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LncRNA ANRIL knockdown ameliorates retinopathy in diabetic rats by inhibiting the NF-κB pathway

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Abstract. – OBJECTIVE: To evaluate the impacts of long non-coding ribonucleic acid (lncRNA) antisense non-coding RNA in the INK4 locus (ANRIL) on diabetic retinopathy (DR) in rats and the underlying mechanism.

MATERIALS AND METHODS: A total of 60 adult male Sprague Dawley (SD) rats were randomly divided into 3 groups: the Sham group (n=20), DR group (n=20) and DR + lncRNA ANRIL knockdown group [DR + lncRNA ANRIL small interfering RNA (siRNA) group, n=20]. DR model in rats was established by intraperitoneal injection of streptozocin (STZ; 60 mg/kg). Meanwhile, a certain dose of lncRNA ANRIL siRNA was added dropwise into rat eyes of DR + lncRNA ANRIL siRNA group during model induction to downregulate lncRNA ANRIL expression in the retina. 16 weeks later, rat retinal tissues were taken to extract total RNA and protein. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was applied to detect the expression levels of lncRNA ANRIL, interleukin-1 (IL-1), IL-6 and monocyte chemotactic protein-1 (MCP-1) in each group of the rat retina. Pathological structure of rat retinal tissues in each group was observed *via* hematoxylin and eosin (H&E) staining. Immunohistochemistry was adopted to measure the expression levels of B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax) and P65 in each group of retinal tissues. In addition, the retinal vascular permeability in each group of rats was detected by fluorescent staining. Finally, Western blotting was utilized to determine the expressions of genes in the P65 signaling pathway.

RESULTS: Compared with rats in the Sham group, lncRNA ANRIL was upregulated in rat retinal tissues harvested from the DR + lncRNA ANRIL siRNA group ($p<0.05$). After knockdown of lncRNA ANRIL in the retinal tissues of DR rats, pathologic damage was alleviated notably, and the levels of inflammatory markers (IL-1, IL-10 and MCP-1) were lowered markedly ($p<0.05$). The protein expressions of Bax and P65 decreased evidently, while the protein level of Bcl-2 increased markedly ($p<0.05$) in DR rats with ANRIL knockdown. Furthermore, Western blotting results revealed that inhibition of lncRNA ANRIL could prominently repress the phosphorylation level of P65 in the retinal tissues of DR rats ($p<0.05$).

CONCLUSIONS: LncRNA ANRIL knockdown can significantly ameliorate the retinopathy in diabetic rats by blocking the nuclear factor-kappa B (NF-κB) signaling pathway.

Key Words:
LncRNA ANRIL, Diabetic retinopathy, NF-κB.

Introduction

Diabetic retinopathy (DR) is a major microvascular complication in diabetic patients, and its incidence rate is rising year by year[1]. DR patients are likely to have hypopsia or even blindness if effective treatment is lacked[2]. The pathogenesis of DR is complex, involving abnormal expressions of multiple genes and proteins[3]. Therefore, it is of great significance for early prevention and precise treatment in the future to further elaborate the pathogenesis of DR.

Studies[4] have manifested that persistent low-grade inflammation is not only the leading cause of DR but also one of the main pathological changes in the retina of DR patients. The inflammatory response is a non-specific reaction to injury or pressure, concerning various inflammatory mediators. In the serum and ocular samples (vitreous body and aqueous humor) of DR patients, expression levels of many inflammatory cytokines [interleukin-1 beta (IL-1β), IL-6, IL-8, tu-

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mor necrosis factor-alpha (TNF-α) and monocyte chemotactic protein-1 (MCP-1)] and chemokines are elevated remarkably[5]. Most of these cytokines are generated by microglia, endothelial cells, macroglia and even neurons. The accumulation of these inflammatory mediators is regarded as an important cause of early death of nerve cells in DR patients[6]. Besides, MIP-1, IL-1, and IL-3 are considered key regulators in the angiogenesis in DR[7,8]. Nuclear factor-kappa B (NF-κB), a key transcription factor responsible for the activation of inflammatory genes in DR, serves as a vital target for the treatment of DR.

