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Original Article

Hypoxia-inducible factor (HIF)-1alpha knockout accelerates intervertebral disc degeneration in mice

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Abstract: Introduction: The abnormality of nucleus pulposus (NP) plays a critical role in intervertebral disc (IVD) degeneration, in which NP cells show apoptosis and fibrosis, leading to the ability of the disc to transfer and distribute loads between the vertebrae is decreased. Considering that hypoxia inducible factor-1α (HIF-1α) is abundantly expressed in NP and that it mediates cell proliferation, migration and apoptosis in various cell types, we hypothesized that NP-HIF-1α plays an important role in NP and evaluate whether NP-HIF-1α is involved in IVD degeneration. Material and methods: Sonic Hedgehog-Cre$^{+/-}$ mice were crossed with HIF-1α$^{flox/flox}$ mice to generate NP specific HIF-1α-deficient (HIF-1α$^{-/-}$) mice. Magnetic resonance imaging (MRI) study was used to evaluate NP dehydration and X-ray study was used to acquire the changes of disc height. Histological changese, content of glycoproteins and the *in situ* expression of aggrecan were evaluated by hematoxylin&eosin (H&E) staining, safranin-O/fast green staining and immunohistochemistry assay, respectively. Western bloting was used to detect the change of extracellular matrix in IVD. Results: Firstly, the results of *in situ* hybridization confirmed that HIF-1α in NP was successfully knocked out in HIF-1α$^{-/-}$ mice. Next, for HIF-1α deficiency mice, imaging study shows IVD was narrowed in X-ray and signal intensity of NP was decreased in MR T2-weight imaging. Accordingly, the size and cell number of NP and proteoglycan content was decreased in NP-HIF-1α$^{-/-}$ mice. Finally, Western bloting shows that protein level of collagen II and aggrecan, two main matrix in disc, were both decreased in NP-HIF-1α$^{-/-}$ mice. Conclusions: The present study demonstrates that HIF-1α is essential for NP development and homeostasis and the deficiency of NP-HIF-1α leads to IVD degeneration in mice.

Keywords: Nucleus pulposus, hypoxia-inducible factor-1α, intervertebral disc degeneration, collagen II, aggrercan

Introduction

Intervertebral discs (IVDs) are complex anatomical structures with specific load-bearing organization that provide the spine with flexibility. As the inner core of IVD, nucleus pulposus (NP) is composed of a gel-like material that consists of mainly water, aggrecan, as well as a loose network of collagen fibers [1]. This elastic inner structure allows the vertebral disc to withstand forces of compression and torsion [2]. The hallmark of progressive IVD degeneration is the incapacity of NP cells to maintain normal homeostatic tissue remodeling, thereby clinically leading to low back pain-a condition that affects up to 80% of adults at least once during their lifetime [3-6]. However, the mechanism underlying how IVD degeneration occurs remains largely unknown.

Hypoxia-inducible factor (HIF), a transcription factor responsive to low oxygen tension, is one of the most important factors that directly mediate cellular response to hypoxia [7-9]. HIF-1 consists of a constitutively expressed subunit, HIF-1β, and a subunit that is tightly regulated by the cellular O_2 concentration, HIF-1α [7, 10]. It has been reported that HIF-1α expression in NP cells occurs independent of oxygen tension, an unique character of NP cells [11]. However, HIF-1α was reported to play an important role in homeostatic maintenance of the NP, the energy metabolism of NP cells, and extracellular matrix metabolism [12-15]. Ha et al. demonstrated that HIF-1α was increased in noncontained herniated lumbar discs patients, and it may contribute to NP cells apoptosis [16]. A Chinese cohort study shows that HIF-1α 1790 A > G polymorphisms may be used as a molecu-

