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Upregulation of FoxO6 in nucleus pulposus cells promotes DNA damage repair *via* activation of RAD51

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Abstract. – OBJECTIVE: DNA damage is an essential risk for intervertebral disc degeneration (IDD). Here, we attempted to uncover the effect of FoxO6 and RAD51 on the DNA damage repair of nucleus pulposus (NP) cells in IDD.

PATIENTS AND METHODS: We collected the human NP tissues of different degeneration degrees and tested the collagen II, FoxO6, and RAD51 expression. Besides, the IL-1β induced NP cell model was also used to elucidate the degenerative progress in vitro. We used Chromatin immunoprecipitation (ChIP) and luciferase reporter assay to confirm whether the FoxO6 protein could enhance the RAD51 expression by binding to its promoter. The FoxO6 gene was upregulated in NP cells by vectors transfection. Immunofluorescence staining was used to measure the RAD51 and γH2AX foci formation. Besides, the typical NP cell gene expression was analyzed by RT-PCR. Cell proliferation was determined by CCK-8, and the cell cycle distribution was determined by flow cytometry.

RESULTS: Like collagen II, FoxO6 and RAD51 expression were all decreased both in the severe degenerated NP tissue and in the IL-1β treated NP cells. Upregulation of FoxO6 gene in NP cells enhanced the RAD51 expression via activating the promoter region and inhibited the DNA damage marker γH2AX formation. FoxO6 upregulation alleviated the loss of collagen II, aggrecan, SOD1, and CAT, and suppressed the increase of collagen I/X, TNF-α, and IL-1β expression, which was affected by IL-1β. Besides, FoxO6 also helped the proliferation and cell cycle of NP cells with the activation of RAD51.

CONCLUSIONS: Upregulation of FoxO6 promotes the DNA repair and maintains the typical phenotype of NP cells, via somehow the mediation of RAD51.

Key Words:
Nucleus pulposus cells, FoxO6, RAD51, DNA damage, Cell cycle.

Introduction

Intervertebral disc degeneration (IDD) is the pathological basis of a series of clinical symptoms. As the main cell component, nucleus pulposus (NP) cells play a pivotal role in the organization of the stability of the extracellular matrix (ECM) and the biological function of the intervertebral disc[1]. The impaired capability of NP cells is currently considered the direct cause of IDD, which is mainly affected by abnormal biomechanics, oxidative stress, inflammatory cytokines, nutritional supply disorders, autoimmunity, and genetic inheritance. At present, the existing treatment for IDD is mainly targeted to clinical symptoms rather than the pathogenesis[2,3]. Differently, changing the degenerative phenotype by novel biological approaches is the treatment devoting to the etiology. Thereinto, keeping the function and population of NP cells within the disc tissue is a crucial strategy for repairing degenerative disc tissue[4].

Forkhead box O6 (FoxO6) is a transcription factor of the O subclass of the forkhead family, which is stably expressed in mammals[5]. The functions of the FoxO transcription factors are complex and diverse, involving oxidative stress, cell metabolism, DNA repair, cell cycle regulation, cell apoptosis, and autophagy[6]. Additionally, it also plays an essential regulatory role in

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the development of age-related diseases, such as cancer, cardiovascular disease, bone loss, nerve tissue degradation. Alvarez-Garcia et al[7] found that FoxO expression was reduced in the lumbar disc during aging. Conditional deletion of all FoxO1/3/4 spontaneously drives severe NP cell loss and leads to IDD in the mouse. Besides, FxO also helps to resist the oxidative and inflammatory response in primary human NP cells[8]. However, how FoxO6 affects the fate of NP cells, and the progress of IDD remains unclear.

DNA is the material basis of heredity, and the high fidelity of its sequence is essential for maintaining normal life activities. DNA damage may occur in the process of self-metabolism and attack by harmful environmental factors[9]. The monitoring and repair system of DNA damage ensure the cellular genome stability, and the abnormal DNA damage repair mechanism will result in the cell senescence or death. Ju et al[10] found that FoxO1 contributes to the DNA damage repair by the JNK pathway in H1299 lung cancer cells. Bigarella et al[11] declared that FoxO3 helps to protect the stability and health of hematopoietic stem and progenitor cells from oxidative DNA damage. Besides, Brenkman et al[12] suggested FoxO4 coordinates with Ku70, a DNA double-strand break repair component, to maintain the survival in mouse embryonic stem cells. Besides, there are few related studies about the function of FoxO6 in the DNA damage process.

To clear the role of FoxO6 in IDD, we texted the FoxO6 gene expression in the disc tissue of different degeneration degrees and used an NP cell degeneration model to explore the potential mechanism related to DNA damage repair. Based on the previous studies[13,14], RAD51 has been mentioned to play a vital role in the process of DNA double-strand recombination repair and cell cycle regulation. Currently, we examined for the first time the effect of FoxO6 on the NP cell degeneration, primarily focusing on the RAD51 involved DNA damage repair procession.