Long non-coding ribonucleic acids (RNAs) are a group of RNAs with a length of over 200 nt incapable of encoding proteins[9]. LncRNA transcripts were once neglected because they were regarded as simple transcriptional "noise" or "cloning relics"[10]. In recent years, lncRNAs in human diseases have been highlighted (e.g., proliferation, angiogenesis, apoptosis, migration and invasion of tumor cells) with the completion of Human Genome Project and the rapid development of molecular genetic techniques[11]. For example, lncRNA HOTTIP can ameliorate the DR by regulating the p38-mitogen-activated protein kinase (p38-MAPK) signaling pathway[12]. Overexpression of lncRNA MEG3 is able to suppress the progression of DR by regulating proteins associated with TGF-β1 and vascular endothelial growth factor (VEGF)[13]. However, the mechanism of lncRNA antisense non-coding RNA in the INK4 locus (ANRIL) underlying the occurrence and development of DR has not been reported yet. In this research, the expression of lncRNA ANRIL in the retinal tissues of DR rats was detected. Subsequently, the DR model in rats with *in vivo* knockdown of ANRIL was established. Potential impacts of ANRIL on pathological changes of rat retinal tissues and inflammatory response were explored. Finally, the mechanism of lncRNA ANRIL in DR was further analyzed by detecting the expression of related pathways.

Materials and Methods

Animal Grouping and Modeling

A total of 60 male Sprague Dawley (SD) rats aged 12-14 weeks and weighing (283.42±3.62) g were randomly divided into 3 groups, including the Sham group (n=20), DR group (n=20) and DR + lncRNA ANRIL knockdown group (DR + lncRNA ANRIL siRNA group, n=20). There were no statistical differences in age, weight and other baseline information among the three groups of rats. The DR modeling processes were as follows: streptozocin (STZ; 60 mg/kg, Solarbio, Beijing, China) was injected intraperitoneally into the rats. 5 days later, the blood glucose was detected for consecutive 16 weeks. Rats with a glucose level >16.7 mmol/L were diagnosed with diabetes, and they were enrolled for further experiments. In the same period, rats in the DR + lncRNA ANRIL siRNA group were administrated a certain dose of lncRNA ANRIL siRNA. 16 weeks later, the eyes were enucleated, and the retinal tissues were isolated. This study was approved by the Animal Ethics Committee of the First Hospital of Xi'an Animal Center.

Hematoxylin and Eosin (H&E) Staining

Retinal tissues in each group were placed in 10% formalin overnight, followed by dehydration and embedding in paraffin blocks. Subsequently, all the conjunctival tissues were sliced to 5-μm-thick sections, fixed on glass slides and dried by baking. According to the instructions, the tissues were soaked in xylene, graded ethanol and hematoxylin and mounted in resin. After air drying, the tissues were observed and photographed under a light microscope (Leica, Wetzlar, Germany). Pathological changes in the conjunctival tissues and the number of eosinophils in the sections were observed.

Detection of Expressions of Related Genes in Conjunctival Tissues via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) The total RNA in the retinal tissues was extracted using the TRIzol reagent method (Invitrogen, Carlsbad, CA, USA), and RNA concentration and purity were measured by an ultraviolet spectrophotometer. RNA with A_{260}/A_{280}=1.8-2.0 was eligible for use. (2) The messenger RNA (mRNA) was synthesized into complementary deoxyribonucleic acid (cDNA) through reverse transcription, and the cDNA was stored in a refrigerator at -80°C. (3) Real Time-Polymerase Chain Reaction (RT-PCR) system: 2.5 μL of 10× Buffer, 2 μL of cDNA, 0.25 μL of forward primer (20 μmol/L), 0.25 μL of reverse primer (20 μmol/L), 0.5 μL of dNTPs (10 mmol/L), 0.5 μL of Taq Polymerase (2×10^6 U/L) and 19 μL of ddH_2O. The RT-PCR amplification system was the same. Primer sequences used in this study were as follows:

lncRNA ANRIL, F: 5'-CAGGAACCCCCTCCTTACTC-3', R: 5'-CTAGGGATGTGTCCGAAGGA-3'; IL-1, F: 5′-ACGATGCACCTGTACGATCA-3′, R: 5'- TCTTTCAACACGCAGGACAG -3'; IL-6, F: 5'-ACTTCTCCACAACGCTCTG-3', R: 5'-CAGAGGGAAGAGTTGTCCAG-3'; MCP-1: F: 5'-GATTCAGCAAATCACATCACTAGAT-3', R: 5'-CGGCTAAGAACTGCATGTC-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Immunohistochemical Staining