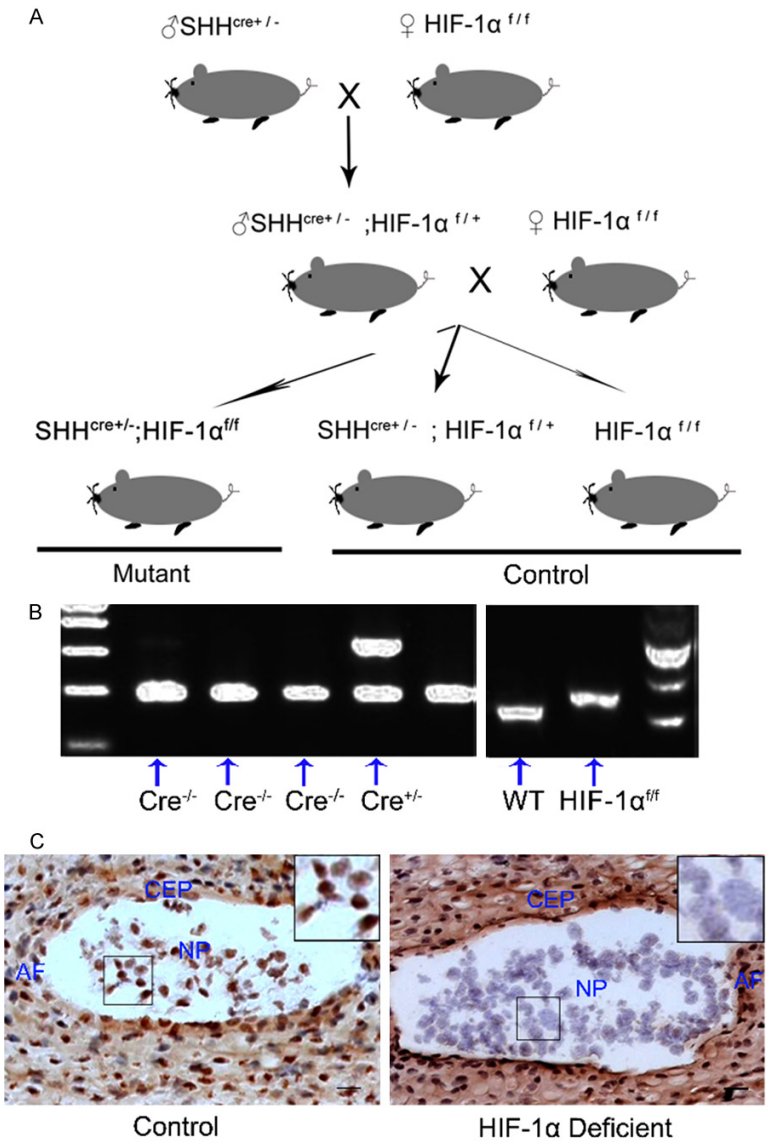

Figure 1. Specificity and deletion efficient of HIF-1α in the nucleus pulposus of mutant mice. A. Generation scheme used to obtain NP-HIF-1α deficiency mice. B. Genotyping of HIF-1α$^{f/f}$ and SHH-Cre$^{+/-}$ mice. There were two strips in Shh-Cre$^{+/-}$ and one strip in Shh-Cre$^{-/-}$ (the left panel), the strip in HIF-1α$^{f/f}$ mice were bigger than that of wild type mice (the right panel). C. In situ hybridization of HIF-1α in control and HIF-1α$^{-/-}$ mice. Signals of brown point indicate the presence and the absence of HIF-1α in NP cells, respectively. Scale bars 50 μm. N = 3. NP: nuclues pulposus, AF: anulus fibrous, CEP: cartilage endplate.

lar marker to determine the susceptibility and severity of disc degeneration [17].

In the present study, we hypothesized that HIF-1α plays a protective effect in IVD, and in order to inquire whether and how HIF-1α affects NP biology *in vivo*, we then used sonic hedgehog (Shh)-driven cre to specifically knockout HIF-1α in notochordal cells and then evaluated the changes of NP. There are multiple measurement techniques available to visualize the degeneration of IVD, ranging from MRI, DHI to histology [18-20]. The decrease of DHI and T2-weighted MR imaging signal intensity indicate dehydratation and degradation of NP. It was found that complete deficiency of HIF-1α resulted in embryonic lethality due to heart deformity in mice at E9.5 [21, 22]. Sonic hedgehog (Shh) protein is expressed in the notochord, and Shh promotor-driven Cre recombinase is only experessed in NP tissue [23, 24]. As expected, normal spine developed mice was successfully established after NP-HIF-1α was specifically deleted using Shh promotor, and the process of IVD degeneration was accelebrated in HIF-1α$^{-/-}$ mice. Our study demonstrates that the deficiency of NP-HIF-1α accelerates the degeneration of IVD in mice, and it indicates that this transcription factor may be involved in IVD degeneration in humans.

Materials and methods

Generation of mice lacking HIF-1α in intervertebral disc

All mice were raised in accordance with Shanghai Jiaotong University School of Medicine Animal Care and Use Committee (SJUSMACUC) Guidelines under specific pathogen-free (SPF) conditions. Adult male Sonic hedgehog (Shh)-Cre$^{+/-}$ mice (Shh promotor-driven Cre recombinase transgenic heterzygote mice, #005622 Jackson Laboratory, Maine, USA) were crossed with adult female HIF-1α$^{flox/flox}$ mice (homozygote for a floxed HIF-1α allele, gift from Dr. Clement) to generate the heterzygote (Shh-Cre$^{+/-}$; HIF-1α$^{f/+}$) male mice. Then these newly generated males were crossed with female HIF-1α$^{flox/flox}$ mice to obtain HIF-1α$^{-/-}$ (Shh-Cre$^{+/-}$; HIF-1α$^{f/f}$) mutant mice. HIF-1α$^{flox/flox}$ mice were used as control

