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Elevated expression of hypoxia-inducible factor-2alpha regulated catabolic factors during intervertebral disc degeneration

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ABSTRACT

Headings aims: The present study determined whether nucleus pulposus (NP) cells express hypoxia-inducible factor-2alpha (HIF-2α) and assessed its role in regulating the expression of catabolic factors during intervertebral disc degeneration.

Materials and methods: Human degenerated NP tissues were acquired to examine the HIF-2α expression levels using immunohistochemistry, western blotting, and Real-time PCR. Human NP cells were cultivated under normoxic or hypoxic conditions, and the HIF-2α expression was determined. Then, human NP cells were treated with HIF-2α plasmids, HIF-2α siRNA, and tumor necrosis factor-alpha (TNF-α) to evaluate the role of HIF-2α in regulating matrix metalloproteinase (MMP) and aggrecanase expression. An in vivo rabbit disc degeneration model was established to demonstrate that HIF-2α plays a critical role in disc degeneration.

Key findings: We found that HIF-2α had a markedly elevated expression in human degenerated discs in the Grade III stage. HIF-2α protein and gene transcript levels in vitro were relatively higher under hypoxic conditions. The expression of MMP-13, ADAMTS-4 was decreased significantly in HIF-2α silencing condition, while the over-expression resulted in significantly increased levels of MMP-13 and ADAMTS-4. When cytokine TNF-α was added, HIF-2α was induced by nuclear factor-κB (NF-κB). The in vivo experiments showed that the HIF-2α controlled the catabolic factors MMP-13 and ADAMTS-4 that regulated the collagen II and aggrecan metabolism in disc degeneration.

Significance: HIF-2α is a catabolic regulator in disc degeneration and directly controls the catabolic genes. The suppression of HIF-2α expression leads to decelerates extracellular matrix degradation that might represent a therapeutic target for the degenerative disc.

1. Introduction

Intervertebral disc degeneration is a major cause of spinal disorders and has been associated with substantial low back pain [1]. The intervertebral disc consists of nucleus pulposus (NP) core, the tough annulus fibrosus, and cartilage endplates. The aggrecan-rich NP is an avascular tissue and sparsely populated with NP cells in a hypoxic environment [2]. The complex structure of the intervertebral disc allows it to absorb stress, bear the weight of the whole body, and support a wide range of movements. Disc degeneration is characterized by a reduction in the number of NP cells and an imbalance between extracellular matrix (ECM) anabolism and catabolism [3], due to which, the aggrecan content and collagen II synthesis decrease. In addition, inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β, stimulate the expression of ECM catabolic enzymes that aggravate disc degeneration [4,5].

When the ECM dynamic balance is damaged in the NP, matrix metalloproteinases (MMPs) and a disintegrin and metallopeptidase with thrombospondin motifs (ADAMTS) contribute substantially to intervertebral disc degeneration [6,7]. MMPs are the main proteinases that degrade the ECM, and ADAMTS belong to a family of zinc-dependent enzymes that is important in degrading the aggrecan. Li et al. [8] showed that with the progression of disc degeneration, the over-expression of MMP-9 leads to the positive damage of ECM, resulting in the declined biomechanics of the intervertebral disc. MMP-13 is a major matrix metalloproteinase that mediates the degradation of matrix components, especially collagen II, and MMP-13 is highly expressed in the early phase of disc degeneration [9]. ADAMTS-4/5 are generally considered as the major aggrecanases in intervertebral disc degeneration due to their high efficiency on cleaving aggrecan. Inflammatory

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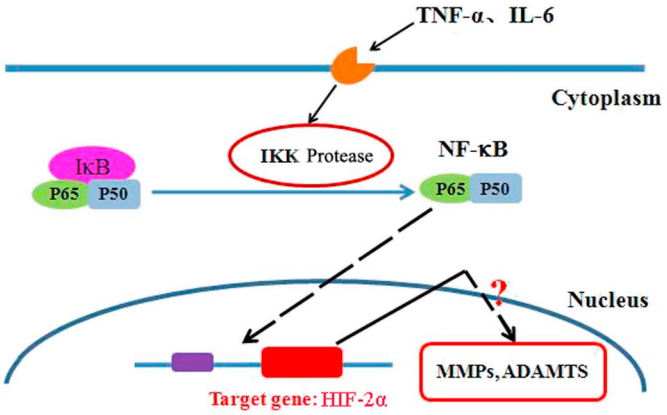

Scheme 1. The hypothesis graph of HIF-2α regulation.

Table 1
Primer sequences used for real-time RT-PCR.

Gene	Sense	Sequence 5′→3′
β-actin (human)	FORWARD	CTCTTCCAGCCTTCCTTCCT
	REVERSE	AGCACTGTGTTGGCGTACAG
HIF-2α(human)	FORWARD	AGCAGCTGGAGAGCAAGAAG
	REVERSE	ATGGAAGAGAGAGGGGTGCT
MMP-9(human)	FORWARD	CAGTCCACCCTTGTGCTCTT
	REVERSE	ATTTCGACTCTCCACGCATC
MMP-13(human)	FORWARD	TTGAGCTGGACTCATTGTCG
	REVERSE	CGCGAGATTTGTAGGATGGT
ADAMTS-4(human)	FORWARD	ACACTGAGGACTGCCCAACT
	REVERSE	GTGTAGCGAGGAACCCAGTC
ADAMTS-5(human)	FORWARD	CAAGGACAAGAGCCTGGAAG
	REVERSE	CTGCATCGTAGTGCTCCTCA
NF-κB(human)	FORWARD	TCAATGGCTACACAGGACCA
	REVERSE	CAGCCTCATAGAAGCCATCC
β-actin (rabbit)	FORWARD	GGCATGGAGTCGTGTGGCATC
	REVERSE	CGTGTTGGCGTACAGGTCCTTG
HIF-2α(rabbit)	FORWARD	CTGCTGTCCTGCCTCATCATCATG
	REVERSE	TGTGGCGGCTCAGGAAGGTC
ADAMTS-4 (rabbit)	FORWARD	CCTGGACAATGGCTATGGACACTG
	REVERSE	CTGGCACTGGCGATCAGCATC
MMP-13(rabbit)	FORWARD	TCTACACCTACACCGGCAAGAGTC
	REVERSE	CGGAGACTGGTAATGGCATCAAGG
COL II(rabbit)	FORWARD	GAAGAACTGGTGGAGCAGCAAGAG
	REVERSE	TGAAGTGGAAGCCGCCATTGATG
Aggrecan (rabbit)	FORWARD	CAACAACGCTCAGGACTACCAGTG
	REVERSE	GCCAGATCATCACCACGCAGTC

factors, such as TNF-α and IL-1β, can mediate the activation of nuclear factor-κB (NF-κB) protein and stimulate the transcription of ADAMTS, which are downstream catabolic mediators in NP cells [10,11].

The hypoxia-inducible factor (HIF) protein family consists of α and βsubunit members that take effect via the formation of heterodimers under hypoxic conditions [12]. HIFs have three αsubunit members, HIF-1α, HIF-2α, and HIF-3α, and they have the same βsubunit that is not affected by hypoxia. The heterodimers stimulate the expression of the target genes by binding the consensus sequence, called the hypoxia-responsive element, in the promoter regions [13]. Because the intervertebral disc is an avascular hypoxic tissue, HIF proteins may play an important role in the function of NP cells. HIF1α is a potent regulator of ECM homeostasis to ensure survival under hypoxic conditions [12,14]. However, HIF2α and HIF1α are not functionally redundant, and HIF-2α (also designated EPAS1), a 50% homolog of HIF-1α, is involved in controlling the hypoxic response via the activation of unique genes among different cell types [15–17]. Moreover, a previous study has indicated that HIF-2α acts as a crucial mediator of cartilage degradation by directly regulating transcriptional targets of catabolic factors, such as MMPs and ADAMTS [18]. However, the role of HIF-2α in regulating ECM catabolism in disc degeneration is still unclear.

