved and uniformly distributed in the degenerated IVDs that received NPCs. Alcian blue staining also showed improvement in the distribution of glycoproteins and cellularity in IVDs transplanted with NPCs than that of degenerated or sham IVDs (Figure 3D). In fact, NPC-transplanted IVDs appeared similar to the healthy IVDs. H&E staining also showed that unlike degenerated IVDs, NPC-transplanted IVDs showed improvement in structural and cellular integrity (Figure 3D).

Immunotracking and Expression of Human-Specific Markers

PKH26-labeled cells were localized in the NP region of the transplanted but not in sham IVDs (Figure 4A). Both transplanted MSCs and NPCs survived in rabbit IVDs. While MSCs remained localized at the transplantation site, NPCs were more dispersed and integrated into the host NP. Expression of chondrogenic genes, *SOX9*, *ACAN*, *COL2*, and NP genes, *FOXF1*, *KRT19*, *PAX6*, *CA12*, and *COMP*, were significantly upregulated in NPC compared to MSC-transplanted IVDs (Figure 4B). In fact, the expression of these genes was significantly higher in NPC following transplantation suggesting that the IVD microenvironment played a role in upregulating the expression of NP-specific genes. Further analysis of IVDs transplanted with MSCs showed high expression of CD90 but lower levels of SOX9, ACAN, and COL2 (Figure 4C). IVDs transplanted with NPCs expressed high levels of both chondrogenic proteins, SOX9, ACAN, and COL2, and the NP protein FOXF1 (Figure 4C). As expected, human housekeeping protein TUBB was expressed in IVDs transplanted with MSCs and NPCs. These results showed the colocalization of human proteins and PKH26 labeling in the NP region of IVDs. Quantification of the mean fluorescent intensity in Figure 4D showed that the intensity of FOXF1 was not detectable in the case of MSC-treated IVDs.

To explore the underlying molecular mechanism involved in the ability of NPCs to regenerate NP in Vivo, various signaling pathways that play a role in the differentiation process were investigated. Transcriptional analysis of the IVDs showed that *TGFβ1*, *TGFβ2*, *SMURF1*, *SMURF2*, *MEKK1*, *SMAD2*, *SMAD3*, *SMAD4*, *SMAD5*, *ERK1*, and *ERK2* were significantly upregulated and *BMP2*, *BMP4*, and *BMP7* were not expressed in the transplanted IVDs as compared to the NPC in Vitro (Figure 5A). Immunocytochemical analysis of NPC-transplanted IVDs revealed the expression of human-specific proteins, TGFβ2 and SMAD2 (Figure 5B) but not TGFβ1 and BMP2 (not shown). These results indicate that TGFβ1/Smad signaling potentially played a role in the ECM production and NP regeneration via TGFβ1 but not the BMP2/4/7 members of this pathway (Figure 5C).



