Arctigenin inhibits apoptosis, extracellular matrix degradation, and inflammation in human nucleus pulposus cells by up-regulating miR-483-3p

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Abstract
Background: Arctigenin (ATG) is the active ingredient of the Chinese herbal medicine Arctium lappa, with anti-inflammatory and antioxidant effects. Excessive inflammation and cell apoptosis are important causes of intervertebral disc degeneration (IDD). Hence, this study probed into the possible role of ATG in IDD.

Methods: Interleukin (IL)-1β (10 ng/ml) was adopted to induce human nucleus pulposus cells (HNPCs) as a cell model for IDD. The effects of different concentrations of ATG (0, 2, 5, 10, 20, 50 μmol/L) on the viability of HNPCs and effects of ATG (10, 50 μmol/L) on the viability of IL-1β-induced HNPCs were detected by cell counting kit-8 (CCK-8). After IL-1β-induced HNPCs were transfected with miR-483-3p inhibitor and/or treated with ATG, cell viability and apoptosis were determined by CCK-8 and flow cytometry; the expressions of miR-483-3p, extracellular matrix (ECM)-related genes, and inflammation-related genes were measured by quantitative real time polymerase chain reaction (qRT-PCR), and expressions of ECM/apoptosis/NF-κB pathway-related proteins were quantified by Western blot.

Results: ATG had no significant effect on the viability of HNPCs but could promote the viability of IL-1β-induced HNPCs. ATG inhibited apoptosis, ECM degradation, inflammation, and activation of NF-κB pathway in HNPCs induced by IL-1β, but promoted the expression of miR-483-3p. MiR-483-3p inhibitor reversed the above-mentioned regulatory effects of ATG.

Conclusion: Arctigenin suppresses apoptosis, ECM degradation, inflammation, and NF-κB pathway activation in HNPCs by up-regulating miR-483-3p.

KEYWORDS
arctigenin, human nucleus pulposus cells, intervertebral disc mutation, miR-483-3p

1 | INTRODUCTION

Intervertebral disc degeneration (IDD) is the basic pathological change that leads to the development of a series of spinal degenerative diseases. These diseases cover many common and frequently occurring diseases in the field of spine orthopedics, such as lumbar disc herniation, lumbar spinal stenosis, and cervical spondylolisthesis. IDD is interpreted as the result of diverse factors in vivo and in vitro,
featured by the reduction of the number of nucleus pulposus cells (NPCs), decomposition in extracellular matrix, and up-regulation of various inflammatory factors in the intervertebral disc. \(^5\) Currently, IDD is treated mainly depending on conservative and surgical therapies; however, the therapeutic efficacy of the two is limited and even the high risk of recurrence would be caused by the surgical therapy. \(^5\) Therefore, the treatment methods for IDD need to be refined.

Traditional Chinese medicine has a long history in the prevention and treatment of IDD due to its small side effects, many targets, and wide effects. \(^6\) *Arctium lappa* is a medicinal and edible plant in China, possessing anti-tumor, anti-inflammatory, immune-regulating, anti-oxidative and hypolipidemic effects. \(^7\) Arctigenin (ATG) is the main active ingredient of *A. lappa* and has a strong anti-inflammatory activity against many acute and chronic inflammations. \(^8\) Studies in the past decade have revealed that the powerful anti-inflammatory effect of ATG is attributable to its regulation on certain important inflammatory factors, including interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-\(\alpha\)), and IL-1\(\beta\). \(^9,10\) Moreover, it has also been reported that ATG can prevent the development of degenerative arthritis by modulating the NF-\(\kappa\)B pathway, a classic inflammation signaling pathway. \(^11\) In addition to its powerful anti-inflammatory effect, ATG has also been proven by Tang et al. to prevent the degradation of extracellular matrix (ECM). \(^11\) These evidences indicated that ATG is likely to be a potential treatment for IDD.

