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Original Article

Identification and quantitative analysis of extracellular matrix in rabbit nucleus pulposus and cartilage

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Abstract: This study aims to establish an identification standard for nucleus pulposus and cartilage tissue by analyzing the extracellular matrix in normal nucleus pulposus and cartilage. Rabbit nucleus pulposus and cartilage tissue were isolated from the New Zealand rabbit for the determination of water content. Furthermore, cells separated from nucleus pulposus and cartilage tissue were cultured respectively, and then the cell morphology, type II collagen content and red O staining results were evaluated and compared. The mRNA expressions of proteoglycan and type II collagen were also determined. Not only the water content in nucleus pulposus was remarkably higher than that in cartilage, but also proteoglycan was significantly higher in nucleus pulposus than in cartilage tissue. However, type II collagen content in nucleus pulposus was lower than that in cartilage tissue. In addition, red O staining showed positive in nucleus pulposus cells but weakly positive in cartilage cells. The ratio values of proteoglycan/type II collagen were 15:1 in nucleus pulposus cells and 1.6:1 in cartilage cells, respectively. The ratio values of proteoglycan/type II collagen were 15:1 in nucleus pulposus cells or nucleus pulposus-like cells, which can be used as an identification standard for nucleus pulposus cells.

Keywords: Nucleus pulposus cells, cartilage cells, proteoglycans, type II collagen, intervertebral disc degeneration

Introduction

Spinal diseases, including lubar intervertebral disc protrusion whose main symptom is lumbago, seriously influence the quality of life of patients all the time [1, 2]. It has been shown that about 70% of the patients with lubar intervertebral disc protrusion will eventually appear the symptom of lumbago [3]. Although the causes are still unclear, lumbar degeneration may play a key role [4]. Most patients with degenerative lumbar spine disease do not need treatment, but about one-third patients need long-term treatment or even in-patient care. At present, the main treatment for lubar intervertebral disc protrusion is to slow down the degeneration and relieve the symptoms caused by degeneration, but few researches focused on the regeneration of intercalated disc. Currently, the main surgical methods in the treatment of lumbar intervertebral disc protrusion are intercalated disc excision and intervertebral fusion [5]. However, although surgical methods can achieve a good short-term clinical result, they change the biomechanical structure of spine and lead to degeneration in the adjacent segmental vertebral body [6]. Owing to the onset position of intervertebral disc degeneration, a lot of researches are now focusing on the tissue engineering of nucleus pulposus and have made great progress in the last decade.

Treatment for intervertebral disc degeneration is still limited now. Compared with the simple surgical treatment, biological treatment has become the latest model. Intervertebral disc regeneration, a method inducing bone marrow mesenchymal stem cells into nucleus pulposus cells by tissue engineering technique and implanting them in the degenerative intervertebral disc to repair or replace the degenerative disc tissue [7], can achieve a long-term and safe therapeutic effect. So far, however, no specific marker for nucleus pulposus cells has yet been found. In addition to the similar components in the extracellular matrix of cartilage tissue and nucleus pulposus tissue [8], it is difficult to identify whether bone marrow mesenchymal stem cells successfully differentiate into nucleus pulposus cells.

In this study, we calculated the ratio value of proteoglycans/type II collagen content (proteoglycan/type II collagen) in normal nucleus pulposus cells and hyaline cartilage cells, and validated whether the proteoglycan/type II collagen ratio value can be an identification standard for nucleus pulposus and hyaline cartilage tissue. In addition, we also qualitatively and quantitatively analyzed and identified nucleus pulposus cells and cartilage cells based on the determination of water content, cell morphology and special dyeing.

Methods

Collection of nucleus pulposus and cartilage tissue from New Zealand rabbit

Six New Zealand white rabbit, aged two months and weighing 2.5 to 3.0 kg, were purchased from the Animal Center of the First Affiliated Hospital of Harbin Medical University (Qualified number: P00102008). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Harbin Medical University. Rabbits were sacrificed by intravenous injection of over dose of 3% pentobarbital sodium. Then the rabbit spines were separated from dorsal midline incision and bilateral knee joints were isolated in sterile condition. Subsequently, gelatin nucleus pulposus from six segmental intervertebral discs were removed by blunt dissection. Cartilage tissues in bilateral knee joint of the rabbits were stripped respectively in size 1 mm × 1 mm using sterile blade.