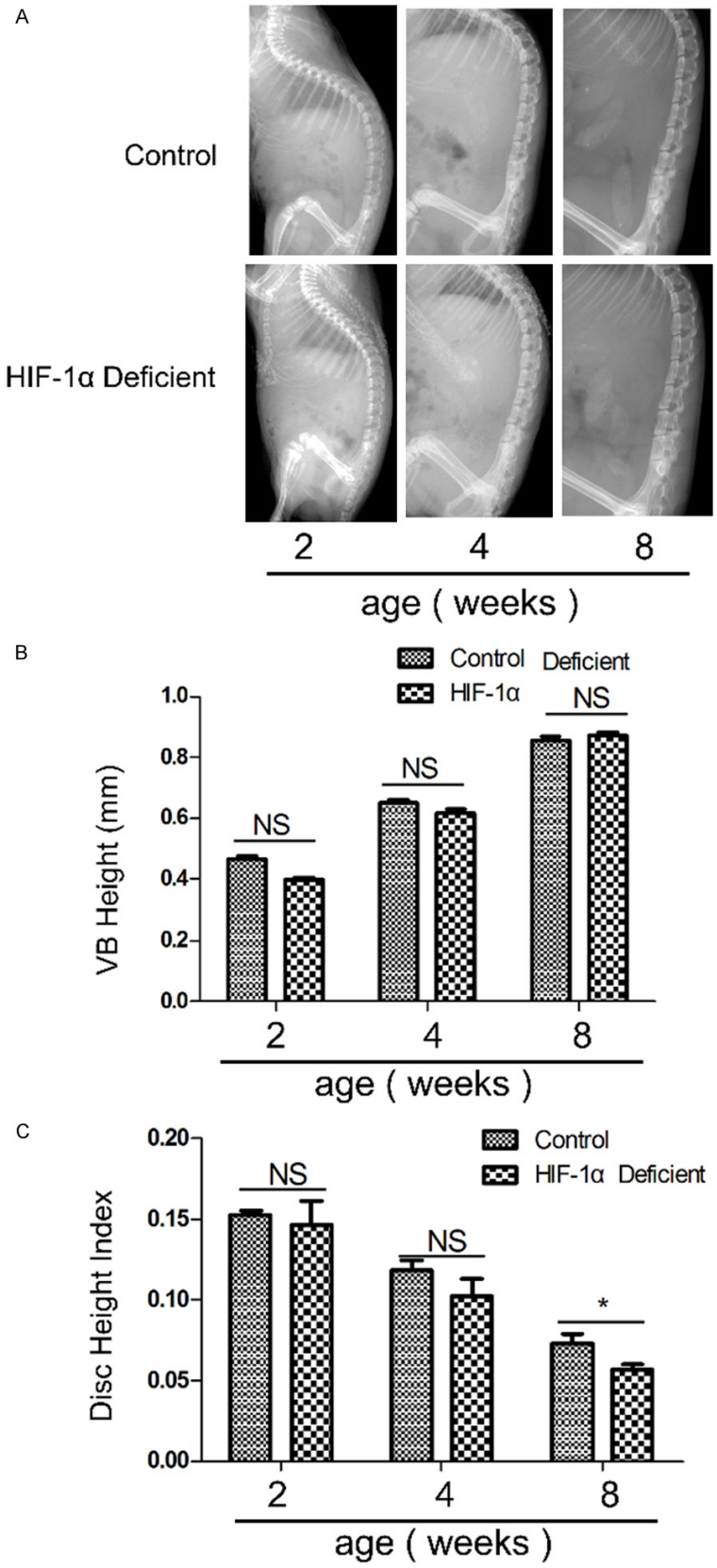

Figure 2. Comparison of X-Ray radiographs in control and HIF-1α$^{-/-}$ mice. HIF-1α$^{-/-}$ mice reached adulthood and did not exhibit obvious spinal structural defects when compared to control mice (A). Vertebral (VB) height was not affected in HIF-1α$^{-/-}$ mice compared to age-matched control (B). There is no significant difference of disc height index (DHI) in 2- or 4-week-old mice between two groups, but 8-week-old HIF-1α$^{-/-}$ mice exibited significant lower DHI value compared to age-matched control (C). N = 3.

(**Figure 1A**). Tail snips DNA was isolated from 2-week-old mice, genotyping was performed with PCR according to the PCR protocol for generic Cre from the Jackson Laboratory. Primers were as follows:

Cre$^{+/-}$: 5'GGTGCGCTCCTGGACGTA3', 5'GGGACAGCTCACAAGTCCTC3'; Cre$^{-/-}$: 5'CTCGGCTACGTTGGGAATAA3', 5'GGGACAGCTCACAAGTCCTC3'; HIF-1α/flox: 5'-GCGTTAAGAGCACTAGTTG-3';5'-GGAGCTATCTCTCTAGACC-3'.