The sliced retinal tissue sections were baked in the oven at 60°C for 30 min, followed by deparaffinization in xylene (5 min × 3 times) and dehydration with 100%, 95% and 70% ethanol 3 times, respectively. The activity of endogenous peroxidase was inhibited by 3% hydrogen peroxide-methanol, and the tissues were sealed in goat serum for 1 h. The anti-B-cell lymphoma-2 (Bcl-2), anti-Bcl-2-associated X protein (Bax) and anti-P65 antibodies were diluted with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) at 1:200 and then incubated at 4°C overnight, followed by washing with PBS on a shaking table 4 times. After incubation with secondary antibodies, the diaminobenzidine was adopted for color development. Then, 6 samples were randomly selected from each group, and 5 fields of vision were randomly selected from each sample for photographing under the light microscope (magnification 400×).

Detection via Western Blotting

After retinal tissues of each group of rats were fully ground in lysis buffer, ultrasonication was performed. Then, the lysis buffer was centrifuged, and the supernatant was aspirated and subpackaged into Eppendorf (EP; Eppendorf, Hamburg, Germany) tubes. Later, the protein concentration was determined through the bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA) and ultraviolet spectrophotometric assay, and the volume of all the sample proteins was maintained at equal concentration. Next, the proteins were subpackaged and preserved in the refrigerator at -80°C. The total protein was extracted and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After that, the protein in the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were incubated with primary antibody at 4°C overnight and goat anti-rabbit secondary antibody in the dark for 1 h. Scanning and quantification of protein bands were achieved using an Odyssey membrane scanner. The level of the protein to be detected was corrected *via* glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Detection of Capillary Permeability

After the rats in each group were anesthetized by zolazepam (Zoletil, Virbac, Carros, France), 100 mg/kg of fluorescein isothiocyanate-bovine serum albumin (FITC-BSA; Sigma-Aldrich, St. Louis, MO, USA) was injected into the left ventricle of the laboratory rats. The tracer solution was circulated for 10 min, and then one eyeball was enucleated to dissect the retina, which was placed flatly on a glass slide, followed by observation and photographing under a fluorescence microscope.

Statistical Analysis

All the data were analyzed using the Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Measurement data were presented as mean ± standard deviation, and *t*-test was performed for comparison of data between the two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Expression of LncRNA ANRIL in Retinal Tissues of Each Group of Rats

The expression levels of lncRNA ANRIL in retinal tissues of control rats and DR rats were detected *via* RT-PCR. As shown in Figure 1, compared with that in the Sham group, the expression of lncRNA ANRIL in retinal tissues of DR rats was up-regulated remarkably, which was about 5.67 times that of control rats ($p<0.05$).

Impacts of LncRNA ANRIL Knockdown on Morphology of Retinal Tissues of DR Rats

The H&E staining results under the light microscope indicated that the thickness of rod and cone photoreceptors, outer nuclear layer, inner nuclear layer, inner plexiform layer and ganglion cell layer in the retinal tissues of DR rats decreased evidently compared with those of the Sham group (Figure 2). However, lncRNA ANRIL knockdown could ameliorate the above-mentioned retinal injuries caused by DR.

