THE EFFECT OF HYALURONIC ACID ON NUCLEUS PULPOSUS EXTRACELLULAR MATRIX PRODUCTION THROUGH HYPOXIA-INDUCIBLE FACTOR-1A TRANSCRIPTIONAL ACTIVATION OF CD44 UNDER HYPOXIA

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Abstract

Intervertebral disc degeneration (IDD) is the leading cause of low-back pain. Implantation of hyaluronic acid (HA) is potentially a therapeutic strategy for IDD, but its pharmacological effects and mechanism under hypoxic conditions remain unclear. In this study, the expression of extracellular matrix genes and proteins were enhanced in nucleus pulposus cells (NPCs) in the presence of HA under hypoxic condition, as shown by real-time reverse transcription-polymerase chain reaction, immunofluorescence staining, and dimethylmethylene blue assays. Moreover, the expression of CD44 was increased in the presence of both HA and hypoxia compared to either alone. Using a bioinformatic database, hypoxia inducible factor-1α (HIF-1α), a key transcription factor in the hypoxic condition, was found to have 4 predicted binding sites on the CD44 promoter. CD44 expression was significantly increased by treatment with cobalt chloride or dimethyloxalylglycine. Over-expression of HIF-1α in NPCs significantly up-regulated the expression of CD44. The binding site of HIF-1α in the CD44 promoter region, was identified by promoter truncation experiments and chromatin immunoprecipitation assays. Taken together, these results indicated that hypoxic conditions positively potentiated the ability of NPCs matrix synthesis in the presence of HA, which correlated with the increasing CD44 expression by HIF-1α transcriptional activation.

Keywords: Intervertebral disc degeneration, hypoxia inducible factor-1α, CD44, hyaluronic acid, gene regulation.

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List of Abbreviations

5′ UTR	5′ untranslated region
ADAMTS4	a disintegrin and metalloproteinase with thrombospondin motifs 4
ANOVA	analysis of variance
BSA	bovine serum albumin
CA12	carbonic anhydrase 12
CCK	cell counting kits
CCN2	cellular communication network factor 2
CD24	a sialoglycoprotein expressed at the surface of most B lymphocytes and differentiating neuroblasts
CD44	a main cell surface receptor of HA
ChIP	chromatin immunoprecipitation
COL II	collagen II
DAPI	4′,6-diamidino-2-phenylindole
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DMMB	dimethylmethylene blue
DMOG	dimethyloxalylglycine
ECL	enhanced chemiluminescence

ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FBS	foetal-bovine serum
GAG	glycosaminoglycan
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HA	hyaluronic acid
HABP2	HA-binding protein 2
HIF-1α	hypoxia-inducible factor-1α
HRE	hypoxia-responsive element
HRP	horseradish peroxidase
IDD	intervertebral-disc degeneration
IF	immunofluorescence
IL-1R1	interleukin 1 receptor, type I
IVD	intervertebral disc
MyD88	myeloid differentiation factor 88
NP	nucleus pulposus
NPCs	NP cells
OCT	optimal cutting temperature
Over-HIF-1α	HIF-1α over-expression construct
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PHD	prolyl-hydroxylases
PMSF	phenylmethylsulphonyl fluoride
RIPA	radio-immunoprecipitation assay
RT-qPCR	real-time reverse transcription PCR
SD	standard deviation
SDC4	syndecan4
SDS	sodium dodecyl sulphate
TBST	tris-buffered saline with polysorbate 20
TSS	transcription start sites

Introduction

Low-back pain is a common health problem that affects up to 80 % of individuals during their lifetime, imposing enormous health and economic burdens on society (Takahashi *et al.*, 2008). IDD is considered to be a primary cause of low-back pain (Vergroesen *et al.*, 2015). Current therapies for IDD range from rehabilitation and medications to invasive surgical interventions. All of them have limited efficacy and can neither address the underlying degeneration nor restore the native function of the IVD (An *et al.*, 2003). For this reason, there is a compelling need to develop a therapeutic approach with improved outcomes.

HA is a major non-sulphated GAG present in the ECM of the IVD. In NP tissue, HA plays a critical role in ECM production, cell migration and phenotypic maintenance, acting as the backbone of proteoglycan aggregates that contain aggrecan molecules (Chung *et al.*, 2008). As IDD progresses, the molecular weight and concentration of HA in the NP decrease, which results in further disc degeneration through enhancement of matrix degradation, inflammatory response, and reduction of water retention in the discs (Quero *et al.*, 2013).

With the development of tissue engineering technology, implantation of HA-based hydrogel is a novel biological treatment for early stage IDD. Various HA hydrogels have been investigated for their potential to replace and regenerate the NP through reducing inflammation response and nociceptive behaviour (Isa *et al.*, 2015; Mohd *et al.*, 2018), promoting ECM synthesis (Kim *et al.*, 2015), and restoring biomechanical properties (Zhou *et al.*, 2014). Furthermore, it has recently been shown that HA hydrogels could promote stem cell differentiation to NP-like cells and improve the efficacy of stem cells for NP regeneration (Chen *et al.*, 2019; Peroglio *et al.*, 2013). Some studies have explored the mechanism by which HA promotes NP regeneration. For example, HA could modulate the inflammatory receptor of IL-1R1, MyD88 and neurotrophin expression (Isa *et al.*, 2015), or downregulate the catabolic marker ADAMTS4 (Kazezian *et al.*, 2017). However, these studies assessing the biological effects and molecular mechanisms of HA for NP regeneration, have been performed under standard normoxic conditions, which is not representative of the physiological hypoxic environment in the intervertebral disc.

CD44, plays an important role in regulating multiple cellular functions (Ishida *et al.*, 1997). It has previously been shown that hypoxia potentiated the anabolic effects of HA, including the redifferentiation of articular chondrocytes and synthesis of ECM, through enhancing CD44 expression and binding affinity (Ichimaru *et al.*, 2016). Furthermore, previous research suggested that hypoxia could enhance the expression of CD44 *via* HIF-1α (Liang *et al.*, 2017). Based on the bioinformatics databases JASPAR (Web ref. 1) and Promoter 2.0 Prediction Server (Web ref. 2), HIF-1α binding sites were found in the human CD44 gene promoter region, which means that HIF-1α may transcriptionally activate CD44, resulting in enhanced CD44 expression (Fornes *et al.*, 2020).

