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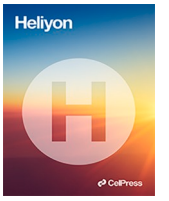

Research article

Puerarin modulates proliferation, inflammation and ECM metabolism in human nucleus pulposus mesenchymal stem cells via the lncRNA LINC01535

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A B S T R A C T

Background: Intervertebral disc degeneration (IVDD) is a highly prevalent musculoskeletal disorder characterized by progressive destruction of the intervertebral disc, leading to chronic low back pain and disability. Emerging evidence suggests that dysregulation of ferroptosis, a recently discovered form of regulated cell death, participates in IVDD pathogenesis. Puerarin, a natural flavonoid compound from *Pueraria lobata*, has shown promise in modulating ferroptosis in various diseases.
Methods: Human nucleus pulposus-derived mesenchymal stem cells (NPMSCs) were isolated and identified by flow cytometry. We investigated the effects of puerarin on human NPMSCs and examined the underlying molecular mechanisms.
Results: Puerarin significantly promoted human NPMSC proliferation, as evidenced by the increased cell viability and colony formation ability. Furthermore, puerarin suppressed the expression of cyclooxygenase-2 and the proinflammatory cytokine interleukin-6 in NPMSCs, demonstrating the anti-inflammatory properties of the compound. Notably, puerarin attenuated ECM breakdown by downregulating the ECM-degrading enzymes MMP3, MMP13 and ADAMTS5, and it increased ECM component synthesis, including collagen type II and aggrecan, by NPMSCs. Moreover, puerarin inhibited ferroptosis in NPMSCs by modulating the expression of key ferroptosis-related genes, including *ACSL4*, *PTGS2* and *GPX4*. Depletion of LINC01535 abolished the effects of puerarin on proliferation, inflammation and ECM metabolism, suggesting a key role of this lncRNA in mediating the effects of puerarin.
Conclusion: Our findings show that puerarin promotes the proliferation of human NPMSCs and ECM synthesis by these cells. Furthermore, puerarin inhibits inflammation and ECM degradation by suppressing ferroptosis via LINC01535. These results provide insights into the molecular mechanisms underlying the therapeutic effects of puerarin in IVDD. Targeting ferroptosis and its regulatory factors, such as LINC01535, may have therapeutic potential for the treatment of IDD and other degenerative disorders of the intervertebral disc. Further studies are needed to uncover the translational potential of puerarin and its downstream targets in preclinical and clinical applications.

1. Introduction

Low back pain is a global public health problem that greatly affects the quality of life of patients and incurs a major economic burden on society [1]. The causes of low back pain are complex, with intervertebral disc degeneration (IVDD) being the most common cause [2]. There are two distinct cell populations in the intervertebral disc (IVD): nucleus pulposus (NP) cells in the central part and

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annulus fibrosus cells in the outer part. NP cells synthesize the extracellular matrix (ECM), namely, proteoglycans (such as aggrecan) and collagens (mainly type II collagen), which play critical roles in disc function. The main cause of IVDD is a decrease in the number and activity of NP cells, followed by a decrease in ECM [3]. Therapies based on mesenchymal stem cell (MSC) transplantation have shown great potential in the treatment of IVDD. Endogenous MSCs have been increasingly used to repair IVDs. In 2007, Risbud et al. isolated and identified nucleus pulposus-derived mesenchymal stem cells (NPMSCs) in degenerative IVD, providing new approaches for the treatment of IVDD [4]. Compared with bone marrow mesenchymal stem cells and adipose mesenchymal stem cells, NPMSCs show a stronger ability to differentiate into NP cells and can better withstand adverse microenvironmental conditions, such as the hypoxia, hypoglycemia and high osmolarity associated with IVDD [5,6]. In IVDD, the proliferative ability of NPMSCs is reduced while the apoptosis rate is increased, leading to a decrease in NPMSCs; this decrease in NPMSC abundance results in the failure of IVD repair, which is considered to be the main cause of IVDD [7]. Therefore, strategies that enhance NPMSC viability and maintain their normal functions are urgently needed to promote endogenous repair in IVDD.

