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Overexpression of MicroRNA-25 by Withaferin A Induces Cyclooxygenase-2 Expression in Rabbit Articular Chondrocytes

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Abstract. Increasing evidence supports the role of microRNAs (miRNA) in the regulation of inflammation in various human disorders. Several recent studies have demonstrated that microRNA-25 (miR-25) has multiple functions, and it affects the expression of inflammatory mediators. Withaferin A (WFA), a natural compound derived from the medicinal plant *Withania somnifera*, has shown the potential to be an effective drug for arthritis treatment in several preclinical and clinical studies. We investigated the role of miR-25 in the WFA-mediated up-regulation of cyclooxygenase-2 (COX-2) expression in rabbit articular chondrocytes. WFA induced COX-2 expression in a dose-dependent manner as analyzed by western blot analysis and immunofluorescence staining in rabbit articular chondrocytes. WFA up-regulated miR-25 expression as determined by real-time PCR. Overexpression of miR-25 in the presence of WFA increased the expression of COX-2 compared to that observed with just WFA treatment alone, as indicated by western blot analysis and Real-time PCR. Moreover, silencing of miR-25 by anti-miR25 inhibited COX-2 expression in a dose-dependent manner. Since miR-25 up-regulation by WFA treatment induced the expression of COX-2 in rabbit articular chondrocytes, these findings collectively suggest that miR-25 mediates the WFA-induced inflammatory responses in chondrocytes.

Keywords: microRNA-25, chondrocyte, withaferin A, cyclooxygenase-2, inflammation

Introduction

MicroRNAs (miRNA) are a family of approximately 22-nucleotide-long noncoding RNAs and have been identified in organisms ranging from nematodes to humans. Many miRNAs are evolutionarily conserved and regulate gene expression by post-transcriptional gene repression. As a result of its high degree of complementarity, the binding of miRNA to its target mRNA can result in either cleavage of the target or in translation repression (1). Several miRNAs show a tissue-specific or developmental stage-specific expression pattern and have been reported to be associated with human diseases such as cancer, leukemia, and viral infections. In addition, a number of studies on osteoarthritis (OA) suggest that miRNA might play a role in OA pathogenesis (2).

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miRNAs play an important role in a variety of human diseases, including rheumatoid arthritis (RA) and OA. OA is a chronic degenerative joint disorder that is more pronounced in the articulating joints. OA is the most frequently encountered condition in rheumatology practice, and its prevalence is rising due to the extension of life span (3). It is characterized by destruction of the articular cartilage, limitation of movement, crepitus, a variable degree of inflammation without systemic effects, subchondral bone alterations, and synovitis. Currently available drugs that are used to treat OA are predominantly directed towards the symptomatic relief of pain and inflammation and do little to prevent the degeneration of the cartilage. Effective prevention of the structural damage must be a key objective of novel therapeutic approaches (4 – 6).

Some of the studies indicate that microRNA-25 (miR-25) is down-regulated by inflammatory stimuli, which in turn affects the expression of inflammatory mediators as well as extracellular matrix (ECM) and contractile proteins.

miR-25 has a major role in regulating airway smooth muscle function by modulating the expression of inflammatory mediators. Since miR-25 targets Krüppel-like factor 4 (KLF4), the inhibition of miR-25 in the cytokine-stimulated airway smooth muscle cells up-regulates KLF4 expression through a post-transcriptional mechanism (7). One of the studies demonstrated that miR-25 and miR-30d directly target the 3′UTR of tumor protein$_{53}$ (TP_{53}) to down-regulate p53 protein levels and reduce the expression of genes that are transcriptionally activated by p53 (8). However, little is known about the role of miR-25 in articular chondrocytes.

The role of miRNAs in maintaining cartilage homeostasis during development and their dysregulation in OA has been reported (6). Recent studies suggest that the expression of miR-146a is induced by IL-1β and is linked to the pain-related pathology of OA. The over-expression of miR-146a was found to be associated with up-regulation of aggrecan and COL2A1 expression in IL-1β–stimulated OA chondrocytes (9). Silencing of miR-34 reduced the IL-1β–induced apoptosis in rat knee chondrocytes (2). The expression of miR-140 was higher in the normal cartilage but lower in OA, and miR-140 knockout mice developed an OA-like pathology with age (10, 11).