Based on these above findings, the hypothesis that hypoxic conditions could enhance the effect of HA for NP regeneration through HIF-1α transcriptional activation of CD44 is proposed. In this study, the aim was to determine the transcriptional activation of CD44 by HIF-1α and explore the ability of NPCs proliferation and matrix synthesis under hypoxic conditions in the presence of HA. The therapeutic potential of HA for the treatment of IDD in hypoxic condition was demonstrated.

Materials and Methods

Human NPCs culture and treatment

NP tissues were obtained during discectomy surgery from 10 patients with lumbar-degenerative diseases (Table 1). All procedures were performed in accordance with the guidance and approval of the research ethics committee of the 7th Affiliated Hospital of Sun Yat-sen University, Shenzhen, Guangdong, China. The tissue of the central portion of NP was washed 3 times with PBS (Gibco) to eliminate blood, and cut into 1 mm^3 fragments. Subsequently, NP tissue was digested for 30 min with 0.25 % trypsin-EDTA (Gibco) followed by 0.25 % collagenase type II for 4 h at 37 °C, with agitation. After filtration

through a 70 μm nylon mesh to remove tissue debris, the NPCs were isolated by centrifugation at 1000 ×*g* for 5 min. The NPCs were maintained in DMEM/nutrient mixture F12 (Gibco) supplemented with 10 % FBS (Gibco) and 50 μg/mL Primocin (InvivoGen, San Diego, CA, USA), and incubated in a humidified atmosphere with 5 % CO_2 at 37 °C. The anti-microbial agents were replaced with 1 % penicillin/streptomycin (Gibco) after the cells reached P2. The cells of passage 3, 4 and 5 were used for the experiments.

For the hypoxia treatment, cells were maintained in a Hypoxia Work Station (Panasonic, Osaka, Japan) with a mixture of 2 % O_2, 5 % CO_2, and 93 % N_2 for 24-72 h. Due to the longer half-life and potential to increase the amount of endogenous HA and exhibit anti-inflammatory activity, high molecular weight HA (MW = 1,800-2,200 kDa) was chosen according to a previous study (Chen *et al*., 2013). Considering that HA degrades when exposed to high temperature, ultraviolet rays or ultrasound (Snetkov *et al*., 2020), high molecular weight HA was placed in an ampoule and the air removed, then sterilised at 121 °C for 15 min, to minimise molecular weight loss. NPCs were cultured in 6-well plate (Corning, NY, USA) (10^5 cells/well) with complete DMEM/F12 containing 2 mg/mL high molecular weight HA (Freda, Jinan, China) for 72 h under normoxic or hypoxic conditions. To induce hypoxic stress, NPCs were treated with 100, 200 or 400 μmol/L $CoCl_2$ (Sigma, St. Louis, MO, USA) for 24 h. To stabilise HIF-1α by inhibition of PHD function, NP cells were treated with 0.5 or 1 mmol/L DMOG (MCE, Monmouth Junction, NJ, USA) for 24 h (Fujita *et al*., 2012).

Plasmids and cell transfection

Human CD44 promoter reporter constructs from nucleotide − 2934 to ATG (2934-pCD 44) and control reporter vector were developed by Genecoepia (Rockville, MD, USA). The plasmid with HIF-1α over-expression construct or control vector were purchased from Genecoepia (Rockville). 2803-pCD44, 908-pCD44, 442-pCD44, 339-pCD44 were generated by restriction enzyme digestion of 2934-pCD44, using the primers listed in Table 2.

Lipofectamine LTX & PLUS Reagent (Invitrogen, Carlsbad, CA,USA) was used in the transient transfection according to the manufacturer's instructions. Briefly, 10^5 cells/well NPCs were transferred to 6-well plates (Corning) in DMEM/F12 with 10 % FBS before transfection. When the cells were at a confluence of 70-80 %, the plasmid with HIF-1α over-expression construct or control vector was diluted in Opti-MEM® reduced serum medium (Invitrogen), and mixed with lipofectamine reagent to prepare plasmid DNA-lipid complexes, then added to cells. Following incubation for 3 d at 37 °C under normoxic or hypoxic conditions, the cells were harvested, and the protein was extracted for the subsequent Western blot analysis.

RT-qPCR

Total RNA was extracted from the treated NPCs using an RNAeasy™ animal RNA isolation Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Reverse transcription was performed using PrimeScript™ RT Master Mix (TaKaRa, Shiga, Japan) according to the manufacturer's instruction. Real-time PCR was performed using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Foster City, CA, USA) in CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA). The total volume (10 μL) of each PCR reaction contained 5 μL SYBR Green Master Mix, 2 μL RNase-free dH_2O, 2 μL cDNA, and 0.5 μL of each forward and reverse primer (Table 3). The housekeeping gene *GAPDH* was used for normalisation, and the relative expression of each target gene was determined using the $2^{-\Delta\Delta Ct}$ method.

Protein extraction and Western blot analysis

Following treatment, NPCs were placed on ice immediately and washed 3 times with ice-cold PBS. Cells were lysed by RIPA lysis buffer (Boster, Wuhan, China) containing 1 % PMSF (Boster) and 1 % Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, Waltham, MA, USA), followed by sonication (Sonics CVX130, Newtown, MA, USA) for 30 s. Then, the protein was isolated by centrifugation at 12,000 ×*g* for 10 min at 4 °C. Protein extracts were resolved by electrophoresis on NuPAGE™ 4-12 % Bis-Tris

Table 1. Clinical patient characteristics.

Patient	Age	Gender	Disc level	Degeneration grade	Experiment
1	40	female	L4/5	III	Cell culture
2	66	female	L4/5	III	Cell culture
3	36	male	L4/5	III	Cell culture
4	63	male	L4/5	III	Cell culture
5	53	male	L3/4	V	RT-qPCR, IF
6	46	female	L5/S1	II	RT-qPCR, IF
7	70	male	L4/5	IV	RT-qPCR, IF
8	24	male	L4/5	III	RT-qPCR, IF
9	73	male	L4/5	IV	RT-qPCR, IF
10	46	male	L4/5	II	RT-qPCR, IF

Table 2. Primer sequences used for vector construction.