Patients and Methods

Patient Tissue Samples Collection

This specimen use of this project was approved by the Ethics Committee of the Affiliated Hospital of Changchun University of Chinese Medicine. Signed written informed consents were obtained from the patients. To obtain the intervertebral disc tissue of different degenerated degrees, we recruited the patients diagnosed with the spine fracture (n=12, age 36-45) or disc herniation (n=16, age 42-51) who needed to undergo the operation to remove the disc. Having no spine pathology history is the inclusion criteria of the fracture cases. The inclusion criteria of the disc herniation patients contain noticeable imaging pathological changes, intolerable pain, and an over three months' disease course[15]. The exclusion criteria of all the patients are that there is no accidental underlying severe disease of the spine. All the tissues were valued by Pfirrmann score[16] according to the patients' Magnetic Resonance Imaging (MRI), of which grades 1 to 5 indicate the severity from mild to severe. We divided the specimens into two groups, a mild degeneration group with the tissues of grade 1 or 2, and an extreme degeneration group with grade 4 or 5. We conserved the tissues in a cold DMEM medium immediately after removing from operations for the following NP cells or RNA isolation.

NP Cells Isolation and Treatments

Human NP cells (n=8) were isolated from the tissue with mild degeneration. After separated from the disc, the NP sample was fragmented and digested with 0.1% type II collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 6 h. The isolated NP cell pellets were re-suspended in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). The primary NP cells were seeded in the 12-well plates. We split them at approximate 80% confluence for the following experiments.

To induce the human NP cells degeneration, we used the cytokine IL-1β (10 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) according to a previous method[17]. We used the FoxO6-coding vector to transfect NP cells to upregulated the FoxO6 expression. Besides, RAD51 active protein (50 ng/mL, ab63808, Abcam, Cambridge, MA, USA) was also used in the culture medium to induce a higher RAD51 expression. To suppress the RAD51 activity, we used the specific inhibitor RI-1 (50 mM, Selleck, Shanghai, China) to stimulate NP cells.

Western blot (WB)

The efficiency of the FoxO6-transfection was determined by WB methods. The total protein of the NP cells with or without transfection was isolated using the ProteoPrep Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA). Protein quality

in each group was measured by a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). An equal amount of protein was separated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking, the membrane was incubated overnight at 4°C with anti-FoxO6 primary antibodies (Thermo Fisher Scientific, Waltham, MA, USA). On the following day, the membrane was incubated for 2 h at room temperature with the secondary antibody. The gray value of blots was measured by Image J software (NIH, Bethesda, MD, USA).

Immunofluorescence (IF)

The RAD51 and γH2AX foci in the nucleus of NP cells were counted by the IF method. After fixed and permeabilized, NP cells were incubated with the primary antibodies: anti-Rad51 (ab133534, Abcam, Cambridge, MA, USA) and anti-γH2AX (ab81299, Abcam, Cambridge, MA, USA). The secondary antibodies used were Alexa Fluor 488 and 568-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA). The cell nucleus was counterstained with 4',6-diamidino-2-phenylindole (DAPI).

RNA Isolation and Gene Expression Analysis

The gene expression of lumbar disc tissue and NP cells were determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted using a Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. After mRNA was reverse-transcribed into complementary deoxyribose nucleic acid (cDNA), the gene expressions of collagen II, FoxO6, RAD51, aggrecan, collagen I, collagen X, MMP3/9, TIMP3, SOD1, CAT, GPX1, TNF-α, IL-1β, and IL-4 were analyzed by normalization to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by using $2^{-\Delta\Delta}$ Ct method using SYBR Green Master (TOYOBO, Osaka, Japan). The primers are listed in Table I.

Chromatin Immunoprecipiation (ChIP)

ChIP assay (#26157, Invitrogen, Carlsbad, CA, USA) was used to confirm the binding between FoxO6 and RAD51 promoter regions. We found the 2000-bp upstream promoter region of RAD51 from the Genome Browser Gateway database (http://genome.ucsc.edu/cgi-bin/hgGateway) and used the JASPAR core database to predict the DNA-binding sites. We found several potential binding sites and chose the highest related ones for verification. Briefly, the anti-FoxO6 antibody (Thermo Fisher Scientific, Waltham, MA, USA) linked beads were used to immunoprecipitation with NP cells' chromatin. Normal goat IgG linked beads were used as a control. After separating the DNA from immunoprecipitated chromatin, PCR was used to determine the binding efficiency. The primer used for PCR was designed by Primer5

