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Original Article

Identification of notochordal cell-specific molecular markers in mouse intervertebral disc

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Abstract: *Objective:* Dehydration and dysfunction of the nucleus pulposus (NP) are the main causes of intervertebral disc (IVD) degeneration, in which the ability of the disc to transfer and distribute loads between the vertebrae is decreased. Notochordal cells are involved in the early stages of NP development, but disappear in severely degenerated discs. These cells might play a protective role in preventing disc degeneration; however, the lack of consensus among researchers over an optimal NP cell marker makes it difficult to identify and isolate notochordal cells from IVDs in mice. This study aimed to identify a marker that distinguishes notochordal cells from annulus fibrosus (AF) cells and endplate chondrocytes. *Methods:* Specimens of intervertebral discs were taken from different age groups of C57BL/6J mice. Routine histological staining was performed to evaluate the changes in the disc matrix and cells. *In situ* immunofluorescence staining was performed to validate the proposed target markers, such as HIF-1α, GLUT-1, aggrecan, collagen II, SHH, brachyury, CD24. *Results:* The healthy NP of 1-week-old mice contained a high volume of notochordal cells in various sizes, which were tightly packed and uniformly arranged. In contrast, the degenerated NP of 22-month-old mice had cavities and clefts, the cells were disorderly, NP volume and cell number were reduced, and the cell shape and phenotype had changed; the cytoplasm showed eosinophilic staining. At last, CD24 was stably expressed on the surface of the notochordal cell membrane, as well as brachyury was expressed in the notochordal cell nucleus, but not in AF cells and endplate chondrocytes. *Conclusion:* IVD degeneration results in a decrease in notochordal cells, and CD24 and brachyury might be the notochordal cell markers. These results could be helpful in isolating and purifying notochordal cells for further studies.

Keywords: Intervertebral disc degeneration, notochordal cell, cellular marker, HIF-1α, GLUT-1, aggrecan, collagen II, SHH, brachyury, CD24

Introduction

Disc degeneration is a chronic progressive disease that can cause low back pain (LBP), which is a crippling condition and socioeconomic burden for the patient [1-4]. Several risk factors, such as age, smoking, obesity, occupation, posture, and genetic inheritance, have been reported to be related to intervertebral disc (IVD) degeneration disease and LBP [5, 6], but the mechanisms involved in this degenerative process have not been fully understood, and therapies are mainly aimed at symptomatic relief [7]. Recent in vitro and in vivo studies on biological therapeutic strategies aimed at regenerating the damaged nucleus pulposus (NP), the inner core of the vertebral disc, have shown promising results [8]. Dehydration and dysfunction of the NP are the main causes of IVD degeneration [9]. The NP is aggrecan-rich hydrated tissue without direct blood vessels [10]. Notochordal cells, which might play a protective role in preventing disc degeneration, are present in the early stages of NP development, but disappear in severely degenerated discs. Because of their role in IVD development and degeneration, these cells have been the focus of research in the tissue-engineering fields [11-13]. Findings suggest that the expression of the following markers differs between NP cells and other related cell types: HIF-1α [14, 15], glucose transporter type I (GLUT-1) [14], MMP-2 [14], VEGF

[16, 17], CD24 [18], collagen II [19], aggrecan [19, 20], brachyury [12, 21], and sonic hedgehog (SHH) [12, 22]; however, a key challenge for these studies in mice is that the lack of a specific cellular marker makes it difficult to identify and isolate their notochordal cells from the IVD. This study aimed to identify a marker that distinguishes notochordal cells from annulus fibrosus (AF) cells and endplate chondrocytes in mice.

Materials and methods

Animals

Wild-type C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and fed a regular chow diet. All mice were raised in accordance with Shanghai Jiao Tong University School of Medicine Animal Care and Use Committee Guidelines under specific pathogen-free conditions.

Five-day-old and 1-week-old mice were euthanized by hypothermia and decapitated. Four-week-old and 22-month-old mice were euthanized using a very high dose of sodium pentobarbital (2.5%) followed by cervical dislocation. All procedures were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine.