Isolation and culture of nucleus pulposus cells and cartilage cells

Preparation of nucleus pulposus cells: Thoracic lumbar spines separated from rabbits were flushed with PBS to remove blood. Then the annulus fibrosus disci intervertebralis was cut by a sharp knife and the intervertebral space was opened. After that, the gelatin nucleus pulposus was scraped out using a sterile curette and placed into a petri dish containing DMEM/F12 medium (Sigma, St. Louis, MO). After being cut into pieces, tissue blocks were transferred into the centrifugal tubes and centrifuged at 1000 r/min for 5 min, followed by digestion with trypsin (Sigma, st. Louis, MO) at 37°C for 10 min. After that, tissues underwent another digestion with 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) at 37°C for 30 min. During the digestions, the cells were shaken gently for several times and observed under inverted microscope to monitor the digestive effect. When the tissues were digested in almost single cell suspension, medium containing serum was added to stop the digestion. Subsequently, cells were filtered by a sterile nylon mesh filter (Ø 75 μm) and centrifuged at 1000 r/min for 5 min. Cells were then resuspended with DMEM/F12 medium containing 15% FBS and cultured at a density of 1×10^4/mL in an incubator containing 5% CO_2 at 37°C. Medium was changed every 3 d. When cells grew to 90% confluence, they were subcultured.

Preparation of cartilage cells: Cartilage tissues from rabbit bilateral knees were isolated under a sterile condition and washed with PBS. Then cartilage tissues were cut into 1 mm × 1 mm × 1 mm pieces in a culture dish containing DMEM/F12 medium and subsequently transferred into centrifugal tubes and centrifuged at 1000 r/min for 5 min. The sediment was digested with trypsin at 37°C for 60 min and stopped by medium containing serum, followed by another digestion with 0.2% type II collagenase at 37°C for 8 h. Then cells were treated with the similar protocol to the nucleus pulposus cells.

Determination of water content in tissues

Nucleus pulposus and cartilage tissue samples were weighed immediately after isolation and then dried in a thermostat at 60°C for 48 h and weighed again. The water content in tissue was defined as the difference between the weights before and after drying divided by tissue weight before drying.

Histological staining

Immunohistochemical staining: Nucleus pulposus and cartilage tissue samples were conventionally dehydrated and waxed and made in blocks. Then they were cut into sections and treated with an immunohistochemical method for detecting the expression of type II collagen by following the procedure introduced in the immunohistochemical kit (Roche, Basel, Switzerland).

Table 1. Primer sequences and PCR product sizes

Primer	Sequence	Product size
COLA1	Forward: CAGCGTCACTGTCGATGGC	150 bp
	Reverse: AGGCGGGAGGTCTGGTG	
GPC	Forward: TCAGTGGGGACTGTGATGATG	147 bp
	Reverse: TGTTTGATGGATTTGCTTTACATCAC	
GAPDH	Forward: TCAATGACAACTTTGTCAAGCTCA	136 bp
	Reverse: GTGGGTGGTCCAGGGTTTCTTACT	

Red O staining: Nucleus pulposus cells and cartilage cells climbing pieces were fixed with 10% neutral formaldehyde and then rinsed with PBS. Subsequently, they were incubated with red O solution (Worthington Biochemical, Lakewood, NJ) for 15 min, followed by staining with fast green for 10 minutes. After washing with PBS and mounting, climbing pieces were observed under a light microscope.

Total RNA extraction and real-time PCR

The third passage of cartilage cells and nucleus pulposus cells were used for total RNA extraction with Ultrapure RNA Extraction Kit (Sigma, St. Louis, MO) following the instruction supplied in the kit. The purity and concentration of total RNA were analyzed by agarose gel electrophoresis and spectrophotometer detection. Then 2 μL RNA was transcribed into cDNA using HiFi-MMLV cDNA synthesis kit (Sigma, St. Louis, MO) following the manufacturer's instruction, and subsequently subjected to PCR amplification using UltraSYBR Mixture (Sigma, St. Louis, MO) according to the instruction. PCR reactions were then carried out in a CFX96 PCR machine (Bio-Rad, USA) for one cycle of 10 min at 95°C and 40 cycles with 15 s at 95°C and 60 s at 60°C. Specific gene primer pairs (**Table 1**) for proteoglycan and type II collagen were synthesized by Shanghai Invitrogen Inc. China. The relative expressions of proteoglycan mRNA and type II collagen mRNA were calculated with the formula $2^{-\Delta\Delta CT}$.