Puerarin, an isoflavonoid extracted from Radix puerariae, is often used as a dietary supplement [8]. Puerarin is widely used in the treatment of many diseases, including cardiovascular disease [9,10], cancers [11] and femoral head osteonecrosis [12], because of its broad pharmacological actions. It also has therapeutic effects in neurological diseases such as cerebral edema and Alzheimer's disease [13]. Chen et al. showed that puerarin suppresses inflammation and ECM degradation induced by IL-1β in mouse chondrocytes [14]. Tang et al. reported that puerarin promotes ECM synthesis in rat NP cells [15]. However, the effect of puerarin on human NPMSCs remains unknown. Iron, a necessary trace element, plays an important role in maintaining health. Under iron overload conditions, the excess iron can cause diseases of the cardiovascular system [16], bone and joint system [17] and digestive system [18]. Many studies have shown that puerarin has therapeutic effects in iron overload-related diseases. For example, Liu et al. found that puerarin suppressed cardiomyocyte loss by inhibiting ferroptosis [19]. Puerarin alleviates autism-related behaviors by suppressing ferroptosis in hippocampal neural stem cells [20]. Song et al. showed that the protective action of puerarin against retinal injury caused by iron overload involved a reduction in iron levels in the serum and retinal cells [21]. These results suggest that puerarin may have therapeutic potential in the treatment of diseases caused by iron overload. Studies have indicated that iron overload is closely related to the occurrence and development of IVDD. Wang et al. reported that iron overload is a risk factor for IVDD and that it accelerates the progression of the disease by promoting oxidative stress and ferroptosis [22]. However, the role of puerarin in regulating iron overload in human NPMSCs remains unclear.

Ferroptosis is a form of programmed cell death triggered by the combination of iron toxicity, lipid peroxidation and plasma membrane damage, and it is usually accompanied by significant iron accumulation and lipid peroxidation [23]. First discovered in 2012 [24], ferroptosis is morphologically characterized by cellular atrophy and a high mitochondrial density. Current research on ferroptosis is mainly focused on the mechanisms of neurodegeneration and the therapeutic action of cancer drugs. Ferroptosis has been reported to be involved in neuronal cell death associated with various neurological diseases, including hemorrhagic stroke [25], ischemic stroke [26], Parkinson's disease [27] and Huntington's disease [28]. Several studies have shown that therapy-refractory cancer cells in specific cellular states are sensitive to ferroptosis. Hangauer et al. reported that drug-resistant cancer cells after targeted therapy are hypersensitive to GPX4 inhibition [29]. Viswanathan et al. showed that drug-resistant and metastasis-prone mesenchymal carcinoma cells are susceptible to ferroptosis [30]. Emerging evidence suggests that ferroptosis has a key role in IVDD. Zhu et al. studied the role of USP11 and SIRT3 in IVDD and revealed that USP11 alleviates IVDD by modulating ferroptosis [31]. Yang et al. demonstrated that polydopamine nanoparticles ameliorate IVDD by targeting ferroptosis and suppressing GPX4 ubiquitination [32]. However, the effects and mechanisms of action of puerarin on ferroptosis in human NPMSCs remain unknown. In this study, we produced an *in vitro* model of IVDD by stimulating NPMSCs with tumor necrosis factor-alpha (TNF-α). We then investigated the effects of puerarin on ferroptosis in these cells, as well as the underlying mechanisms of action.

2. Materials and methods

2.1. Culture of human NPMSCs

NP tissues were donated by a patient, who was diagnosed with lumbar hemivertebral deformity and underwent hemivertebrectomy. NP tissues were obtained intraoperatively. All procedures were approved by the Ethics Committee of Beijing Jishuitan Hospital, Capital Medical University. NPMSCs were isolated and cultured according to previously reported methods [33]. Briefly, NP tissues were carefully separated with a dissecting microscope and then washed with phosphate-buffered saline (PBS; Hyclone, USA). Next, NP tissues were minced and digested with 0.2 % type II collagenase (Sigma, USA) at 37 °C under 5 % CO_2 for 6 h. The obtained cells were cultured in mesenchymal stem cell (MSC) complete medium (Hyclone) at 37 °C in a humid atmosphere containing 5 % CO_2. The medium was changed every 3 days, and when the cells reached 80%–90 % confluency, they were passaged at a 1:3 ratio. Third-generation NPMSCs were used for the following experiments.