Jones Watkins et al. investigated the expression profile of 157 human miRNAs isolated from OA and normal cartilage and identified 17 miRNAs that showed differential expression. Among the 17 miRNAs, we selected highly expressed in normal tissues of the two miRNAs and in the arthritis of the two miRNAs. The expression of miR-149 and miR-130b was more elevated in normal tissue than in OA tissue. In contrast, the expression of miR-200a and miR-25 was more elevated in OA tissue than in normal tissue (12).

Cyclooxygenases (COXs) are rate-limiting enzymes for the generation of prostaglandin metabolites and their expression has been linked to inflammatory diseases, including RA and OA (13 – 16).

The COX isoforms, COX-1 and COX-2, encoded by distinct genes have been identified in eukaryotic cells. COX-1 is constitutively expressed in most cell types and facilitates the maintenance of physiological function. In contrast, COX-2 is generally an inducible enzyme that is activated by pro-inflammatory cytokines, tumor promoters, oncogenes, and growth factors; and it is involved mainly in the modulation of inflammatory responses in many types of cells, such as monocytes, fibroblasts, and endothelial cells. COX activity and expression is regulated at the levels of transcription, post-transcription, and translation during inflammation. COX-2 transcription is induced by a variety of exogenous stimuli that modulate the intracellular signaling pathway, which in turn modulates the activity of transcription factors. Post-transcriptional stabilization and nuclear export of COX-2 mRNA are necessary processes for maximal induction of COX-2 (17).

Withaferin A (WFA), a natural compound isolated from the medicinal plant *Withania somnifera*, has been safely used for centuries in Indian Ayurvedic medicine for its anti-inflammatory effects. It has been reported to possess various medicinal properties, including anti-tumor, anti-inflammatory, and immunomodulatory activities (18). Several studies have reported its anti-tumor effects, including the inhibition of cell growth, angiogenesis, and metastasis (19). Recent studies have reported that WFA induces anti-proliferative effects by inducing G2/M phase cell cycle arrest (20), inhibits cell adhesion molecules by inactivating Akt and NF-κB (21), inhibits colon cancer survival by down-regulating Notch, and induces apoptosis by generating reactive oxygen species (ROS) (22). In addition, WFA inhibits cell migration/invasion via the down-regulation of STAT3 activity and induces apoptosis in human breast cancer cells (18).

However, the molecular mechanisms by which WFA induces the inflammatory response in chondrocytes have not been fully understood. In previous studies, we found that the WFA-induced increase in the expression of COX-2 was strongly related to the inflammation and cartilage degeneration in chondrocytes (23). In addition, we investigated the effects of WFA on inflammation in rabbit articular chondrocytes with specific focus on the regulatory role of miRNA. The findings of this study indicate that miR-25 expression in the presence of WFA induces the expression of COX-2, suggesting that miR-25 affects the WFA-induced expression of COX-2. Our results collectively indicate that miR-25 influences COX-2 expression and highlights its utility as a potential target for OA treatment.

Materials and Methods

Culture of primary chondrocytes

Rabbit articular chondrocytes from joint cartilage slices of 2-week-old New Zealand white rabbits (KOATECH, Pyeongtaek-si, Kyunggi-do, South Korea) were dissociated for 6 h in 0.2% type II collagenase (381 units/mL; Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA). After collecting individual cells by brief centrifugation at 230 × g for 10 min at 20°C, the cells were suspended in DMEM supplemented with 10% (v/v) bovine calf serum (Invitrogen), 50 μg/mL streptomycin (Sigma), and 50 units/mL penicillin (Sigma). The cells were then plated on culture dishes at a density of 5×10^4 cells/cm^2. After 3 days in culture, the medium was

replaced with a glucose-free medium, and cells were treated with the pharmacological reagents. This study was approved by the ethics committee of Kongju National University, Gongju, Republic of Korea.