Statistical analysis

Data are shown as mean ± standard deviation (SD) and processed using SPSS v11.5 statistical software. Comparisons among several groups were carried out using analysis of variance, while comparisons between two groups were performed using *t* test. Intra-group comparisons were carried out using *q* test. A value of $P < 0.05$ was considered statistically significant.

Results

Cell morphology of cartilage cells and nucleus pulposus cells

As observed under an inverted phase contrast microscope, cartilage cells adhered at 12 h after the inoculation and completed deformation at 24 to 48 h after the inoculation. Most primary cartilage cells showed polygonal, abundant cytoplasm, round and centered nucleus with 2 or 3 apparent nucleoli and appeared obvious slabstone-like shape with the increasing cell number (**Figure 1A** and **1B**). However, nucleus pulposus cells adhered more slowly than cartilage cells. They adhered at 3 d after inoculation and part of them appeared fusiformate with clear outline and obvious nucleus with 1 or 2 nucleoli. After 14-day growth, nucleus pulposus cells began to transform gradually to long spindle and irregular shape and finally 80% of them transformed into long spindle shape (**Figure 1C** and **1D**).

Water content in cartilage and nucleus pulposus tissues

Water contents before and after drying revealed that water content in cartilage was about 41.42%, significantly lower than that in nucleus pulposus (about 82.34%, $P < 0.05$. **Table 2**).

Histological observation

Immunohistochemical staining showed that type II collagen was granularly positive in the cytoplasm of cartilage cells, especially surrounding the nuclei, but less in nuclei (**Figure 2A**); however, it was weakly positive in the cytoplasm of nucleus pulposus cells (**Figure 2B**). Red O staining revealed that gathering proteoglycan was positive in nucleus pulposus cells (**Figure 2C**) but weakly positive in cartilage cells (**Figure 2D**). The cytoplasm of nucleus pulposus cells was stained in pink, with brown granular materials surrounding the nuclei.

The mRNA expression of type II collagen and gathering proteoglycan in cartilage cells and nucleus pulposus cells

The mRNA expression of type II collagen and gathering proteoglycan in 6 cases of cartilage