2.2. Isolation of human NPMSCs

To purify human NPMSCs, the culture medium was aspirated, and the cells were washed with PBS and digested with 0.25 % trypsin (Invitrogen, USA). The cell suspension was centrifuged, the supernatant was discarded, and the cell pellets were resuspended in cold PBS. Then, CD73-PE, CD90-APC, CD45-BV510, CD105-PE, CD34-APC and CD31-PE-Cy7 were added to the cell suspensions and incubated for 30 min at 4 °C in the dark. Next, staining buffer was added, and PBS was added for diluting. The suspension was centrifuged, and the supernatant was removed. The cell pellets were resuspended in 100 μl of PBS and detected by flow cytometry.

2.3. Cell proliferation assay

Cell proliferation was assessed using a previously published method [34]. Briefly, the cells were seeded in a 96-well plate at a density of 1×10^4 cells per well. After 12 h, fresh complete media containing different concentrations of puerarin were added to the wells, and the plates were incubated for 72 h in an incubator at 37 °C and 5 % CO_2. Then, the medium was discarded, and 10 μl of cell counting kit-8 solution (CCK-8, Dojindo, Japan) was added to each well and incubated for 2 h at 37 °C while avoiding exposure to light. The absorbance values of the wells were measured with a spectrophotometer (Infinite M200 PRO, Tecan, Switzerland) at a wavelength of 450 nm and a reference wavelength of 650 nm.

2.4. Colony formation assay

Cells in the logarithmic growth phase were digested with 0.25 % trypsin, resuspended in complete medium to obtain a cell suspension and counted with a cell counter. The cells were seeded in 6-well plates at a density of 400 cells per well, and the medium was discarded after 24 h. Then, fresh media containing different concentrations of puerarin were added. The cells were cultured for 14 days, and the medium was changed every 3 days during the observation period. After colonies were obtained, the cells were washed with PBS. Next, the cells were fixed by adding 1 mL of 4 % paraformaldehyde for 30 min and washed once. Then, the colonies were incubated with crystal violet staining solution for 20 min, the plates were washed three times with PBS and photographed, and the colonies were counted.

2.5. Cell transfection

LINC01535 was synthesized by Sangon Biotech (Shanghai, China). Scrambled small interfering RNA (siRNA) as the Control (siControl) and siRNA against LINC01535 (siRNA LINC01535) were synthesized by Sangon Biotech (Shanghai, China) and transfected into human NPMSCs using Lipofectamine 3000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Third-generation NPMSCs were seeded in six-well plates, and the medium was replaced after 24 h with medium containing TNF-α (50 ng/mL; Peprotech, USA) and different concentrations of puerarin. The cells were divided into the following five groups: Control, TNF-α, TNF-α + puerarin (PUR)-75 μM, TNF-α + PUR-150 μM and TNF-α + PUR-300 μM. The cells were collected after 24 h and washed three times with cold PBS. RNA was extracted using an RNA Extraction Kit (Takara, Japan) according to the manufacturer's instructions. A NanoDrop One spectrometer (Thermo Fisher, USA) was used to measure the concentration and purity of the RNA. DNA contamination was eliminated using gDNA Eraser (Takara), and 1 μg of RNA was then reverse-transcribed into cDNA using the PrimeScript RT reagent Kit (Takara). TB Green Premix Ex Taq (Takara) was used for qRT-PCR on a Real-Time PCR System (Applied Biosystems, USA). Relative gene expression was measured using the $2^{-\Delta\Delta CT}$ method, and *GAPDH* was used as the internal reference gene. The sequences of the gene primers are listed in Table 1.

2.7. Western blot analysis

The cells were divided into the five groups as described above and collected after 24 h of treatment. The cells were lysed with lysis buffer containing PMSF, and protein concentrations were measured with a BCA protein assay kit (Beyotime, China). Then, 4 × loading buffer was added to the lysate, and the samples were heated at 99 °C for 10 min. Proteins were separated on a NuPAGE 4%–12 % Bis-Tris Gel (Invitrogen, USA) and transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare Life Science, USA) using the wet transfer method. The PVDF membranes were blocked with 5 % bovine serum albumin and incubated on a shaker for 1 h at room temperature to block nonspecific protein binding sites. The membranes were then incubated at 4 °C overnight with the following