Some studies believed that the anti-tumor and anti-inflammatory effects of ATG may be achieved by regulating microRNAs (miRNAs). \(^12,13\) MiRNA is widely present in eukaryotic organisms and mainly plays a role in post-transcriptional regulation of genes. \(^14\) Wang and his colleagues used bioinformatics analysis to find 40 miRNAs that are clearly dysregulated in IDD, and these miRNAs are likely to be vital targets for the treatment of IDD. \(^15\) For instance, Ji et al. have demonstrated that miR-141 promotes the progression of IDD by inducing apoptosis in NPCs. \(^16\)

Sherafatian et al. found that miR-483 expression is usually down-regulated in IDD. \(^17\) Our preliminary experiments identified that ATG has a regulatory effect on the expression of miR-483-3p. Combined the above findings together, it is worthy of fathoming whether ATG regulates the development of IDD through miR-483-3p, and the corresponding mechanism will be expounded in this study.

### 2 | MATERIALS AND METHODS

#### 2.1 | Cell culture and treatment

Human NPCs (HNPCs, CP-H097; Procell) were grown in HNPCs Complete Medium (CM-H097; Procell) at 37°C with 5% CO\(_2\) in an incubator (Forma Steri-Cycle, Thermo Scientific).

ATG (IA0450; Solarbio) was dissolved in dimethyl sulfoxide (DMSO, D8371; Solarbio). In order to evaluate the effect of ATG on HNPCs, different concentrations of ATG (2, 5, 10, 20, and 50 \(\mu\)mol/L) were used to treat HNPCs for 24 h or 48 h. Then, the viability of HNPCs was detected.

To assess the effect of ATG on IL-1\(\beta\) (12,393; Merck)-induced HNPCs, HNPCs were exposed to 10 ng/ml IL-1\(\beta\) (refer to previous research) \(^18\) with or without ATG (10 or 50 \(\mu\)mol/L) for 24 h. Finally, changes in cell biological behaviors were observed.

For determining whether ATG exerts effects through miR-483-3p, HNPCs were transfected with miR-483-3p inhibitor (I) or inhibitor control \(^19\) and treated with IL-1\(\beta\) and/or ATG for 24 h to observe the changes in cell biological behaviors.

#### 2.2 | Cell counting kit-8 (CCK-8)

The cell viability test was carried out with the help of CCK-8 kit (E-CK-A362; Elabscience). Cells (5000 cells/well) were seeded in a 96-well plate and incubated in a cell culture incubator for 24 h. After drug stimulation according to different experimental requirements, 10 \(\mu\)l CCK-8 solution was added to each well for continuously 2 h of incubation. The absorbance at 450 nm on the microplate reader (SpectraMax5; Molecular Devices) was recorded to calculate the relative viability of the cells.

#### 2.3 | Cell apoptosis

Annexin V-FITC/PI Apoptosis Detection Kit (E-CK-A211; Elabscience) was exploited to detect cell apoptosis. After being induced to undergo apoptosis according to the experimental protocol, the cells were collected by centrifugation at 300g for 5 min. 5 \(\times\) 10\(^4\) cells were resuspended in 500 \(\mu\)l Binding buffer and then reacted with 5 \(\mu\)l Annexin V-FITC and 5 \(\mu\)l PI staining solution for 15 min. Finally, 400 \(\mu\)l Binding buffer was mixed with the stained sample. The results were observed by a flow cytometer (DxFLEX; Beckman).

#### 2.4 | Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA or miRNA from cells was extracted by Total RNA Purification Kit (17,200; NORGEX) or microRNA Purification Kit (21,300; NORGEX). The reverse transcription and qPCR process were completed in one step with the assistance of the TaqMan One Step RT-qPCR Kit (T2210; Solarbio) or All-in-One miRNA qRT-PCR Detection Kit 2.0 (OP115; GeneCopoeia). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6 was employed as the reference gene as needed. The data were shown in real-time PCR Detection system (CFX96; Bio-rad). The primers are listed in Table 1. The relative expression was standardized using \(2^{-\Delta\Delta Cq}\) method. \(^20\)
TABLE 1