Routine histological staining

For light microscopy, tissues from 1-week- and 22-month-old mice were fixed in 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS) (pH 7.4) for 24-48 h at 4°C followed by decalcification in fresh 10% ethylenediaminetetraacetic acid solution (pH 7.4) at 4°C for up to 10 d for 1-week-old specimens and 6 weeks for 22-month-old specimens. The specimens were then dehydrated in ethanol, transferred to xylene, and embedded in paraffin according to standard histological procedures. Sections of 5 μm thickness were cut at several levels of the IVDs from different specimens and used for hematoxylin and eosin (H&E) staining according to standard protocols. The morphology of the NP was examined and assessed according to histological characteristics, such as mucoid degeneration, cell death, tears and clefts, and granular changes [23] as well as changes in cell volume, shape, and vacuoles.

Cryosections

Specimens of the lumbar intervertebral discs from 5-day- and 4-week-old mice were fixed in 4% PFA/PBS (pH 7.4) at 4°C overnight, followed by dehydration and storage in 70% ethanol at 4°C. IVDs from the superior and inferior vertebrae of 4-week-old mice were carefully separated. The specimens were then immersed in 20% sucrose/PBS (0.1 M) solution for 2-3 h, placed in 30% sucrose/PBS (0.1 M) solution at 4°C overnight, and then preserved in an embedding medium at optimum cutting temperature (OCT) at -20°C until use. Sections 10 μm thick were cut at several levels of IVDs from different OCT blocks. This procedure was performed at -25°C using the Leica Cryostat (Leica Biosystems, Inc., Buffalo Grove, IL, USA). Sections were then used for immunofluorescence staining or stored at -80°C.

In situ immunofluorescence staining

For *in situ* immunofluorescence staining of 5-day-and 4-week-old sections, the cryosections were treated with 0.25% Triton X-100 for 10 min to permeabilize the cells and incubated with the following primary antibodies: 1:150 anti-HIF-1-alpha (Abcam, Cambridge, UK), 1:200 anti-brachyury (Abcam, Cambridge, UK), 1:150 anti-collagen I (Abcam, Cambridge, UK), 1:150 anti-col2a (Abcam, Cambridge, UK), 1:150 anti-collagen III (Abcam, Cambridge, UK), 1:150 anti-SHH (Abcam, Cambridge, UK), and 1:150 anti-aggrecan (Abcam, Cambridge, UK) overnight at 4°C. The cryosections without permeabilization were incubated with 1:150 anti-CD24 (Abcam, Cambridge, UK) and 1:150 anti-GLUT-1 (Abcam, Cambridge, UK) overnight at 4°C. The sections were then treated with each of the following: anti-HIF-1-alpha, anti-collagen II, anti-SHH, and anti-CD24 antibodies were incubated with the secondary antibody (labeled with fluorescein isothiocyanate [FITC]) in 1.0% bovine serum albumin (BSA) in phosphate buffered saline with Tween (PBST) in a humidified chamber for 1 h in the dark at room temperature. The sections, treated with each of the following: anti-brachyury, anti-collagen I, anti-collagen III, anti-aggrecan, and anti-GLUT-1 antibodies, were incubated with the secondary antibody (labeled with CY3) in 1.0% BSA in PBST in a humidified chamber for 1 h in the dark at room temperature. The sections were