Vector name	Organism	Sense primer	Antisense primer
2803-pCD44	*Homo sapiens*	ACTAGTTTCAGGTGGGAGTTACGGGT	CATATGCATGCAGTAAGCACTTTAAA
908-pCD44	*Homo sapiens*	ACTAGTGGATGGGCGGATGGAAGGAT	CAGCTGCTCGCCAGCGGTGC
442-pCD44	*Homo sapiens*	ACTAGTGCCAGCGGGAGAAGAAAGCC	CAGCTGCTCGCCAGCGGTGC
339-pCD44	*Homo sapiens*	ACTAGTGGCAGCCCCGATTATTTACA	CAGCTGCTCGCCAGCGGTGC

Table 3. Primer sequences used for RT-qPCR experiments.

Gene transcript	Organism	Sense primer	Antisense primer
CD44	*Homo sapiens*	TGGCATCCCTCTTGGCCTTGG	TGAGACTTGCTGGCCTCTCCGT
Aggrecan	*Homo sapiens*	TCCCCTGCTATTTCATCGAC	CCAGCAGCACTACCTCCTTC
Collagen II	*Homo sapiens*	GGAGCAGCAAGAGCAAGGAGAA	AGCAGGCGTAGGAAGGTCATCT
GAPDH	*Homo sapiens*	CGGAGCCAAAAGGGTCATCA	GGGGGGCTAAGCAGTTGGTG

gels (Invitrogen) and transferred by electroblotting to PVDF membrane (Invitrogen). The membrane was rinsed in water and blocked with 5 % non-fat dry milk in TBST (Leagene, Beijing, China) for 2 h at room temperature with constant agitation. The membrane was then incubated overnight at 4 °C in TBST with antibodies against CD44 (1 : 2,000, ab157107, Abcam, Cambridge, UK), HABP2 (1 : 2,000, ab181837, Abcam), β-actin (1 : 2,000, Cell Signaling Technology, Danvers, MA, USA). After washing the membrane 3 times with TBST, goat anti-mouse HRP (1 : 4000, ab205719, Abcam) or goat anti-rabbit HRP (1 : 4000, ab205718, Abcam) was added and incubated at room temperature for 2 h. Following washing 3 times with TBST, the immunoblots were visualised using an ECL reagent (Beyotime). The protein band densities were quantified using Image J software and presented as relative level to β-actin. The experiment was repeated 3 times.

IF staining

Human NP tissues were fixed in 4 % paraformaldehyde for 24 h and dehydrated in graded sucrose solutions. Tissues were embedded in OCT compound (Sakura, Torrance, CA, USA) and then cut into 10 μm-thick cryosections. The sections were permeabilised in PBS containing 0.3 % Triton X-100 (BioFroxx, Shanghai, China) for 30 min, and then blocked in PBS containing 5 % BSA (BioFroxx) and 0.1 % Triton X-100 (BioFroxx) for 1 h. Subsequently, sections were incubated overnight at 4 °C in dilutions of the antibodies against CD44 (1 : 500, ab157107, Abcam) and/or HIF-1α (1 : 200, ab1, Abcam). After thoroughly washing the sections with TBST, sections were incubated with Alexa Fluor-488 conjugated anti-rabbit and/or Fluor-594 conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Inc., West Grove, PA, USA) at 1 : 300 dilution for 1 h at room temperature, and then washed with TBST before counterstaining with DAPI (Abcam) for 5 min. The staining was visualised using a laser confocal microscope (LSM 880 with Airyscan, Zeiss Microscopy GmbH, Jena, Germany).

For cultured cell immunofluorescence staining, NPCs were seeded on glass coverslips. After the treatment, cells were washed with PBS, fixed in 4 % paraformaldehyde for 20 min, then permeabilised and blocked with PBS containing 5 % BSA and 0.3 % Triton X-100 for 1 h at room temperature. Subsequently, cells on coverslips were incubated with antibodies against CD44 (1 : 500, ab157107, Abcam), HIF-1α (1 : 200, ab1, Abcam), COL II (1:200, Cell Signaling Technology), aggrecan (1 : 200, ab3778, Abcam). The following immunostaining protocols were identical to cryosections as described above. The ImageJ software was used to semi-quantitative analysis of the fluorescence intensity (Jensen *et al*., 2013).

Cell viability analyses

To evaluate NPC proliferation in the presence of HA under hypoxic conditions, the CCKs (MCE) were used. Briefly, NPCs were seeded in 24-well plates (2×10^4 cells/well) in the presence or absence of HA under normoxic or hypoxic conditions. Following culturing for 72 h, 50 μL CCK solution was added to cells, and monitored spectrophotometrically at 450 nm after incubation for 2 h.

Biochemical analyses

To quantify the amount of GAG in NPCs, a DMMB assay was performed using GAG detection kits (Genmed Scientifics Inc., Arlington, MA, USA) according to the manufacturer's instructions. Briefly, the soluble GAG was extracted with high-salt solution and combined with DMMB dye. After adding a propanol dissociation solution, the purple dye was released and monitored spectrophotometrically (SynergyH1, BioTek, Winooski, VT, USA). The total amount of GAG was calculated by comparison with standard samples provided by the GAG detection kits (50 ng/μL) and normalised to the total amount of DNA (NanoDrop One, Thermo Fisher Scientific, MA, USA) as measured by the spectrophotometer in the same sample (Stone *et al*., 1994).

Dual-luciferase reporter assay

To confirm the direct activation of CD44 by HIF-1α, dual-luciferase reporter assay was performed. HEK 293 cells were seeded in 96-well plates (5×10^3 cells/

well) in DMEM/F12 with 10 % FBS, 1 d before transfection. Cells were transfected with HIF-1α over-expression construct and human CD44 promoter reporter construct (2934-pCD44, 2803-pCD44, 908-pCD44, 442-pCD44, 339-pCD44) or control vector. After incubation for 48 h, the firefly and Renilla luciferase activities were detected by dual-luciferase reporter assay system (Promega, Madison, WI, USA). The relative luciferase activity was determined by the ratio of firefly luciferase : Renilla luciferase activity (internal control) (Gao *et al.*, 2018).