Table I. Primer sequences for RT-PCR.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
FoxO6	GGCCGCGCTCGTGTACC	TACACGAGCGCGGCCG
RAD51	CAACCCATTTCACGGTTAGAGC	TTCTTTGGCGCATAGGCAACA
Aggrecan	ACTCTGGGTTTTCGTGACTCT	ACACTCAGCGAGTTGTCATGG
Collagen I	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
Collagen X	ATGCTGCCACAAATACCCTTT	GGTAGTGGGCCTTTTATGCCT
MMP3	AGTCTTCCAATCCTACTGTTGCT	TCCCCGTCACCTCCAATCC
MMP9	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
SOD1	GGTGGGCCAAAGGATGAAGAG	CCACAAGCCAAACGACTTCC
CAT	TGGAGCTGGTAACCCAGTAGG	CCTTTGCCTTGGAGTATTTGGTA
GPx1	CAGTCGGTGTATGCCTTCTCG	GAGGGACGCCACATTCTCG
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
TIMP4	CACTACCATCTGAACTGTGGCTG	GCTTTCGTTCCAACAGCCAGTC
IL-4	CCAACTGCTTCCCCCTCTG	TCTGTTACGGTCAACTCGGTG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR: Reverse Transcription-Polymerase Chain Reaction.

Table II. Primer sequences for PCR.

Predicted sequence	Start	End	Score	Primer sequences for PCR
ATAAATA	4	10	6.878	Sense:5'-GCCAGGACCATTCCGACAGC-3'; Anti-sense:3'-AAACGAGGAGCACGGAACCC-5
GTAAACA	1671	1677	13.257	Sense:5'-CATTACCTCTTGGGAGTCGTGG-3'; Anti-sense:3'-GGACTTTAGGGAGCGGGGTG-5'

software (Table II). The whole chromatin was used as Input DNA templates.

Cell Transfection and Luciferase Assays

We used Dual-Luciferase assays to confirm that FoxO6 promotes the RAD51 expression by the promoter activation. We purchased the pCS2-Vector with empty or FoxO6 coding, basic pGL3-Vector with original or Mut FoxO6 promoter sequence, and pRL-Renilla Vector from Genechem (Shanghai, China). For plasmid transfection, NP cells were transfected using Lipofectamine 2000 (Beyotime, Shanghai, China) according to the manufacturer's advice. After 24 h transfection, the firefly Luciferase activity was normalized by Renilla with a Dual-Luciferase Reporter Assay System.

Cell Cycle Analysis

After treatments, the cell cycle of NP cells was evaluated by the method of Propidium iodide staining (ab139418, Abcam, Cambridge, MA, USA). After washing twice with phosphate-buffered saline (PBS), 1×10^5 NP cells in each group were incubated with Propidium iodide (PI; 50 μg/mL) at 4°C for 30 min. Then, the processed NP cells were subjected to flow cytometry.

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation was determined by the CCK-8 assay (Sigma-Aldrich, St. Louis, MO, USA). NP Cells were seeded with 5000 cells/well in a 96-well plate and incubated with CCK8 reagent at 37°C for 2 h. The product intensity was measured at 570 nm using a microplate reader.

Statistical Analysis

All statistical analyses and cartogram generation were performed by Prism 8 software (La Jolla, CA, USA). Data were reported as the mean ± standard deviation (SD). Differences between the two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was made using a One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). *p*-values less than 0.05 were considered significant.

Results

FoxO6 and RAD51 Expression are Decreased Degenerated Human NP Cells

To determine whether the expression of FoxO6 and RAD51 were related to the NP cell degeneration, we tested the mRNA gene expression of FoxO6 and RAD51 in the human disc tissue, and the IL-1β treated NP cells *in vitro*. As collagen II gene is the specific marker of NP cells, which is reduced during its degenerative progress. As the only cell type in the NP tissue, NP cells's RNA map can be used to present the gene expression of the NP tissue. In the human tissue, the collagen II expression in Pfirrmann grade 1 or 2 (G1/2) disc tissues was significantly higher than the grade 4 or 5 (G4/5) (Figure 1A). Meanwhile, both FoxO6 and RAD51 mRNA expression were also higher in the G1/2 group than G4/5 (Figure 1B, 1C). We isolated the NP cells from the G1 NP tissues and treated them with 10 ng/ml IL-1β for 3 days to trigger the degeneration. After treatment, collagen II, FoxO6, and RAD51 mRNA were all significantly reduced compared to the control cells (Figure 1D-1F). Therefore, FoxO6 and RAD51 gene expression was consistently suppressed when NP cell goes to degeneration.

