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Up-regulated HIF-2α contributes to the Osteoarthritis development through mediating the primary cilia loss

Qining Yang, Yongwei Zhou, Pengfei Cai, Weicong Fu, Jinhua Wang, Qiang Wei, Xiaofei Li*

Department of Joint Surgery, Jinhua Municipal Central Hospital, Jinhua City 321000, Zhejiang Province, PR China

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ABSTRACT

Backgrounds: Up-regulated HIF-2α (hypoxia induced factor 2) had been demonstrated to contribute to Osteoarthritis (OA) development via inducing the expression of matrix-degrading enzymes. However, the HIF-2α also could promote primary cilia loss through HIF-2α/AURKA (Aurora kinase A)/NEDD9 pathway. And the primary cilia dysfunction is another characteristic of the OA. Thus, we investigated here whether the HIF-2α also contributes the OA development through mediating the primary cilia loss.

Methods: The primary chondrocytes were isolated from the experimental OA mice induced by destabilization of the medial meniscus (DMM). Chondrocytes were cultured under normoxia (21% O_2) or hypoxia (2% O_2) conditions. The HIF-1α and HIF-2α expressions were assessed by western blot. The cilia formation was counted by immuno-staining the acetylated tubulin. The contribution of HIF-1α or HIF-2α to the primary cilia loss was assessed by knocking-down the HIF-1α or HIF-2α individually. The HIF-2α/AURKA/NEDD9 pathway was validated through over-expressing or knocking-down specific components of the pathway and then counting the primary cilia number. Finally, the pathway was further confirmed in the OA mice.

Results: Hypoxia could induce the expression of both HIF-1α and HIF-2α, and also reduce the number of primary cilia on the chondrocytes isolated from the experimental OA mice. Knocking-down or over-expressing HIF-1α or HIF-2α individually showed that the HIF-2α could induce the primary cilia reduction rather than the HIF-1α. Manipulating the HIF-2α expression could positively affect the AURKA and NEDD9 expression. Manipulating the AURKA and NEDD9 expressions could reverse the function of HIF-2α on primary cilia. In the mice, knocking-down both AURKA and NEDD9 could alleviate the OA development significantly.

Conclusion: Up-regulated HIF-2α contributes to the Osteoarthritis development through mediating the primary cilia loss, which might be developed as therapeutic targets for OA treatment.

1. Introduction

OA (Osteoarthritis), a prevalent degenerative disease, is characterized by the cartilage destruction, synovial inflammation, osteophyte formation and subchondral bone sclerosis, resulting in chronic pain, restriction of joint mobility, and disability [1–5]. The pathogenic mechanisms that underlie these morphological phenotypes remain largely unknown. There is an urgent need for additional studies to identify therapeutic targets to prevent and treat OA disease, as there are currently no effective treatment options beyond total joint replacement.

Articular cartilage is a connective avascular, aneural, and aliphatic tissue that works hydrodynamically to support and distribute the load of the body and provide an almost friction-free movement in joints [6]. Since articular cartilage has no capillary networks, the cartilage microenvironment is hypoxic. As a consequence, the hypoxia-inducible factors (HIFs) are induced [7]. And the loss of VHL, an important regulator of HIFs, in adult articular cartilage is associated with earlier dysregulation of cartilage homeostasis, and the accelerated age-related and surgery-induced OA development [8].

The transcription factor hypoxia inducible factor HIF-1α is of pivotal importance for survival and growth arrest of chondrocytes during cartilage development as well as energy generation and matrix synthesis of chondrocytes in healthy as well as osteoarthritic cartilage [9]. HIF-1α regulates chondrogenesis by regulating SOX9 expression in the genetic level. HIF-1α also serves to regulate both autophagy and apoptosis. Therefore, HIF-1α may protect articular cartilage by promoting the chondrocyte phenotype, maintaining chondrocyte viability, and supporting metabolic adaptation to a hypoxic environment [10].

* Corresponding author at: Department of Joint Surgery, Jinhua Municipal Central Hospital, No. 365 Renmin East Road, Wucheng District, Jinhua City 321000, Zhejiang Province, PR China.
E-mail address: taoligao1966@163.com (X. Li).