Transfection of microRNAs and treatment of chondrocytes

Chondrocytes were transfected with miR-25 mimics (5′-CAUUGCACUUGUCUCGGUCUGA-3′) for 6 h and miR-25 inhibitors (5′-UCAGACCGAGACAAGUG CAAUG-3′) for 1.5 h (Genolution, Seoul, South Korea). The miR-25 mimics and miR-25 inhibitors in 100 μL of serum-free antibiotic-free medium were mixed with 2 μL of Turbofect transfection reagent (Fermentas, Glen Burnie, MD, USA) and allowed to stand at room temperature for 20 min. The resultant 100-μL transfection solution was then added to wells containing 1 mL of medium. After 6 h or 1.5 h, the cultures were replaced with 1 mL fresh medium supplemented with 10% (v/v) bovine calf serum, 50 μg/mL streptomycin, and 50 units/mL penicillin. After transfection, the cells were treated with WFA (Calbiochem, San Diego, CA, USA or Enzo Life Sciences, Farmingdale, NY, USA).

Western blot analysis

Whole cell lysates were prepared by extracting proteins using a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS supplemented with protease inhibitors and phosphatase inhibitors. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% non-fat dry milk in Tris-buffered saline and incubated with the following primary antibodies: anti-COX-2 (Cayman Chemical, Ann Arbor, MI, USA) and anti-actin (Santa Cruz Biotechnologies, San Francisco, CA, USA). Blots were developed using a peroxidase-conjugated secondary antibody and a chemiluminescence system.

Immunofluorescence staining

Cells were fixed in PBS containing 3.5% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100. The fixed cells were washed three times with PBS and incubated for 2 h with antibodies against COX-2 (Cayman). The cells were washed and incubated with secondary antibodies for 1 h and washed with PBS. The cells were observed under a fluorescence microscope and images were obtained.

Quantitative real-time RT-PCR

Total RNA was isolated from chondrocytes using TRIZOL reagent (Invitrogen) following the manufacturer's protocol. For cDNA synthesis, total RNA was reverse-transcribed using the Mir-X miRNA First-Strand Synthesis Kit (Clontech, Mountain View, CA, USA) or RevoScript RT PreMix (INtRON Biotechnology, Seongnam-si, Kyunggi-do, South Korea) following the manufacturer's protocol. MiR-25 reverse transcription (RT) was performed in a 10-μL mixture containing 5 μL of mRQ Buffer (2 ×), 3.75 μL of template RNA (0.25 – 8 μg), and 1.25 μL of mRQ Enzyme at 37°C for 1 h and at 85°C for 5 min, and then the cDNA product was maintained at 4°C. COX-2 gene reverse transcription (RT) was performed in a 20-μL mixture containing 1 μg of total RNA and RNase-free water. Quantitative real-time RT-PCR analysis was performed using a SYBR Green assay using the Eco real time PCR system (Illumina, San Diego, CA, USA). The miR-25 reaction mixture contained 10 μL of SYBR Green (Philekorea Technology, Daejeon, South Korea), 1 μL of miR-25 primer (Qiagen, Valencia, CA, USA; MS00003227), 0.5 μL mRQ 3′ primer (containing Mir-X miRNA First-Strand Synthesis Kit), and 1 μg of cDNA template, and this mixture was made up to a final volume of 20 μL with distilled water. The reactions were performed with an initial activation at 95°C 10 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 20 s. The COX-2 gene reaction mixture contained 10 μL of SYBR Green, 1 μL of forward primer (5′-CAAACTGCTCCT GAAACCCACTC-3′), 1 μL of reverse primer (5′-GCT ATTGACGATGTTCCAGACTCC-3′), and 3 μg of cDNA template, and this mixture was made up to a final volume of 20 μL with distilled water. The reactions were performed with an initial activation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 15 s. All reactions were performed in triplicate. The levels of gene expression were calculated by relative quantification using GAPDH (Forward: 5′-TCACCATCTTCCAGGAGCGA-3′, Reverse: 5′-CACAATGCCGAAGTGGTCGT-3′) or U6 snRNA (containing Mir-X miRNA First-Strand Synthesis Kit) as the endogenous housekeeping genes. The threshold cycle (C_t) values were obtained in the exponential phases of amplification, and the relative expression levels were quantified by the $\Delta\Delta$Ct method. The value of each control sample was set at 1 to calculate the fold change in mRNA/miRNA expression.