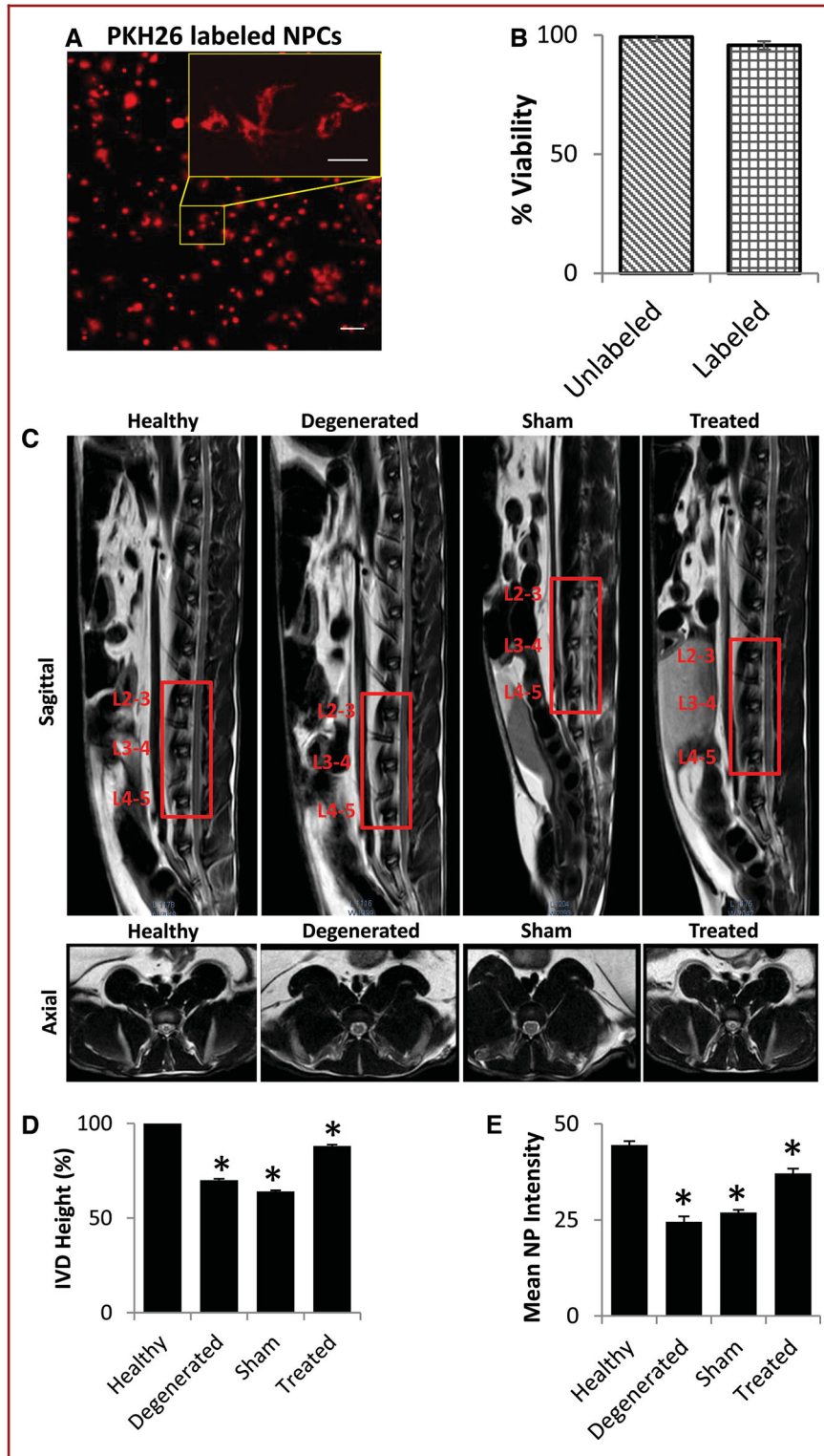


FIGURE 2. Transplantation of NPCs into IVDs in a rabbit model of DDD. **A,** Confocal image of the NPCs labeled with PKH26-Cy3 dye. Scale bars represent 100 μm (magnification: 10 \times). Insert represents higher magnification, 100 μm (magnification: 20 \times). **B,** Trypan blue assay on the labeled NPCs depicting the viability of the cells. **C,** Sagittal T2-weighted and axial view MRI of the rabbit lumbar spine comparing healthy and degenerated IVDs, and degenerated IVDs followed by treatment with medium alone (sham) or NPCs as determined 6 wk post-transplantation. Sagittal view images of healthy disc with normal height, degenerated and sham discs with reduced height and treated disc with improvement in the height. Axial view of the NP also showed normal NP intensity in the healthy disc, which was significantly decreased upon degeneration and sham treatment. NPC-treated discs showed significant improvement in NP intensity. **D,** Quantification of disc height of healthy, degenerated, sham and treated IVDs using Philips DICOM viewer software (Philips Healthcare). **E,** Quantification of mean NP signal intensity of healthy, degenerated, sham and treated IVDs using Philips DICOM viewer software.