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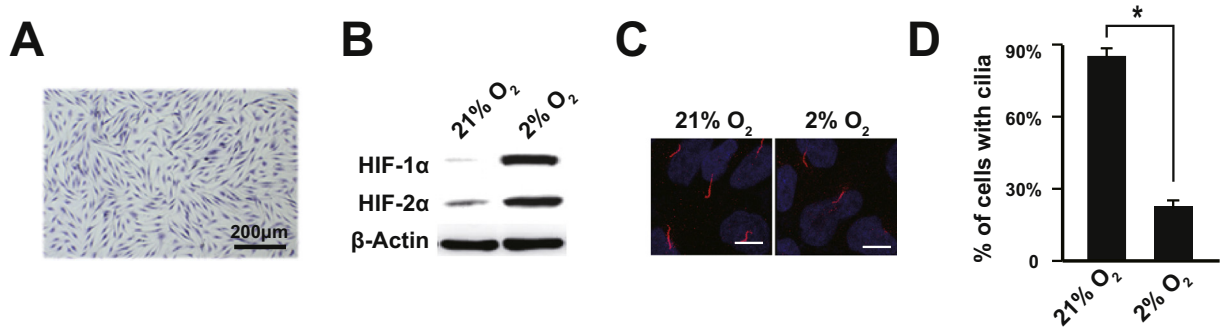

Fig. 1. Hypoxia induced primary cilia reduction in chondrocytes isolated from OA mice. (A) Primary chondrocytes isolated from OA mice, stained with Alcian Blue, the chondrocytes marker. (B) Western blot showed the up-regulation of HIF-1α and HIF-2α under hypoxia, 2% O_2. (C) Representative figures for primary cilia stained with anti-acetylated tubulin. (D) Percentage of chondrocytes with primary cilia (n = 3). Scale bar: 200 μm. * indicates $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