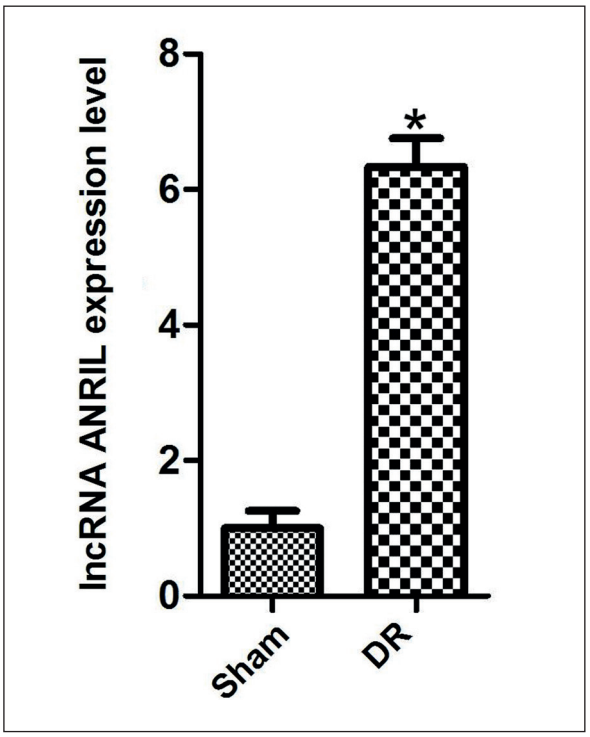

Figure 1. Expression of lncRNA ANRIL in retinal tissues of each group of rats. Sham group, DR group, *p<0.05 *vs.* Sham group, with a statistical difference.

Impacts of LncRNA ANRIL Knockdown on Cell Apoptosis in Retinal Tissues of DR Rats

Protein expressions of relevant apoptotic molecules in the retinal tissues of each group of rats were detected *via* immunohistochemistry (Figure 3). The results indicated that lncRNA ANRIL knockdown could prominently reverse DR-induced up-regulation of Bax, a pro-apoptotic protein, and down-regulation of Bcl-2, an anti-apoptotic protein, in the retinal tissues of rats (p<0.05).

Impacts of LncRNA ANRIL Knockdown on Inflammation in Retinal Tissues of DR Rats

The mRNA expressions of related inflammatory markers in the retinal tissues of each group of rats were detected. The results manifested that expression levels of IL-1, IL-6 and MCP-1 in the retinal tissues in the DR + lncRNA ANRIL siRNA group declined notably compared with those in the DR group (p<0.05), illustrating that silence of lncRNA ANRIL can lower the inflammation level in the retinal tissues of DR rats (Figure 4).

Impacts of LncRNA ANRIL Knockdown on Vascular Function of Retinal Tissues of DR Rats

To explore the impacts of lncRNA ANRIL on the vascular function of DR rats, FITC-BSA injection and immunolabeling technique were applied to determine the microvascular permeability in each group of rats. It was shown that the vascular fluorescence intensity increased markedly in the DR group, indicating the improved vascular permeability and declined vascular function (Figure 5). However, after silence of lncRNA ANRIL, the degree of vascular permeability was repressed significantly. These results suggested that lncRNA ANRIL knockdown can improve the vascular function of DR rats.

Immunohistochemical Staining for P65 in Retinal Tissues of the Three Groups of Rats

Moreover, the distribution and expression of P65 in the retinal tissues of the three groups of rats were measured by immunohistochemistry. It was manifested that lncRNA ANRIL knockdown can prominently suppress the DR-induced activation of P65 in the retinal tissues of rats (Figure 6).

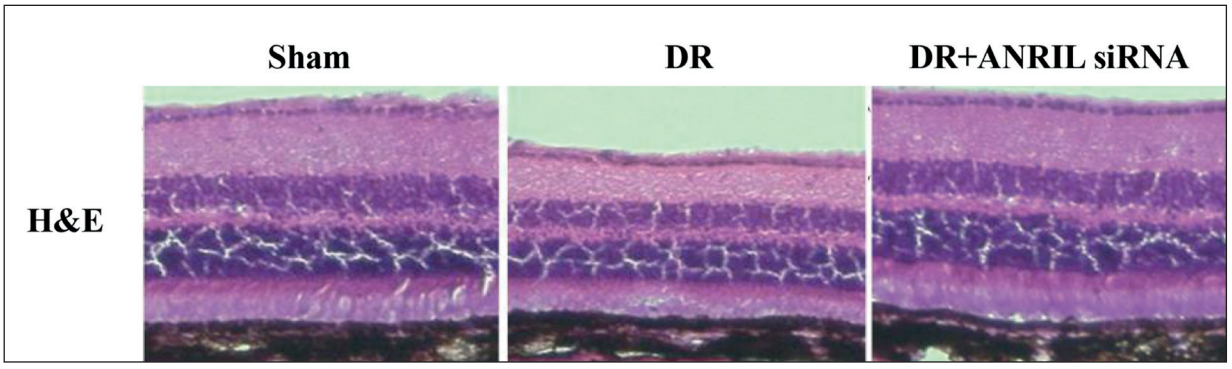

Figure 2. Impacts of lncRNA ANRIL knockdown on morphology of retinal tissues of DR rats (magnification × 40). Sham group, DR group, DR + lncRNA ANRIL siRNA group.