Hence, the present study aimed to determine the relationship between disc degeneration and HIF-2α expression, clarify the role of HIF-2α in controlling the expression of MMP and ADAMTS catabolic factors in vitro and ex vivo, and elucidate the mechanism of HIF-2α regulation in response to inflammatory stimulation (Scheme 1).

2. Materials and methods

2.1. Plasmids and reagents

Inhibitors of HIF-2α (HIF-2α-IN-1, Cat#:HY-19949) and NF-kB (JSH-23, Cat#:HY-13982) were purchased from MedChemExpress, Ltd. (Maryland, USA). HIF-2α expression plasmid EPAS1 NM and control plasmid pCMV6 were purchased from GeneCopoeia Inc. (Maryland, USA). Human HIF-2α siRNA and control siRNA duplexes were purchased from Sangon Biotech Co., Ltd. (China). Lipofectamine 3000 was purchased from Invitrogen (California, USA). The reporter was obtained from GeneCopoeia and contained MMP and ADAMTS promoters translated into the pGL3-Basic luciferase reporter vector. Vector pRL-TK (Promega, USA) containing the *Renilla reniformis* luciferase gene was used as an internal transfection control.

2.2. Collection and grading of human NP tissues

Our study protocol was approved by the Ethics Committee of Beijing Jishuitan Hospital and informed written consent for sample collection was obtained from all patients. Human NP samples were collected from patients with degenerated intervertebral discs, who underwent discectomy and fusion surgical procedures between December 2016 and June 2017. The mean age of the patients was 43 years old (range: 22–65 years), and there were 20 males and 16 females. The degenerated discs were assessed and the samples were subdivided into four groups (Grade II, III, IV, V) using the Pfirrmann grading system [19]. All NP samples were immediately dissected under microscopic observation and either placed in serum-free Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, USA), or frozen in liquid nitrogen, or fixed in 4% paraformaldehyde.

2.3. Human NP cell culture and treatment

The degenerated NP tissue samples were diced into small pieces about 1 mm^3 in size and digested with 0.15% collagenase II (Invitrogen) for 4 h at 37 °C. The NP cells were collected and resuspended in DMEM medium supplemented with 15% fetal bovine serum (FBS) with a density of $3 \times 10^4/cm^2$ in a humidified atmosphere containing 5% CO_2 at 37 °C. After a 90% confluence, the cultured cells were detached using 2.5 g/l trypsin (Invitrogen), and were passaged two to three times for use in the following experiments. For the hypoxia treatment, human NP cells were cultured in a hypoxia work station (Invivo2300, Ruskinn Technology, UK) with a mixture of 2% O_2, 5% CO_2, and 93% N_2 for

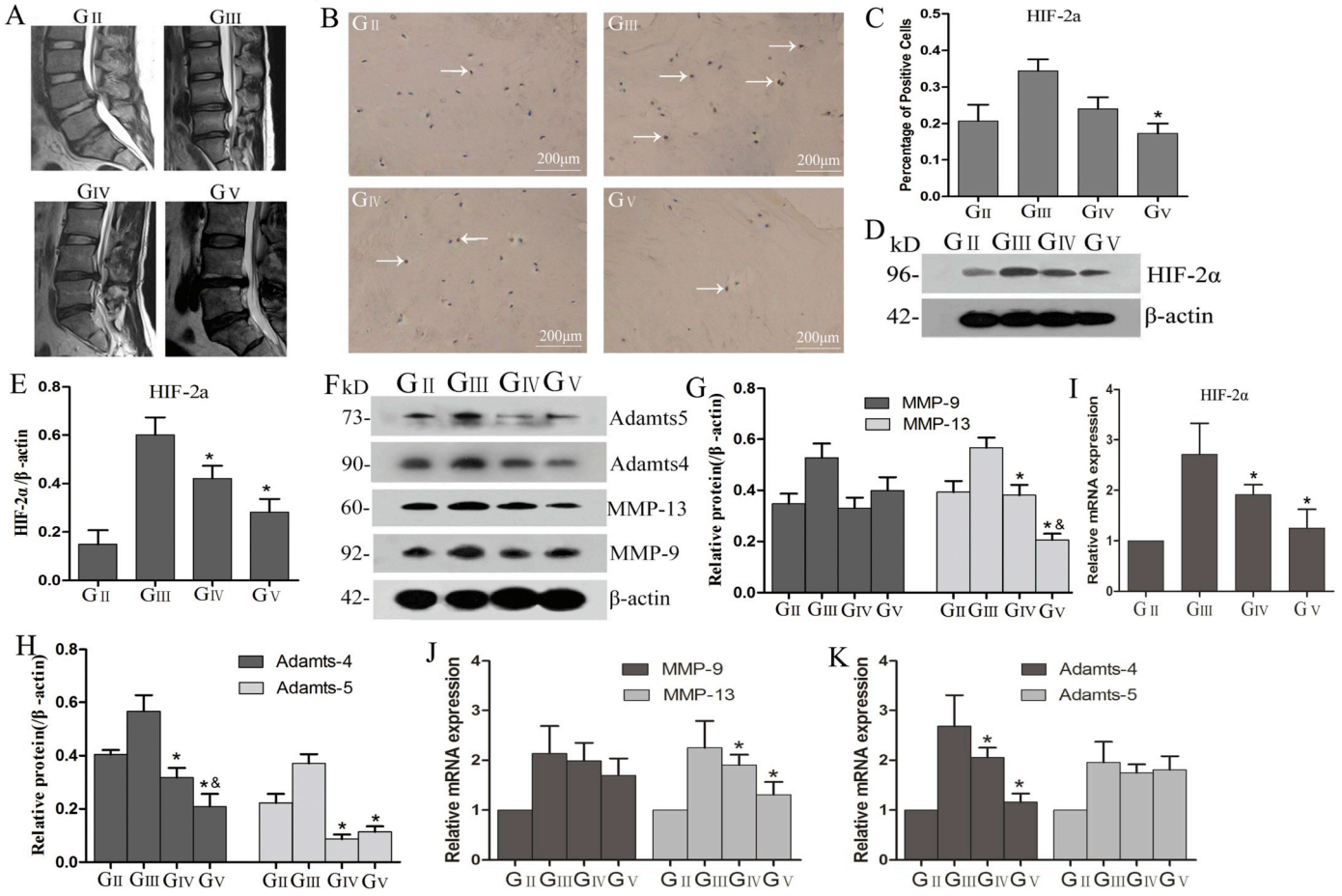

Fig. 1. Evaluation of human degenerated NP samples by MRI, immunohistological analysis, western blotting, and real-time RT-PCR. (A) T2WIs of patients with different grades of intervertebral disc degeneration. (B,C) Immunohistological staining of the degenerated samples with anti-HIF-2α antibody to test the HIF-2α expression (× 200). Scale bar: 200 μm. White arrow:HIF-2α protein expression. (D-H) Western blot and (I-J) Real-time PCR results for HIF-2α, MMP-9/13, and ADAMTS-4/5 protein and gene expressions in the degenerated intervertebral disc samples. (*$p < 0.05$ compared with G III group ; per group $n = 9$; per repetition $r = 4$.)