Data analyses and statistics

The results are expressed as mean values with standard deviation. Values were calculated from the specified number of determinations. The data were subjected to an analysis of variance (ANOVA) using Tukey's test to analyze differences. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

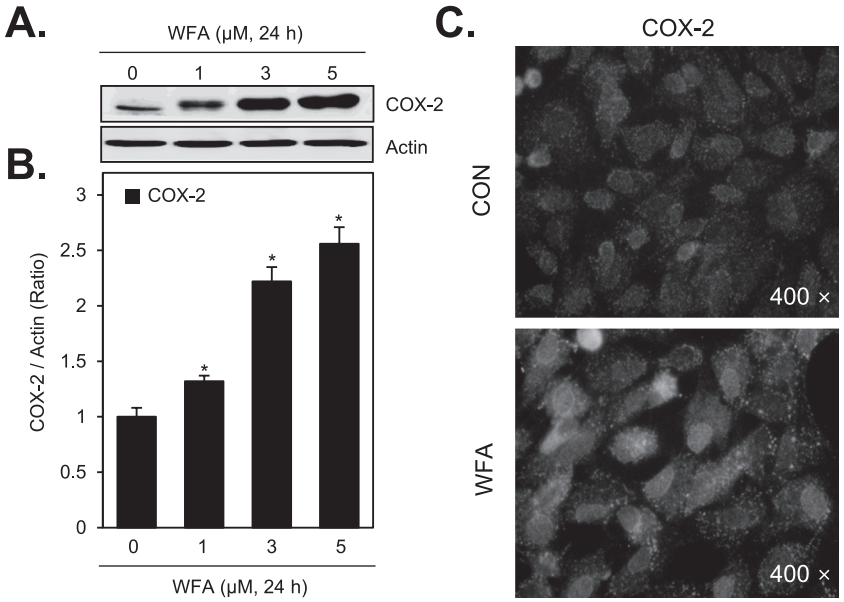

Fig. 1. WFA induces expression of COX-2 in rabbit articular chondrocytes. A) Chondrocytes were treated with the indicated concentrations of WFA for 24 h. COX-2 expression was detected using western blot analysis. Actin was used as the loading control. B) The relative amounts of COX-2 were quantified by densitometric measurement (ImageJ). C) Chondrocytes were treated with or without 3 μM WFA for 24 h. Distribution of COX-2 was detected by immunofluorescence. The data represent the results of a typical experiment. *$P < 0.05$, compared to the control.

Results

WFA induces expression of COX-2 in rabbit articular chondrocytes

We investigated whether WFA regulates the expression of COX-2 in rabbit articular chondrocytes. In previous studies, we found that WFA induced COX-2 expression from 3 h to 24 h. Therefore we treated the cells with WFA for 24 h (23). Cells were treated with various concentrations of WFA (Fig. 1: A and B) or with WFA at 3 μM for 24 h (Fig. 1C). WFA dramatically increased COX-2 expression in a dose-dependent manner, as determined by western blot analysis (Fig. 1: A and B). Our previous data showed that treatment with WFA markedly increased PGE_2 in chondrocytes (23). We used actin as a loading control. However, treatment with more than 5 μM WFA was cytotoxic to chondrocytes. In further studies, we decided to treat the cells with 3 μM WFA. Consistent with the western blot analysis data, immunofluorescence staining indicated that WFA induced COX-2 expression (Fig. 1C).