ChIP

To confirm HIF-1α activated CD44 expression by the predicted site, ChIP assays were performed using the ChIP Assay Kit (Beyotime) according to the manufacturer's protocol. Briefly, following culture in 100 mm dishes under hypoxic conditions for 3 d, NPCs were incubated in 1 % formaldehyde solution at 37 °C for 10 min, and glycine solution was added to stop the reaction. After washing in PBS with 1 % PMSF and lysis with SDS buffer, the cells was treated by sonication (Sonics CVX130, city, state, USA) to cut genomic DNA. ChIP grade antibody against HIF-1α (1 : 200, ab1, Abcam) was used to bind the protein-DNA complex. Following washing and unlinking by 5 mol/L NaCl, the DNA fragments were purified using a DNA Purification Kit (Beyotime). Finally, the purified DNA was amplified by PCR with the primers listed in Table 4. The predicted binding site 1 and 2 were too close to design primers separately, so the designed primers 'ChIP site 12' contained the predicted binding site 1 and 2.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 statistical software. Quantitative data were presented as means ± SD. Data distribution was checked with Shapiro–Wilk normality test, and non-normally distributed data were examined by using the Mann-Whitney test. Differences among the groups were identified by *t*-test or one-way ANOVA. In all analyses, $p < 0.05$ was considered statistically significant.

Results

Expression of HIF-1α and CD44 in NPCs and in NP tissues

Human NP tissues were divided into the mild degeneration group (II -III grade) and the severe degeneration group (IV-V grade), according to the Pfirrmann grading system (Pfirrmann *et al.*, 2001). The immunofluorescence staining and RT-qPCR results showed that CD44 expression progressively decreased from the mild degeneration to severe degeneration group ($p < 0.05$) (Fig. 1**a**,**b**). In NPCs, the confocal images showed the co-expression of HIF-1α and CD44 (Fig. 1**c**). To explore the distribution of HIF-1α and CD44 in NP tissues, immunofluorescence co-staining was performed and showed that HIF-1α and CD44 co-expressed in NP tissues (Fig. 1**d**).

Hypoxia with HA potentiates NPCs proliferation and the synthesis of ECM

To determine the metabolic effects of exogenous HA and hypoxia on the ECM, human NPCs were cultured for 72 h in the presence or absence of HA under normoxic or hypoxic conditions. In the presence of HA under hypoxic condition, the cell proliferation was significantly enhanced ($p < 0.01$) (Fig. 2**a**), and the total GAG amount was increased as observed by DMMB assays ($p < 0.05$) (Fig. 2**b**), in contrast to in the absence of HA under normoxic condition. Immunofluorescence staining results showed that the level of COL II and aggrecan expression were significantly higher in NPCs treated with HA under hypoxic conditions than in the absence of HA under normoxic condition ($p < 0.05$) (Fig. 2**c-f**). The aggrecan mRNA expression was increased in the presence of HA under hypoxic condition, but the change was not statistically significant (Fig. 2**h**) ($p > 0.05$), and the COL II mRNA expression showed no obvious change (Fig. 2**g**) ($p > 0.05$).

The expression of CD44 and HABP2 were enhanced by hypoxia with HA

To explore the mechanism by which HA potentiates the synthesis of ECM under hypoxic conditions, CD44 and HABP2 expression were detected. The expression of CD44 protein in NPCs increased with HA treatment and hypoxia. Importantly, the expression of CD44 protein increased in the presence of both HA and hypoxia compared to either of them alone. The level of HABP2 was increased in the presence of HA under hypoxic conditions, relative to the level in the absence of HA under normoxic condition ($p < 0.05$) (Fig. 3**a**-**d**). The gene expression of CD44 also appeared to be higher in the presence of HA under hypoxic condition than relative to its level in the absence of HA under normoxic conditions, but the difference was not statistically significant ($p > 0.05$) (Fig. 3**e**).

Table 4. Primer sequences used for ChIP assays.

Gene transcript	Sense primer	Antisense primer
ChIP site 12	ATCCCTGGAGGTTGCATGAG	ATGACTCAAAGAGCCGCCC
ChIP site 3	CATGTGTGTGGAGAGAGGTGC	GTGGAAAGAGGGAGAGCTCATT
ChIP site 4	GCCAAAGGCTGAACCCAATG	GTGCTCTGCTGAGGCTGTAA