12–48 h. The recombinant TNF-α (Invitrogen) was added to the culture medium and NP cells were incubated in different concentration gradients of TNF-α (5, 10, 20 ng/ml) for 6, 12, and 24 h. Next, the culture medium containing TNF-α (10 ng/ml) and NF-κB inhibitor (JSH-23, MCE, USA) or HIF-2α inhibitor (HIF-2α-IN-1, MCE) was added to determine the downstream gene and protein expression.

2.4. Immunofluorescence microscopy

NP cells were harvested from donors and cultured in 96-well plates (5×10^3/well) for 24 h under normoxic or hypoxic (2% O_2) conditions. Subsequently, NP cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 10 min, blocked with 5% FBS, and incubated overnight at 4 °C with anti-HIF-2α antibody (Cat#Ab131743, Abcam, UK) at a dilution of 1:200. The cells used as negative control were reacted with isotype IgG under similar conditions. After washing, the cells were incubated with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen) at a dilution of 1:100 for 1 h at room temperature, and 4′,6-diamidino-2-phenylindole (DAPI) were redyed the cell nucleus. Then they were imaged with a laser scanning confocal microscope (FluoView, Olympus, Japan). HIF-2α expression was performed semi-quantitative analysis according the fluorescence intensity values.

2.5. Transfections and dual luciferase assay

After culturing in 24-well plates for 24 h, the NP cells were treated with 500 ng pHIF-2α and control plasmid using the transfection reagent Lipofectamine 3000 (Invitrogen). For gene suppression experiments, we silenced HIF-2α expression in NP cells using siRNA technology. 300 ng siHIF-2α and control siRNA were premixed with the Lipofectamine 3000 reagent and transfected into NP cells for 24 h. Then, the cells were

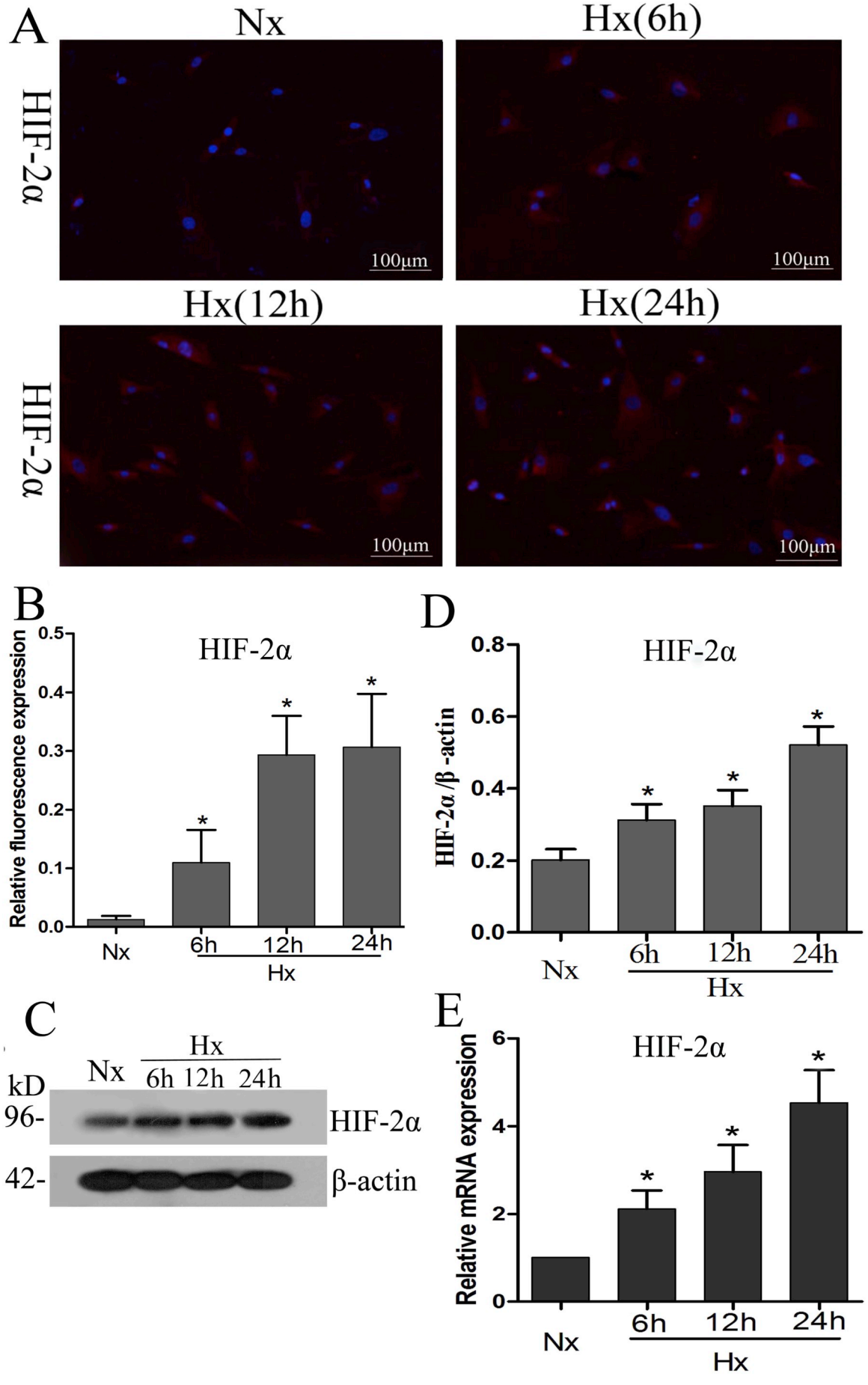

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Fig. 2. Hypoxic regulation of HIF-2α expression in NP cells. (A, B) Immunofluorescence analysis of NP cells under hypoxic and normoxic conditions in different time-points.The cells showed increased HIF-2α expression in 6 h and time dependence after treatment. Scale bar: 100 μm. (C,D) Western blot analysis of HIF-2α protein expression and (E) Real-time PCR of HIF-2α RNA expression by NP cells cultured under hypoxic conditions from 6 to 24 h. (Nx: normoxic; Hx: hypoxic, $*p < 0.05$ compared with normoxic group; per repetition $r = 4$.)

transferred to hypoxic conditions (2% O_2) or were maintained under normoxic conditions (21% O_2) for an additional 24 h. The Dual Luciferase Reporter Gene Assay Kit (Cat#16186, Invitrogen, USA) was used to test the luciferase activity.The NP cells were harvested and Cell Lysis Buffer was added to fully lyse the cells, then the suspension was centrifuged for 2 min to take the supernatant. The Luciferase Assay Reagent and Renilla Luciferase Assay solution were configured and applied. A dual Luciferase reporter assay system (Promega) was used for sequential measurements of firefly and *R.reniformis* luciferase activities. A luminometer (TD-20/20, Turner Designs, USA) was used to quantify luciferase activity and calculate the relative ratios.