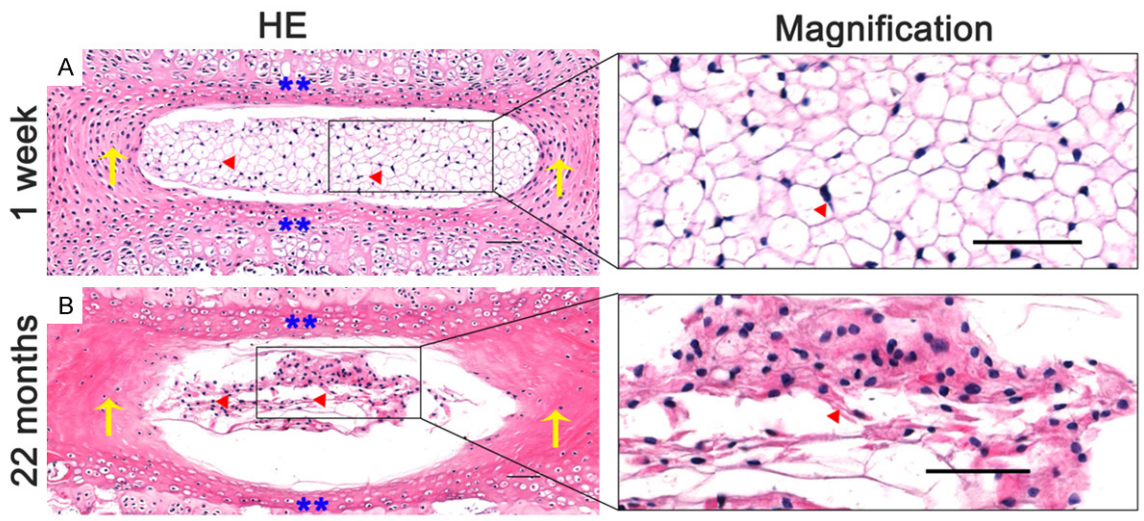

Figure 1. Hematoxylin and eosin (H&E) staining of intervertebral discs (IVDs) of (A) 1-week- and (B) 22-month-old mice. The size of the nucleus pulposus (NP) was significantly smaller in 22-month-old mice than that in 1-week-old mice. In 1-week-old mice, the NP contained a large volume of notochordal cells of various sizes, which had large vacuoles in the cytoplasm and the nuclei were aside. The cells were tightly packed and uniformly arranged. In 22-month-old mice, a cavity within the inner disc, disorderly cells, decreased NP volume and cell number, and changed cell shape and phenotype were observed. Magnification ×20; scale bars 100 μm; N = 3. The triangle symbol indicates notochordal cell, the arrow indicates annulus fibrosus cell, the asterisk symbol indicates endplate chondrocyte.

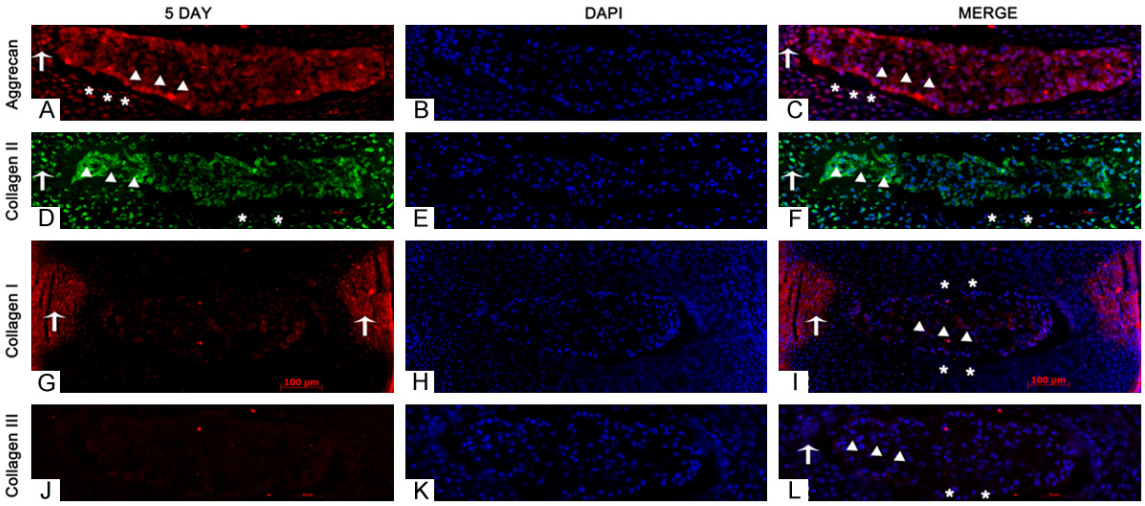

Figure 2. In situ immunofluorescence assay of protein markers related to the notochordal cells of 5-day-old mice. A-C: Aggrecan is more highly expressed in notochordal cells than in annulus fibrosus (AF) cells and endplate chondrocytes. D-F: Collagen II is evenly expressed in the IVD. G-I: Collagen I is expressed near the periphery of (AF). J-L: Collagen III is not expressed in the IVD. Scale bars 100/50 μm; N = 3; red/green signals are positive. NP: nucleus pulposus, AF: annulus fibrosus, CE: cartilaginous endplates. The triangle symbol indicates notochordal cell, the arrow indicates annulus fibrosus cell, the asterisk symbol indicates endplate chondrocyte.

then washed clean in the dark with cold 0.1% PBS and mounted on a slide with a drop of SlowFade® Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (Life Technologies, Grand Island, NY, USA).