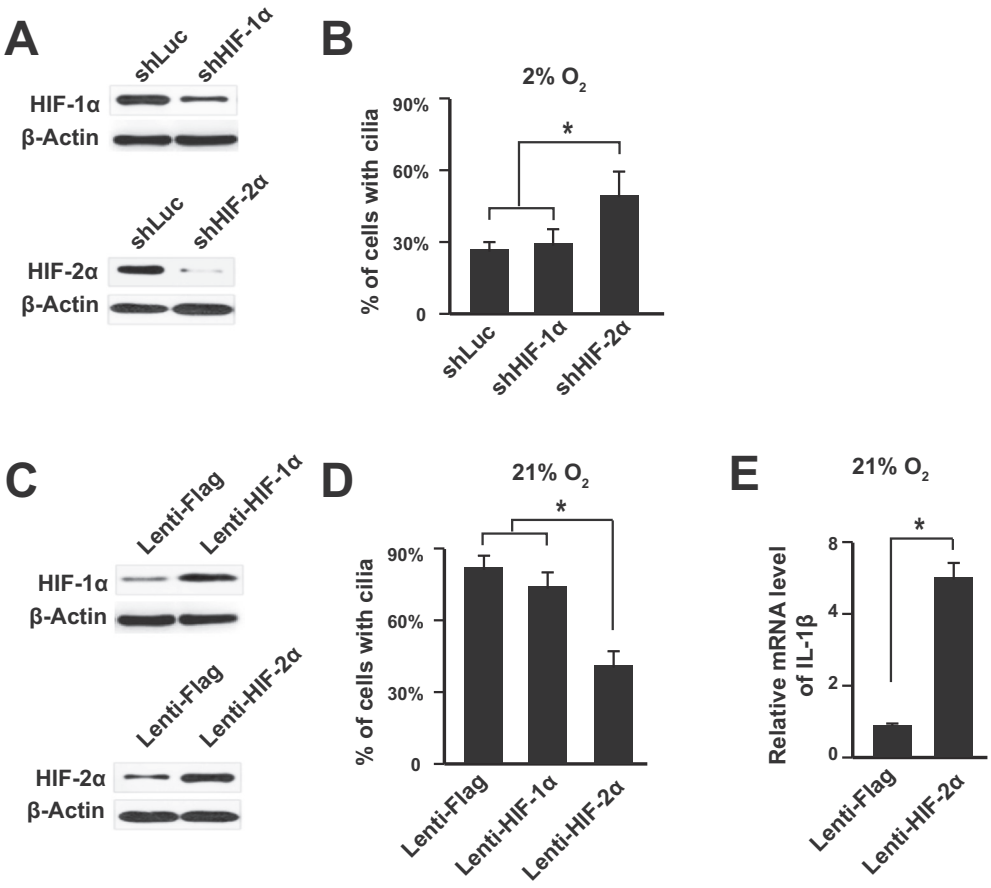

Fig. 2. HIF-2α induced the primary cilia reduction rather than HIF-1α. (A) Western blot showed that knocking-down HIF-1α or HIF-2α via lentiviral shRNAs under hypoxia. (B) Percentage of chondrocytes with primary cilia after knocking-down HIF-1α or HIF-2α under hypoxia (n = 3). (C) Western blot showed that up-regulation of HIF-1α or HIF-2α via lentiviral over-expression under normoxia. (D) Percentage of chondrocytes with primary cilia after overexpressing the HIF-1α or HIF-2α under normoxia (n = 3). (E) The mRNA level of IL-1β (Interleukin-1β) was measured by qPCR after overexpressing the HIF-2α in chondrocytes under normoxia (n = 3). * indicates $P < 0.05$.

In contrast with HIF-1α, HIF-2α (also recognized as endothelial PAS domain protein-1) is a catabolic factor in the osteoarthritic process [11]. HIF-2α directly induces the expression of catabolic factors in chondrocytes, and HIF-2α enhances Fas expression to mediate chondrocyte apoptosis and regulates autophagy in maturing chondrocytes [12,13]. Furthermore, the HIF-2α expression was higher in osteoarthritic cartilages versus nondiseased cartilages of mice and humans [14]. HIF-2α triggers osteoarthritic cartilage destruction by upregulating chondrocyte expression of matrix-degrading enzymes [13,15–17]. These studies indicated that HIF-2α may be a potential target for the treatment of OA [18,19]. And Intra-articular delivery of anti-HIF-2α siRNA by chondrocyte-homing nanoparticles could prevent cartilage degeneration in arthritic mice [20].

In addition, HIF-1α and HIF-2α are also involved into the primary cilia regression process [21]. Primary cilia are singular, cytoskeletal organelles present in the majority of mammalian cell types [22,23], including chondrocytes [24], the functional cell population located in the articular cartilage [25]. They are present in each of the chondrocytes and mediate mechanical and chemical signals [23,26–30]. Deletion the gene IFT88 or BBS (BardeteBiedl Syndrome), the main components of the primary cilia, in mouse showed signs of early OA [26,31,32]. Thus we conducted the present study to investigate whether the HIF-primary cilia pathway also involved into the OA development.