| Name        | Primer sequences (5′-3′)                      |
|-------------|-----------------------------------------------|
| MMP3        | Forward: CTCCTATGAGCTTCACCAAGAATC              |
| MMP3        | Reverse: GTGCTGACTGCACTGAAGGACAAA             |
| MMP13       | Forward: CTGGCCCTGCTGCTGCTATGCC               |
| MMP13       | Reverse: CCTCAGAAAGAGAGCATCGATAG              |
| COL2A1      | Forward: CAGGAATTGTGTTGGACATAGGG              |
| COL2A1      | Reverse: TGGCCTGAGCTGACTGAAGGACAAA           |
| Aggrecan    | Forward: CAGATACCTGCACAGACACCCAA              |
| IL-6        | Forward: GACAGCCACTACACCTCTTCA                |
| IL-6        | Reverse: TTTACAGGGAGTTCTCTCTTC                |
| TNF-α       | Forward: GTCAGATCATCTTCTCGACC                 |
| TNF-α       | Reverse: CAGATAGATGGGCGCTATACC                |
| COX-2       | Forward: GCAAAATCTGGTCTGTTCC                  |
| COX-2       | Reverse: GGAGGAAAGGCTCTAGTA                   |
| iNOS        | Forward: CTTACGAGGCAGAAGGAGGACAG              |
| iNOS        | Reverse: CAGTTTGAGAGAGGAGCTCCCG               |
| GAPDH       | Forward: CAATGACCCCTCTATTGACC                 |
| GAPDH       | Reverse: TTGATTTTGAGGAGCTCTCG                 |
| hsa-miR-483-3p | Forward: ATCACTCTCTCTCCCCCGTC               |
| hsa-miR-483-3p | Reverse: TATGGTTTCTCTGCTCTTCTTTC            |
| U6          | Forward: CTCGCTTCGGCAGCACA                    |
| U6          | Reverse: AGCGTCTGAGGCTCGGT                    |

2.5 Western blot

Western blot was performed as previously described.19 The protein was extracted from HNPCs by protein extraction kit (BC3790; Solarbio). After the protein was quantified with the BCA protein assay kit (PC0020; Solarbio), the protein was sequentially subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransfer process. After being blocked by 5% bovine serum albumin (BSA) for 1 h, the membrane was reacted with primary antibodies at 4°C overnight. The primary antibodies used were those against IκBa (1:1000, 39 kDa, #9242, Cell Signaling Technology), β-actin (1:1000, 42 kDa, ab8226, Abcam), p65 (1:2000, 64 kDa, ab16502, Abcam), Lamin B (1:10000, 68 kDa, ab16048, Abcam), matrix metalloproteinase 3 (MMP3, 1:1000, 50 kDa, ab52915, Abcam), MMP13 (1:1000, 54 kDa, ab51072, Abcam), collagen type II alpha1 (COL2A1, 1:1000, 141 kDa, ab188570, Abcam), Aggrecan (1:1000, 250 kDa, ab3778, Abcam), GAPDH (1:1000, 36 kDa, ab8245, Abcam), Bcl-2 (1:1000, 26 kDa, ab59348, Abcam), Bax (1:1000, 21 kDa, ab32503, Abcam), and cleaved caspase-3 (1:500, 17 kDa, ab2302, Abcam). GAPDH, β-actin, or Lamin B was served as the internal control. Then, the membrane was cultured with a secondary antibody, goat anti-rabbit (1:5000, ab6721, Abcam) or goat anti-mouse (1:5000, ab6788, Abcam) antibody, for 2 h. The protein bands were visualized by iBright CL750 (Thermo Fisher) with an ECL Western Blotting Substrate (ab65623, Abcam). The relative expressions of proteins were quantitated by ImageJ2x (Rawak Software).

2.6 Cell transfection

MiR-483-3p inhibitor (miR200002173-1-5) and inhibitor control (miR20000002-1-5) were purchased from Ribobio (https://www.ribobio.com). The transfection of miR-483-3p inhibitor or inhibitor control was accomplished with the help of lipofectamine 3000 (L3000-015, Thermo Fisher). Post 24-h transfection, the result of transfection was evaluated by qRT-PCR.

2.7 Statistical analysis

Data were analyzed by GraphPad Prism v8.0 (Graphpad Software) and represented as mean ± standard deviation. Differences among multiple groups were analyzed by one-way analysis of variance. p < 0.05 was accepted to be statistically significant.