FoxO6 Binds to RAD51 Promoter

Since FoxO6 and RAD51 decreased simultaneously in the degeneration of nucleus pulposus cells, we wondered whether there might be an absolute correlation between the two in transcriptional expression. FoxO6 belongs to the Forkhead family of transcription factors, which is characterized by a conserved DNA-binding domain. Therefore, we collected the 2000bp upstream of the promoter sequences of RAD51, and predicted the binding sites of FoxO6 protein in the promoter using JASPAR. Finally, we found two likely putative binding sites with the highest predictive

score. As shown in Figure 2A, the putative binding sites were marked in blue (S1) and red (S2). The blue sequence 'ATAAATA' was repeated twice at the beginning of the promoter, and the red sequence 'GTAAAC' was in the last region of the promoter. Furthermore, we used anti-FoxO6 and IgG antibody linked beads to pull down the indicted protein in the chromatin of the NP cells. The total DNA from the cell lysis without immunoprecipitation was used as Input. The IgG linked beads were used as a negative control. After the immunoprecipitated DNA was separated from the beads, it was amplificated by PCR. As shown in Figure 2B, the PCR primers could amplify these two predicted promoter regions (lane 1), and no extra binding RNA was observed from the IgG linked beads (lane 3). The anti-FoxO6 antibody can pull down the S2 site (lane 2), suggesting that the red sequence 'GTAAAC' might be the binding site between FoxO6 protein and RAD51 DNA promoter.

FoxO6 Promotes RAD51 Expression by Activating its Promoter

To confirm that FoxO6 could promote RAD51 expression by activating the promoter, the Dual-Luciferase reporter gene assay was performed. The NP cells were transfected with FoxO6-coding pCS2 Vector to upregulate FoxO6 protein expression. Meanwhile, the pGL3 Luciferase Reporter Vector containing the binding promoter sequence (WT) or containing a mutant binding promoter sequence (Mut) was also used to upregulate the RAD51 expression. The empty pCS2 Vector and basic pGL3 Luciferase Reporter Vector were used as a negative control. Additionally, the pRL-Renilla Luciferase Vector was used to normalize the Luciferase of pGL3. The cartoon of the vectors was shown in Figure 2C. The result of WB analysis suggested an excellent efficiency of FoxO6-vector transfection. Interestingly, we found that FoxO6 protein overexpression could significantly active the pGL3-WT Luciferase activity but not the