WFA increases expression of miR-25 more than any other OA-related miRNAs

Our results indicated that WFA increased the expression of COX-2, which has been previously reported to be a direct target of various miRNAs, including miR-143, miR-16, miR-199a*, miR-101, and miR-146a (6, 14, 24 – 26). Therefore, we further investigated whether WFA treatment influences the impact of miRNA on COX-2 expression. As shown in the introduction, the 4 selected miRNAs (miR-200a, miR-25, miR-149, and miR-130b) have been subjected to intense scrutiny. Our results suggest that treatment with WFA up-regulated the expression of 4 miRNAs, as demonstrated by real-time PCR (Fig. 2). Since the expression of miR-25 was more significantly up-regulated than 3 miRNAs, we further investigated the role of this miRNA.

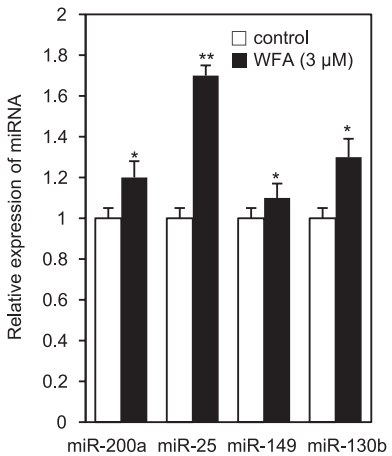

Fig. 2. WFA increases expression of miR-25 more than any other OA-related miRNAs. Chondrocytes were treated with or without 3 μM WFA for 24 h. Expression levels of miR-25 was determined by real-time PCR. Expression of U6 snRNA was used as an endogenous control. The data are the typical results from 4 independent experiments with similar results. *$P < 0.05$, **$P < 0.01$, compared to the control.

miR-25 facilitates the WFA-mediated expression of COX-2

Our results suggest that the impact of WFA on miR-25 expression is more pronounced than its effect on other OA-related miRNAs. To determine whether the WFA-