3 RESULTS

3.1 ATG regulated cell viability, apoptosis, and expressions of ECM degradation-related genes and inflammatory factors, and NF-κB pathway in IL-1β-induced HNPCs

The chemical structure of ATG is depicted in Figure 1A. We evaluated the effects of different concentrations of ATG (0, 2, 5, 10, 20, and 50 μmol/L) on the viability of HNPCs after 24- and 48-h treatments. The results indicated that regardless of 24-h or 48-h treatment, different concentrations of ATG generated no obvious inhibitory effect on normal HNPCs (Figure 1B). In order to accurately evaluate the effect of ATG on IL-1β-induced HNPCs, we chose the two concentrations, 10 and 50 μmol/L, for follow-up experiments. After HNPCs were processed by a pro-inflammatory factor, IL-1β, the viability of HNPCs was dampened and apoptosis was facilitated (Figure 1C,D, p < 0.001). However, ATG attenuated the regulation of IL-1β on the viability and apoptosis of HNPCs in a concentration-dependent manner (Figure 1C,D, p < 0.05). Not only that, compared with the control group, the mRNA levels of MMP3, MMP13, IL-6, TNF-α, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) in the IL-1β group were notably elevated, while the mRNA levels of COL2A1 and Aggrecan were sharply lessened (Figure 1E,F, p < 0.001). Similarly, ATG neutralized the regulation of IL-1β on ECM degradation-related genes and inflammatory factors in a concentration-dependent manner (Figure 1E,F, p < 0.001). IL-1β treatment resulted in down-regulation of IκBa in the cytoplasm of HNPCs and up-regulation of p65 in the nucleus, but the two trends were also offset by ATG (Figure 2, p < 0.001).
FIGURE 1 Effects of ATG on the viability, apoptosis, and expressions of ECM-related genes and inflammatory factors in IL-1β-induced HNPCs. (A) The chemical structural formula of ATG. (B) After HNPCs were treated with different concentrations of ATG (0, 2, 5, 10, 20, and 50 μmol/L) for 24 h or 48 h, cell viability was detected by CCK-8. (C) The viability of HNPCs treated with 10 ng/ml IL-1β and/or ATG (10, 50 μmol/L) for 24 h was detected by CCK-8. (D) The apoptosis of HNPCs treated with 10 ng/ml IL-1β and/or ATG (10, 50 μmol/L) for 24 h was detected by flow cytometry. (E,F) The mRNA levels of ECM-related genes (MMP3, MMP13, COL2A1, and Aggrecan) and inflammatory factors (IL-6, TNF-α, COX-2, and iNOS) in HNPCs treated with 10 ng/ml IL-1β and/or ATG (10, 50 μmol/L) for 24 h were detected by qRT-PCR. GAPDH was used as the internal control. Quantified values were described as mean ± standard deviation of at least three independent experiments. ***p < 0.001 vs. control group. ^p < 0.05, ^^p < 0.001 vs. IL-1β group. ATG, arctigenin; CCK-8, cell counting kit 8; COL2A1, collagen type II alpha 1; COX-2, cyclooxygenase-2; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNPCs, human nucleus pulposus cells; IL, interleukin; iNOS, inducible nitric oxide synthase; MMP, matrix metalloproteinase; qRT-PCR, quantitative real time polymerase chain reaction; TNF, tumor necrosis factor.

FIGURE 2 ATG regulated NF-κB pathway in IL-1β-induced HNPCs. The protein levels of IκBα and p65 in HNPCs treated with 10 ng/ml IL-1β and/or ATG (10, 50 μmol/L) for 24 h were detected by Western blot. β-Actin and Lamin B were used as internal controls. Quantified values were described as mean ± standard deviation of at least three independent experiments. ***p < 0.001 vs. control group. ^p < 0.05, ^^p < 0.001 vs. IL-1β group. ATG, arctigenin; HNPCs, human nucleus pulposus cells; IL, interleukin.
3.2 | MiR-483-3p inhibitor offset the impacts of ATG on the viability and apoptosis of IL-1β-induced HNPCs

We unveiled that IL-1β can induce HNPCs to express less miR-483-3p, while ATG augmented the expression level of miR-483-3p in HNPCs induced by IL-1β (Figure 3A, p < 0.001). This meant that the regulation of ATG on IL-1β-induced HNPCs may be related to miR-483-3p. Therefore, we transfected HNPCs with miR-483-3p inhibitor. 50 μmol/L ATG worked more obviously, so this concentration was selected for subsequent experiments. Figure 3B presented that after the transfection of miR-483-3p inhibitor, the ability of ATG to increase the level of miR-483-3p in IL-1β-induced HNPCs was partially inhibited (p < 0.001). In addition, miR-483-3p inhibitor also weakened the impacts of ATG on IL-1β-induced HNPC viability (Figure 3C, p < 0.001) and apoptosis (Figure 3D, p < 0.001).