The PCR products were run on a 1.5% agarose gel. As shown in **Figure 1B**, there were two strips in Shh-Cre$^{+/-}$ and one strip in Shh-Cre$^{-/-}$, the strip in HIF-1α$^{f/f}$ mice were bigger than that of wild type mice. For animals, euthanization was performed by very high dose of sodium pentobarbital (2.5%) or cervical dislocation.

In situ hybridization

In situ hybridization was performed using a HIF-1α in situ hybridization kit (Boster, Hubei, P. R. China). In brief, paraffin-embedded disc sections were deparafinized, rehydrated, and fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline and treated with proteinase-K (2 g/mL). The sections were then hybridized with DIG-labeled RNA probes in hybridization buffer at 42°C overnight. After hybridization, the sections

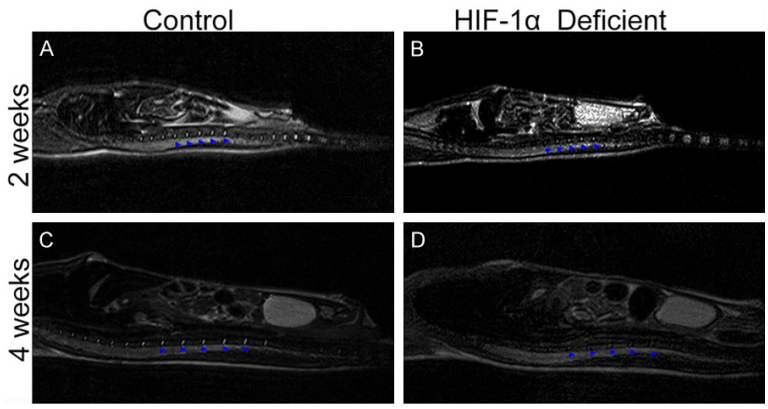

Figure 3. T2-weighted MRI analysis of 2- and 4-week-old control and HIF-1α$^{-/-}$ mice. The arrows indicate signals in the nucleus pulposus area were decreased and became "dark disc" in both 2- (B) and 4-week-old (D) HIF-1α knockout mice compared to age-matched control (A, C). N = 3.

were washed and blocked at 37°C for 30 min, then incubated with a biotinylated mouse anti-digoxigenin antibody at 37°C for 60 min, followed by incubated in biotinylated horseradish peroxidase (Bio-HRP) at 37°C for 20 min. The color reaction was elicited by DAB solution at last.

Imaging studies of mice skeleton and IVD

X-ray imaging: Animals were put under general anesthesia using a 2.5% sodium pentobarbital. Data were then acquired using a low energy X-ray (Faxitron MX-20, IL, USA) with an exposure time of 30 s (30 kV) on animals. Lateral/Anteroposterior (AP) radiographs of spine were collected. The disc height was divided by the height of adjacent vertebral body, and expressed as the Disc Height Index (DHI) [19]. Lateral x-ray images of the spine were taken at 2× using a Faxitron® MX-20 (Faxitron MX-20, IL, USA). Disc Height Index (DHI) = 2(DH1+DH2+DH3)/(LB1+LB2+LB3+UB1+UB2+UB3). UB and LB represent the length of the uper vertebrae body and the lower vertebrae body, respectively. DH represents the disc height between adjacent vertebrae. The age of 2-, 4- and 8-week-old control and HIF-1α deficiency mice were examined in this experiment, at least 3 mice in each group.

MRI was acquired using a 3.0T MR (Trio Tim; Semens, Germany) equipped with a 50-mm millipede scanner coil. T2-weighted sections in the sagittal plane were obtained as a series of multiple two-dimensional slices. The anesthetized mouse was placed supine on a tray.

Histological study

The specimens of lumber IVD were fixed in 4% buffered formalin overnight and then decalcified for 1 month in 10% EDTA solution, and then dehydrated in ethanol, transferred to xylene, and embedded in paraffin. Five μm paraffin sections were used for hematoxylin and eosin staining. Morphology of the cartilage endplate (CEP), anulus fibrous (AF), and NP was examined according to Boos' classification system to evaluate IVD degeneration [20]. Five histological markers including cell proliferation, mucoid degeneration, cell death, tears and clefts, and granular changes were assessed in NP/AF, and 6 markers including cell proliferation, cartilage disorganization, cracks, microfracture, new bone formation, and bony sclerosis were assessed in EP, in which a higher score indicated a more severe stage of disc degeneration. The sections underwent double blind examinations by at least 2 individuals independently.

Safranin-O/Fast green staining was used to quantify proteoglycan (PG) density. Paraffin sections were stained with Safranin-O/Fast green according to standard protocols [25].