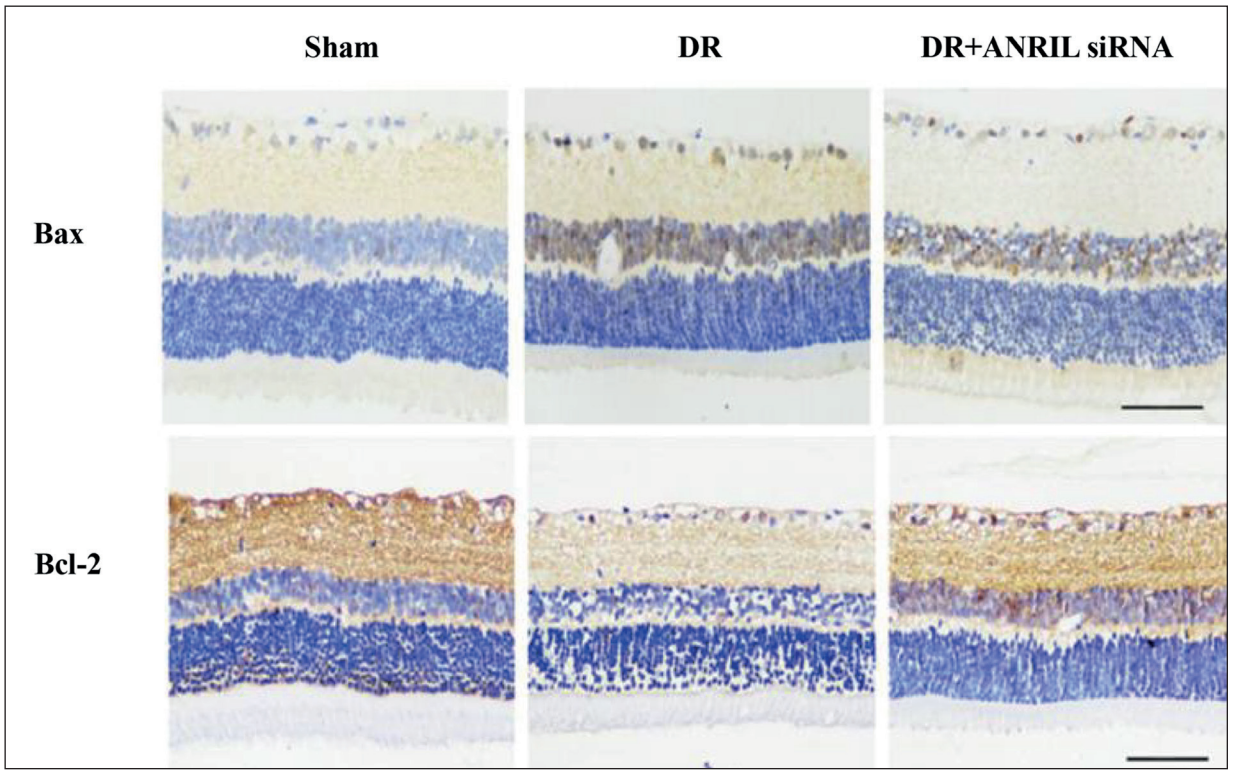

Figure 3. Impacts of lncRNA ANRIL knockdown on cell apoptosis in retinal tissues of DR rats (magnification × 400). Sham group, DR group, DR + lncRNA ANRIL siRNA group.