Image acquisition

Images were captured using a Carl Zeiss CFM-500Z compound microscope (Carl Zeiss CFM-500Z, Germany). For fluorescent images, cryo-

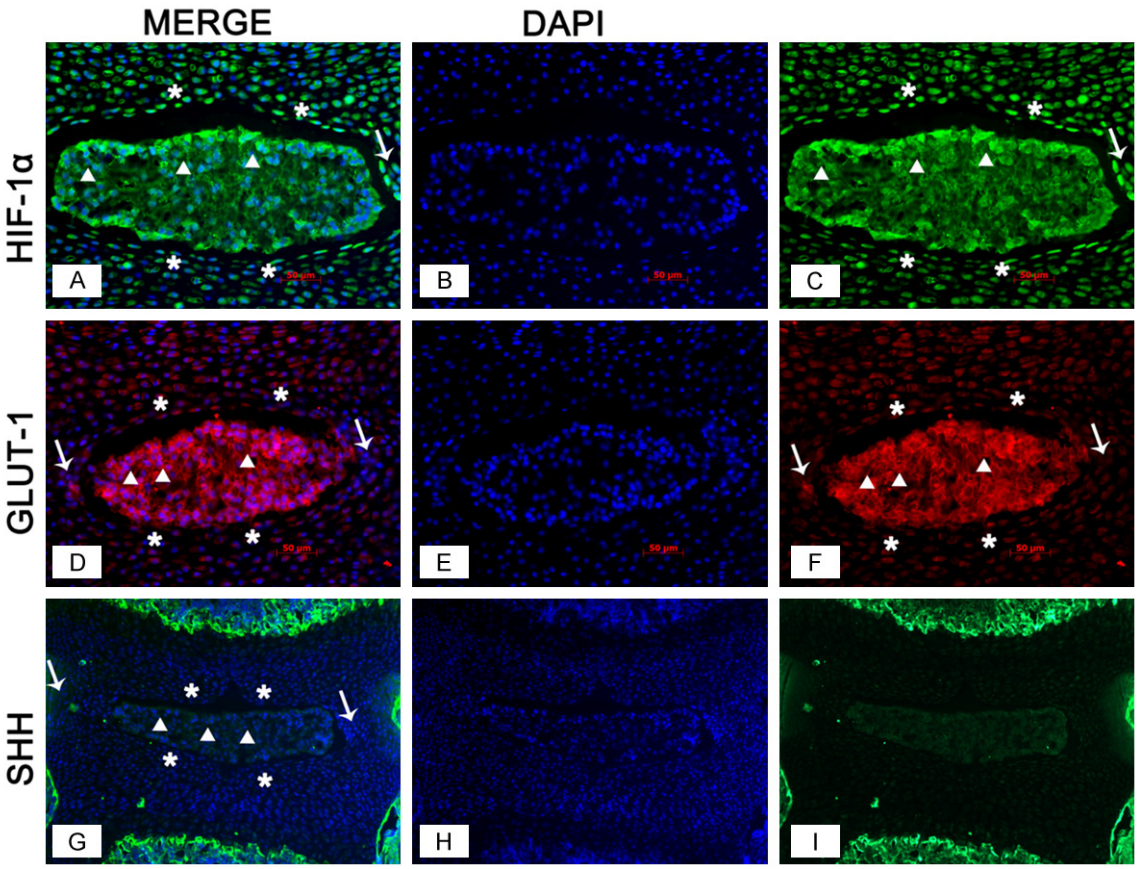

Figure 3. In situ immunofluorescence assay of protein markers related to notochordal cells of 5-day-old mice. A-C: HIF-1α is evenly expressed in the intervertebral disc (IVD). D-F: GLUT-1 is more highly expressed in notochordal cells than in AF cells and endplate chondrocytes. G-I: SHH is not expressed in the IVD. Scale bars 50 μm; N = 3; red/green signals are positive. NP: nucleus pulposus, AF: annulus fibrosus, CE: cartilaginous endplates. The triangle symbol indicates notochordal cell, the arrow indicates annulus fibrosus cell, the asterisk symbol indicates endplate chondrocyte.

sections were stored in advance in the dark in a humidified chamber at 4°C, and photos were taken under dim light. Pictures were captured using filters for red fluorescent protein and FITC.