Immunohistochemistry

The sections were deparaffinized, rehydrated and treated with 3% hydrogen peroxide in methanol for 30 min for blockade of endogenous peroxidase. Antigen retrieval was performed by boiling samples in 10 mM citrate buffer (pH 6.0) for 30 min followed by incubation with aggrecan antibodie (Abcam, Cambridge, UK; 1:150) overnight at 4°C. Control and HIF-1α deficiency mice at the age of 2-, 4- and 8-week-old (3 mice in each group) were examined in this experiment.

Western blotting

Total protein collected from 6 IVD samples was extracted using protein lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St.

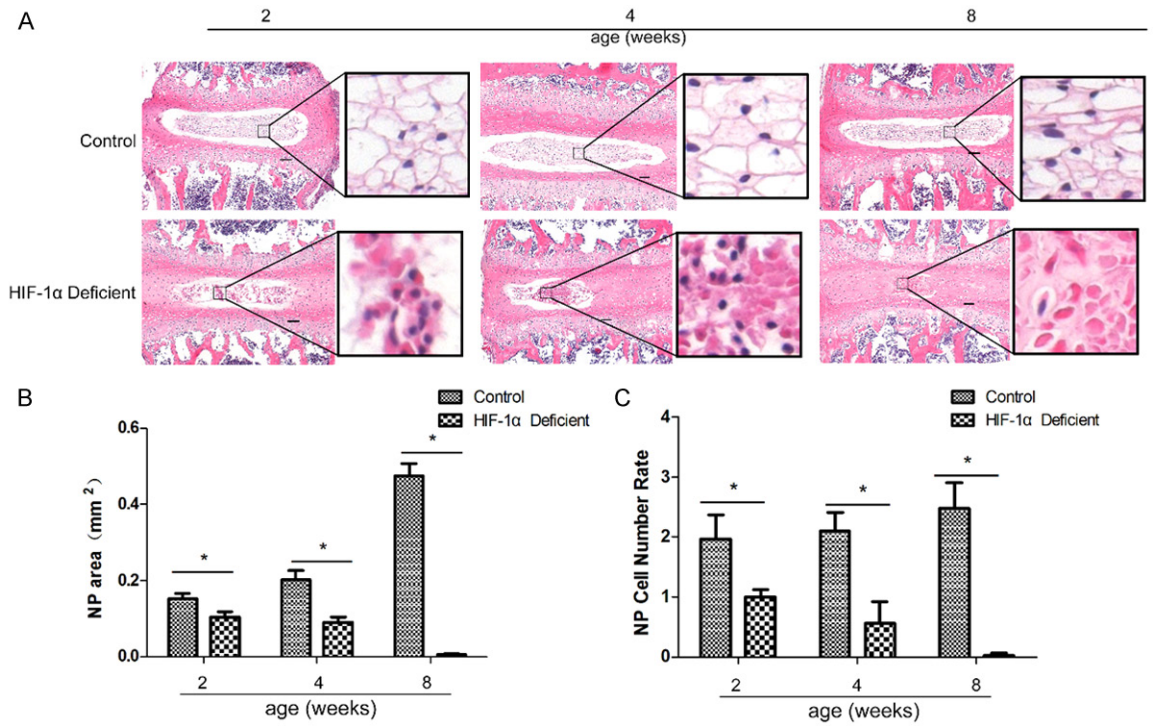

Figure 4. HE staining of IVD in 2, 4- and 8-week-old HIF-1α$^{-/-}$ and age-matched control mice. Representative images showing HE staining of IVD in 2-, 4- and 8-week-old HIF-1α$^{-/-}$ and age-matched control mice (A). Summarized data showing the size of NP (B) and NP cell number (C) in 2-, 4- and 8-week-old HIF-1α$^{-/-}$ and age-matched control mice. Magnification x20; scale bars 100 μm. N = 3.

Louis, MO, USA). Ten μg protein was subjected to 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) for β-actin, aggrecan and collagen II respectively. The samples were then transferred onto nitrocellulose membranes (Millipore, Amsterdam, The Netherlands) . Transfer membranes were immunostained with 1st antibodies to β-actin (Anbo Biotechnology, San Francisco, USA), aggrecan and col2α (Abcam, Cambridge, UK), and peroxidase-labeled 2nd antibodies (Bioworld Technology, St. Louis Park, MN, USA) and visualized by the enhanced chemiluminescence method (Amersham, Arlington Heights, IL).

Statistics analysis

Statistical significance among the differences in the means of western blot assay, Boss score and DHI data was analyzed by one-way ANOVA (SPSS 10.0, Chicago, IL). Results are presented as mean ± standard deviation from three independent experiments, performed in triplicate (n = 3). A *P* value < 0.05 was considered statistically significant.