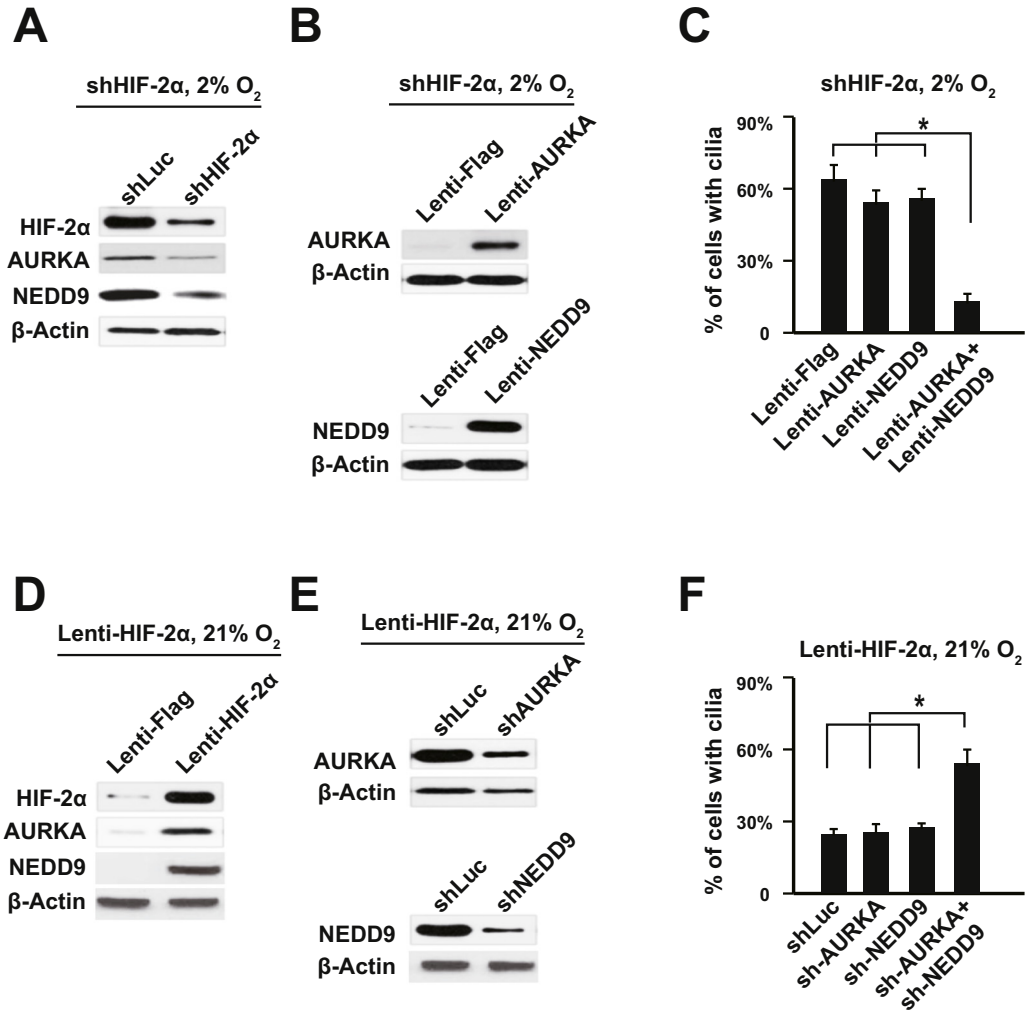

Fig. 3. AURKA/NEDD9 pathway was involved into the primary cilia reduction induced by HIF-2α. (A) Western blot showed that knocking-down HIF-2α suppressed the expression of AURKA and NEDD9 under hypoxia. (B) Western blot showed that AURKA and NEDD9 were successfully over-expressed by lentivirus. (C) Percentage of chondrocytes with primary cilia after knocking-down HIF-2α and over-expressing the AURKA, NEDD9 or AURKA/NEDD9 together under hypoxia (n = 3). (D) Western blot showed that overexpressing HIF-2α up-regulated the expression of AURKA and NEDD9 under normoxia. (E) Western blot showed that AURKA and NEDD9 were successfully knocked-down by lentiviral shRNAs. (F) Percentage of chondrocytes with primary cilia after overexpressing HIF-2α and knocking-down the AURKA, NEDD9 or AURKA/NEDD9 together under normoxia (n = 3). (G) Immuno-staining of NEDD9 on articular cartilage of healthy (Ctrl) and OA mice. * indicates $P < 0.05$.

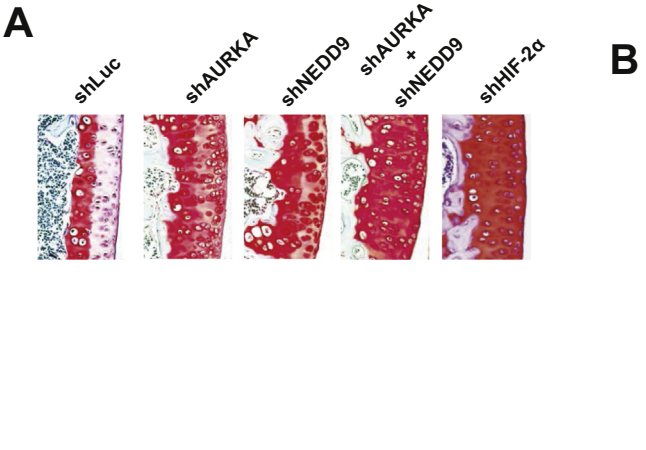

Fig. 4. Targeting the HIF-2α/AURKA/NEDD9 pathway could alleviate the symptoms of OA mice. (A) Representative figures of Safranin-O staining of the cartilage tissues of the OA mice after intra-articular injection of lentiviral shRNAs for knocking-down HIF-2α/AURKA/NEDD9. (B) OARSI grading of the cartilage tissues of the OA mice after intra-articular injection of lentiviral shRNAs for knocking-down HIF-2α/AURKA/NEDD9 (n = 8). * indicates $P < 0.05$.