Results

H&E staining

H&E staining showed that the size of the NP decreased in the 22-month-old mice compared with that in the 1-week-old mice (**Figure 1**). In the 1-week-old mice, the NP contained with a large volume of notochordal cells of various sizes that had large vacuole-like formations in the cytoplasm. The cells were tightly packed and uniformly arranged (**Figure 1A**).

In the 22-month-old mice, a cavity was seen within the inner disc, cells were disorderly, the NP volume and cell number were decreased, and the cell shape and phenotype were changed. The IVD of older mice showed the following degenerative changes that were not present in 1-week-old mice: the NP became smaller and more compressed, the large-vacuolated cells were replaced by small and irregular cells, and cell density and cell death increased (**Figure 1B**).

Expression of markers in 5-day-old mice that relate to notochordal cells

Aggrecan, collagen II, HIF-1α, and GLUT-1, but not SHH and collagen III, were expressed in the notochordal cells, AF cells, and endplate chondrocytes (**Figures 2**, **3**). However, the expression of aggrecan and GLUT-1 was more pronounced in the notochordal cells than in the AF cells and endplate chondrocytes (**Figures 2A-C**,

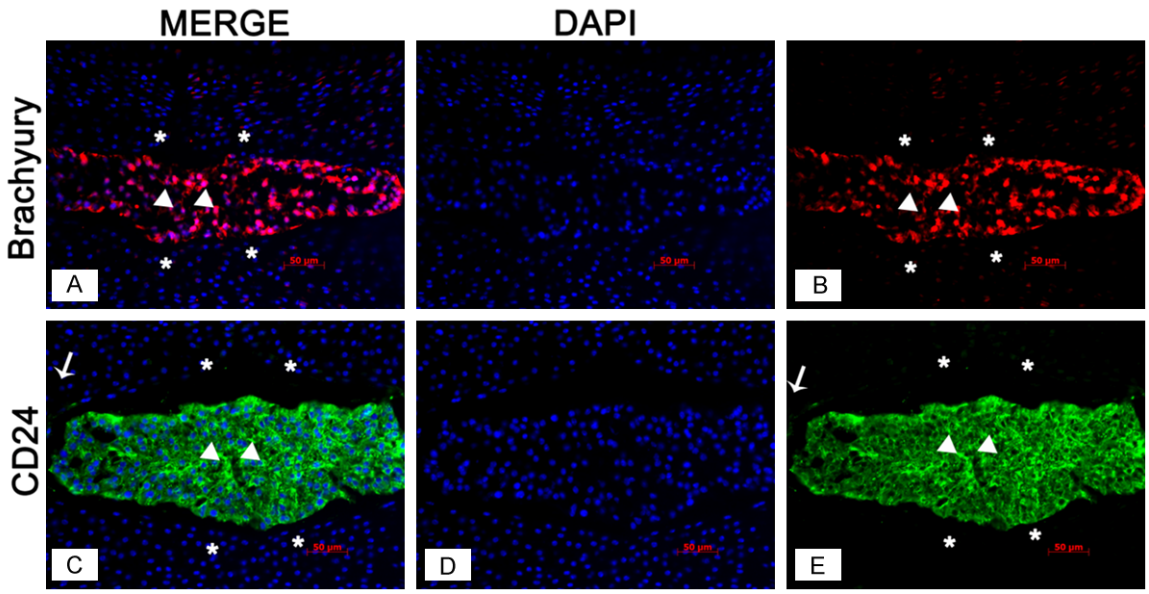

Figure 4. In situ immunofluorescence assay of protein markers that relate to notochordal cell of 5-day-old mice. A, B: Brachyury protein was expressed in the notochordal cell nucleus. C-E: CD24 protein was expressed and stable on the notochordal cell membrane. Scale bars 50 μm; N = 3; red/green signals are positive. NP: nucleus pulposus, AF: annulus fibrosus, CE: cartilaginous endplates. The triangle symbol indicates notochordal cell, the arrow indicates annulus fibrosus cell, the asterisk symbol indicates endplate chondrocyte.