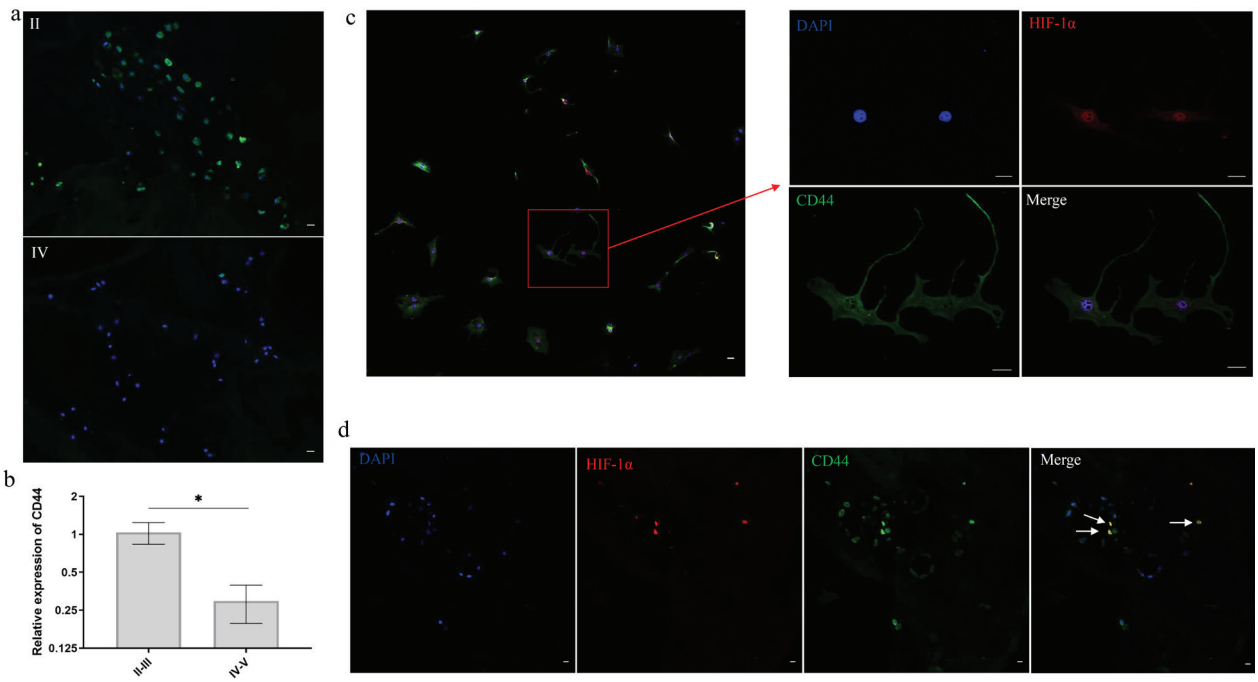

Fig. 1. Expression of HIF-1α and CD44 in NPCs and in NP tissues. Human NP tissues were divided in the mild degeneration group (II -III grade) and the severe degeneration group (IV-V grade) according to the Pfirrmann grading system. (**a**) The CD44 protein expression between 2 groups was analysed by immunofluorescence staining. (**b**) The *CD44* mRNA expression between 2 groups was detected by RT-qPCR. (**c**) The passaged cells obtained from the mild degeneration disc, were cultivated in hypoxic condition for 3 d. The immunofluorescent co-staining results showed the expression pattern of HIF-1α and CD44. CD44 (green); HIF-1α (red); nuclei, DAPI (blue). (**d**) The immunofluorescent co-staining results showed the expression pattern of HIF-1α and CD44 in the mild degeneration NP tissues. Data represent average values of 3 independent experiments, error bars represent SD, * $p < 0.05$. Scale bar: 10 μm.

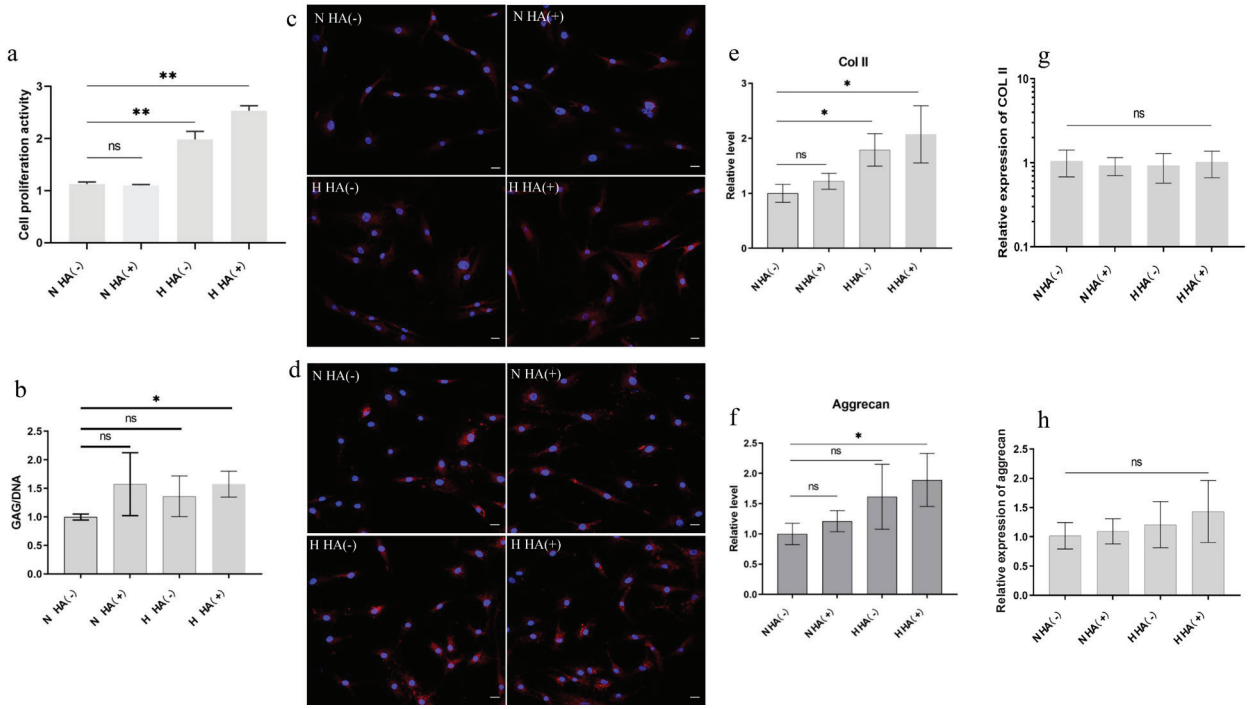

Fig. 2. Hypoxia with HA potentiates NPC proliferation and synthesis of ECM. Human NPCs were cultured for 72 h in the presence (+) or absence (−) of HA under normoxic (N) or hypoxic (H) conditions. (**a**) The cell proliferation was tested using CCK-8. (**b**) The total amount of GAG in each sample was quantified by DMMB assay and normalised relative to DNA level. The COL II (**c**) and aggrecan (**d**) expression were detected by immunofluorescence staining. (**e**,**f**) Semi-quantification of 3 repetitions of the immunofluorescence staining results. The COL II (**g**) and aggrecan (**h**) mRNA expression was detected by RT-qPCR. Data represent average values of 3 independent experiments, normalised to the value of the control group [N HA (-)], error bars represent SD. ns, not significant, * $p < 0.05$, ** $p < 0.01$. Scale bar: 20 μm.