Results

Generation and characterization of HIF-1α knockout mice

Adult male sonic hedgehog (Shh)-Cre$^{+/-}$ mice were crossed with adult female HIF-1α$^{flox/flox}$ mice to generate the heterzygote (Shh-Cre$^{+/-}$; HIF-1α$^{f/+}$) male mice. These newly generated males then were crossed with female HIF-1α$^{flox/flox}$ mice to obtain HIF-1α$^{-/-}$ (Shh-Cre$^{+/-}$; HIF-1α$^{f/f}$) mutant mice (**Figure 1A**), and the knockout efficiency was evaluated by *in situ* hybridization. **Figure 1B** show the genotyping results that there were two strips in Shh-Cre$^{+/-}$ and one strip in Shh-Cre$^{-/-}$, the strip in HIF-1α$^{f/f}$ mice were bigger than that of wild type mice. As shown in **Figure 1C**, HIF-1α mRNA was expressed in the cells of region AF, NP and EP in control mice (Brown staining), but no expression in region of NP in HIF-1α$^{-/-}$ mice, indicating that NP-HIF-1α was sucessfully deleted. Importantly, these NP-HIF-1α null mice were viable and did not display obvious spinal structural defects when compared to age-matched control mice by X-ray imaging (**Figure 2A**), and the vertebral height

Table 1. Boos' score of control and *HIF-1α* KO mice

Age	2 week		4 week		8 week	
Type	Control	KO	Control	KO	Control	KO
NP/AF	0.80±0.30	2.87±0.32*	1.03±0.32	5.53±0.45*	1.51±0.14	8.37±0.42*
CEP	0.11±0.04	0.12±0.03	0.12±0.03	0.43±0.06*	0.34±0.07	1.82±0.45*
Total	0.91±0.34	2.99±0.34*	1.16±0.32	5.96±0.51*	1.85±0.20	10.18±0.86*

Note: KO, HIF-1α Knockout; NP, Nucleus Pulposus; AF, Annulus Fibrosus; EP, end plate. At each time point, scores of control and KO mice were significantly different. *$P < 0.05$ vs age-matched control mice.

showed no difference between two groups (**Figure 2B**).

Image-study of mice axial skeleton and MR imaging

DHI is commonly used as a relative measurement of disc height across species to evaluate the differences in animal size. **Figure 2C** shows that there is no significant difference of DHI in 2- or 4-week-old HIF-1α$^{-/-}$ mice compared to age-matched control. In contrast, 8-week-old HIF-1α$^{-/-}$ mice exibited significant lower DHI value compared to age-matched control (**Figure 2C**), indicating that narrowing of IVD occurred at 8-week-old HIF-1α$^{-/-}$ mice. **Figure 3** shows that signal intensity of NP on T2-weighted MR imaging was decreased and the "light disc" gradually became "dark disc" in both 2- and 4-week-old HIF-1α$^{-/-}$ mice compared to age-matched control. These results indicate that NP degeneration and dehydratation occurred in 4-week-old NP-HIF-1α$^{-/-}$ mice.

H&E staining

H&E staining was used to detect histological changes in HIF-1α$^{-/-}$ mice. **Figure 4** shows that the large NP cell disappeared and NP was replaced with a fibrocartilaginous tissue at last in HIF-1α$^{-/-}$ mice. The size of NP and the number of NP cell were significant decreased (**Figure 4B**, **4C**) and Boos' scores of IVDs were significantly increased (**Table 1**) in 2-, 4- and 8-week-old HIF-1α$^{-/-}$ mice compared with age-matched control. These results show that mutant NP cells died in the hypoxic environment and ECM was decreased, which accelerated degeneration of IVD.

Safranin-O/fast green staining and immunochemistry

Safranin-O/fast green staining was used to evaluate the content of neutral glycoproteins in IVD. The **Figure 5A** shows that NP size and cell number were increased in NP of control mice, while decreased in HIF-1α$^{-/-}$ mice. By 8 week the nomal NP tissue had virtually disappeared and replaced with fibrocartilaginous tissue at last. The strong Safranin O staining suggests that these area were replaced with fibrocartilaginous tissue, which is resembling the inner-layer tissue of AF in HIF-1α$^{-/-}$ mice (**Figure 5A**).

As demonstrated in **Figure 5B**, the size and aggrecan expression of NP were decreased in 4- and 8-week-old HIF-1α$^{-/-}$ mice compared with age-matched control. Interestingly, the replaced tissue (fibrocartilaginous tissue) was rarely express aggrecan in HIF-1α$^{-/-}$ mice (**Figure 5B**), indicating these substituted fibrocartilaginous tissue has abnormal features compared with nomal NP.