DISCUSSION

We have previously shown that mouse ESC-derived chondroprogenitors exhibited promising results in the regeneration of the degenerated IVD.²¹ However, human ESC-based therapies face ethical and moral challenges as well as pose the risk of teratoma formation.^{37,38} MSCs from adult sources have been tested for their therapeutic potential to treat DDD.^{18,19,26,28,30} However, isolation of MSCs from adult sources requires invasive procedures, and they have limited proliferation and differentiation potential due to aging.³⁹⁻⁴¹ Several studies have been attempted to investigate the use of perinatal MSCs to regenerate IVDs,^{23,27,32,42} which are more advantageous since they can be obtained noninvasively in large numbers. Furthermore, they display a lower risk of graft vs host disease compared to bone marrow MSCs.^{43,44} Nevertheless, donor cells/tissue matching with the recipient would be beneficial. We hypothesized that MSCs differentiated into the chondrogenic lineage could be more effective in regenerating NP, since chondroprogenitors are capable of producing sGAG and glycoproteins in animal models.^{21,24,45,46}

The results showed that MSCs differentiated in DM produced ECM and expressed NP-specific genes and proteins. This is supported by the studies that TGF β and IGF, components of DM promote differentiation of MSCs into NP lineage.^{35,47-53}

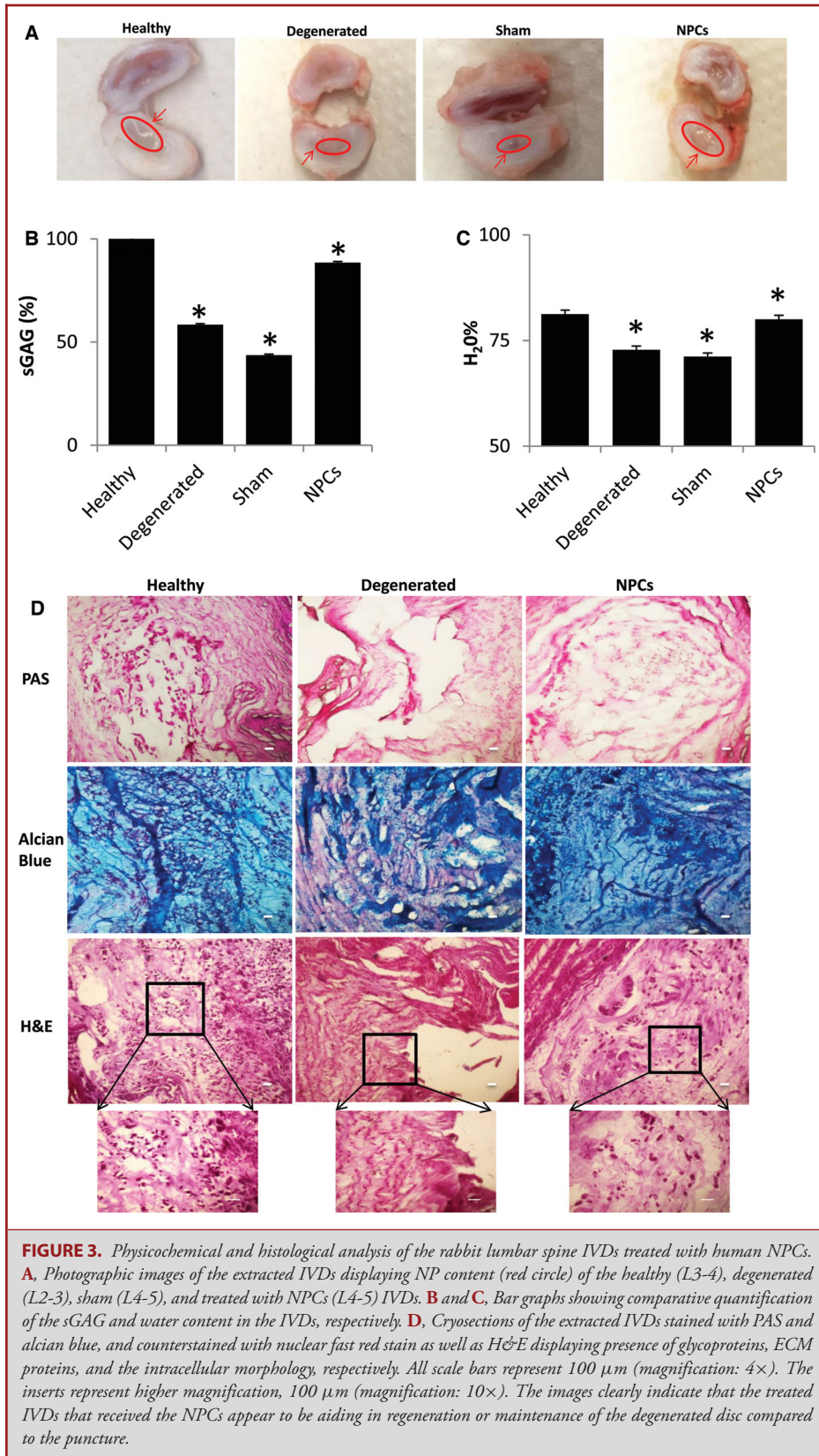
In this study, we differentiated MSCs into NPCs to test their efficacy for regeneration of damaged NP using the rabbit model for DDD. Histological analysis provided evidence of increase in the amount and distribution of ECM, proteoglycans, and glycoproteins as well as enhanced cellularity in the NP of the IVDs transplanted with NPCs. Furthermore, improvement in the structural integrity of NP was observed. These results are in agreement with other studies that showed improvements in cellularity and glycoproteins using cell therapy in IVDs of animal models.^{23,26,30,32} In addition, the transplanted IVDs showed a significant increase in the amount and distribution of sGAG in the NP, suggesting that the human NPCs xenogenetically influenced the metabolic activity of the rabbit IVDs. In our animal model, no Modic changes were observed in the degenerated or regenerated IVDs, perhaps because Modic changes are usually