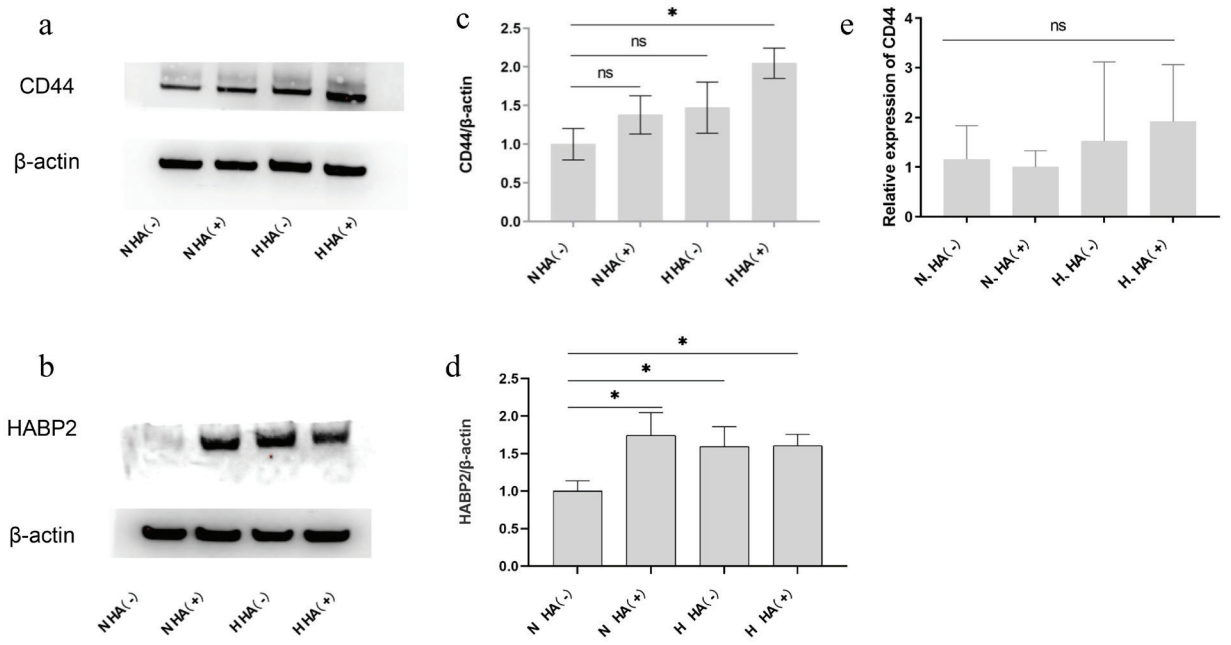

Fig. 3. **The expression of CD44 and HABP2 was enhanced by hypoxia with HA**. Human NPCs were cultured for 72 h in the presence (+) or absence (-) of HA under normoxic (N) or hypoxic (H) conditions. The protein levels of CD44 (**a**) and HABP2 (**b**) were detected by Western blotting, and 3 repetitions of the western blot results were analysed quantitatively by Image J software (**c**,**d**). (**e**) The relative mRNA expression of *CD44* was detected by RT-qPCR. Mean ± SD, * $p < 0.05$.

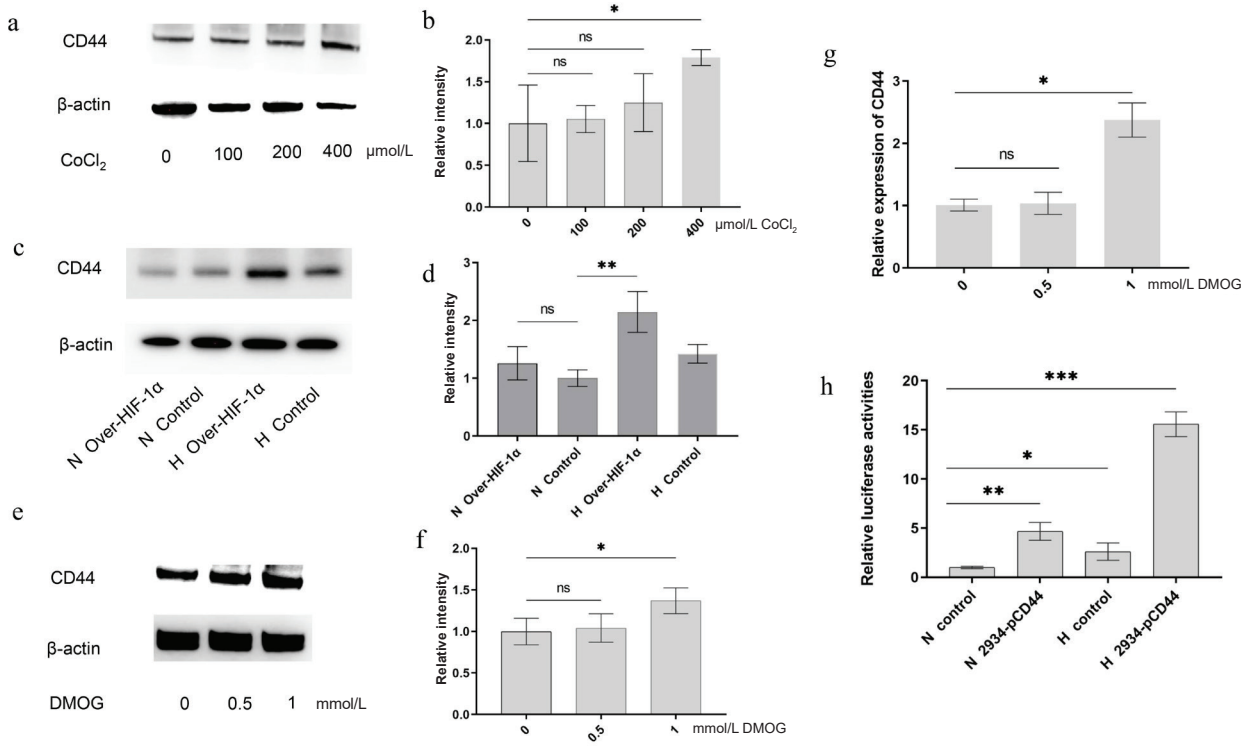

Fig. 4. ***CD44*** **expression was induced by hypoxia and regulated by PHD/HIF-1α**. (**a**,**b**) NPCs were treated with $CoCl_2$ for 24 h, the protein level of CD44 was detected by Western blotting. (**c**,**d**) NPCs were transfected with Over-HIF-1α or basic vector (control) and cultured under normoxic (N) or hypoxic (H) conditions for 72 h, the protein level of CD44 was detected by western blotting, the group (N control) was control group. NPCs were treated with DMOG for 24 h, the expression of CD44 protein (**e**,**f**) and mRNA (**g**) were detected by western blotting and RT-qPCR. (**h**) 293T cells were transfected with human CD44 promoter reporter vector (2934-pCD44) or control reporter vector (control) and cultured under normoxic (N) or hypoxic (H) conditions for 48 h, the relative luciferase activities were detected. Data represent average values of 3 independent experiments. Error bars represent SD. ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CD44 expression was induced by hypoxia and regulated by PHD/HIF-1α