2. Methods and materials

2.1. Mice and OA induction

All animal procedures were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China. The protocol was approved by the Committee on Animal Research and Ethics of Jinhua Municipal Central Hospital. Male C57BL/6 mice (18–22 g, 8-week-old) were purchased from the Experimental Animal Center (Tongji Hospital, Wuhan, China). After one week of acclimation, the mice were anesthetized via intraperitoneal injection of pentobarbiturate (0.5 mg/10 g body weight). Experimental OA was induced by DMM (destabilization of the medial meniscus) in the right knee [33]. Briefly, the joint capsule was opened with an incision just medial to the patellar tendon and the medial meniscotibial ligament was sectioned with microsurgical scissors on the right knee of the mice. In sham surgery group, surgery was performed on right knee joints, but the ligaments were visualized but not transected. All mice were allowed to move freely within their cages after surgery. They were maintained in the same housing conditions with free access to food and water. Cartilage destruction was examined by safranin-O staining and scored using the Osteoarthritis Research Society International (OARSI) grading system.

2.2. Chondrocytes isolation and expansion

Chondrocytes were isolated from the femoral condyles and tibial plateaus of OA mice (8 weeks after OA induction). Chondrocytes were isolated by carefully dissecting articular cartilage from a relatively lesion-free area, followed by sequential digestion with a protease (8 μg/mL) from Streptomyces griseus for 1 h and with collagenase (4 μg/mL) from Clostridium histolyticum and hyaluronidase (0.2 μg/mL) from bovine testes (Sigma-Aldrich) for 2 h. Chondrocytes were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. First-passage cultured chondrocytes were used within 7–10 days after seeding.

2.3. Western blot

Proteins were isolated using a total protein extraction kit (Pierce) according to the manufacturer's instructions. The protein concentration was determined via the bicinchoninic acid assay (Pierce) using BSA as a standard. Protein (20 mg) of each sample was heated to 100 °C for 5 min and then resolved on an 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The proteins were transferred to methanol-wetted polyvinylidene difluoride (PVDF) membranes in Tris/Glycine transfer buffer. Subsequently, the membranes were blocked for 1 h at room temperature in blocking buffer (5% skim milk powder, 0.5% Tween-20 in trisbuffered saline; TBS). Blots were incubated with antibodies of HIF-1α, HIF-2α, AURKA, NEDD9 and beta-Actin (Abcam, Cambridge, UK) followed by an HRP-conjugated secondary antibody. Immunore-active proteins were visualized by Western Blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoblot bands were quantitated with the Tocan 190 protein assay system (Bio-Rad, USA).

2.4. Primary cilia staining and counting

Chondrocytes were plated onto 0.1% gelatin-coated coverslips for 48 h, fixed with 4% paraformaldehyde (Sigma) for 10 min, permeabilized with 0.2% Triton-X 100 in 10% FBS for 30 min, and incubated with mouse monoclonal anti-acetylated tubulin (Sigma) in blocking buffer overnight at 4 °C. Cells were washed five times with PBST (0.1% Triton-X 100 in PBS) for 5 min each time and then incubated with secondary antibody Alexa Fluor® 488 conjugate for 1 h. The cells were further counterstained and mounted with SlowFade® Gold antifade reagent with DAPI (Invitrogen, life technologies, Carlsbad, CA). Cilia were measured by using ImageJ (rsb.info.nih.gov/ij) software. For cilia quantification, four fields in each coverslip were imaged and the number of cilia and number of chondrocytes in each field were quantified. For each sample, 10 coverslips were analyzed. The percent of ciliated cells was then averaged across the samples.