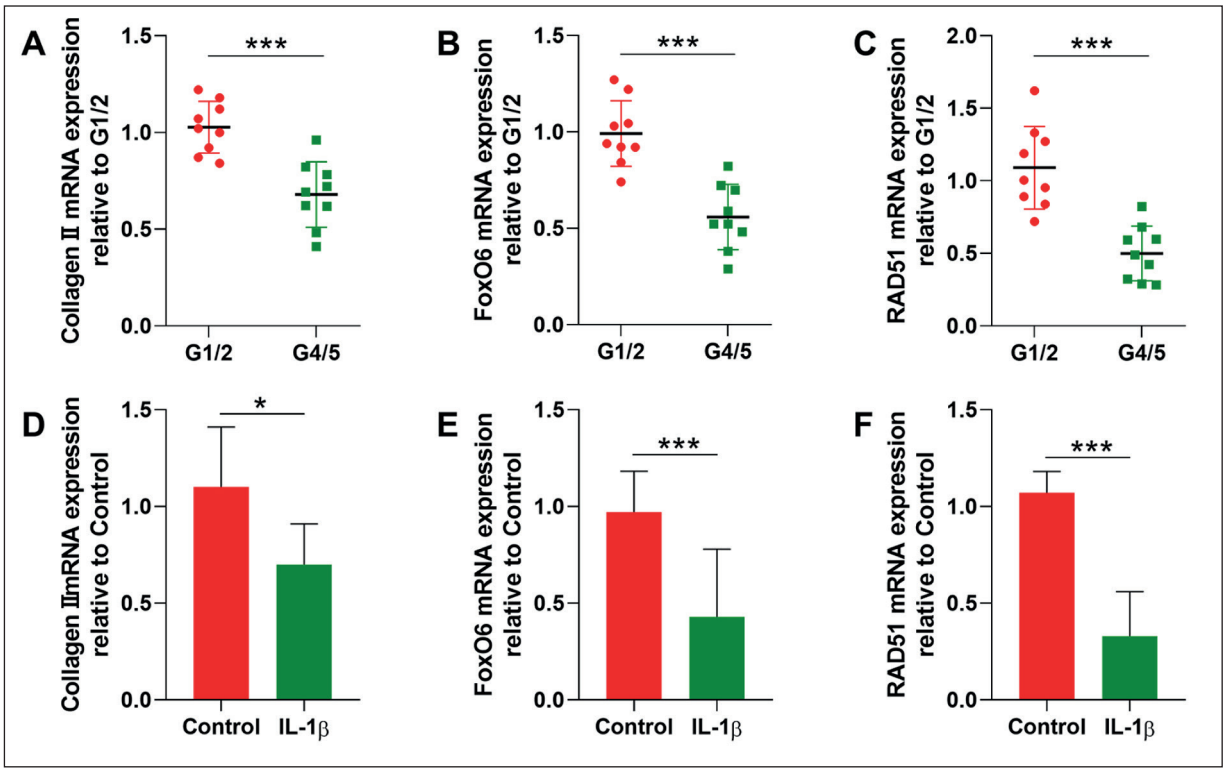

Figure 1. FoxO6 and RAD51 expression are decreased degenerated human NP cells. The human NP tissues were divided into two groups, a mild degeneration group with the tissues of grade 1 or 2 (G1/2), and an extreme degeneration group with grade 4 or 5 (G4/5). **A-C,** RT-PCR analysis for collagen II, FoxO6, and RAD51 of NP tissue by normalization to GAPDH expression. NP cells were isolated from the mild degeneration tissue and treated with 10 ng/ml IL-1β for 3 days. **D-F,** RT-PCR analysis for collagen II, FoxO6, and RAD51 of NP cells by normalization to GAPDH expression. Results are expressed as mean ± SD. (*$p < 0.05$, ***$p < 0.001$).

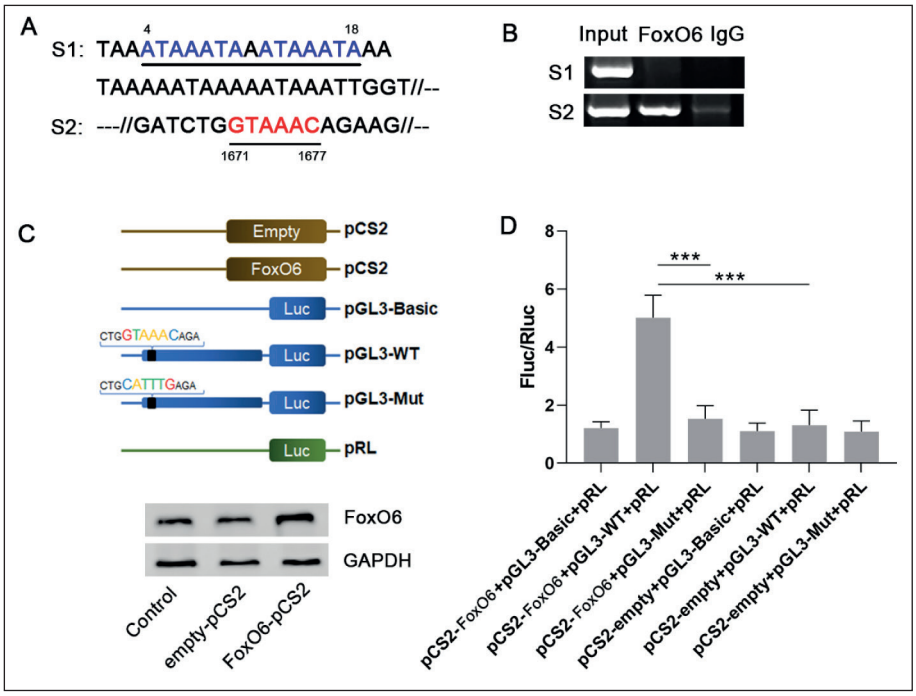

Figure 2. FoxO6 binds to the promoter of RAD51 and promotes its expression. **A,** Two predicted binding sites (S1, S2) in the RAD51 promoter region (2000 bp). **B,** S1 and S2 sequences were recovered by PCR from FoxO6 immunoprecipitates but not from IgG immunoprecipitates. **C,** The cartoon of the vectors transfected to NP cells, and the WB analysis of FoxO6 expression after transfection. **D,** Luciferase activity was driven by the S2 predicted promoter, which was more dramatic following FoxO6 protein upregulation, and no significant difference in Luciferase activity was observed S2 sequence was mutated. Results are expressed as mean ± SD. (***$p < 0.001$).

pGL3-Mut Luciferase activity. Besides, the Luciferase activity of pGL3-WT transfected NP cells was not significantly increased when the FoxO6 was not upregulated (Figure 2D). Therefore, we concluded that FoxO6 could promote the RAD51 expression by binding to its promoter.

FoxO6 Overexpression Suppresses the DNA Damage by Upregulating RAD51

To determine the effect of RAD51 on the DNA damage caused by IL-1β, we tested the expression of DNA damage marker γH2AX in the nucleus. NP cells were divided into the following groups: culture without any treatments defined as control; culture with IL-1*β* to induce degeneration; culture with IL-1β and RAD51 protein; FoxO6 vector-transfected and cultured with IL-1β; FoxO6 vector-transfected and cultured with IL-1β and RI-1. All cells were cultured for three days. Compared to control, IL-1β suppressed the RAD51 but increased γH2AX expression. However, under the IL-1β treatment, FoxO6 transfection maintained the content of RAD51 foci and suppressed γH2AX foci, which was as efficient as the RAD51 protein supplement. When the FoxO6 transfected NP cell was treated with RI-1, RAD51 foci were significantly suppressed, and the γH2AX foci were increased inversely (Figure 3A, 3B). Therefore, RAD51 upregulation played a role in the suppression of DNA damage in NP cells, and FoxO6 upregulation suppressed the DNA damage mediated by RAD51 activation.