To examine the relationship between CD44 and relative hypoxic conditions in NPCs, NPCs were treated by $CoCl_2$, a chemical reagent known to induce hypoxic stress, whereby the CD44 expression was dose-dependently induced by $CoCl_2$ (Fig. 4**a**,**b**). To investigate the effect of HIF-1α regulation on CD44 expression, the Over-HIF-1α or the control vector were transfected into NPCs for 72 h under normoxic or hypoxic conditions. The results showed that over-expression of HIF-1α in NPCs up-regulated CD44 expression, especially under the hypoxic conditions ($p < 0.01$) (Fig. 4**c**,**d**). Further, to determine that the CD44 expression was regulated by PHD/HIF-1α, NPCs were treated by DMOG, a PHD inhibitor. Thereby, the mRNA expression and protein level of CD44 were increased, as observed by western blotting and RT-qPCR (Fig. 4**e**-**g**). Dual-luciferase reporter assay results showed that hypoxic conditions could significantly increase the luciferase activity in 293T cells transfected with the human CD44 promoter reporter vector, when compared with the control group transfected with control reporter vector ($p < 0.001$) (Fig. 4**h**).

Activation of CD44 promoter by HIF-1α

To gain further insight into the mechanisms of transcriptional regulation of CD44 by HIF-1α, the first 3 kb of the Human CD44 promoter were obtained from the UCSC Genome Browser (Web ref. 3). TSS were predicted by the Promoter 2.0 Prediction Server, results showed that a "highly likely prediction" site located position − 300 (Fig. 5**a**) (Web ref. 2). Next, the first 3 kb of the human CD44 promoter was scanned for the presence of HREs using the JASPAR core database (Web ref. 1), at an 80 % profile score threshold, 4 putative HRE sites were reported, as shown in Fig. 5**b**. Luciferase reporter constructs with the 2934 bp promoter sequence of CD44 were constructed for measuring the effect of hypoxia and HIF-1α on promoter activity. Dual-luciferase reporter assays showed that HIF-1α significantly increased the activity of the human CD44 promoter fragments (Fig. 5**c**). To further define the binding sites, serial deletion analysis of the Human CD44 promoter showed that luciferase activity of 2803-pCD44, 908-pCD44, 442-pCD44, 339-pCD44 were significantly higher than the control group ($p < 0.01$) (Fig. 5**d**). ChIP results showed that HIF-1α could bind any of the 4 predicted sites (Fig. 5**e**).

Discussion

Previous studies revealed that hypoxic conditions enhanced the anabolic effects of HA in articular chondrocytes (Ichimaru *et al.*, 2016). However, the role of hypoxia for HA treatment of NPCs has not been determined. In the current study, the expression of ECM genes and proteins were enhanced in the presence of HA under hypoxic condition, which was correlated with the increasing CD44 expression. Furthermore, it was found that CD44 was a hypoxia-sensitive gene, and the regulation was dependent on the PHD - HIF-1α system, especially transcriptional activation by HIF-1α.

High molecular weight HA has been reported to promote ECM synthesis, increase matrix content, and enhance mechanical properties under normoxic

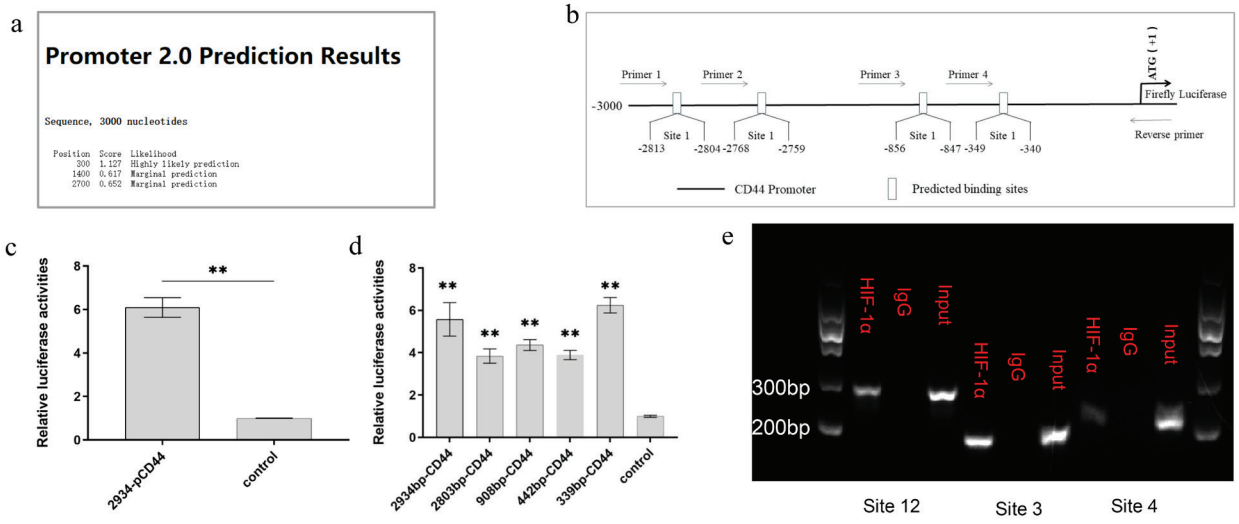

Fig. 5. Activation of CD44 promoter by HIF-1α. (**a**) Prediction of CD44 transcription starting sites using the database of the Promoter 2.0 Prediction Server. (**b**) Schematic diagram showing the luciferase construct with predicted binding sites of HIF-1α on the CD44 promoter acquired from the JASPAR database. (**c**-**d**) Relative luciferase activities in 293T cells transfected with Over-HIF-1α and Human CD44 promoter vector (2934-pCD44, 2803-pCD44, 908-pCD44, 442-pCD44, 339-pCD44) or control reporter vector (control). (**e**) ChIP results of NPCs showing the regulating sites of HIF-1α on CD44. Each experiment was repeated 3 times. ** $p < 0.01$.