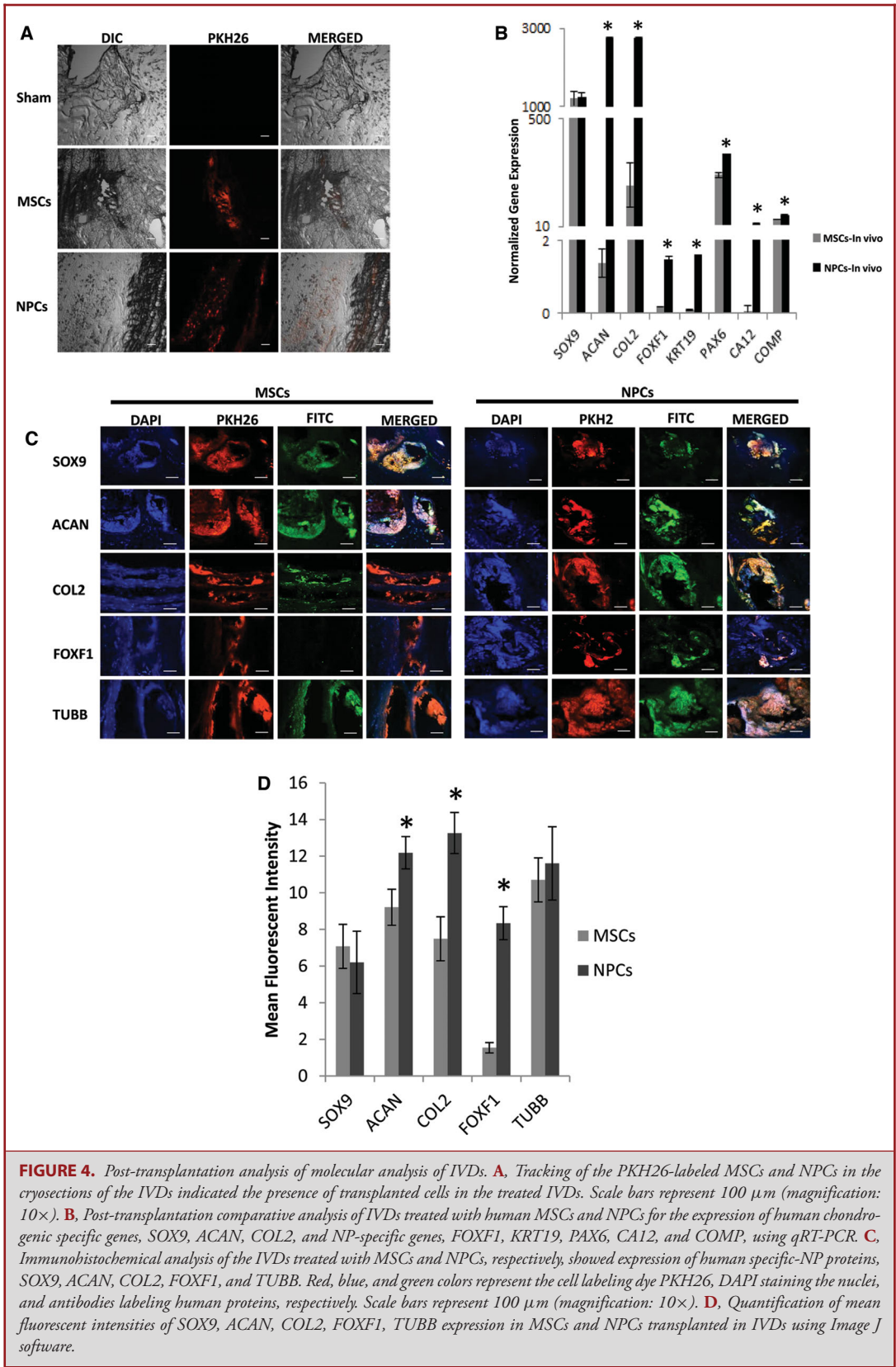
associated with chronic degeneration of the IVD.⁵⁴ A long-term study would be needed to explore the effects of cell therapy on Modic changes.