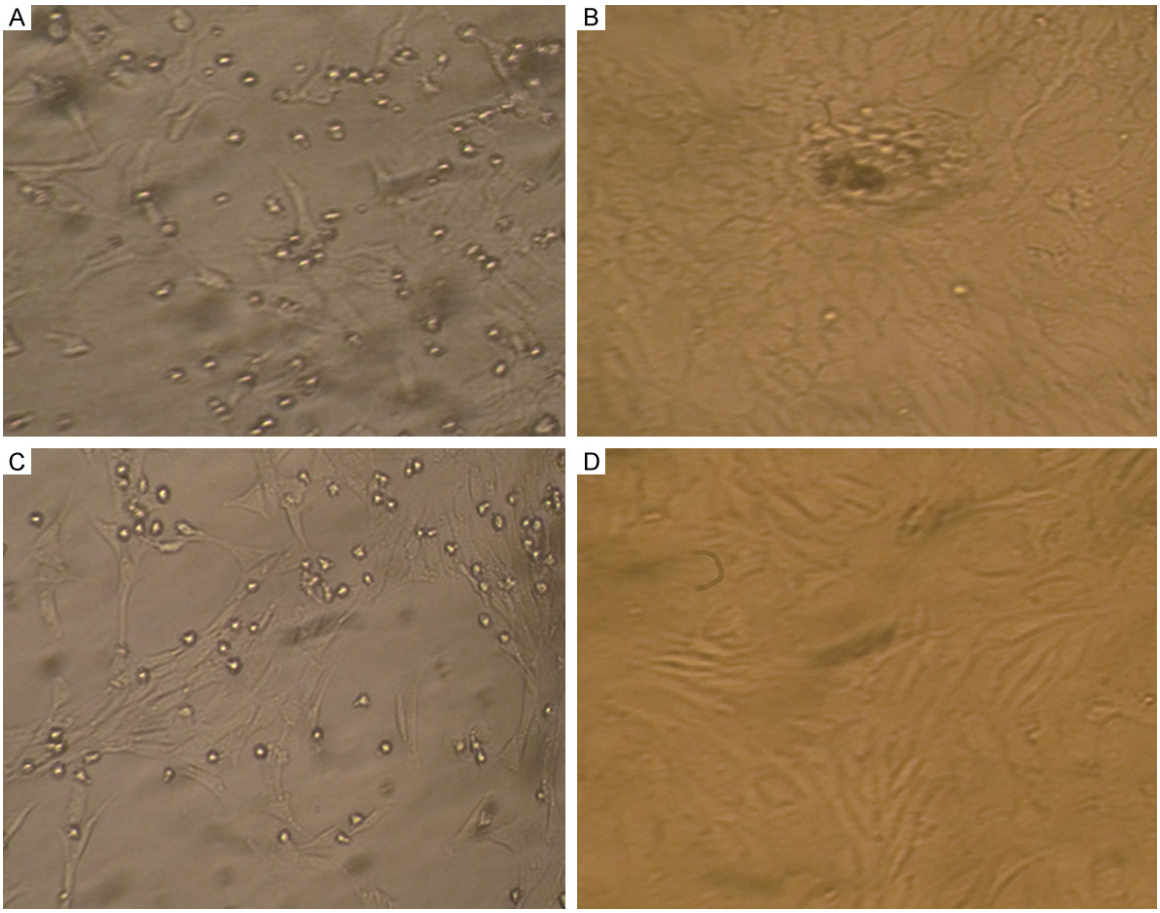

Figure 1. Cell morphology of cartilage cells and nucleus pulposus cells. A. Primary cartilage cells at 48 h showed polygon; B. Primary cartilage cells at 4 d showed obvious slabstone-like appearance; C. Primary nucleus pulposus cells at 3 d showed fusiformate; D. Primary nucleus pulposus cells at 14 d showed obvious long spindle.

Table 2. Water content in cartilage and nucleus pulposus tissues

Group	Weight before drying (g)	Weight after drying (g)	Mean water content (%)
Nucleus pulposus	0.0956	0.0182	82.34%
	0.0974	0.0174	
	0.1024	0.0166	
	0.1047	0.0168	
	0.1006	0.0190	
	0.0904	0.0178	
Cartilage	0.0873	0.0513	41.42%
	0.0904	0.0520	
	0.0913	0.0524	
	0.0885	0.0526	
	0.0890	0.0527	
	0.0895	0.0530	

cells and 6 cases of nucleus pulposus cells were determined by real-time PCR. The mRNA expression of gathering proteoglycan was significantly higher in nucleus pulposus cells than in cartilage cells, while type II collagen mRNA expression was remarkably higher in cartilage cells than in nucleus pulposus cells (**Table 3**). The proteoglycan/type II collagen ratio value in cartilage cells was 1.6/1, which was prominently lower than that in nucleus pulposus cells (15/1, $P < 0.05$).

Discussion

Currently, tissue engineering has become a hot research topic for the treatment of intervertebral disc degeneration. However, how to accurately identify the nucleus pulposus cells remains a problem, although lots of methods are still used, such as cell morphological identification, cell surface markers, cytokines, levels of proteins, genetic