Table 1
Primer sequences.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
ACSL4	CATCCCTGGAGCAGATACTCT	TCACTTAGGATTTCCCTGGTCC
PTGS2	CTGGCGCTCAGCCATACAG	CGCACTTATACTGGTCAAATCCC
GPX4	GAGGCAAGACCGAAGTAAACTAC	CCGAACTGGTTACACGGGAA
COL2A1	TGGACGCCATGAAGGTTTTCT	TGGGAGCCAGATTGTCATCTC
ACAN	ACTCTGGGTTTTCGTGACTCT	ACACTCAGCGAGTTGTCATGG
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
COX-2	GCACCCCGACATAGAGAGC	CTGCGGAGTGCAGTGTTCT
MMP3	CTGGACTCCGACACTCTGGA	CAGGAAAGGTTCTGAAGTGACC
MMP13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
ADAMTS5	GAACATCGACCAACTCTACTCCG	CAATGCCCACCGAACCATCT
LINC01535	CGAGCTCCTGTGGGGATGGAAGTGTGA	GCTCTAGATGGGAGGGAGATAAGGAAAATG

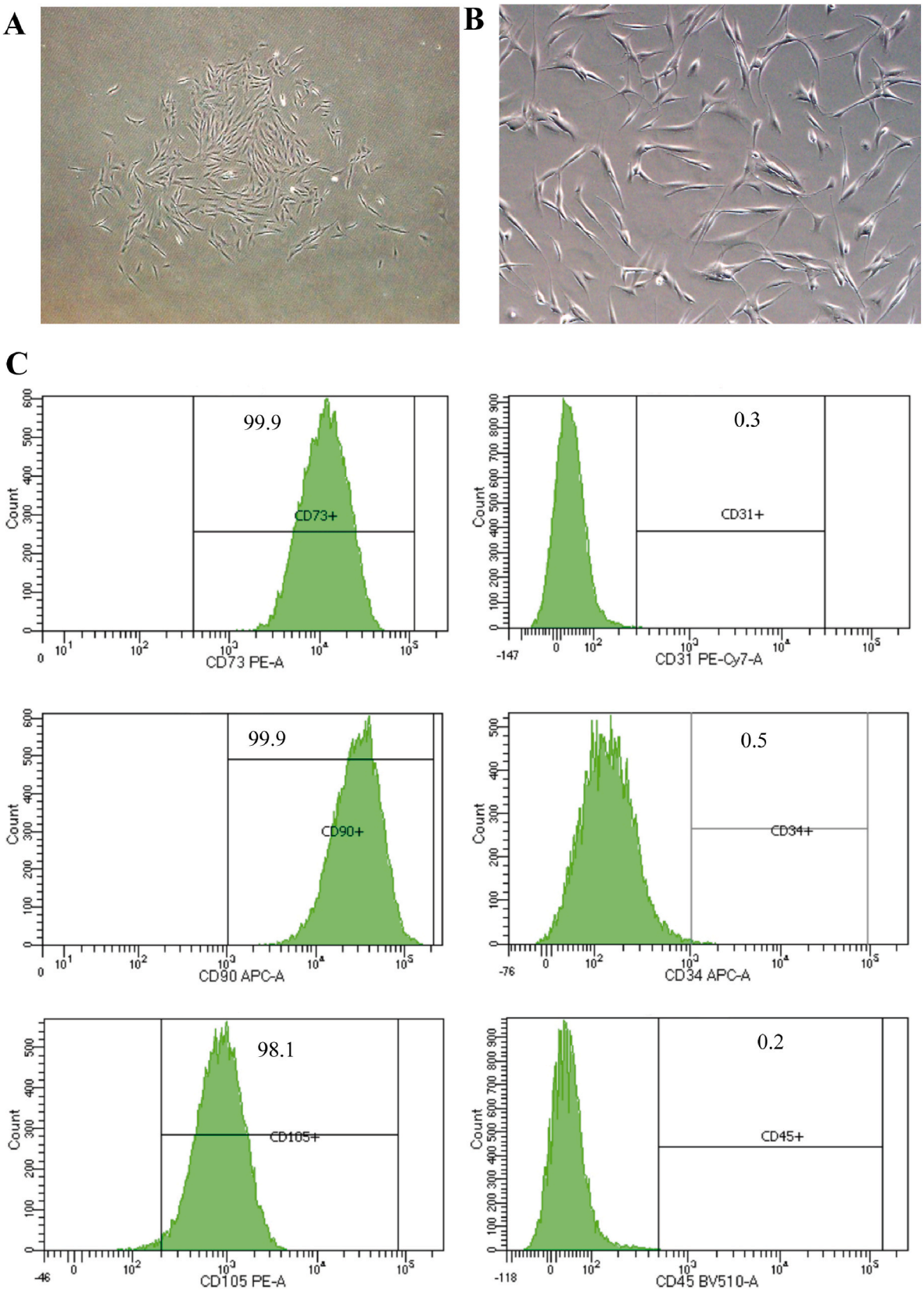

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Fig. 1. Morphology and flow cytometry characterization of NPMSCs. (A) Photomicrographs of NPMSCs (40 ×). (B) Photomicrographs of NPMSCs (100 ×). (C) The cell surface antigens CD31, CD34, CD45, CD73, CD90 and CD105 detected by flow cytometry. (A) and (B) are photomicrographs of NPMSCs from different cell communities and different fields of view.

primary antibodies: GAPDH rabbit mAb (1:1,000; ab181602, Abcam, UK), matrix metalloproteinase 3 (MMP3) rabbit mAb (1:1,000; ab52915, Abcam) and cyclooxygenase-2 (COX-2) mouse mAb (1:1,000; ab300668, Abcam). Thereafter, the membranes were washed with TBST and incubated with secondary antibody at room temperature for 1 h. After washing, immunoreactive bands were visualized using an enhanced chemiluminescence kit (Thermo Fisher, USA). The gray values of the target proteins were quantified using ImageJ software (National Institutes of Health, USA). Relative protein expression was obtained after normalizing to GAPDH.

2.8. Statistical analysis

All data are expressed as the mean ± standard deviation. All experiments were independently repeated at least three times. Differences between two groups were analyzed by two-tailed Student's *t*-tests, and $p < 0.05$ was regarded as statistically significant. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software Inc., USA).