Transplantation of NPCs helped improve the disc height and physicochemical characteristics of the NP as determined by MRI analysis. The fact that degenerated and sham IVDs showed that calcification and partial loss of NP was inhibited by NPC treatment provides preliminary indication of halting the degeneration process. The functional and metabolic restoration of the NP depends entirely on sGAG, NP content, and disc hydration.⁵⁵ Quantitative analysis of the NP of the rabbit IVDs revealed that sGAG, water content, and physical size of the NP were significantly improved due to augmentation with NPCs.

Expression of human specific markers, SOX9, ACAN, COL2, and FOXF1 in the NP of the treated IVDs, suggested that the transplanted human NPCs were viable and functionally active in the rabbit IVD. In fact, expression of acknowledged NP-specific genes, *FOXF1*, *KRT19*, *PAX6*, *CAI2*, and *COMP*,^{9,56,57} was higher in the NP tissue of the IVDs transplanted with NPCs when compared to MSCs. Interestingly, *KRT19* was found to be poorly expressed in NPCs in Vitro as discussed above. *KRT19* is known to be involved in structural integrity⁵⁸; therefore, it may have a role in the histological improvement of the NP in Vivo. These results suggest a possible role of microenvironment of IVDs in the survival and activity of the NPCs.

Our results also showed increased transcript levels of several intermediates of the TGF β /Smad pathway, *TGF β 1*, *TGF β 2*, *SMURF1*, *SMURF2*, *MEKK1*, *SMAD2*, *SMAD3*, *SMAD4*, *SMAD5*, *ERK1*, and *ERK2*, which could be the reason for the production of enhanced levels of sGAG and water contents as well as ECM proteins in the IVDs recipient of NPCs. Because *TGF β 1* was not expressed in the NP of IVDs transplanted with NPCs, TGF β signaling appeared to occur by the binding of TGF β 1 to TGF β 2 that triggered the upregulation of SMADs. It has been previously reported that *TGF β 1* gene therapy increased proteoglycan synthesis.⁵⁹ These results are supported by the reports that the members of the TGF β superfamily play an important role in chondrogenic differentiation⁶⁰ and the expression of TGF β is decreased in IVD cells upon