FoxO6 Overexpression Alleviates the IL-1β Induced NP Cells Degeneration

To determine the effect of FoxO6 on the NP cell fate, we tested the collagen II, aggrecan, and some ECM gene expression. NP cells were divided into the following groups: culture without any treatments defined as control; culture with IL-1β to induce degeneration; FoxO6 vector-transfected and cultured with IL-1β; FoxO6 vector-transfected and cultured with IL-1β and RI-1. All cells were cultured for three days. As shown in Figure 4A, IL-1β decreased the collagen II, aggrecan, and TIMP3 RNA expression, and increased the collagen I, collagen X, MMP3, and MMP9 RNA expression. However, the FoxO6 vector-transfected

NP cells presented a strong ability to resist these effects caused by IL-1β, except TIMP3 expression. Apart from this, the collagen I, collagen X, MMP3, and MMP9 expression was increased again when RAD51 was suppressed. Therefore, FoxO6 protected the stability of ECM *via* the upregulation of RAD51. Additionally, some antioxidative genes and inflammatory genes were also analyzed to explore the potential molecular mechanism further. We found that FoxO6 overexpression increased the SOD1 and CAT expression and inhibited the TNF-α and IL-1β expression. However, only the SOD1 and CAT expression were affected when RAD51 was suppressed, suggesting the anti-inflammatory function of FoxO6 is not dependent on RAD51 (Figure 4B). We tested cell proliferation using the CCK-8 assay, and the result indicated that FoxO6 upregulation protected the proliferation of NP cells, which was abolished after ARD51 was suppressed (Figure 4C). The typical excess of the cell cycle is a necessary guarantee to maintain the level of cell proliferation. We further tested the distribution of the cell cycle phases (G1, S, G2/M). Under the IL-1β treatment, FoxO6 upregulation could promote cells pass through the G2/M to the G1 phase. However, when RAD51 was suppressed, more cells were arrested in the G2/M phase again (Figure 4D).

Discussion

FoxO transcription factors have been mentioned to participate in the mediation of longevi-