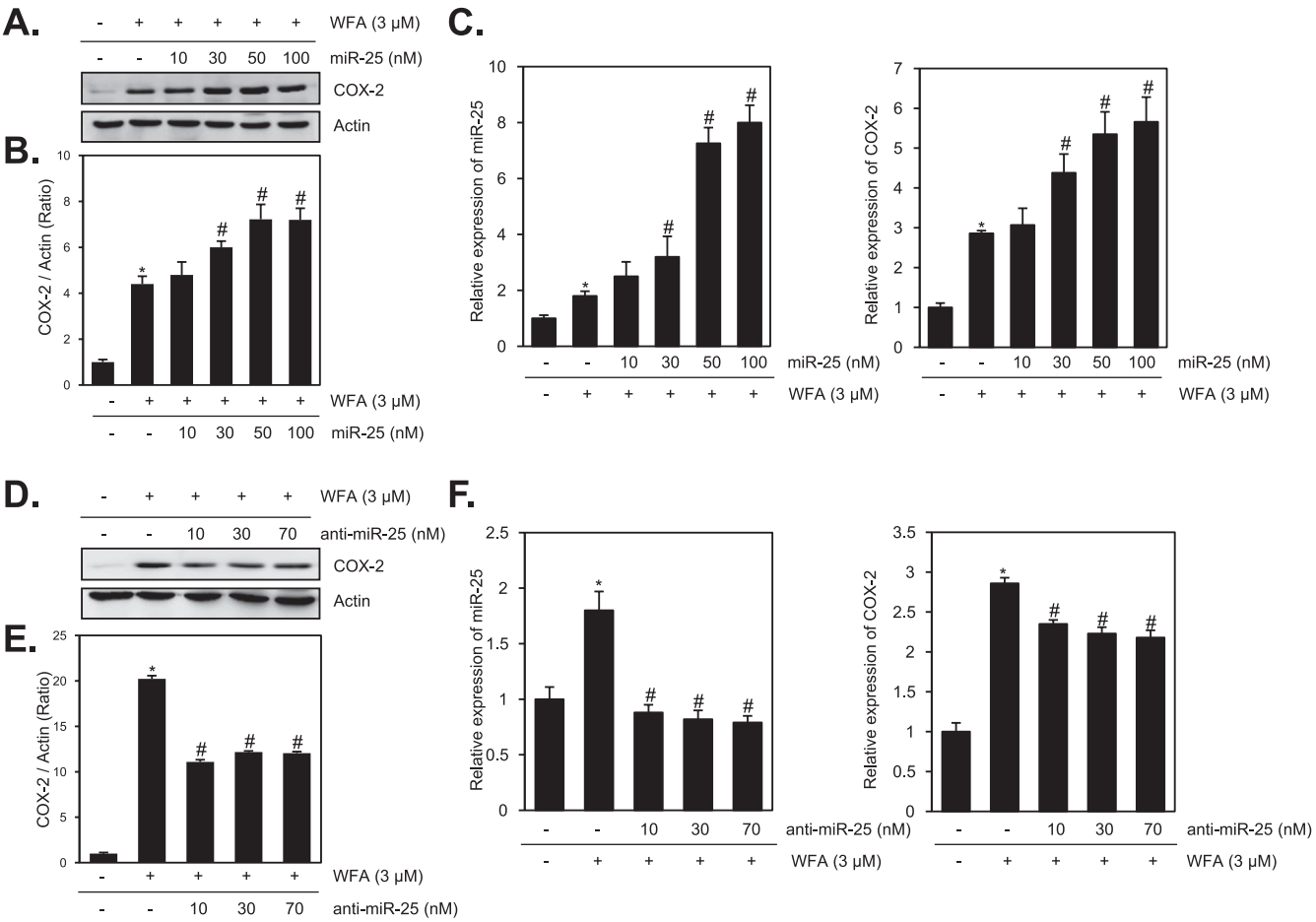

Fig. 3. miR-25 facilitates the WFA-mediated expression of COX-2. A – C) Chondrocytes were transfected with miR-25 mimics for 6 h and then treated with or without 3 μM WFA for 24 h. D – F) Chondrocytes were transfected with miR-25 inhibitor for 1.5 h and then treated with or without 3 μM WFA for 24 h. A, D) Expression of COX-2 was detected using western blot analysis. Actin was used as the loading control. B, E) The relative amounts of COX-2 were quantified by densitometric measurement (ImageJ). C, F) Levels of miR-25 and COX-2 were determined by real-time PCR. U6 snRNA was used as an endogenous control. The data are the typical results from 4 independent experiments with similar results. $^{*}P < 0.05$, compared to the control; $^{\#}P < 0.05$, compared to the treatment with WFA.

induced expression of COX-2 is influenced by miR-25 in the chondrocytes, expression of miR-25 and COX-2 were analyzed by real-time PCR and western blot analysis. For these studies, cells were transiently transfected with miR-25 or anti-miR-25 and were then treated with or without WFA. The overexpression of miR-25 dramatically enhanced the expression of COX-2 in a dose-dependent manner (Fig. 3: A and B). Consistent with the western blot data, real-time PCR showed that the overexpression of miR-25 led to a dramatic increase in the WFA-induced increase in COX-2 and miR-25 expression (Fig. 3C). On the other hand, repression of miR-25 reduced the expression of COX-2 (Fig. 3: D and E). Consistent with the western blot data, real-time PCR showed that the ectopic expression of anti-miR-25 decreased the expression of COX-2 and mir-25 (Fig. 3F). These results demonstrate that WFA up-regulated the expression of miR-25, and miR-25 influences the WFA-induced COX-2 expressions.