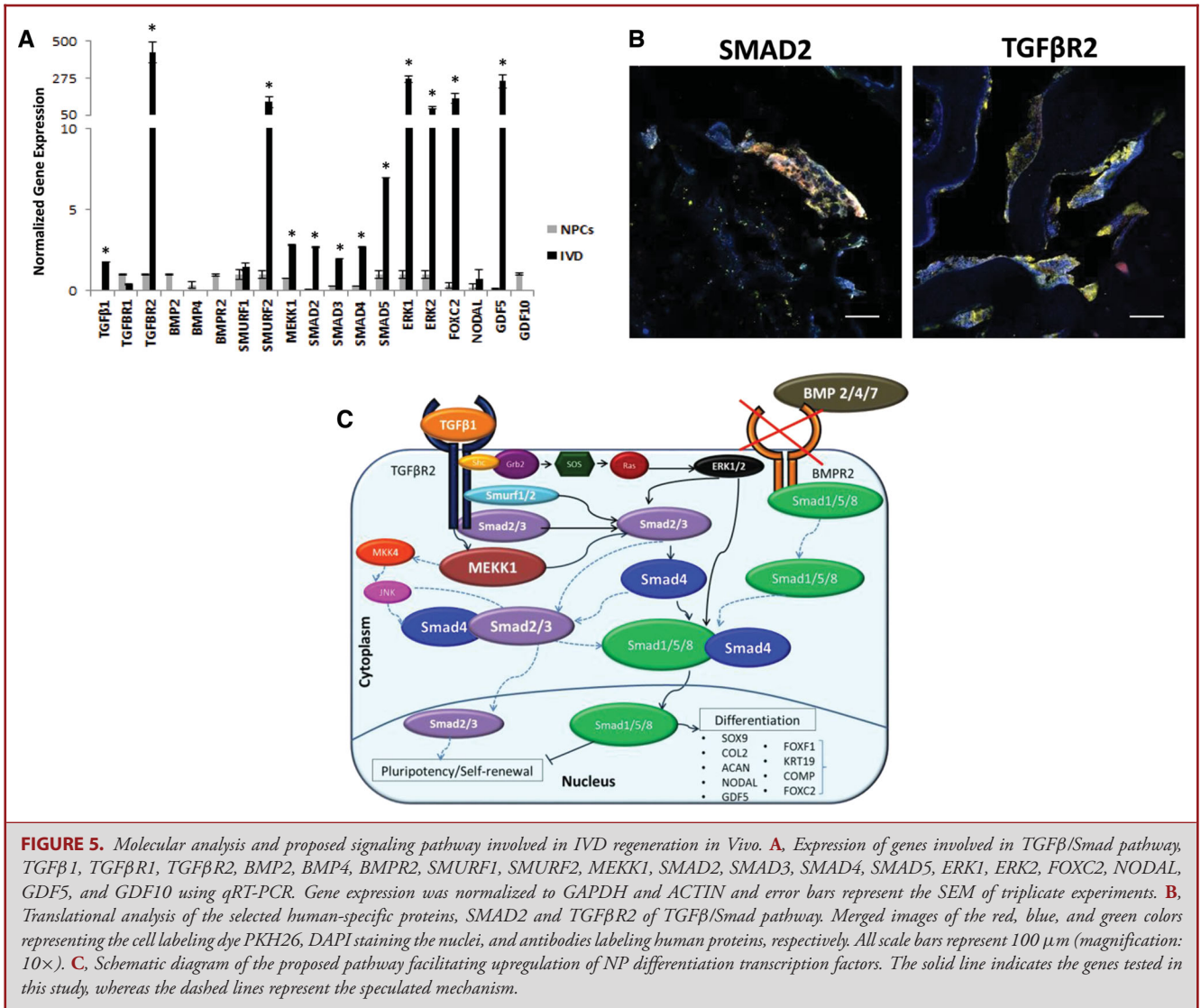


FIGURE 5. Molecular analysis and proposed signaling pathway involved in IVD regeneration in vivo. **A**, Expression of genes involved in TGFβ/Smad pathway, TGFβ1, TGFβR1, TGFβR2, BMP2, BMP4, BMPR2, SMURF1, SMURF2, MEKK1, SMAD2, SMAD3, SMAD4, SMAD5, ERK1, ERK2, FOXC2, NODAL, GDF5, and GDF10 using qRT-PCR. Gene expression was normalized to GAPDH and ACTIN and error bars represent the SEM of triplicate experiments. **B**, Translational analysis of the selected human-specific proteins, SMAD2 and TGFβR2 of TGFβ/Smad pathway. Merged images of the red, blue, and green colors representing the cell labeling dye PKH26, DAPI staining the nuclei, and antibodies labeling human proteins, respectively. All scale bars represent 100 μm (magnification: 10×). **C**, Schematic diagram of the proposed pathway facilitating upregulation of NP differentiation transcription factors. The solid line indicates the genes tested in this study, whereas the dashed lines represent the speculated mechanism.

aging.⁶¹ Based on these observations, a molecular mechanism involved in the functioning of NPCS and regeneration of NP in the IVDs via TGFβ1 is proposed. However, additional experiments are warranted to determine the signaling mechanism.

Due to the fact that NP is an avascular tissue,⁶² no inflammatory response was observed in the transplanted IVDs. Furthermore, perinatal stem cells have low risk of rejection-making NPCS derived from umbilical cord MSCs an ideal source for treating DDD in humans.

Limitations

The results of this study provide more compelling evidence for the efficacy of MSC derivatives for devising strategies to treat not only DDD but also other degenerative diseases. However,

this study was carried out using a limited number of animals and further blinded investigations utilizing a greater number of animals or use of a large animal model would be helpful. Nevertheless, the significance of our results is strengthened by a recent clinical study,⁴² reporting treatment of 2 patients with chronic discogenic low back pain using umbilical cord cells resulting in alleviation of pain and functional improvement.

CONCLUSION

This study demonstrates for the first time that NPC-derived MSCs were functionally active in the rabbit IVDs and showed the potential to repair or regenerate the degenerated NP tissue. The results further provide the impetus and basis for clinical trials for treating DDD using MSC-derived NPCS.

Disclosures

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COMMENT

The current published study is an extremely important piece of work demonstrating that human-derived umbilical mesenchymal stem cells that are grown in differentiated medium express specific nucleus pulposus genes and proteins that ultimately can repair the damaged nucleus pulposus in a rabbit model of degenerative disc disease. The study end-points are comprehensive and include histological and imaging criteria of success.

The above animal studies are critical elements required to move stem cell injections to treat degenerative disc disease in humans (a ubiquitous disease) in a non-invasive fashion. Due to the relative avascular nature of the nucleus pulposus – the current study suggests that immune rejection from MHC disparities will pose less of a problem. We look forward to more data from this group as this exciting area of research moves forward.

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