2.6. Western blotting

NP cells or tissues were collected and incubated in Radio Immunoprecipitation Assay (RIPA) buffer (Bio-Rad, USA) on ice for 30 min, followed by centrifugation at 12,000 rpm for 15 min to extract the supernatant. The proteins were electrophoresed on a 10% SDS-PAGE gel and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk in Tris-HCl Tween(TBST)at room temperature for 1 h, and then probed with antibodies (diluted 1:500, Abcam) against β-actin (Cat#Ab8226), NF-κB (Cat#Ab131546), HIF-2α (Cat#Ab131743), MMP-9/13 (Cat#Ab38898/Ab39012), ADAMTS-4/5 (Cat#Ab185722/Ab41037), aggrecan(Cat#Ab3773), or collagen II(Cat#Ab34712) at 4 °C overnight, followed by the secondary antibodies (diluted 1:2000; ZSGB-BIO, China) at 37 °C for 2 h. Subsequently, the membranes were washed with TBST buffer and followed by immunolabeling detection with enhanced chemiluminescent (ECL) reagent (Millipore, USA). Finally, the protein bands were scanned with an imaging system (Image Quant LAS 4000 mini, GE, USA).

2.7. Real-time RT PCR analysis

Total RNA was extracted from NP tissues or treated NP cells using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's protocol. Complement DNA was synthesized from extracted RNA with the High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, USA) using Real-time PCR system (SuperScriptIII Synthesis System, Thermo Fisher Scientific). The reactions were set up in microcapillary tubes of 96-well plates using 2 μL cDNA with SYBR Green PCR Master Mix (10 μL; Thermo Fisher Scientific) to which gene-specific forward and 8 μL reverse PCR primers were added (Table 1). β-actin was the housekeeping gene and the quantity of each target gene was normalized to β-actin expression. All primers were synthesized by Integrated DNA Technologies (Sangon Biotech). The PCR reactions were performed in Real-time PCR system (ABIPrism 7500, Applied Biosystems, USA) according to the manufacturer's instructions. Data was analyzed by use of ΔΔCt and results were expressed as $2^{\Delta\Delta Ct}$ [18].

2.8. Histological studies

Human NP tissues of 36 samples were fixed in 4% paraformaldehyde for 2 days and rabbit disc of 14 samples were decalcified in 30% formic acid for 1 week. Then, the tissues were dehydrated in graded ethanol solutions and embedded in paraffin. In order to examine the morphology and matrix degeneration in rabbit intervertebral disc samples, the samples were sectioned into 4 μm slices and stained with Masson trichrome and safranin O staining following the standard protocols. For localizing HIF-2α, the sections of human and rabbit tissues were incubated with the anti-HIF-2α antibody (diluted 1:200, Abcam) in 2% bovine serum albumin at 4 °C overnight, followed by the secondary antibodies (diluted 1:500, ZSGB-BIO) for 30 min at room temperature. The specimens were examined and photographed using a fluorescence microscope (FluoView, Olympus) and HIF-2α protein was performed semi-quantitative analysis according counting the percentage of positive cells (10 high-power fields and calculation the average number of positive cells in each section).

2.9. Rabbit model

Twenty-eight female New Zealand White rabbits (2.5–3.0 kg body weight) were used. All procedures of the animal study were approved by the Animal Research Committee of Beijing Jishuitan Hospital (No.2017-12). The rabbits were randomly divided into three different groups: control group ($n = 8$); model group whereby the fifth lumbar disc was punctured ($n = 10$) and drug group treating with HIF-2α inhibitor (n = 10).

The animals were subjected to general anesthesia by intramuscular injection with compound ketamine in 0.3 ml/kg of body weight per rabbit before the puncture process. A 20-gauge puncture needle (Spinocan® B, Germany) was used to induce annulus fibrosus injury at the fifth lumbar (L5) level using a posterolateral approach until the needle was advanced through the annular fibrosus to the nucleus pulposus based on resistance [20]. For each puncture, the operator visualized the needle in the nucleus pulposus by radiography. After three month of puncture, the rabbits of drug group were given the HIF-2α inhibitor (HIF-2α-IN-1,10 μg/kg) by intraspinal administration.

For imaging procedures, MRI were performed to evaluate of the degeneration process on 28 rabbits at 6 months after the puncture. Sagittal of the spines were taken with exposure of 100 mAs and penetration power of 45 kVp by a magnetic resonance machine (CareStream Health, Rochester, NY, USA) to obtain T2-weighted images (TE = 100 ms; TR = 4000 ms).

2.10. Statistical analysis

All statistical analyses were performed using SPSS version 19.0 (IBM, USA) and results were expressed as mean ± standard deviation. To test for significance, data were analyzed using Student's *t*-test between two groups. Analysis of variance was conducted to compare