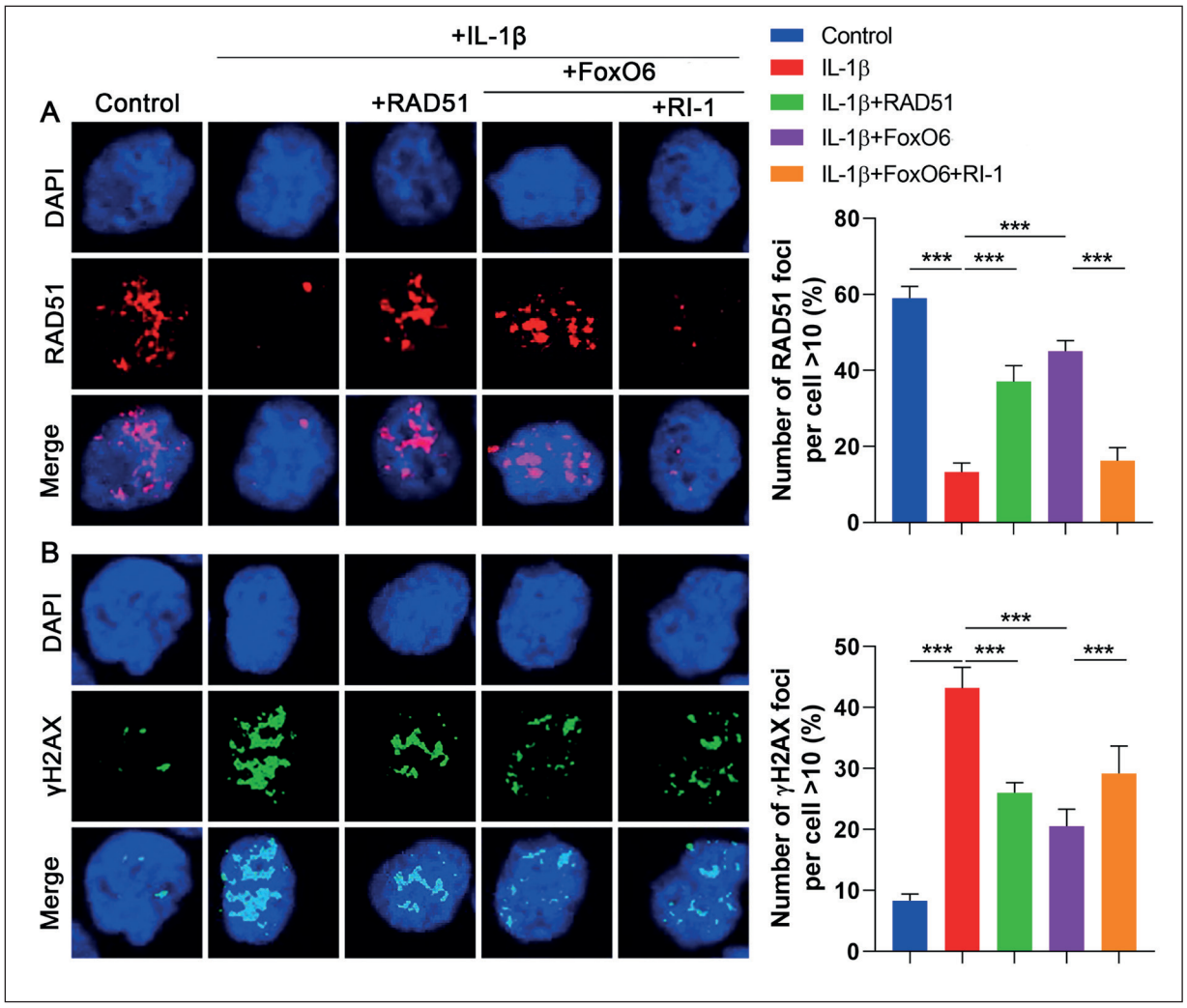

Figure 3. FoxO6 overexpression suppresses the DNA damage by upregulating RAD51. **A-B,** Immunofluorescence analysis of RAD51 and γH2AX foci formation (magnification: 400×) and quantification by counting the foci of more than 10 per nucleus. Results are expressed as mean ± SD. (***$p < 0.001$).

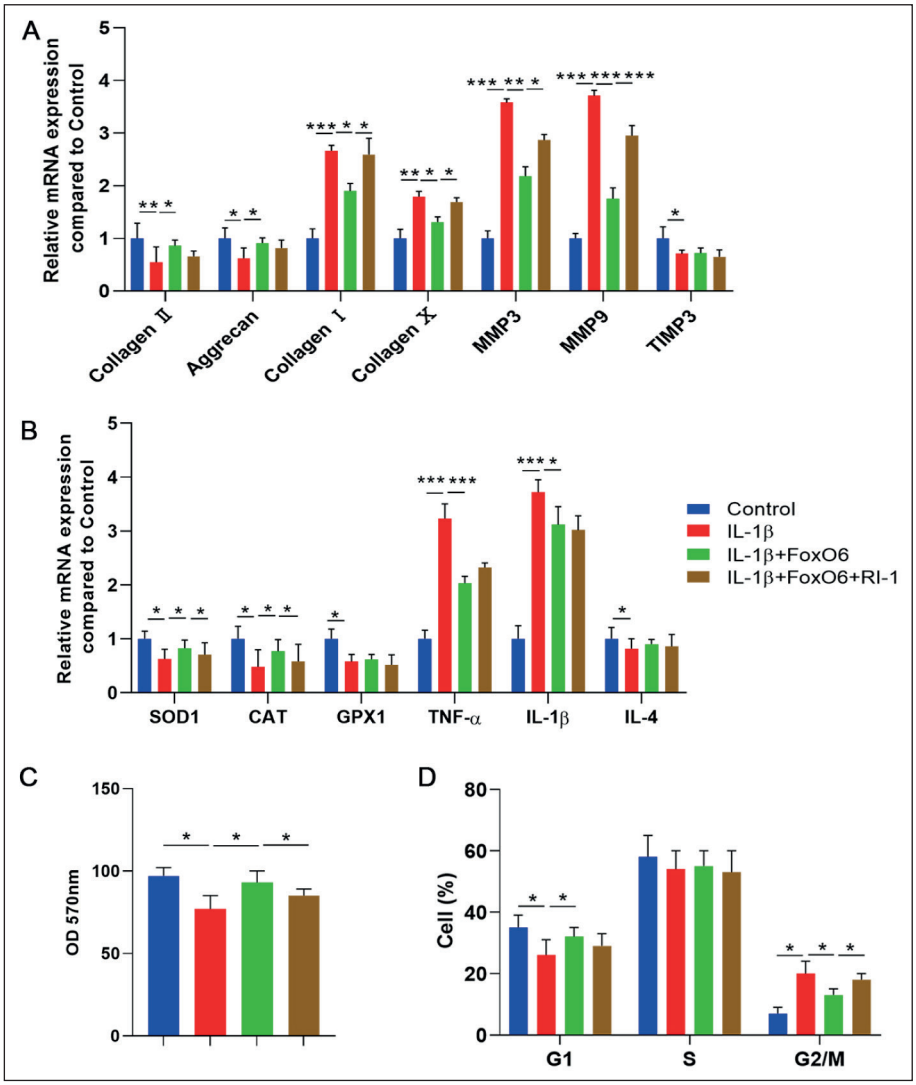

Figure 4. FoxO6 overexpression alleviates the IL-1β induced NP cell degeneration. Normal NP cells were subjected to 10 ng/mL IL-1β for 3 days as control. The FoxO6-overexpressed NP cells were subjected to 10 ng/mL IL-1β with or without the RI-1 stimulation for 3 days. **A-B,** RT-PCR analysis for indicated RNA expression by normalization to GAPDH expression. **C,** Cell proliferation assay by CCK-8. **D,** Distribution of cell cycle. Results are expressed as mean ± SD. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