3D-F). Collagen I was expressed only in the outer AF cells (**Figure 2G-I**), CD24 was specifically expressed in the notochordal cell membrane (**Figure 4C-E**), and brachyury was expressed only in the nucleus of the notochordal cells (**Figure 4A**, **4B**).

Expression of markers in 4-week-old mice that relate to notochordal cells

In 4-week-old mice, the expression of HIF-1α increased in the notochordal cells as well as in the AF cells and endplate chondrocytes (**Figure 5D-F**). The expression of GLUT-1 markedly increased in the notochordal cells over that in the AF cells and endplate chondrocytes (**Figure 5A-C**). The protein expressions of CD24 and GLUT-1 were robust and stable on the notochordal cell membrane (**Figure 6**). SHH protein was not expressed in any cells of the intervertebral discs of 4-week-old mice (**Figure 5G-I**).

Discussion

In the present study, we observed that notochordal cells degraded with aging from comparison between 1-week-old and 22-month-old mice by HE staining, and then validated several proposed notochordal cell markers by immunofluorescence staining in IVDs of 5-day-old and 4-week-old mice. Our main finding was that the protein of CD24 was specifically expressed in the notochordal cell membrane, and brachyury was expressed in the notochordal cell nucleus. These results indicate that both CD24 and brachyury have the potential to serve as notochordal cell markers; while CD24 may help to distinguish notochordal cells from AF cells and endplate chondrocytes in young mice.

The IVD consists of three distinct tissue parts-the outer fibrocartilaginous AF, the superior and inferior cartilaginous endplates, and the completely enclosed gel-like NP [24]. The hallmark of progressive IVD degeneration is the incapacity of NP cells to maintain normal homeostatic tissue remodeling, thereby clinically leading to LBP, a crippling condition that affects up to 80% of adults at least once during their lives [25, 26] with significant health and economic consequences [27].

NP cells are derived from the embryonic notochord and reside in a hypoxic and hyperosmotic niche [28, 29]. In the early stages of intervertebral disc development, the NP is filled with a large volume of notochordal cells; however, as IVD degeneration progresses, the number of