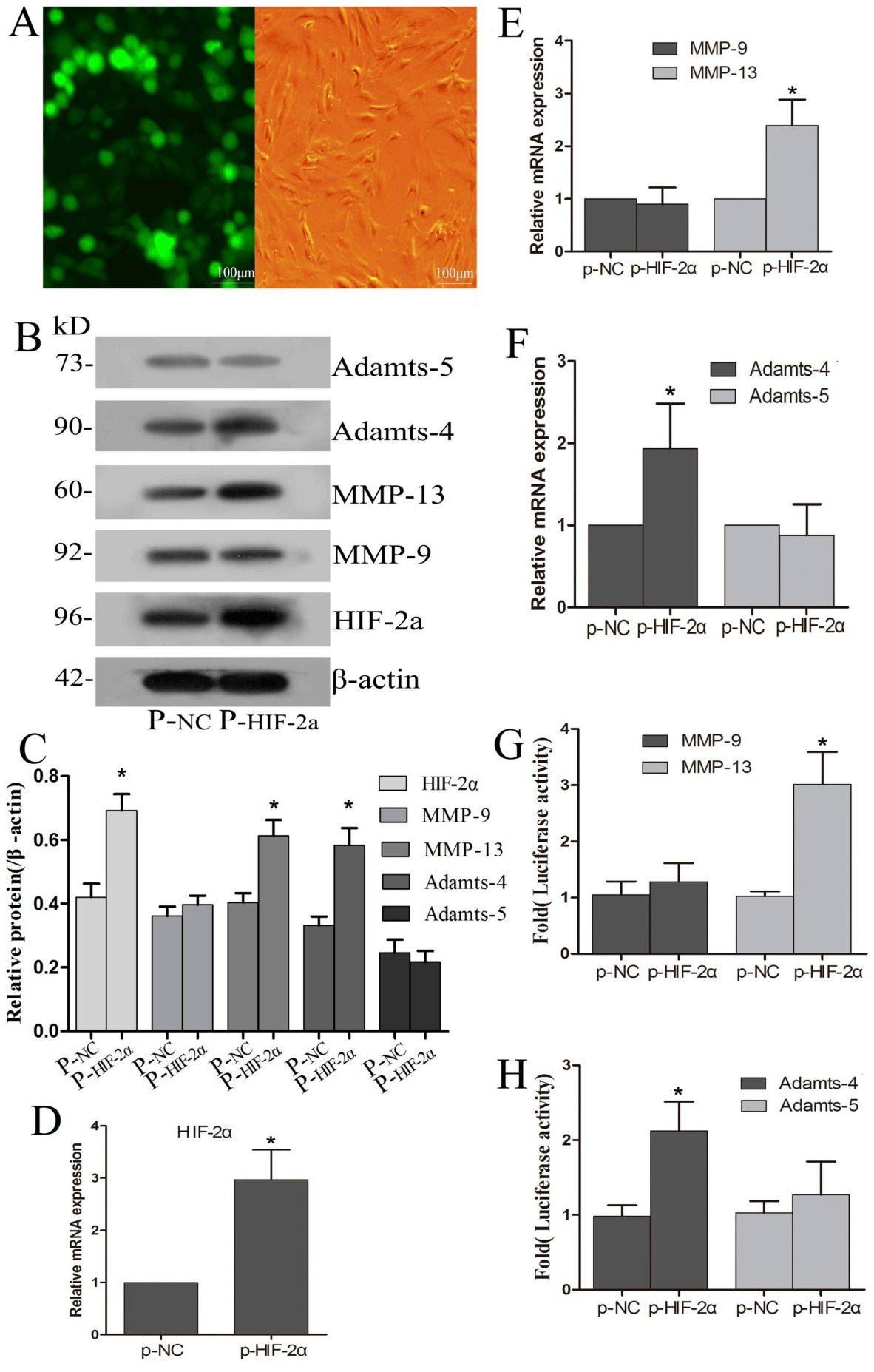

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Fig. 3. Over-expression of HIF-2α in regulating MMP and ADAMTS. (A) NP cells were transfected with pHIF-2α and cultured under hypoxic conditions to observe the transfection efficiency (right control without GFP). (B,C) Western blot analysis of MMP-13 and ADAMTS-4 protein and (D-F) Real-time RT-PCR analysis of mRNA expression levels increased by over-expression of HIF-2α. (G,H) Using the Luciferase reporter gene assay to test the effect of HIF-2α on MMP and ADAMTS promoter activity in NP cells transfected with pHIF-2α under hypoxic conditions. (NC: normal control, *p < 0.05 compared with p-NC group; per repetition r = 4.)

trends among different groups and the Fisher's LSD was used between groups, with the level of significance set at $p < 0.05$.

3. Results

3.1. Evaluation of human degenerated NP samples

The expression levels of HIF-2α and MMP and ADAMTS catabolic factors in human degenerated disc tissues were evaluated according to the Pfirrmann grading system (Fig. 1A). With the increase of grading, T2 weighted signal intensity was decreased and the disc space became narrower. Immunohistological analysis revealed that HIF-2α was expressed higher in degenerated NP tissue, especially in the early stages (Grade II,III) of degeneration, while in the later stages (Grade IV, V), the NP cell numbers and HIF-2α expression were significantly decreased (Fig. 1B,C). Western blot analysis showed that HIF-2α protein expressions were elevated in grade III, IV and V. The highest HIF-2α expression was observed in grade III with a gradual decreased in grade IV and V. The MMP-13 and ADAMTS-4 proteins showed similar results that grade III tissues owed the most proteins while progressively smaller reductions in grade IV and V periods (Fig. 1D-H). The Real-time PCR results showed the same trend in HIF-2α (Fig. 1I), MMP-13, and ADAMTS-4 (Fig. 1J, K) that the highest mRNA expression was observed in grade III tissues and a gradual decreased mRNA expression was observed with the increasing of grades.

3.2. HIF-2α expression under hypoxic condition

To further evaluate the relationship between HIF-2α regulation and oxygen tension in NP cells, we determined HIF-2α expression under normoxic and hypoxic conditions. As shown in Fig. 2A, the human NP cells cultured under hypoxic tension showed higher expression levels of HIF-2α protein compared with the cells under normoxic condition in an immunofluorescence assay and semi-quantitative analysis, especially in 12 h and 24 h (Fig. 2B). Western blotting and Real-time PCR showed that HIF-2α expressions were relatively lower under normoxic condition and was elevated in 6 h after treatment under hypoxic condition, and HIF-2α proteins and mRNA were increased with the extended response time(Fig. 2C–E).

3.3. Over-expression of HIF-2α in regulating the catabolic factors

To determine the effect of stable over-expression of HIF-2α in regulating MMP and ADAMTS levels, we transfected NP cells with plasmid-HIF-2α. By evaluating the cells with a GFP positive control, we confirmed that the transfection efficiency was > 70% (Fig. 3A). As expected, Western blotting demonstrated an increase in HIF-2α protein levels in the transfected NP cells compared with the control group (Fig. 3B, C). Moreover, the HIF-2α-transfected cells showed increased MMP-13 and ADAMTS-4 protein expression, but no change in MMP-9 and ADAMTS-5 expression. The real-time RT PCR results showed that the mRNA levels of HIF-2α, MMP-13, and ADAMTS-4 were increased significantly compared with the control group (Fig. 3D–F). We evaluated the role of HIF-2α in regulating MMP and ADAMTS promoter activity further by using the Dual Luciferase Reporter Gene Assay. In NP cells, over-expression of HIF-2α effectively induced MMP-13 and ADAMTS-4 promoter activity under hypoxic conditions, but there was no significant effect on the induction of MMP-9 and ADAMTS-5 promoter activity (Fig. 3G,H).

3.4. Suppression of HIF-2α in regulating catabolic factors

To further assess the role of HIF-2α in controlling catabolic factors, we performed loss function experiment in NP cells using siRNA silencing of HIF-2α under hypoxic condition. The high transfection efficiency of lentiviral si-HIF-2α is shown in Fig. 4A and the suppression efficiency of genes was confirmed by western blotting and Real-time PCR. The HIF-2α-silenced NP cells showed a decrease in protein and mRNA expressions of HIF-2α (Fig. 4B-D) compared with control siRNA, while the HIF-2α levels were relatively higher under hypoxic conditions compared with normoxic conditions. Notably, the target gene expression of MMP-13 and ADAMTS-4 was significantly decreased in NP cells that were transfected with HIF-2α siRNA. In contrast, the HIF-2α knockdown did not affect MMP-9 and ADAMTS-5 expression under hypoxic condition (Fig. 4E, F). Compared with control siRNA under normoxic and hypoxic condition, HIF-2α silencing strongly inhibited MMP-13 and ADAMTS-4 promoter activity, but had no obvious correlation on MMP-9 and ADAMTS-5 promoter activity (Fig. 4G,H).

3.5. TNF-α stimulation on HIF-2α and signal pathway regulation

TNF-α was a regulator of catabolic factors that might be regulated by HIF-2α mediators in cultured NP cells. In the present study, TNF-α moderately induced expression of HIF-2α protein and gene levels in NP cells that were positively correlated with the concentration and time (Fig. 5A-D). Compared with the control group, the TNF-α induced groups showed the higher expression of the NF-κB protein and RNA, accompanied by the activation of the MMP-13 and ADAMTS-4 catabolic factors, however, no obvious correlation with MMP-9 and ADAMTS-5 expressions was observed (Fig. 5E–I). To clarify the mechanisms involved, we treated the NP cells with NF-κB and HIF-2α inhibitors and examined the effects on the production of HIF-2α, MMP, and ADAMTS. HIF-2α protein and gene levels were down-regulated in the NF-κB inhibitor group, and similar decrease was showed on MMP-13 and ADAMTS-4 expressions in the NF-κB and HIF-2α inhibitor groups compared with the control group, but no significant difference in MMP-9 and ADAMTS-5 expressions (Fig. 5J–N). These results proposed that HIF-2α mediates up-regulation of MMP-13 and ADAMTS-4 by potentiating the TNF-α/NF-κB signaling, which in turn, plays an essential role in regulating catabolism-promoting gene expression in NP cells.