Repression of miR-25 inhibits the COX-2 expression

Our findings suggest that miR-25 expression is correlated with COX-2 expression in chondrocytes. We investigated whether inhibition of miR-25 interferes with WFA-induced expression of COX-2. The expression of COX-2 was potentiated by the overexpression of miR-25 in the presence of WFA compared to treatment with WFA alone, and was reduced by the repression of miR-25 in the presence of WFA, as detected by western blot analysis (Fig. 4: A and B). Although the expression of miR-25 was increased by WFA treatment, anti-miR-25 and WFA co-treatment decreased the expression of

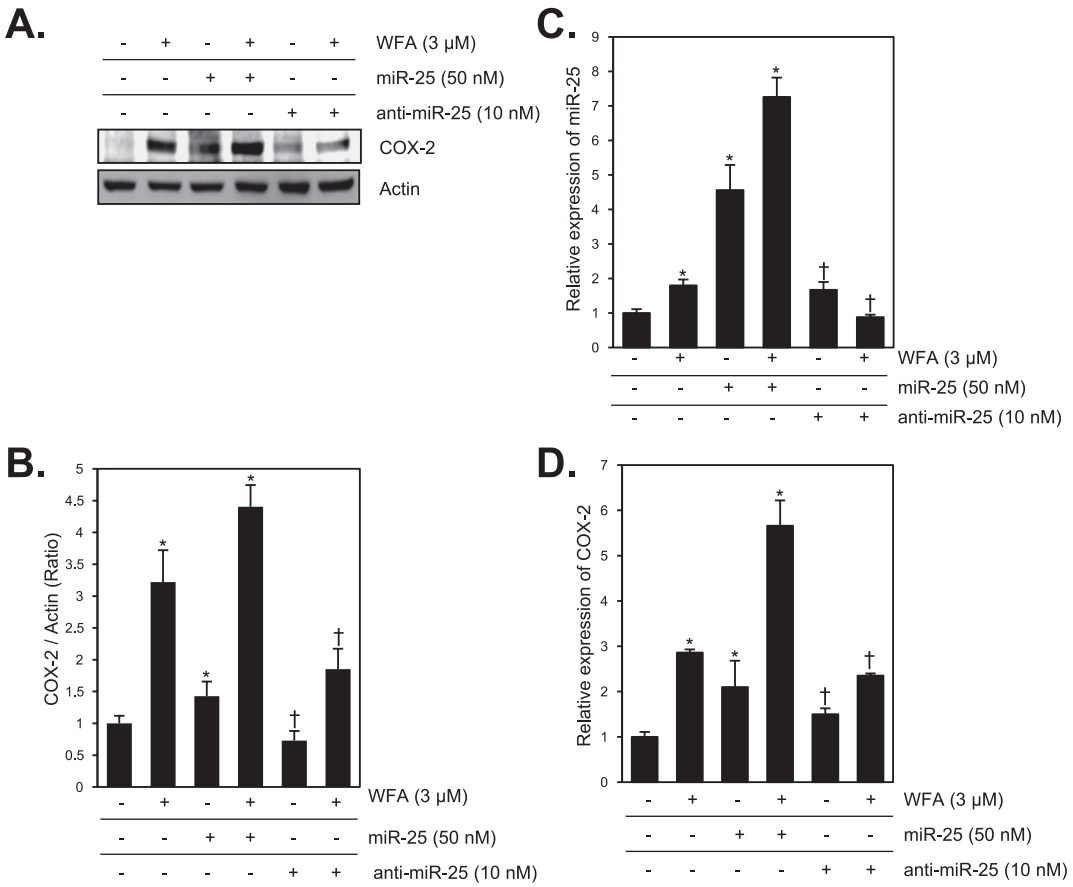

Fig. 4. Repression of miR-25 inhibits the COX-2 expression. A) Primary chondrocytes were transfected with miR-25 mimics for 6 h or miR-25 inhibitors for 1.5 h. The chondrocytes were then treated with or without 3 μM WFA for 24 h. COX-2 expression was detected using western blot analysis. Actin was used as the loading control. B) The relative amounts of COX-2 were quantified by densitometric measurement (ImageJ). C, D) miR-25 and COX-2 levels were measured by real-time PCR. U6 snRNA was used as a housekeeping gene. The data represent the results of the typical experiments. *$P < 0.05$, compared to the control; $^{\dagger}P < 0.05$, compared to the co-treatment with WFA and miR-25.

miR-25, as indicated by real-time PCR (Fig. 4C). The COX-2 levels, quantified by real-time PCR, were consistent with that observed during the western blot analysis (Fig. 4D). Here, we found that anti-miR-25 interfered with the function of miR-25.