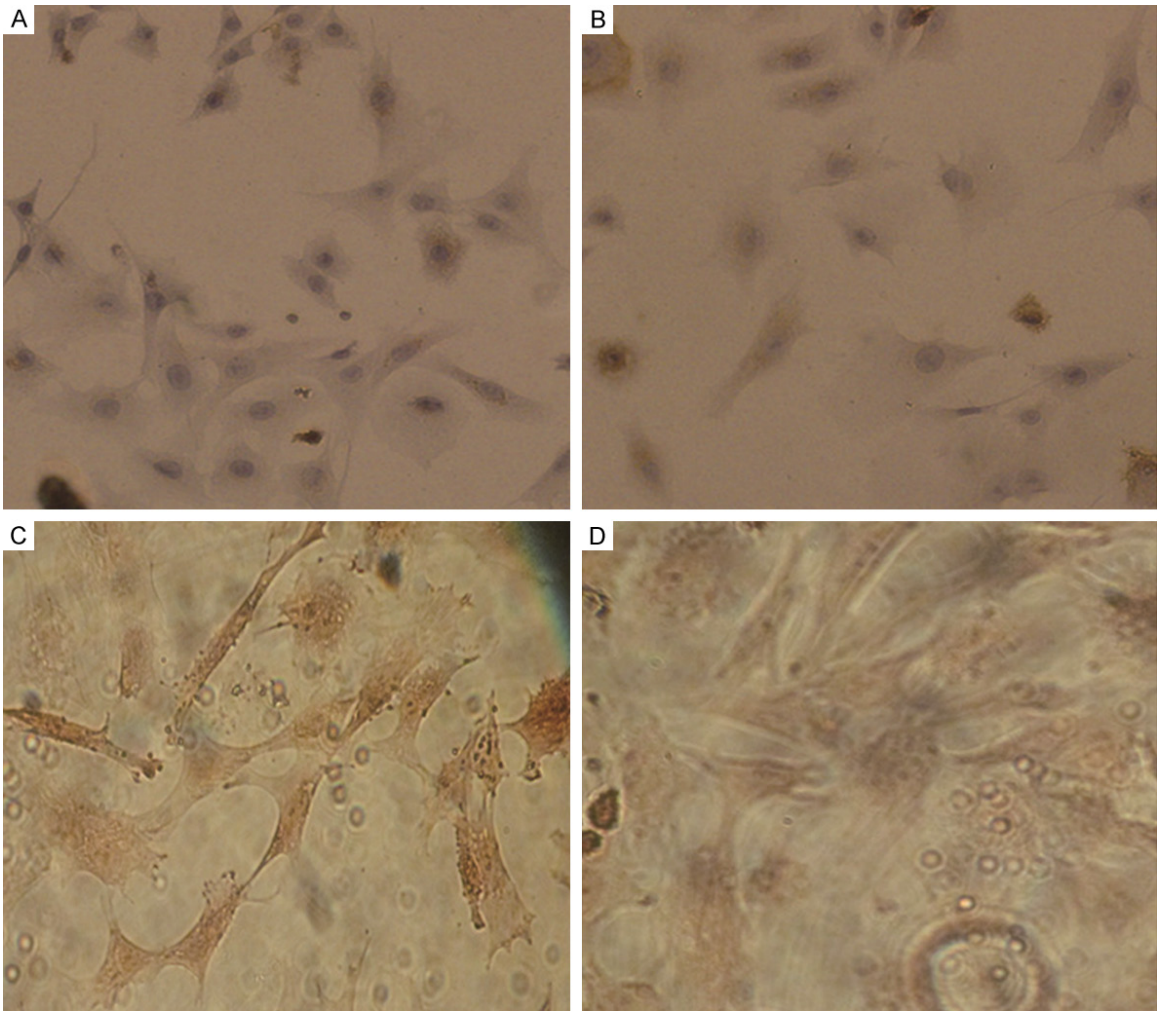

Figure 2. Expression of type II collagen and gathering proteoglycan in cartilage cells and nucleus pulposus cells. A. Type II collagen was positive in cartilage cells detected by IHC; B. Type II collagen was weakly positive in nucleus pulposus cells detected by IHC; C. Gathering proteoglycan was positive in nucleus pulposus cells determined by red O staining; D. Gathering proteoglycan was weakly positive in cartilage cells determined by red O staining.

Table 3. The mRNA expression of gathering proteoglycan and type II collagen in cartilage cells and nucleus pulposus cells

Group	Gathering proteoglycan		Type II collagen	
	mRNA expression	Ct value	mRNA expression	Ct value
Cartilage cells	1.00 ± 0.32	36.61 ± 0.38	1.00 ± 0.80	36.25 ± 1.12
Nucleus pulposus cells	36.34 ± 5.17	35.98 ± 0.20	0.34 ± 0.09	39.58 ± 0.00

Data are shown as mean ± SD.