3.6. The role of HIF-2α in the rabbit degenerated disc model

In order to further explore the potential protective effects of HIF-2α

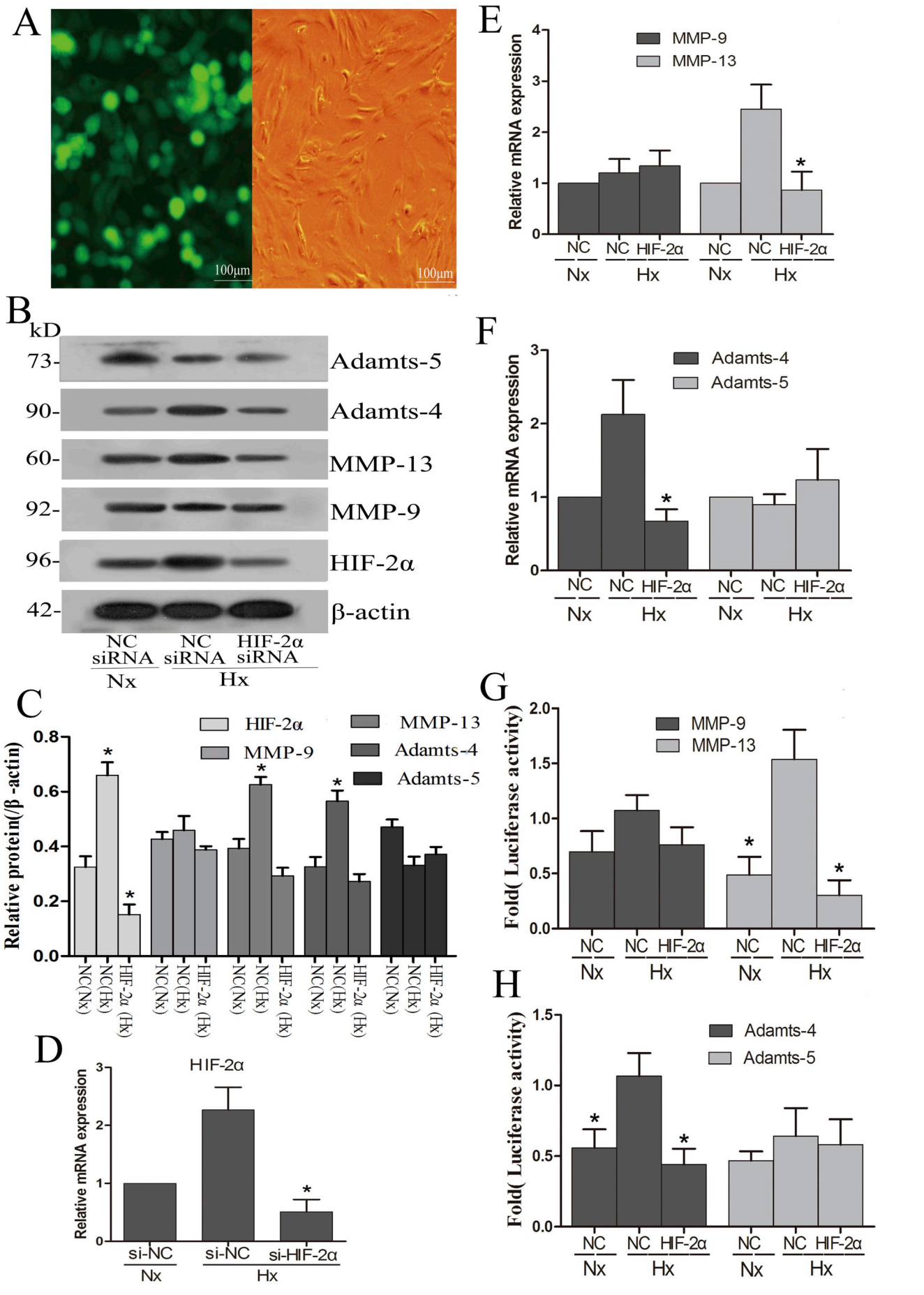

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Fig. 4. Effect of HIF-2α suppression on MMP and ADAMTS expression. (A) Cells were transfected with HIF-2α-siRNA and cultured under hypoxic conditions to observe the transfection efficiency (right control without GFP). (B,C) Western blot analysis and (D-F) real-time RT-PCR detection of NP cells treated with control siRNA or HIF-2α siRNA under normoxic and hypoxic conditions. (G,H) Using the Luciferase reporter gene assay to test the effect of HIF-2α silencing on MMP and ADAMTS promoter activity in NP cells under normoxic and hypoxic conditions. (Nx: normoxic; Hx: hypoxic, NC: normal control, *$p < 0.05$ compared with Hx-NC group; per repetition r = 4.)

in degenerative disc disease, a rabbit degenerated disc model was established. The MRI T2WIs of the degenerated disc model showed that the signal intensity of NP tissue was decreased in model group compared with the control and treatment groups (Fig. 6A). Masson and safranin O (SO) staining showed that the ECM was significantly degraded in the model group, and In-HIF-2α treatment effectively slowed down the degradation of ECM, but was partly decreased compared with control group (Fig. 6B). Immunohistochemical staining of HIF-2α protein showed that HIF-2α expression in the degenerated disc model group was markedly increased, whereas the control and treatment groups had less HIF-2α protein in the NP tissue (Fig. 6B, C). Western blot analysis and Real-time PCR showed that the expressions of HIF-2α protein and catabolic factors MMP-13 and ADAMTS-4 were clearly elevated and the ECM expressions of aggrecan and collagen II were significantly decreased in the model group, which was partially restored in the treatment group(Fig. 6D–G).

4. Discussion

Among the sequential steps of investigation on NP cell activity and NP tissue degradation, our study revealed for the first time that HIF2α is an important regulator of catabolic factors in degenerated intervertebral discs and may be a marker for disc degeneration. HIF2α was increased and led to MMP-13 and ADAMTS-4 expression in intervertebral discs degradation tissue, and the levels of positive regulator proteins were correlated with the severity of the human disc degeneration. In-HIF2α treatment could partially block and reverse the upregulation of MMP-13 and ADAMTS-4 proteins. Altering the balance of anabolism and catabolism in the ECM of NP tissues by modulating HIF-2α signaling may be a suitable therapeutic strategy to mitigate and prevent the effects of disc degeneration.

HIF2α possesses about 50% amino acid homology with HIF1α, a potential regulator of NP homeostasis, and the NP cells maintain their bio-synthetic activities by constitutive expression of HIF-1α and HIF-2α [21,22]. However, valid evidences showed a discrete expression form and functions between the two proteins that HIF-1α regulates aggrecan expression and induces synthesis of collagen II in chondrocytes by inducing the SOX9 promoter sequence, whereas HIF-2α is a transcription factor in the development of osteoarthritis [18,23,24]. The current study showed that HIF2α was expressed from early stage disc degeneration and particularly higher in the grade III stage and then decreased slightly with the severity of grades. In the later or severe stage, the reduction of HIF2α expression might have closely connection with the apoptosis of NP cells that apoptosis was recognized as an important patterns of cellular programmed death in the disc degeneration [25]. HIF2α activities were increased by the stimulation of hypoxia and the inflammatory factor TNF-α in NP cells that were time and dose dependent. It had been verified in another tissue that TNF-α regulation of the hypoxia-induced erythropoietin expression was mediated primarily by HIF2α rather than HIF1α [26]. Likewise, the animal model of disc degeneration confirmed that HIF2α levels were markedly increased in NP tissues and direct treatment with In-HIF2α effectively mitigated the disc degeneration progression in the early stages, supporting the pathological significance of HIF2α expression. HIF2α protein have been previously reported expressing in nucleus pulposus of intervertebral disc and Yang et al. also demonstrated that HIF-2a is elevated in degenerative arthritis and causes serious cartilage destruction [18,27]. Although our results were different from those reported by Agrawal et al. [28] and Yang et al. [29], that may be use of different cell lines and different culture time.