2.5. Immunohistochemistry

The right knee joint from each group were fixed in 10% neutral buffered formalin for 24 h, decalcified in TBD-2 (Thermofisher) for 48 h, followed by paraffin embedding. Serial sections (4 mm) were cut. For NEDD9 immunostaining, a microwave-based antigen retrieval process was employed with EDTA buffer, pH 8.0, for 30 min. After the sections had been cooled, endogenous peroxidase was inhibited with 3% hydrogen peroxide for 10 min at room temperature. Non-specific binding was blocked with fetal calf serum for 15 min before incubation of the sections with mouse anti-NEDD9 antibody (Abcam) at 4 °C overnight. As a negative control, sections were incubated with normal mouse IgG. After being incubated with the primary antibodies, the sections were then incubated with horseradish peroxidase (HRP)-labeled anti-mouse IgG at 37 °C for 30 min, followed by visualization with 3, 3-diaminobenzidine (DAB) and counterstaining with Mayer's hematoxylin. Desired color reaction was observed when monitored with the microscope.

2.6. Histopathological evaluation for articular cartilage degeneration

The right knee joint from each group were fixed in 10% neutral buffered formalin for 24 h, decalcified in TBD-2 (Thermofisher) for 48 h, followed by paraffin embedding. Serial sections (4 mm) were cut. Sections were stained with safranin O (Thermofisher) and then graded by three independent observers using the Osteoarthritis Research Society International (OARSI) scoring system [34]. The scores are defined as follows: 0: normal; 0.5: loss of toluidine blue without structural changes; 1: small fibrillations without loss of cartilage; 2: vertical clefts extending from the articular surface down to the layer immediately below the superficial tangential zone with some loss of surface lamina; 3: vertical clefts/erosion extending down to the calcified articular cartilage comprising < 25% of the quadrant width; 4: vertical clefts/erosion extending down to the articular calcified cartilage comprising 25–50% of the quadrant width; 5: vertical clefts/erosion extending down to the calcified articular cartilage comprising 50–75% of the quadrant; and 6: vertical clefts/erosion extending down to the calcified articular cartilage comprising > 75% of the quadrant width. Three non-consecutive sections were scored in each mouse and the scorers were blinded to treatment.

2.7. Lentivirus preparation

shRNAs were cloned into lentiviral pLKO.1-puro vector. The following shRNA sequences were used: control Luc shRNA, 5′-ACTCAAA AGGAAGTGACAAGA-30; HIF-1α shRNA, 50-CTGATGACCAGCAACT TGA-30; HIF-2α shRNA-2, 5′-CAGCATCTTTGATAGCAGT-3′; NEDD9 shRNA, 5′-CACCCAAGAACAAGAGGTA-3′; AURKA shRNA, 5′-ATGCCC TGTCTTACTGTCA-3′. For gene overexpression, the NEDD9 (isoform 1, NM_006403.3), AURKA (isoform 1, NM_198433.1), HIF-1α (carrying mutations at prolines 402 and 564) [21] and HIF-2α (prolines 405 and 531) [21] were cloned into the pLVX-puro lentiviral vector. Lentiviruses were prepared using 293T cells according to the manufacturer's instructions. The lentivirus was purified with Fast-Trap Lentivirus Purification and Concentration Kit (Merk).

2.8. Treatment of OA with intra-articular injections of lentivirus

Ten microliters of lentivirus solution (1×10^6 infectious lentivirus particles) was injected into the intra-articular space using a 29-gauge needle. The injection was performed 4 weeks after surgery, corresponding to the early onset of OA. Mice were sacrificed at 12 weeks after DMM surgery. The knee joints were harvested, and the joint tissue was subjected to histological evaluation, gene and protein expression analyses.

2.9. Statistics

Data were expressed as mean (± SE) and analyzed by a SPSS software package (SPSS Standard version 13.0, SPSS Inc., USA). Unpaired Student's *t*-test and one way ANOVA were used as appropriate to assess the statistical significant of difference. *P* values under 0.05 were considered statistically significant.

3. Results

To verify that the HIF-primary cilia pathway was also involved into the OA development, the chondrocytes were isolated from the OA mice (Fig. 1A), 8 weeks after the OA induction. Under hypoxia (2% O_2), both HIF-1α and HIF-2α were up-regulated (Fig. 1B). And as expected, the hypoxia could significantly inhibit the primary cilia formation (Fig. 1C, D). To clarify which HIF protein contributes to the reduction of primary cilia under hypoxia, the expression of HIF-1α or HIF-2α was knocked-down via lentiviral expression of shRNAs. Data showed that knocking-down HIF-2α could increase the percentage of the chondrocytes with primary cilia under hypoxia, when comparing to the negative control (shLuc) or knocking-down HIF-1α (Fig. 2A, B). Under normoxia condition, over-expressing the HIF-1α did not affect the primary cilia formation. However, HIF-2α overexpression significantly decreased the cilia formation efficiency (Fig. 2C, D). Furthermore, the IL-1β (Interleukin-1β), which is an important pro-inflammatory factor in OA development [35,36], was up-regulated by HIF-2α (Fig. 2E).