Collagen II and aggrecan expression

Figure 6 shows that the protein level of both collagen II and aggrecan were decreased in 4-week-old HIF-1α$^{-/-}$ mice compared with age-matched control. These results indicate that the expession of collagen II and aggrecan may be regulated by HIF-1α, and the decreased protein expression may be responsible for the NP degeneration and dehydratation occurred in 4-week-old HIF-1α$^{-/-}$ mice at T2-weighted MR imaging.

Discussion

In this study, the results indicate that HIF-1α plays a protective role in NP development and homeostasis, since the NP virtually disappeared and had been replaced with the fibrocartilaginous tissue in NP-HIF-1α deficiency mice. Firstly, we generated specific NP-HIF-1α knockout mice and found the height of IVD narrowed in X-ray and signal intensity of NP decreased in T2-weighted MRI, main matrixes in disc were decreased in IVD, as well as NP underwent progressive disappearance and was replaced with fibrocartilage tissue in NP-HIF-1α$^{-/-}$ mice compared with age-matched control mice.

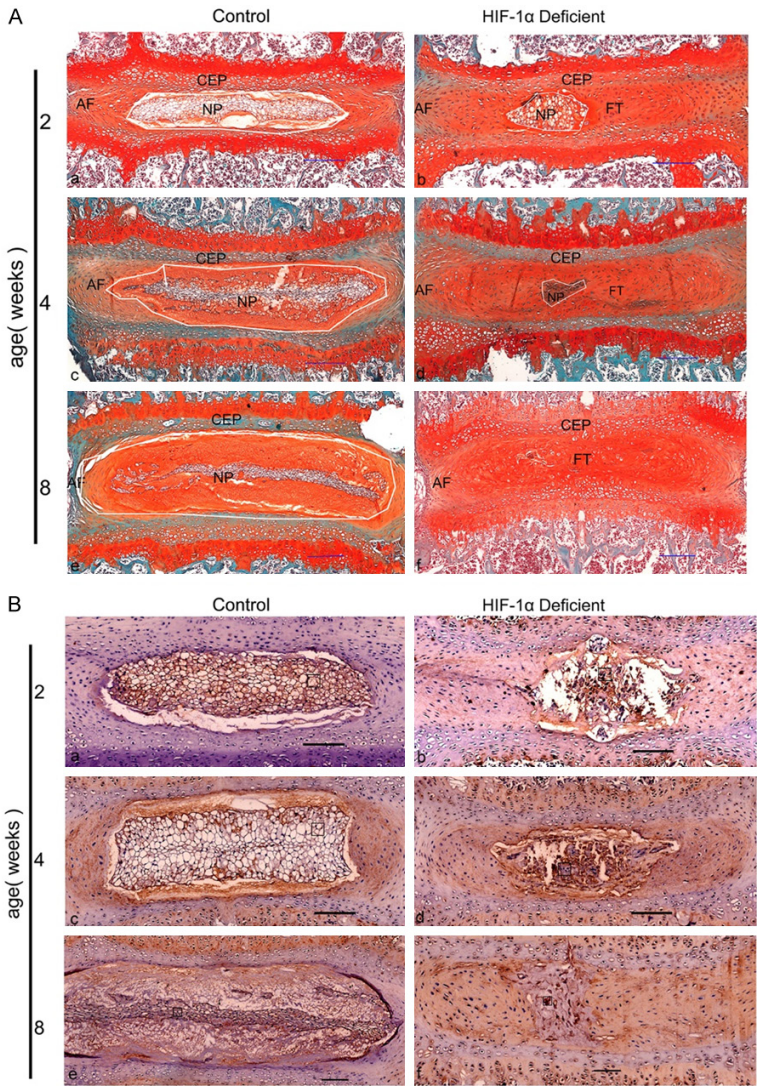

Figure 5. Safranin O/Fast green staining and immunohistochemistry of aggrecan of IVD in 2-, 4- and 8-week-old HIF-1α$^{-/-}$ and control mice. Safranin O staining shows that glycosaminoglycans of IVD was decreased. The NP was replaced with a fibrocartilaginous tissue that strongly stained for Safranin O in HIF-1α$^{-/-}$ mice (A). Aggrecan is highly expressed in NP aera, and its expression was decreased in HIF-1α$^{-/-}$ mice compared with age-matched control (B). Brown signals (aggrecan positive) were highly expressed in NP aera. The fibrocartilaginous tissue (FT) rarely express aggrecan in 4- and 8-week-old HIF-1α$^{-/-}$ mice. FT: fibrocartilaginous tissue, NP: nuclues pulposus, AF: anulus fibrous, CEP: cartilage endplate. Magnification x20; scale bars 200 μm. N = 3.