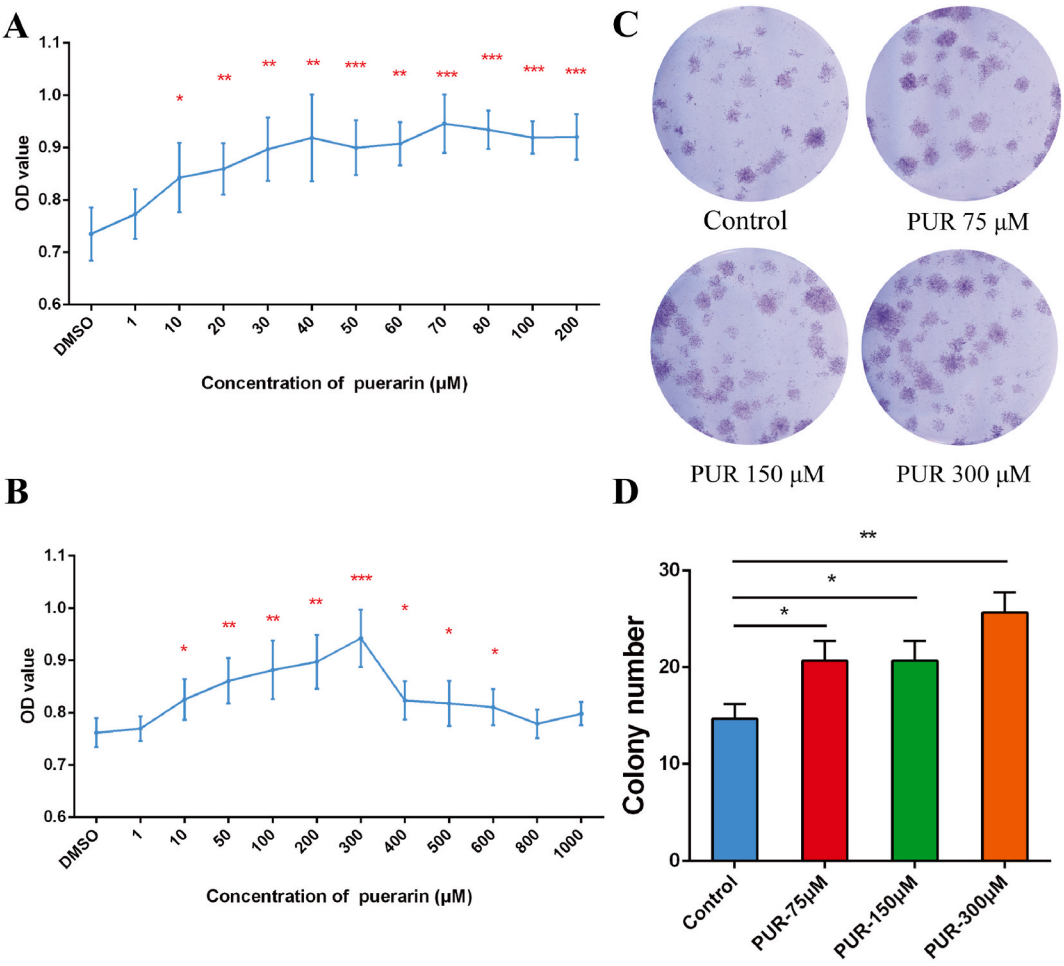

Fig. 2. Puerarin promotes proliferation and colony formation of human NPMSCs. (A) The effect of different concentrations (1–100 μM) of puerarin on the proliferation of NPMSCs evaluated with the CCK-8 assay. (B) The effect of different concentrations (1–1,000 μM) of puerarin on the proliferation of NPMSCs evaluated with the CCK-8 assay. (C) The effect of puerarin on the colony formation ability of NPMSCs assessed with the colony formation assay. (D) Number of colonies. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 3$. PUR: puerarin.

3. Results

3.1. Identification of NPMSCs

The NPMSCs isolated from human NP tissues grew while adhering to the wall, exhibited a spindle shape, and mainly grew in a swirling pattern once the cells had reached 90 % confluency (Fig. 1A and B). The isolated cultured cells highly expressed CD73 (100 %), CD90 (99.7 %) and CD105 (99.6 %) and weakly expressed CD31 (0.5 %), CD34 (0.2 %) and CD45 (0.5 %), which is phenotypically consistent with MSCs (Fig. 1C).

3.2. Puerarin promotes proliferation and colony formation by human NPMSCs

To investigate the effects of puerarin on the proliferation of NPMSCs, the cells were incubated with various concentrations of puerarin for 24 h and then were evaluated by the CCK-8 assay. Puerarin was dissolved in DMSO, and therefore, the DMSO group was used as a control. Compared with NPMSCs in the DMSO group, those in the group treated with puerarin were significantly proliferated at puerarin concentrations of 10–1,000 μM (Fig. 2A and B). Puerarin at 300 μM had the most significant effect on cell proliferation. Puerarin promoted the proliferation of NPMSCs in a dose-dependent manner at concentrations of 75–300 μM (Fig. 2A). To further examine the effect of puerarin on the proliferation of NPMSCs, colony formation was assessed. NPMSCs were treated with various concentrations of puerarin for 10 days. The colony formation assay showed that NPMSCs treated with puerarin at concentrations of 75, 150 and 300 μM generated more and larger colonies than those in the Control group (Fig. 2C and D). These results reveal that puerarin