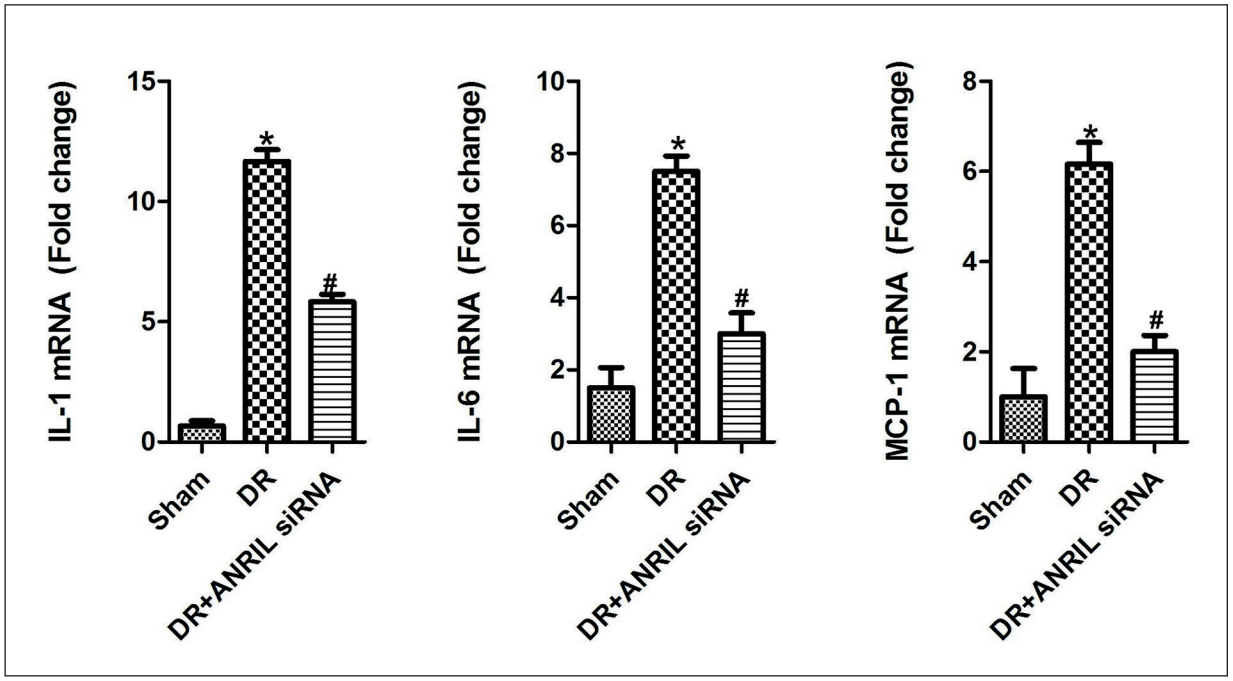

Figure 4. Impacts of lncRNA ANRIL knockdown on inflammation in retinal tissues of DR rats. Sham group, DR group, DR + lncRNA ANRIL siRNA group, *p<0.05 *vs.* Sham group, #p<0.05 *vs.* DR group, with a statistical difference.

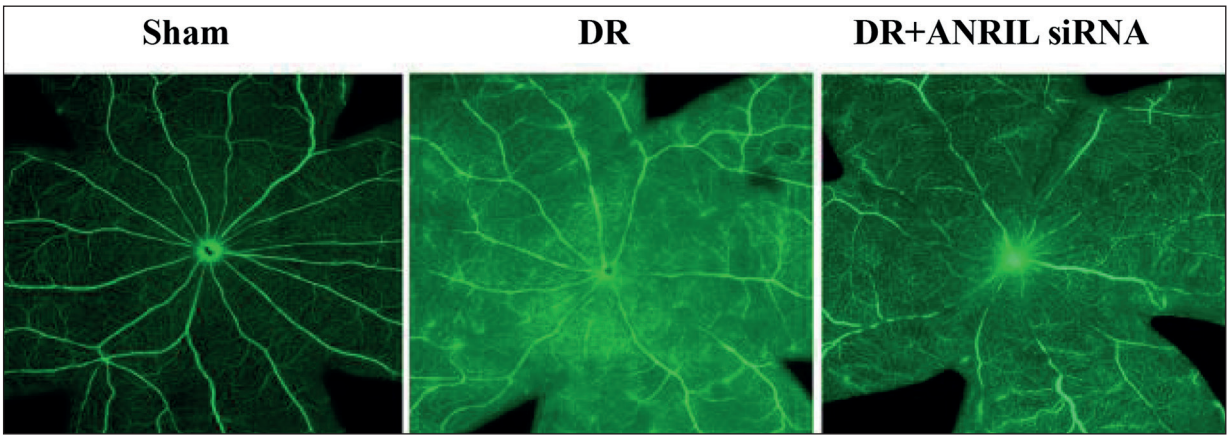

Figure 5. Impacts of lncRNA ANRIL knockdown on vascular function of retinal tissues of DR rats (magnification × 400). Sham group, DR group, DR + lncRNA ANRIL siRNA group.