ty phenomenon, of which FoxO1, FoxO3, FoxO4, and FoxO6 exist in mammals. They were correlated with the growth, differentiation, metabolism, and apoptosis in cells[18]. However, the objective function of FoxO6 in the intervertebral disc is still unknown. This is the first study that describes the existing expression pattern of FoxO6 in human NP cells briefly. We found there is a significant reduction of FoxO6 expression in the degenerated NP tissue. In addition, using the NP cell degeneration model, we also demonstrated that FoxO6 expression was suppressed when the NP cell underwent dysfunctional progress.

After being subjected to endogenous or exogenous damage factors, especially ionizing radiation, cells are prone to DNA double-strand break (DSB)[19], impacting a biologically significant range of cytological events. Un-repaired DNA damages can induce cell aging or even death, which as an inducer of IDD, has been supported by evidence in humans and mice, relating to ionizing radiation and tobacco smoking[20-22]. RAD51 is one of the most important genes involved in DNA homologous recombination[23] involved in the complex signaling pathways of DSB repair and cell cycle[24,25]. Our data showed that RAD51

expression in mild degenerated NP tissue was much higher than that of severe degenerated NP tissue. After being treated with IL-1β, the RAD51 gene expression was also significantly reduced. Due to the synchronous reduction of FoxO6 and RAD 51 in the degenerated NP cell, we verified whether the FoxO6 transcriptionally regulated RAD51 expression. Luckily, we found some potential binding sites between FoxO6 protein and the RAD51 DNA promoter region. In addition, the dual-luciferase reporter gene assay showed that the overexpressed FoxO6 was efficient to activate the RAD51 promoter expression, indicating a positive relationship between the RAD51 and the FoxO6 level.

The formation and loss of DNA damage foci have been elucidated in lots of established cell lines, monitored by the fluorescent protein staining[23] among which γH2AX foci formation is overwhelmingly believed as a strong correlation with DSB[26]. In our study, IL-1β was proved to induce the DNA damage of NP cells with an increase of γH2AX foci expression. However, the upregulation of RAD51 foci, resulting from both exogenic RAD51 protein supplement and FoxO6 gene transfection, suppressed the formation of γH2AX foci, suggesting a mechanism underlying how FoxO6 helps the DNA damage repair. In addition, the upregulated FoxO6 gene enhanced NP cells to alleviate the loss of collagen II and aggrecan and confront the production of collagen I/X and MMP3/9. Typical ECM components in NP is consist of collagen II and aggrecan to maintain the hydrostatic pressure to withstand compressive loading stresses. As IDD, the ECM distribution is replaced by collagen I/III/X and is degraded by the accumulation of MMPs. Keeping the ECM stable is the basis of resisting the dysfunction of intervertebral disc[27]. Since most of the ECM molecules are secreted by NP cells, it is incredibly essential to maintain the normal secretory phenotype of NP cells. Our data verified that FoxO6 gene upregulation supported NP cells resisting the effects of IL-1β, which was somehow dependent on the activation of RAD51.

Oxidative stress and inflammation are the classical pathological process in IDD[28]. Therefore, we also measured the RNA levels of relative antioxidative enzymes and inflammatory factors to clear whether FoxO6 also plays a role in keeping the balance of oxidative stress and inflammatory response. In the present study, we found that FoxO6 upregulation in NP cells increased the SOD1 and CAT gene expression, which was abolished when RI-1 suppressed RAD51. Therefore, the RAD51 is a vital mediator for FoxO6 to protect the antioxidative function in NP cells. However, more evidence should be supplied to confirm FoxO6 upregulation also suppressed the inflammation via the RAD51 manner. Previous studies[29,30] have mentioned that FoxO and RAD51 all regulate the cell cycle concerning ensure normal cell physiological activities. Our data revealed that FoxO6 could promote the cell cycle to go through the G2/M checkpoint to the G1 phase, which helped the proliferative progress of NP cells. These results suggest that FoxO6 expression truly protects the function and activity of NP cells within the damage caused by IL-1β.

Conclusions

For the first time we demonstrated that FoxO6 attenuates NP cell degenerative phenotype and DNA damage by activating RAD51 expression. The protective role of FoxO6 in the NP cells may involve keeping ECM and oxidative enzyme stability and anti-inflammation, providing new knowledge to understand better how FoxO6 acts during IDD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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