markers and so on. Cell surface markers may have certain significance for the identification of nucleus pulposus cells, but it should be based on the exclusion of all other cells expressing the same markers. In addition, detection of cell surface markers is complex and expenses more resources. Cytokines detection also plays some roles in the identification of nucleus pulposus cells. It has been reported that the expressions of hypoxia inducing factor 1 (HIF-1), glucose transporter protein 1 (GLUT-1) and matrix metalloproteinase-2 (MMP-2) in nucleus pulposus cells and cartilage cells are significantly different [9], thus combined application of indexes can improve the identification rate of nucleus pulposus cells. But this method produces plenty data and lacks of specificity. In terms of gene identification, there are 24 genes showing significantly different expression

between nucleus pulposus cells and cartilage cells, for example, PAX1, FOXF11, CA12, OVOS2 and other genes are higher expressed in nucleus pulposus cells than in cartilage cells [10]. However, the detection method has complex procedures and lacks a specific data support.

Intervertebral disc is mainly composed of water, gathering proteoglycan and collagen fibers, which obviously differently distribute in the different parts of intervertebral disc. Collagen fiber distributes most in the outer layer of anulus fibrosus and least in nucleus pulposus cells, while protein and water distribute most highly in nucleus pulposus. The proteoglycan and type II collagen expressed in nucleus pulposus cell play a crucial role when intervertebral disc is under pressure [11]. Nucleus pulposus cells has the strongest ability to synthesize sulfate proteoglycan, which is helpful to balance the extracellular matrix of nucleus pulposus and maintains the biomechanical function of intervertebral disc [12]. Intercalated disc tissue is constituted by peripheral anulus fibrosus and central nucleus pulposus, among which anulus fibrosus is rich of collagen and nucleus pulposus is rich of proteoglycan [13-15]. Compared with articular cartilage, the nucleus pulposus tissue contains richer protein polysaccharide composition with resistance to pressure [16]. Both tissues contain proteoglycan which has a specific interaction with collagen [17, 18]. Eight kinds of collagen constituents have been found in nucleus pulposus tissue, including collagen type I, II, III, V, VI, IX, X and XI, among which type II collagen is the main composition in nucleus pulposus and articular cartilage tissue [19-21]. Considering these characteristics of nucleus pulposus cells and the complexity in its discrimination from cartilage cells, this study expected to use proteoglycan/type II collagen ratio value as an identification criterion for the nucleus pulposus and hyaline cartilage tissue.

In this study, we directly isolated nucleus pulposus cells and cartilage cells from rabbit and cultured them for identification. The results showed that the water content in nucleus pulposus was significantly higher than that in cartilage, which is consistent with the characteristics that nucleus pulposus cells contain the highest content of protein and water. Immunohistochemical staining on the climbing pieces showed that nucleus pulposus cells expressed more proteoglycan but less type II collagen than cartilage cells, which indicated the ratio value of proteoglycan/type II collagen in nucleus pulposus cells would be significantly higher than that in cartilage cells, consistent with our quantitative results.

Red O, a basic stain, is one kind of good cationic dyes for histochemically quantifying the proteoglycan in cell matrix [22]. It can bind to polyanion in proteoglycan but not bind to collagen, thus it can indirectly reflect the content and distribution of proteoglycan in the matrix. This study showed that proteoglycan is positive in nucleus pulposus cells but weakly positive in cartilage cells, suggesting that the expression of proteoglycan in nucleus pulposus cells was obviously higher than that in cartilage cells.

We also detected the mRNA expression of proteoglycan and type II collagen in nucleus pulposus cells and cartilage cells by real-time PCR and the result showed the ratio value of proteoglycan/type II collagen was 15:1 in nucleus pulposus cells and 1.6:1 in cartilage cells, indicating that this ratio can provide more intuitive basis for the identification of nucleus pulposus cells.

Biological treatment for degenerative intervertebral disc is the application of tissue engineering technology, briefly, bone marrow mesenchymal stem cells is induced into nucleus pulposus cells and implanted into the degenerative intervertebral disc to repair or replace the degenerative disc tissues [7]. *In vitro* inducing system may have slight influence on the component expression, thus there are certain limitations in this study. To sum up, changing the cell culture model, further identifying the quantity and quality of the nucleus pulposus cells and providing high quality nucleus pulposus cells for intervertebral disc transplantation still need lot of researches.

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Disclosure of conflict of interest

None.

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