The main catabolic factors, MMPs and ADAMTS, play a key role in the ECM degeneration of the NP tissues that degrade the key ECM components of aggrecan and collagen II [5,30]. Interestingly, in vitro studies of NP cell cultures, human and animal model NP samples suggested that disc degeneration was partially caused by the high expression of MMP-13 and ADAMTS-4 proteins and the expression was positively correlated with the HIF2α levels. Meanwhile, silencing the HIF2α decreased MMP-13 and ADAMTS-4 expressions, whereas the over-expression of HIF2α is sufficient to trigger higher expressions of MMP-13 and ADAMTS-4. Aggrecan degradation is mainly mediated by ADAMTS-4 and MMP-13 is one of the most predominant collagenases targeting type II collagen [31,32]. In osteoarthritis cartilage , HIF2α is highly expressed and causes progressive cartilage damage and upregulation of multiple degradative enzymes, including MMP and ADAMTS [33]. These results suggested that these two metabolic genes are regulated in an HIF-2α-dependent manner and inhibiting HIF2α protein may effectively decrease catabolic factor expression and alleviate disc degeneration. Furthermore, elevated expression of HIF2α stimulated MMP-13 and ADAMTS-4 promoter activity and mutation of HIF2α abolished the responsiveness of the two promoters to changes, indicating that HIF2α proteins are required for the regulation of MMP-13 and ADAMTS-4 promoter activity at the transcriptional level. Previous study have revealed that HIF2α functions as an extensive transcriptional regulator of the central step and MMP-13 are the direct transcriptional target [24].

Inflammation is correlated with disc degeneration and multiple genes are associated with the genetic predisposition to disc degeneration, including the inflammatory factors COX-2, IL-1β, and TNF-α [34,35]. TNF-α is a pro- inflammatory factor that contributes to the loss of the disc matrix and promotes cellular apoptosis via cytotoxic effects in human NP tissues [36]. In the present study, we demonstrated that TNF-α effectively induced the expression of HIF2α gene, which was closely associated with elevating MMP-13 and ADAMTS-4 expression. Among the signaling pathways activated by TNF-α, those involving NF-κB is the most potent regulators of HIF2α expression [37]. NF-κB was previously reported as a major meditator in disc degeneration, which reversed the changes in the expression of ADAMTS and MMPs [38,39]. Our results indicated that the amount of NF-κB protein was increased by

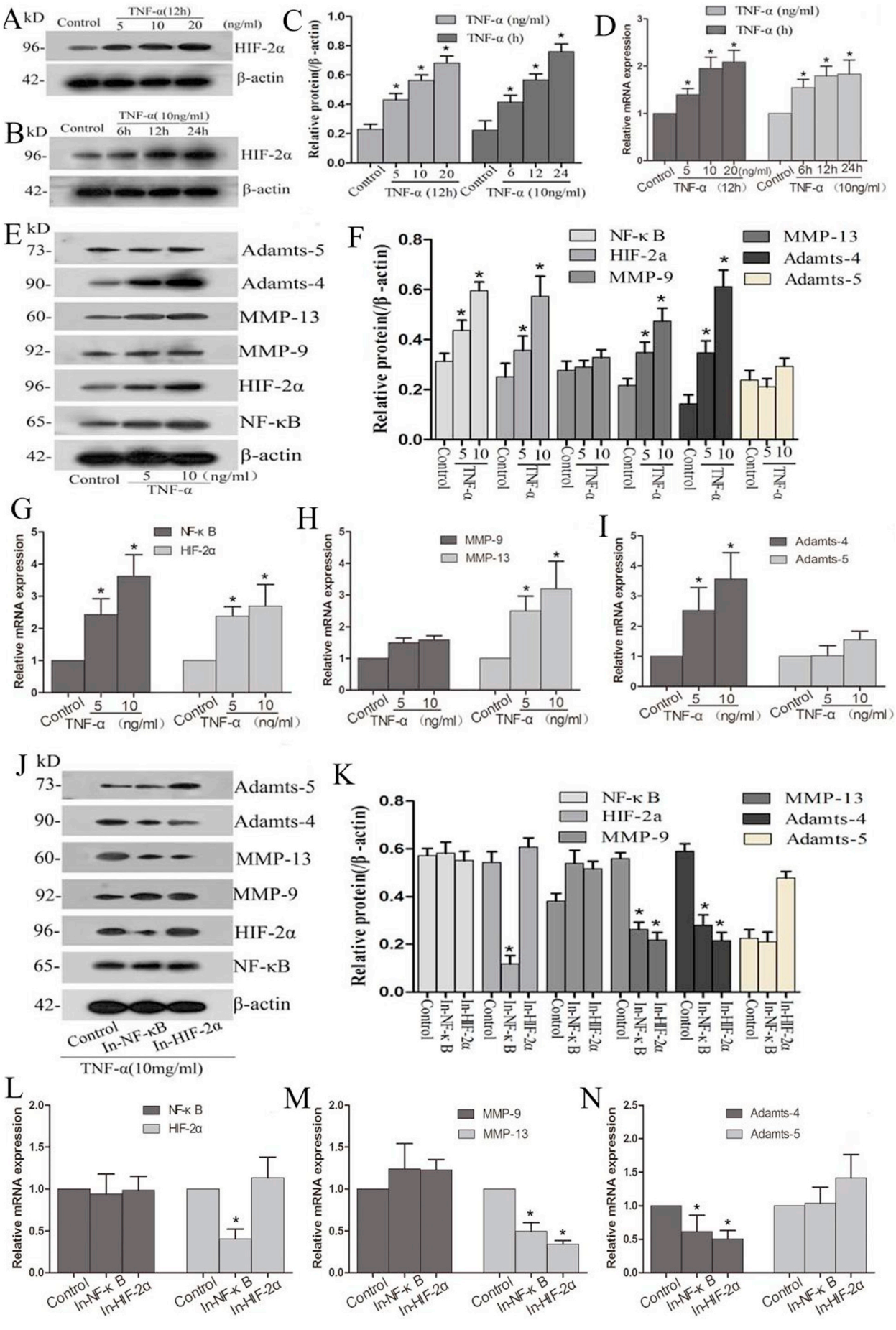

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Fig. 5. TNF-α stimulates NF-κB dependent expression of HIF-2α and catabolic factors in NP cells. (A-D) NP cells were exposed to TNF-α with different concentration gradients of TNF-α (5, 10, 20 ng/ml) for 6,12, and 24 h, after which (A-C) western blot analysis and (C) real-time RT-PCR were performed to monitor the expression of HIF-2α. (*E*-I) NF-κB, HIF-2α, MMP, and ADAMTS expression was stimulated by TNF-α to observe the relationship between the proteins and genes via (E,F) western blot analysis and (G-I) Real-time PCR. (J-N) NF-κB and HIF-2α inhibitors were used to detect the effect on downstream gene and protein expression via (J,K) western blot analysis and (L-N) Real-time PCR. (In-NF-κB:NF-κB inhibitor; In-HIF-2α:HIF-2α inhibitor; *$p < 0.05$ compared with control group; per repetition r = 4.)