conditions (Kazezian *et al.*, 2017; Kim *et al.*, 2015), which related to high expression of CD44 receptor after HA treatment (Isa *et al.*, 2015). In addition, compared with normoxia, hypoxia has been reported to promote ECM synthesis and enhance the expression of genes encoding NP ECM (Feng *et al.*, 2013), which through HIF-1α regulate some target genes, such as *CCN2* and *CA12* (Chen *et al.*, 2016; Tran *et al.*, 2013). Although HA and hypoxia have been individually reported to enhance matrix metabolism in NPCs, their combined effects remain unclear. In this study, although there was no significant increase in the expression of aggrecan and COL II mRNA, the GAG content and the protein of COL II, aggrecan and HABP2 were increased in the presence of HA under hypoxic condition compared to normoxic conditions in the absence of HA. These findings suggested that hypoxic conditions positively potentiated the ability of NPCs matrix synthesis in the presence of HA, which was consistent with previous results in articular chondrocytes (Ichimaru *et al.*, 2016).

As the most important receptor for hyaluronic acid, CD44 plays an important role in regulating cell adhesion, growth, survival, migration, differentiation and HA metabolism (Ponta *et al.*, 2003). Moreover, CD44 is important in intervertebral disc formation by anchoring hyaluronan to the cells (Stevens *et al.*, 2000). It was found that the expression of CD44 gene and protein were decreased in the severely degenerated disc, as shown by RT-qPCR and immunofluorescence staining, which means that the lack of expression of CD44 may be linked to IDD. Previous studies have revealed that HA promotes NP regeneration by enhancing the expression of CD44 and binding to CD44 in NPCs (Isa *et al.*, 2015). In this study, the expression of CD44 was increased in the presence of both HA and hypoxia compared to either alone, suggesting that the possible mechanism of hypoxic conditions potentiate the ability of NPCs matrix synthesis was by enhancing the expression of CD44.

HIF-1α, a key transcription factor in hypoxic condition, plays an important role in promoting cell survival, matrix synthesis, and regulating energy metabolism, in the disc under hypoxic condition (Choi *et al.*, 2015; Risbud *et al.*, 2006). Through HIF-1α, hypoxia controls a host of factors, such as SDC4 (Fujita *et al.*, 2014), CA12 (Chen *et al.*, 2016) and CD24 (Liu *et al.*, 2018), which contribute to maintain homeostasis of NP and protect against degeneration in hypoxic condition of the disc. Although there was no evidence to conclude that HIF-1α could enhance the expression of CD44 in NPCs, several researchers have shown that expression of CD44 correlated with hypoxia-induced gene signatures in tumours. Previous research found that CD44 expression was associated with hypoxia-induced gene signatures and poor survival in human glioblastoma multiforme (Pietras *et al.*, 2014). In breast cancer, HIF-1α increased the expression of CD44 molecules and the percentage of CD44 positive cells under hypoxia condition, and a higher expression of CD44 was observed in the hypoxic tumour regions *in vivo* (Krishnamachary *et al.*, 2012). In addition, hypoxia enhanced CD44 expression *via* HIF-1α to promote cell proliferation and invasion, in gastric cancer cells (Liang *et al.*, 2017).

In the current study, it was shown that CD44 expression was induced by hypoxia and selectively regulated by the PHD - HIF-1α system in NP cells. Firstly, to induce hypoxic stress, NPCs were treated with $CoCl_2$, a chemical inducer of HIF-1α, and CD44 expression was dose-dependently induced. We further found that CD44 expression was increased by transfection with the HIF-1α over-expression plasmid in the hypoxic condition. However, HIF-1α did not modulate CD44 expression under normoxia, which may be related to rapid degradation of HIF-1α in normoxic condition (Fujita *et al.*, 2012). Subsequently, PHDs was inhibited by DMOG, a PHD inhibitor, and it was found that CD44 expression was dose-dependently induced, which demonstrated the importance of PHDs in HIF-1α-induced CD44 expression.

HIF-1α regulates several genes by binding to a specific site in the promoter region, termed HRE (Semenza, 2000). Based on the Promoter Prediction server and JASPAR core database, 4 HREs were predicted in the human CD44 promoter. Dual-luciferase reporter assay results demonstrated that hypoxia and HIF-1α could regulate the expression of CD44. Moreover, the promoter truncation experiments and ChIP assays further confirmed that HIF-1α could transcriptionally activate *CD44* expression by binding to any of the predicted sites. Taken together, the results clearly indicated that *CD44* was a hypoxia-sensitive gene, and the regulation was dependent on the PHD - HIF1 system, especially the transcriptional activation by HIF-1α. After truncating all 4 predicted binding sites, the activity of luciferase was still markedly higher than that of the control group, which indicated that HIF-1α could activate *CD44* expression by binding to the left sequence, most of which belongs to the 5′ UTR. Several studies have found that 5′ UTR functions as a regulatory element in certain genes (Bonnet-Magnaval *et al.*, 2016; Gao *et al.*, 2018). More detailed studies would be carried out to identify the regulatory elements in the 5′ UTR of CD44 in the future.

This study had some limitations. Only the synergistic effect of hypoxia and HA on the synthesis of ECM in 2D cell culture was explored, which is not close to the physiological environment in the disc. To address this limitation, the effect of HA will further be tested in organ models or in animal models. Another limitation to this study was that a HA concentration of 1 μmol/L was selected, because most therapeutic pharmaceutical formulations are administered at this concentration (Rayahin *et al.*, 2015), which may be not the optimal concentration in the hypoxic microenvironment of the disc.

Conclusions

Together, results from this study clearly indicated that hypoxic conditions enhanced the ability of matrix synthesis of NPCs in the presence of HA, which was nicely correlated with up-regulated *CD44* expression upon transcriptional activation of HIF-1α. Therefore, genetic or pharmacological modulation of HIF-1α/CD44 activity combined with high molecular weight HA may be a novel therapeutic approach to degenerative disc disease.

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