3.3 | MiR-483-3p inhibitor reversed the regulation of ATG on expressions of ECM degradation/apoptosis/inflammation-related genes and activation of NF-κB pathway

From a molecular point of view, ATG could effectively inhibit expressions of ECM degradation-related enzymes (MMP-3, MMP-13) (Figure 3E,F, p < 0.001), apoptosis-related proteins (Bax, cleaved caspase-3) (Figure 3G, p < 0.05) and inflammatory factors (IL-6, TNF-α, COX-2, and iNOS) (Figure 4A, p < 0.001), but promote expressions of the main components of ECM (COL2A1 and Aggrecan) and Bcl-2 protein (Figure 3E-G, p < 0.001). miR-483-3p inhibitor weakened the impacts of ATG on the expressions of ECM degradation/apoptosis/inflammation-related genes (Figures 3E-G and 4A, p < 0.05). Besides, the impacts of ATG on expressions of IkBα and p65 in HNPCs induced by IL-1β was also offset by miR-483-3p inhibitor (Figure 4B, p < 0.001).

4 | DISCUSSION

Intervertebral disc degeneration is the pathological basis of a series of diseases in spinal surgery, the exact mechanism of which has not yet been elucidated. Our research confirmed that the mechanism of the traditional Chinese medicine extract ATG in the treatment of IDD is related to the expression of miR-483-3p, which provides a new direction for the treatment of IDD.

In our research, we used IL-1β to induce HNPCs as a cell model for IDD, because IL-1β has a strong pro-inflammatory activity. A study has reported that IL-1β is involved in multiple pathological processes of IDD, including inflammation, ECM degradation, NPC apoptosis, and oxidative stress.21 In the study of Wei et al., IL-1β also acts as an inducer of IDD.22

The change in ECM during IDD is one of the significant pathological changes. NPCs mainly secrete and synthesize type II collagen, while during IDD, the type II collagen in the nucleus pulposus tissue is gradually decreased.23 Aggrecan, as a kind of proteoglycan, is a component of ECM, whose loss is often accompanied by the progress of IDD.24 MMPs participate in the degradation of ECM, of which MMP3 and MMP13 are instrumental in the IDD process. MMP-3 mainly degrades non-collagen matrix proteins, while MMP-13 degrades collagen.25 Our study verified that ATG treatment brought about down-regulations of MMP-3 and MMP-13 yet up-regulations of COL2A1 and Aggrecan in HNPCs induced by IL-1β. A similar phenomenon has also been reported in the study of osteoarthritis.11 This indicates that ATG may have the potential to influence the development of IDD by restoring the balance between the synthesis and decomposition of ECM.

Different from the way that MMPs directly degrade ECM, inflammatory factors indirectly destroy the integrity of the intervertebral disc by promoting the production of inflammatory mediators in cells. Inflammatory mediators such as TNF-α and IL-1β can induce the expressions of COX-2 and iNOS, thereby triggering neuropathic pain.26,27 Deng et al. believed that the expression level of IL-6 is related to IDD.28 Inhibiting the expressions of these inflammatory factors and MMPs is conducive to improving IDD. It has been found that apigenin, luteoloside, naringin, and other substances alleviate IDD by reducing the levels of TNF-α, IL-1β, IL-6, COX-2, iNOS, and MMPs in NPCs induced by IL-1β.29–31 In our study, ATG produced an inhibitory effect on the inflammatory response of HNPCs induced by IL-1β, which also could regulate the activation of the NF-κB pathway. NF-κB is involved in all aspects of cellular inflammatory response, and plays an extremely critical role in modulating the inflammatory response. Blocking the activation of the NF-κB pathway is one of the strategies for the treatment of IDD. Berberine and Stachydrine are considered as potential drugs for the treatment of IDD because they inhibit the NF-κB pathway in IDD.32,33 In addition, inhibiting the apoptosis of NPCs is also considered to be the key for treating IDD.34–36 A study has pointed out that activation of NF-κB can up-regulate the expression of anti-apoptotic family member Bcl-2.37 However, Kaltschmidt et al. affirmed that NF-κB exerts a pro-apoptotic or anti-apoptotic function depending on the nature of the signal caused by the death inducer.38 The above findings manifested that there is no contradiction between ATG-induced inactivation of NF-κB and suppression of cell apoptosis, because IL-1β may induce NF-κB to play a pro-apoptotic effect.