Previous studies have shown that HIF-1α is a critical mediator in cellular adaptation to hypoxic stresses and NP cells were derived from notochord in mice and experience oxygen and nutrient deprivation [7, 26-28]. In the present study, we used Shh driven cre to specifically knockout HIF-1α in notochordal cell. In the results, cre expression driven by the Shh promoter seems to be highly specific for the NP cells, since *in situ* hybridization assay shows there was no HIF-1α signal in NP, but HIF-1α signal could be detected in region of AF and CEP in HIF-1α$^{-/-}$ mice, as well as in NP, AF and CEP in control mice. These results show that HIF-1α was efficiently and specifically knocked out in NP cells, indicating the Shh-lineage notochordal cell became NP cell in late development, as suggested by previous studies [8, 24, 28-31]. Although there still is a debate in origin of NP cell whether is notochordal cell in mammal [29], but the analysis of the NP-HIF-1α deficiency mice further corroborates the notion that cells of the NP, or at least the vast majority, are derived from notochord in mice and had adapted to survive in the hypoxic condition [31-33].

Merceron et al. used Cre driven by Foxa2 to specifically knockout HIF-1α in notochordal cells and then also obtain the NP-HIF-1α deficiency mice, and they found that NP cell did not transdifferentiate into chondrocyte-like cell but underwent massive died, and NP tissue were completely replaced by fibrocartilagenous tissue, which cell population belonging to a lineage distinct from the notochordal one [8]. In the present study we got the similar results by another way that used the cre driven by Shh. The results corroborate the truth that HIF-1α is essential for NP homeostasis and play a protective role in IVD degeneration [8, 14, 34]. We further find that HIF-1α may regulate the expression of aggrecan and collagen II, since the loss of HIF-1α leads to the reduction of collagen II and aggrecan proteins exprssion in IVDs.

In the present study, imaging studies indicate that MR imaging is more sensitive for early

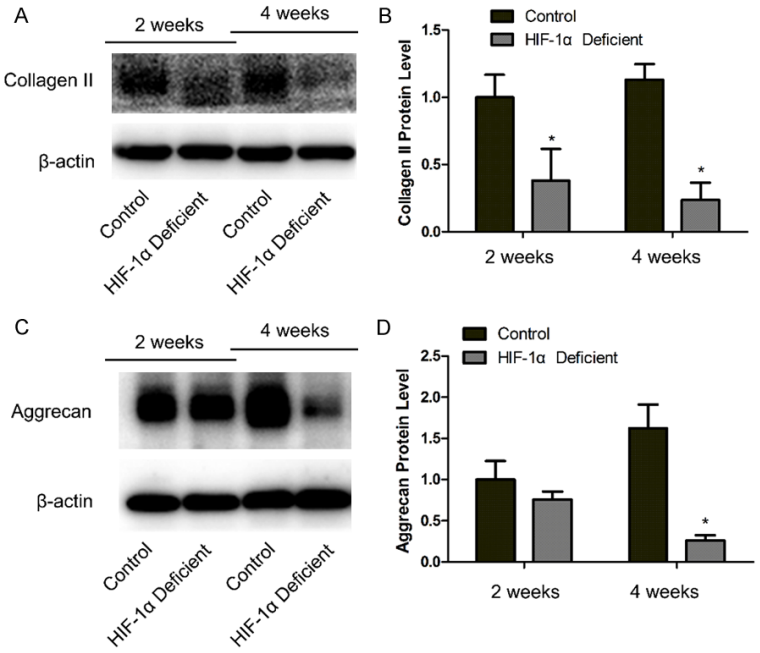

Figure 6. Representative Western blot image (A, C) and summarized data (B, D) showing collagen II (A, B) and aggrecan (C, D) levels in 2- and 4-week-old HIF-1α$^{-/-}$ and control mice. N = 3, *P < 0.05 vs age-matched control mice.

stages of IVD degeneration than DHI in X-ray. Clinically, we can predict the IVD situation of the patient by the advantage of MRI [18, 35]. In this animal model, we observed that when the signal intensity of IVD on T2-weighted MRI was decreased, the corresponding histological studies showed that cell number and size of NP exhibited decreased, and fibrotic changes were taking place in NP. Therefor we can assume that the decreased of MRI signal indicates the IVD has undergone progressive degenerative changes histologically. In order to improve the hypoxic environment in IVD and slow down IVD degeneration, every patient with decreased signal intensity on T2-weighted MRI should be advised to restrict some activities, such as to avoid strenuous exercises and maintaining a sedentary posture.

In summary, HIF-1α is critical for maintenance of NP cell survival, functional activities including proteoglycan matrix synthesis, and it plays an protective role in IVD degeneration. Since the novel model is similar to the IVD degeneration process in human, it can be used in further study of hypoxia related degenaration mechannisms in IVD, and to search for novel therapeutical approaches for the treatment of IVD degeneration.

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Disclosure of conflict of interest

None.

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