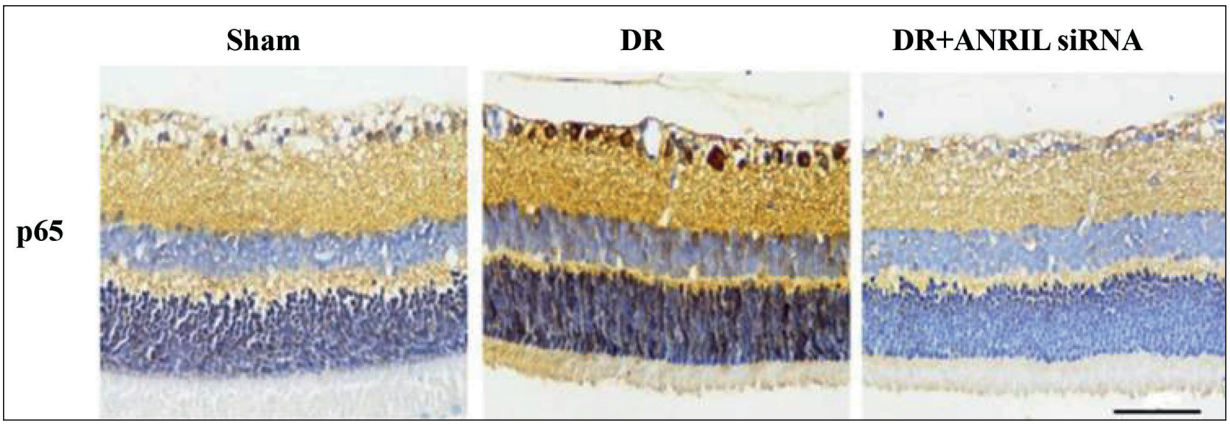

Figure 6. Immunohistochemical staining for P65 in retinal tissues of the three groups of rats (magnification × 400). Sham group, DR group, DR + lncRNA ANRIL siRNA group.

Impacts of LncRNA ANRIL Knockdown on the NF-κB Signaling Pathway

Finally, expressions of phosphorylated P65 (p-P65) and total P65 (t-P65) in the retinal tissues of the three groups of rats were detected and quantified. It was revealed that knockdown of lncRNA ANRIL could remarkably repress the phosphorylation level of P65 in the retinal tissues of DR rats and reduce the p-P65/t-P65 ratio ($p<0.05$; Figure 7).

Discussion

Diabetes mellitus is one of the most common metabolic diseases in developing and developed countries. According to epidemiological data[14], about 10% population suffer from diabetes globally. It is estimated by the World Health Organization that the number of diabetic patients will be up to 366 million by 2030[15]. In the meantime, the International Diabetes Federation (IDF) proposed that the number will reach 693 million by 2045[16]. The persistent high glucose in the blood can affect cell function and damage the great vessels and microvessels of various organs. The IDF declares that in comparison with such high-risk infectious diseases as AIDS and tuberculosis, approximately 5 million people die from specific complications of diabetes, including DR and diabetic nephropathy[17]. Therefore, DR is becoming a leading cause of visual impairment among the elderly in the majority of regions. Large quantities of clinical studies have demonstrated that sugar/glucose imbalance can injure the retinal microvessels and further result in rupture, effusion and intra-retinal hemorrhage of the blood-retinal