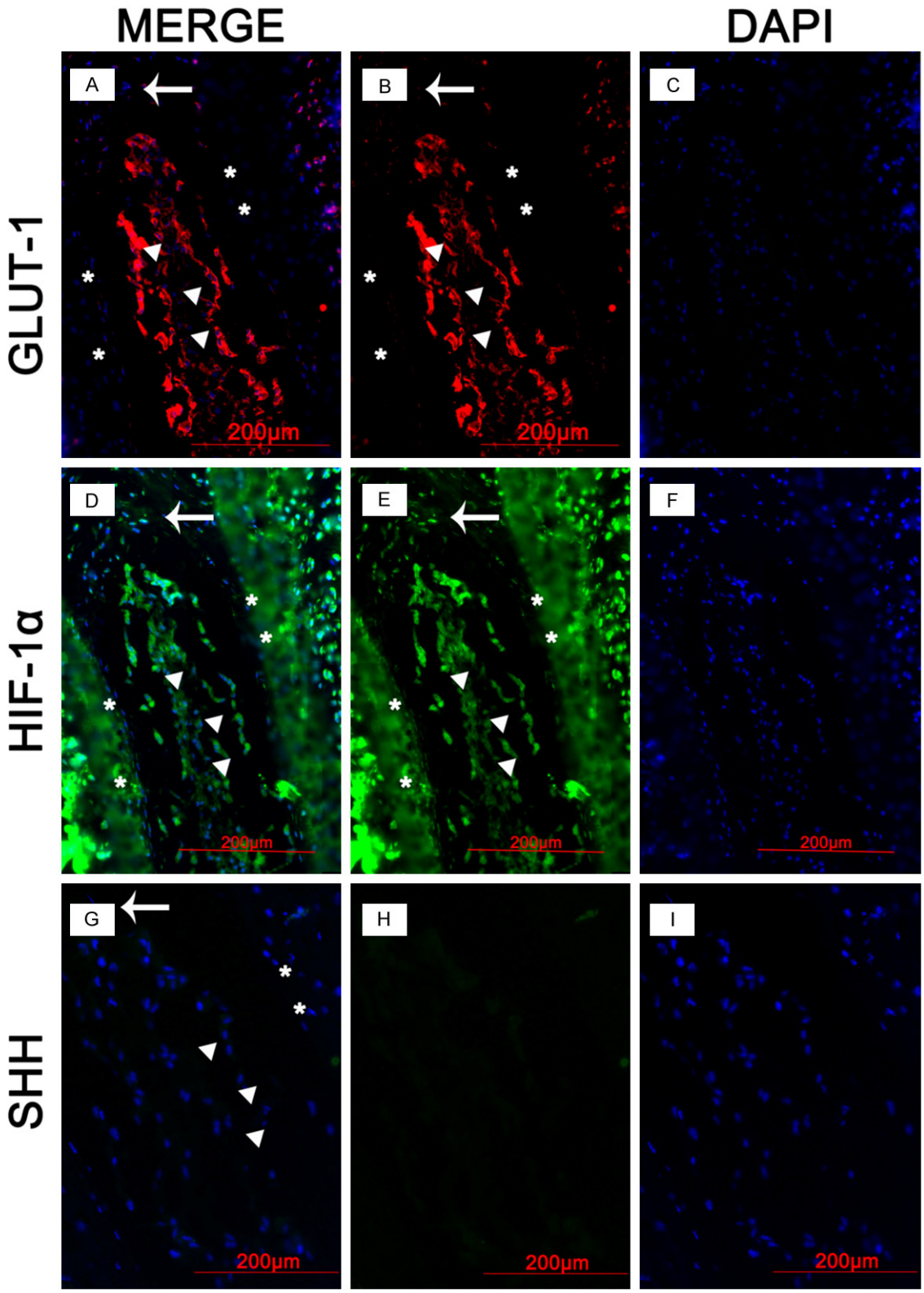

Figure 5. In situ immunofluorescence assay of protein markers that relate to notochordal cells of 4-week-old mice. A-C: GLUT-1 protein is more highly expressed in notochordal cells than AF cells and endplate chondrocytes. D-F: HIF-1α protein is evenly expressed in the IVD. G-I: Sonic hedgehog (SHH) is not expressed in the IVD. Scale bars 50 μm; N = 3; red/green signals are positive. NP: nucleus pulposus, AF: annulus fibrosus, CE: cartilaginous endplates. The triangle symbol indicates notochordal cell, the arrow indicates annulus fibrosus cell, the asterisk symbol indicatesendplate chondrocyte.

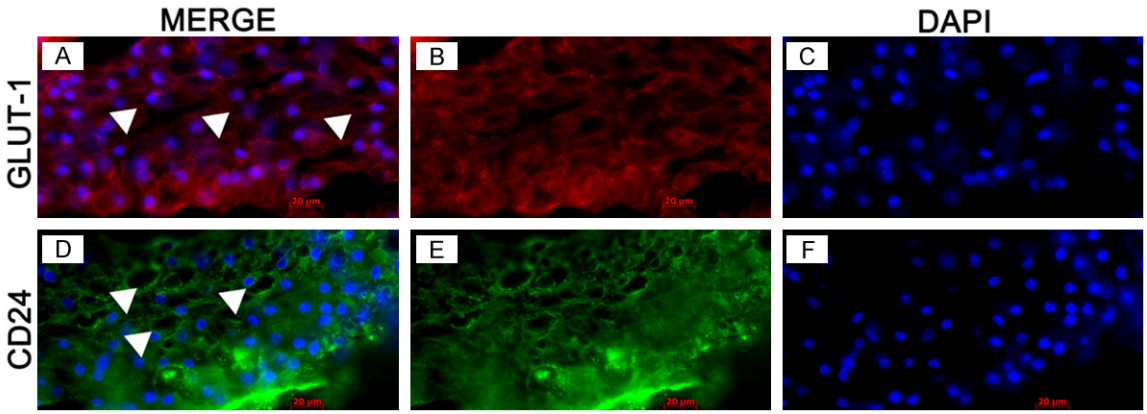

Figure 6. In situ immunofluorescence assay of protein markers that relate to notochordal cells of 4-week-old mice. A-C: GLUT-1 protein is expressed in the notochordal cell membrane. D-F: CD24 protein is expressed and stable on the notochordal cell membrane. Scale bars 20 μm; N = 3; red/green signals are positive. NP: nucleus pulposus, AF: annulus fibrosus, CE: cartilaginous endplates. The triangle symbol indicates notochordal cell.