Discussion

In this study, we identified a correlation between miRNA and WFA-induced COX-2 expression in articular chondrocytes. miR-25 is known to be associated with the inflammatory response, and co-treatment with WFA accelerated the WFA-induced expression of COX-2. Hence, our findings suggest that miR-25 influences the expression of COX-2.

In the previous studies, treatment of WFA inhibited inflammatory cytokine secretion in human and mouse islets (27). Also, WFA inhibited inflammation via inhibition of NO production and iNOS expression by blocking Akt and subsequently down-regulating NF-κB activity in RAW 264.7, a mouse leukemic monocyte macrophage cell line (28). In other research, WFA inhibited LPS-induced PGE_2 production and COX-2 expression by blocking STAT1 and STAT3 activation in microglial cells (18). In most studies, WFA induced anti-inflammatory effects in various cell types. However, our results suggested that WFA induced inflammation via increase of COX-2 expression in chondrocytes. Our group investigated the properties of WFA on inflammatory effects with treatment at low and high dose. Treatment with low doses (0.1, 0.3, 0.5 μM) inhibited IL-1β–induced COX-2 expression and PGE_2 synthesis but treatment with high doses (1, 3, 5 μM) induced expression of COX-2 in chondrocytes (23). Therefore, our results also induced COX-2 expression by treatment with high doses of WFA in chondrocytes.

In other studies, miR-25 is decreased in human colon cancer tissues. Functional studies reveal that reinstatement of miR-25 expression inhibited cell proliferation and migration. Moreover, miR-25 inhibition could promote these effects. They also identified Smad7 as a direct target of miR-25. These results suggest that miR-25 may function as a tumor suppressor by targeting Smad7 in colon cancer (29). MiR-25 was up-regulated in 60 esophageal squamous cell carcinoma (ESCC) tissues compared with matched adjacent non-cancer tissues. Moreover, up-regulation of miR-25 is significantly correlated with the status of lymph node metastasis and the Tumor, Node, and Metastasis (TNM) stage. Furthermore, over-expression of miR-25 markedly promoted migration and invasion of ESCC cells. In addition, miR-25 directly targeted E-cadherin 3′-untranslated region (3′UTR) and repressed the expression of E-cadherin (30).

miRNAs have been extensively studied in chondrocytes. However, the effect of miR-25 on the articular chondrocyte has not been reported. In this study, we have, for the first time, revealed a role for miR-25 in the physiology of rabbit articular chondrocytes. Based on publicly available algorithms (PicTar, TargetScan, miRBase, miRNA.org, and Patrocles), several miRNAs have been predicted to target multiple mRNA transcripts associated with COX-2. However, COX-2 was not listed as a target gene of miR-25 in these algorithms. Nevertheless, we identified that miR-25 affects the expression of COX-2 in rabbit articular chondrocytes. This could be an indirect effect of miR-25 on COX-2 expression in chondrocytes. In a previous study, they show that miR-29 suppresses DNMT activity and thus induces expression of COX-2 and PGE_2. These findings reveal a novel proinflammatory cascade in the control of influenza A virus infection (31).

Similarly, we identify that miR-25 in the presence of WFA inhibits the expression of specific target genes and induces the expression of COX-2 in articular chondrocytes. Further research is needed to find the direct mRNA target of miR-25 and clarify the miR-25 signaling cascade involved in the WFA-induced inflammatory response.

In conclusion, we have shown that the expression of miR-25 was increased by WFA, and miR-25 in the presence of WFA increased the WFA-induced expression of COX-2. This demonstrated that miR-25 influences the WFA-induced inflammatory response. Our results provide a novel insight into how miR-25 works in chondrocytes and provide clues for the development of novel therapeutic strategies for OA.

Acknowledgments

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Conflicts of Interest

The authors declare that they have no competing interests.

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