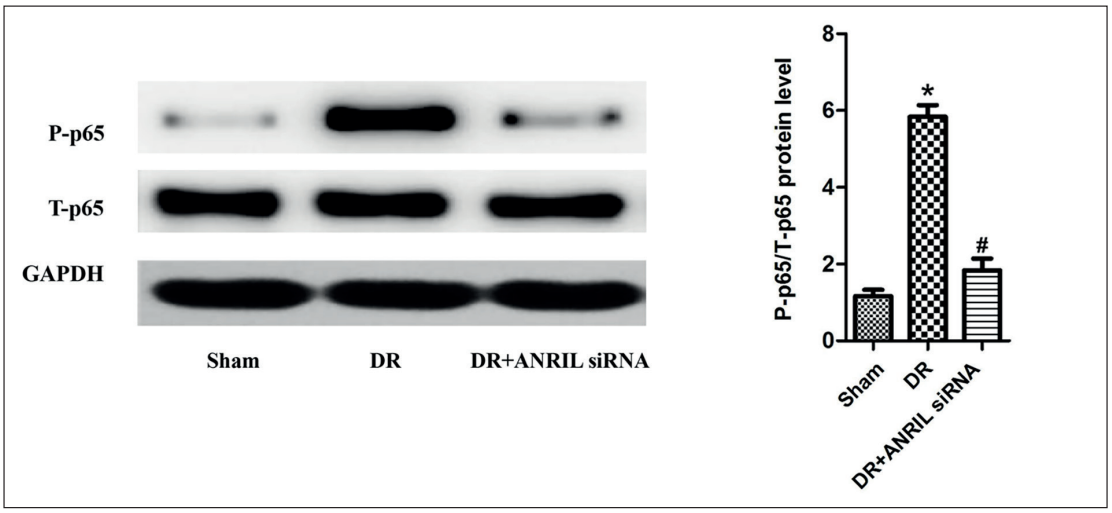

Figure 7. Impacts of lncRNA ANRIL knockdown on the NF-κB signaling pathway in retinal tissues of DR rats. Sham group, DR group, DR + lncRNA ANRIL siRNA group, *p<0.05 *vs.* Sham group, #p<0.05 *vs.* DR group, with a statistical difference.

barrier (BRB) in the early non-proliferative phase of DR. These pathological lesions lead to retinal neovascularization in the proliferative phase of DR[18]. In addition, chronic low-grade inflammation has a close correlation with the pathogenesis of DR by activating innate immunity. Long-term hyperglycemia will destroy the integrity of BRB, eventually causing ocular complications. In this case, neutrophils can infiltrate into the retina and choroid through the injured BRB and adhere to the vascular endothelial cells[14].

Although it has been verified that many inflammatory mediators and cytokines are involved in the occurrence and development of DR, there is little knowledge about the activation of the signaling pathways in the pathogenesis of DR. NF-κB can be activated by a variety of signals, such as hypoxia, bacterial and viral infections and pro-inflammatory cytokines[19]. NF-κB represents a protein family with similar structure and conserved function evolution, including Rel (c-Rel), RelA (P65), RelB, NF-κB1 (P50 and its precursor P105) and NF-κB2 (P52 and its precursor P100)[20]. NF-κB signal is abnormally activated in the occurrence and development of DR, which is used as a target for intervention in several studies, and preferable effects have been obtained. For instance, the activation of the NF-κB signaling pathway and cell apoptosis are repressed remarkably in the retinal tissues of DR rats after the administration of resveratrol[21]. Naringin can also alleviate DR by inhibiting inflammation, oxidative stress and NF-κB activation[22]. In high glucose-induced lens cells, NF-κB can aggravate cell damage by regulating iNOS expression[23]. In this research, it was discovered for the first time that lncRNA ANRIL regulated NF-κB in the rat model of DR. The results indicated that the expression level of lncRNA ANRIL in the retinal tissues of DR rats was elevated notably. Knockdown of ANRIL could significantly relieve pathologic damage to the retina of rats and markedly decreased the inflammation level, manifesting as decreased expression levels of IL-1, IL-6 and MCP-1. Moreover, apoptosis in the retinal tissues of rats was suppressed significantly, and the microvascular function was improved remarkably. Furthermore, it was revealed that the expression and activation levels of P65 in the retinal tissues of DR rats declined prominently after lncRNA ANRIL knockdown. Nonetheless, there were still some limitations in this research: (1) *In vitro* experiments for underlying the role of ANRIL in DR are lacked; (2) Direct targets of lncRNA ANRIL are not found; and (3) this research only investigated the impacts of lncRNA ANRIL on P65, a classical NF-κB signaling pathway, instead of non-classical pathways.

Conclusions

The inhibition on lncRNA ANRIL can significantly ameliorate DR in rats by inhibiting P65, the classical NF-κB signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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