Thus the HIF pathway was involved into the primary cilia formation in chondrocytes under hypoxia. And HIF-2α rather than HIF-1α contributed to the cilia formation suppression.

As the HIF/AURKA (Aurora kinase A)/NEDD9 pathway had been demonstrated to promote primary cilia regression [21], we then tested whether this pathway was also involved into the primary cilia reduction in chondrocytes. Data showed that knocking-down HIF-2α could suppress the expression of both AURKA and NEDD9 (Fig. 3A), which is in accordance with previous investigation [21]. And over-expression these two genes together could abolish the primary cilia formation recovery resulting from the HIF-2α knocking-down under hypoxia (Fig. 3B, C). This was further confirmed that knocking-down the AURKA and NEDD9 together while overexpressing the HIF-2α under normoxia could significantly promote the primary cilia formation in chondrocytes (Fig. 3D–F). Thus the HIF-2α/AURKA/NEDD9 pathway also contributed to the primary cilia reduction in OA chondrocytes under hypoxia. The AURKA was highly expressed in OA tissues and cells. The animal experiments proved that depleting AURKA could repress the occurrence of OA. [37]. Furthermore, the NEDD9 also up-regulated in the OA tissues (Fig. 3G), indicating that this pathway might also be involved into the primary cilia dysfunction in vivo.

Then the HIF-2α/AURKA/NEDD9 pathway was knocked-down in the OA mice model through intra-articular injection of lentiviral expressing shRNAs. Knocking-down either AURKA or NEDD9 reduced the OARSI score significantly and combining them together could further alleviate the OA symptoms (Fig. 4A, B). However, the OA mice with knocking-down both AURKA and NEDD9 still had higher OARSI score than the OA mice with knocking-down HIF-2α, indicating that the HIF-2α also contribute to the OA development via other pathways, such as the upregulation of matrix-degrading enzymes [13,15–17].

4. Discussion

Hypoxia inducible factors play an important role in cartilage development and homeostasis maintenance because of the hypoxic microenvironment of the cartilage [7]. It has been demonstrated that the HIF-1α maintains the chondrocytes healthy under hypoxia [9], while the HIF-2α contributes to the Osteoarthritis development via inducing the expression of matrix-degrading enzymes [13,15–20]. However, the HIF-2α also could promote primary cilia loss through HIF-2α/AURKA/NEDD9 pathway [21]. And primary cilia dysfunction is another characteristic of the OA [23,26–32]. Furthermore, it has been demonstrated that the cilia frequency decreased in the deep zone of OA [6]. Thus, we studied whether the HIF-2α also contributes the OA development through mediating the primary cilia loss.

Our data here demonstrated that the hypoxia could induce the expression of both HIF-1α and HIF-2α, and also reduce the number of primary cilia on the chondrocytes isolated from the experimental OA mice. Knocking-down or over-expressing HIF-1α or HIF-2α individually showed that the HIF-2α could induce the primary cilia reduction rather than the HIF-1α. Manipulating the HIF-2α expression could positively affect the AURKA and NEDD9 expression. Manipulating the AURKA and NEDD9 expressions could reverse the function of HIF-2α on primary cilia. In the OA mice, knocking-down both AURKA and NEDD9 could alleviate the OA development significantly. Thus up-regulated HIF-2α contributes to the Osteoarthritis development through mediating the primary cilia loss, which might be developed as therapeutic targets for OA treatment.

Authors' contributions

Q.Y. conducted the western blot, primary cilia counting, wrote the manuscript draft; Y.Z. isolated and cultured the chondrocytes; P.C. conducted the mice experiments; W.F., J.W. and Q.W. conducted the OARSI grading; X.L. supervised the project and corrected the manuscript.

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Data accessibility statement

All data have been presented in the figures. And other related information is available under request to the corresponding author.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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