TNF-α stimulation, whereas inhibiting NF-κB decreased the TNF-α-induced expression of HIF2α protein and catabolic factors MMP-13 and ADAMTS-4 sharply. Therefore, TNF-α was an initial factor in disc degeneration and the NF-κB signal pathways were activated to accelerate degeneration by regulating the HIF2α transcription factor and genes encoding the catabolic factors.

5. Limitations

Although interesting findings were appeared, Our research still had certain limitations. Firstly, what we chose was the degenerated disc in human, so the grades have errors for NP cells. Secondly, our research only studied the human NP tissues, the annulus fibrosus and cartilage endplates needed further study. Although we had found the effect of In-HIF2α on ECM catabolism in animal model, the safety need further evaluation if applied in human.

6. Conclusions

In conclusion, we revealed that HIF2α was a potently induced catabolic factors of MMP-13 and ADAMTS-4 expressions in human NP cells and tissues, and that HIF2α acts as a crucial mediator of disc degeneration. Additionally, the inflammatory factor TNF-α stimulated HIF2α expression and facilitated ECM catabolism by up-regulating the activation of the NF-κB pathway. Therefore, genetic treatment of inhibiting HIF2α activity might be a novel and potential therapeutic approach to degenerative disc disease.

Abbreviations

NP	nucleus pulposus
HIF-2α	hypoxia-inducible factor-2alpha
TNF-α	tumor necrosis factor-alpha
MMP	matrix metalloproteinase
ADAMTS-4	disintegrin and metallopeptidase with thrombospondin motifs-4
NF-κB	nuclear factor-κB
ECM	extracellular matrix
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
RIPA	Radio Immunoprecipitation Assay
ECL	enhanced chemiluminescent

Author contribution to study form

Tian Wei contributed to the conception of the study.

Huang Yuelong contributed significantly to analysis and manuscript preparation;

Wang Ying performed the data analyses and wrote the manuscript;

Wu Chengai helped perform the analysis with constructive discussions.

Declarations

Ethical approval and consent to participate

Our study protocol was approved by the Ethics Committee of Beijing Jishuitan Hospital and informed consent for sample collection was obtained from all patients.

Consent for publication

All the authors agree the consent for publication. Informed consent for publication of individual details and images were obtained from all patients.

Availability of supporting data

All the data and material were Available.

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Authors' contributions

Huang Yuelong carried out the all experiment and drafted the manuscript; Wang ying and Wu chengai participate in real-time PCR and Western blotting. Tian wei conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Authors' information

The information of authors provided in the first page.

Declaration of Competing Interest

All the authors declared that they have no conflicts of interest to this work and do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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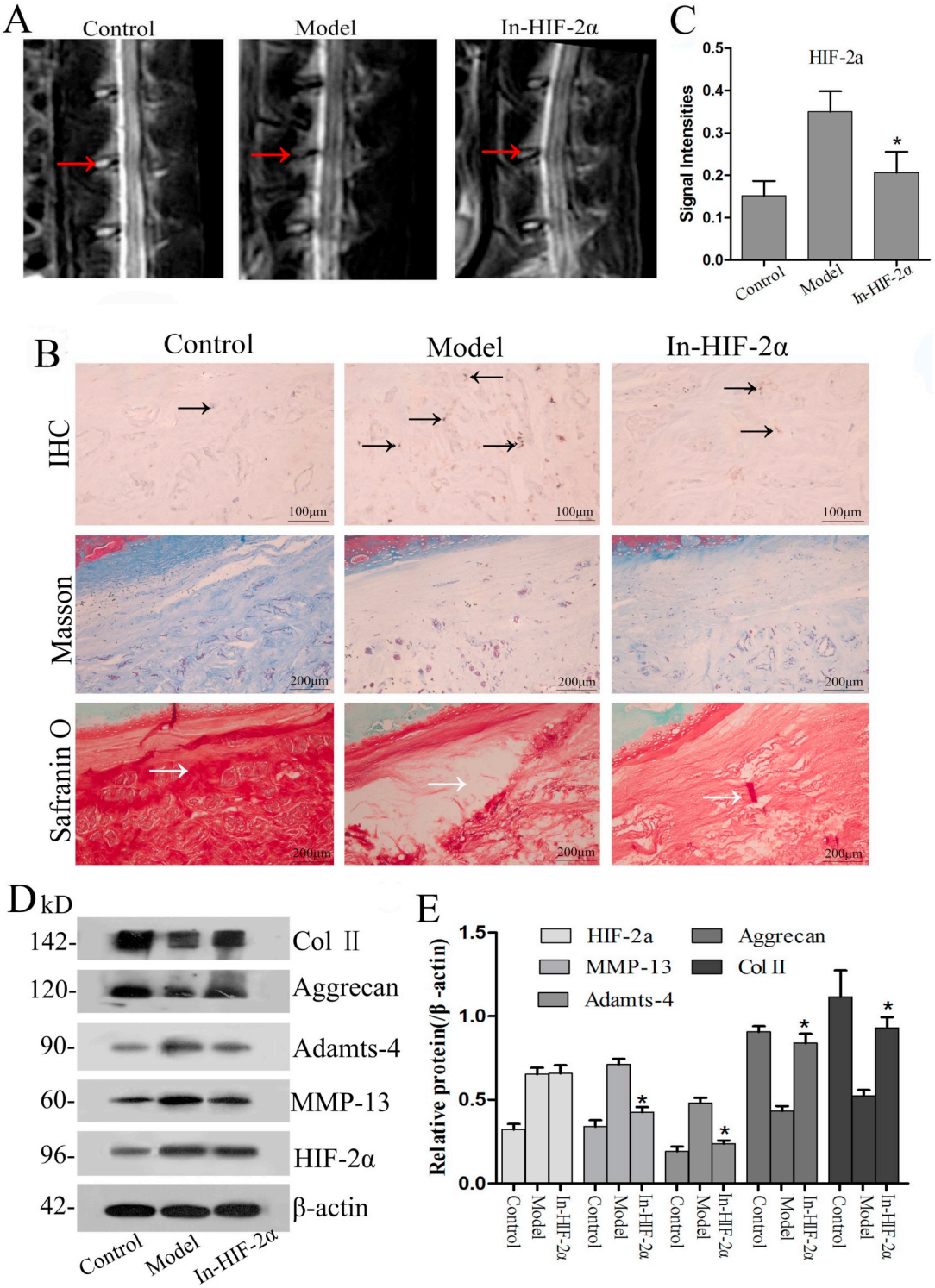

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Fig. 6. The role of HIF-2α in the rabbit degenerated disc model. (A) T2WIs of the control (n = 8), degenerated disc model (n = 10), and In-HIF-2α-treated groups (n = 10).Red arrow:degenerated disc (B,C) Immunohistochemical (anti-HIF-2α antibody), Masson, and Safranin O staining of an intervertebral disc treated with In-HIF-2α respectively show HIF-2α expression, collagen II content and aggrecan content difference in different groups. Black arrow:HIF-2α protein expression; White arrow:aggrecan content. (D,E) Western blot analysis of HIF-2α, MMP-13, ADAMTS-4, aggrecan, and collagen II protein expression. (F, G) Real-time PCR analysis of HIF-2α, MMP-13, ADAMTS-4, aggrecan, and collagen II RNA expression in different groups. (In-HIF-2α: HIF-2α inhibitor; *p $<$ 0.05 compared with model group; per repetition r = 4 or 5.) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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