We also unraveled that ATG exerts a therapeutic effect on IL-1β-induced HNPCs by up-regulating the level of miR-483-3p. This coincides with the research of Yang et al.39 who augmented the level of miR-483-3p through traditional Chinese medicine formula, thereby promoting the proliferation of NPCs and restoring the balance of ECM decomposition and synthesis. However, HNPCs cultured in vitro cannot completely mimic the situation in vivo, so follow-up experiments will be conducted with animal experiments to further explain the mechanism of ATG in the treatment of IDD.
FIGURE 3 MiR-483-3p inhibitor reversed the regulation of ATG on IL-1β-induced HNPC viability, apoptosis, and expressions of ECM-related genes and apoptosis factors. (A) The expression of miR-483-3p in HNPCs treated with 10 ng/ml IL-1β and/or ATG (10, 50 μmol/L) for 24 h was detected by qRT-PCR. U6 was used as the internal control. (B) The expression of miR-483-3p inhibitor/inhibitor control and treated with 10 ng/ml IL-1β and/or ATG (50 μmol/L) for 24 h was detected by qRT-PCR. U6 was used as the internal control. (C) HNPCs were transfected with miR-483-3p inhibitor/inhibitor control and then treated with 10 ng/ml IL-1β and/or ATG (50 μmol/L) for 24 h, and cell viability was tested by CCK-8. (D) After HNPCs received miR-483-3p inhibitor/inhibitor control transfection and 10 ng/ml IL-1β and/or ATG (50 μmol/L) treatment, cell apoptosis was detected by flow cytometry. (E) qRT-PCR was conducted to quantify MMP3, MMP13, COL2A1, and Aggrecan expressions in HNPCs after miR-483-3p inhibitor/inhibitor control transfection and 10 ng/ml IL-1β and/or ATG (50 μmol/L) treatment. GAPDH was used as the internal control. (F-G) the protein levels of MMP-3, MMP-12, COL2A1, Aggrecan, Bcl-2, Bax, and cleaved caspase 3 in HNPCs transfected with miR-483-3p inhibitor/inhibitor control and treated with 10 ng/ml IL-1β and/or ATG (50 μmol/L) for 24 h were detected by Western blot. Quantified values were described as mean ± standard deviation of at least three independent experiments. *** p < 0.001 vs. control group. ^^^ p < 0.001 vs. IL-1β group. +++p < 0.001 vs. IL-1β + IC group. #p < 0.01, ####p < 0.001 vs. ATG 50 + IC group. ATG, arctigenin; COL2A1, collagen type II alpha 1; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNPCs, human nucleus pulposus cells; I, inhibitor; IC, inhibitor control; IL, interleukin; MMP, matrix metalloproteinase; qRT-PCR, quantitative real time polymerase chain reaction.
CONCLUSIONS

In a word, our research corroborates that ATG inhibits ECM degradation, inflammation, apoptosis, and activation of NF-κB pathway in HNPCs induced by IL-1β through up-regulating the level of miR-483-3p, which indicates that ATG may be a potential therapeutic drug for IDD.

AUTHOR CONTRIBUTIONS

ZJ and RG had made substantial contributions to conception and design and also contributed to drafting of the article or critically revising it for important intellectual content. ZM and HL contributed to data acquisition, data analysis, and interpretation. All authors contributed to the final approval of the version to be published. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

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How to cite this article: Ji Z, Guo R, Ma Z, Li H. Arctigenin inhibits apoptosis, extracellular matrix degradation, and inflammation in human nucleus pulposus cells by up-regulating miR-483-3p. \textit{J Clin Lab Anal}. 2022;36:e24508. doi: 10.1002/jcla.24508