notochordal cells decreases, and the cells slightly adapt to the unique environment by changing their phenotype from larger vacuolated cells to smaller, irregular cells, which are considered "chondrocyte-like" [12] (**Figure 1**).

HIF-1α is indispensable for NP cell survival because NP is the largest avascular tissue in the body and is physiologically hypoxic [30]. On removal of HIF-1α protein from the mouse notochord, large vacuolated cells became smaller and non-vacuolated, and massive postnatal apoptotic cell death was observed [31]. Our results showed that other cells, including endplate chondrocytes and AF cells, also constitutively express HIF-1α in large amounts; therefore, HIF-1α cannot be a marker for notochordal cells.

SHH is required for the formation of the notochordal sheath and for patterning the NP [22] and is specifically expressed in the notochord of embryos [22]; however, SHH expression decreases as the embryo develops. Our results show that no SHH protein was expressed in the notochordal cells of 5-day- and 4-week-old mice.

Brachyury is necessary for development of the embryonic mesoderm, especially the morphogenesis of the notochord [12]. Our results showed that the brachyury protein is expressed particularly in the notochordal cell nucleus of 5-day and 3-week-old mice. As a nucleus protein, it might merely be used to identify the notochordal cells through the permeabilized membrane, in which the tissue should be fixed in advance. Nevertheless, the brachyury protein should be considered as a physiologically relevant notochordal cell marker.

Aggrecan and collagen II, two main extracellular matrix proteins, are responsible for maintaining hydration [32], which is necessary in NP to resist compressive forces and maintain disc height [33]. These secreted proteins are closely connected to cell function and survival and are regarded as cell phenotype markers. Our results showed that both notochordal cells and endplate chondrocytes both express aggrecan and collagen II. Collagen I protein was found only in the outer AF cells, and the collagen III protein was largely expressed in skin tissue but not in the IVD. The notochordal cells expressed more collagen II and aggrecan than the endplate chondrocytes and AF cells.

GLUT-1 is a uniporter protein that facilitates the transport of glucose across the plasma membranes of mammalian cells [34]. Our results showed that GLUT-1 is widely expressed in notochordal cells, AF cells, and endplate chondrocytes and its expression increases in the notochordal cells as they age. High-level expression of GLUT-1 in the cell membranes might be induced by an inadequate supply, which can aid in the uptake of more glucose for cell survival. The expression of GLUT-1 protein in the notochordal cell membrane is more than that in the

AF cells and endplate chondrocytes; therefore, the GLUT-1 might be an alternative cell membrane marker for identifying and isolating pure notochordal cells.

CD24 is a cell-adhesion molecule; its expressed protein is anchored through a glycosyl-phosphatidylinositol link to the cell surface [35]. The results of this study suggest that the protein of CD24 is specifically expressed on the notochordal cell membrane, and not in the adjoining AF cells, or endplate chondrocytes. The CD24 protein is robust and stable and can be the target molecule to distinguish thenotochordal cells from AF cells and endplate chondrocytes in mice.

This study validated several mouse notochordal cell markers using in situ immunofluorescence staining. The results showed that CD24 protein, which is robust and stable, is expressed on the notochordal cell membrane and brachyury proteins are expressed in the nucleus of notochordal cells within 4 weeks. These proteins might serve as notochordal cell-specific markers because they distinguish notochordal cells from AF cells and endplate chondrocytes. In particular, CD24 protein can help to distinguish notochordal cells from AF cells and endplate chondrocytes in mice: therefore, they should be regarded as the characteristic cell membrane marker for notochordal cells. This finding could be beneficial not only for isolating and purifying the notochordal cells but also for developing new therapeutic and regenerative strategies to treat pathological IVD degeneration.

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Disclosure of conflict of interest

None.

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