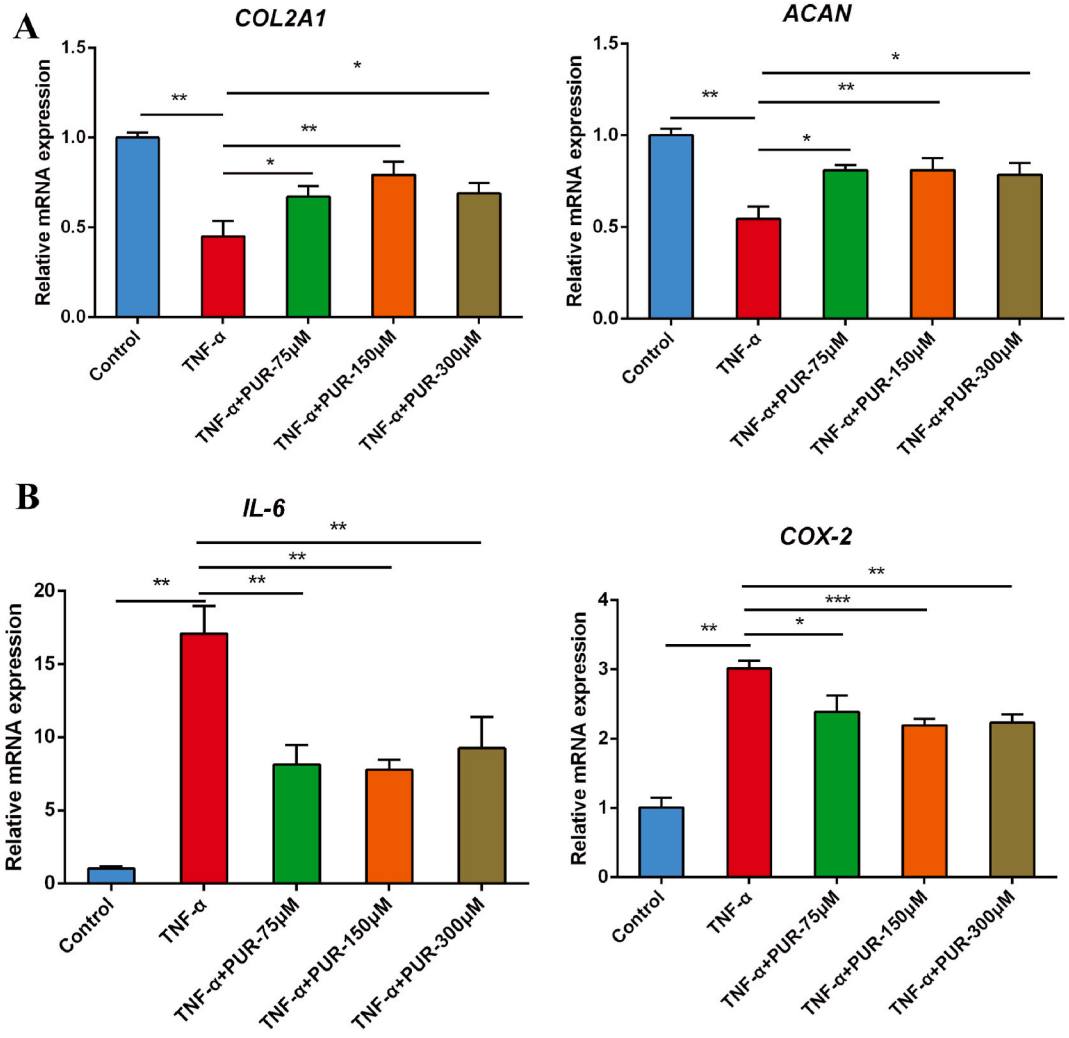

Fig. 3. Puerarin promotes ECM synthesis and inhibits the inflammatory response in human NPMSCs. (A) The mRNA expression levels of *COL2A1* and *ACAN* measured by qRT-PCR. (B) The mRNA expression levels of *IL-6* and *COX-2* measured by qRT-PCR. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 3$. PUR: puerarin.

promotes the proliferation of NPMSCs and colony formation by these cells.

3.3. Puerarin promotes ECM synthesis and inhibits the inflammatory response in human NPMSCs

To investigate the effect of puerarin on ECM synthesis and the inflammatory response, we measured the expression of type II collagen (Col2a1), aggrecan (Acan), interleukin-6 (IL-6) and cyclooxygenase-2 (COX-2) in human NPMSCs. qRT-PCR analysis showed that compared with expression levels in the Control group, the mRNA expression levels of *COL2A1* and *ACAN* were significantly decreased, while the mRNA expression levels of *IL-6* and *COX-2* was increased in the TNF-α group (Fig. 3A and B). The mRNA expression levels of *COL2A1* and *ACAN* were higher in the TNF-α + PUR-75 μM, TNF-α + PUR-150 μM and TNF-α + PUR-300 μM groups than in the TNF-α group (Fig. 3A). Compared with levels in the TNF-α group, the mRNA expression levels of *IL-6* and *COX-2* were significantly decreased in the TNF-α + PUR-75 μM, TNF-α + PUR-150 μM and TNF-α + PUR-300 μM groups (Fig. 3B).

The protein expression levels of COX-2 were examined in human NPMSCs by Western blot analysis. The protein expression levels of COX-2 in the TNF-α group were higher than those in the Control group (Fig. 4B). Compared with protein levels in the TNF-α group, the protein expression levels of COX-2 were significantly reduced in the TNF-α + PUR-150 μM and TNF-α + PUR-300 μM groups (Fig. 4B). These results demonstrate that puerarin promotes the synthesis of ECM components and inhibits the increase in inflammatory mediators induced by TNF-α in human NPMSCs.

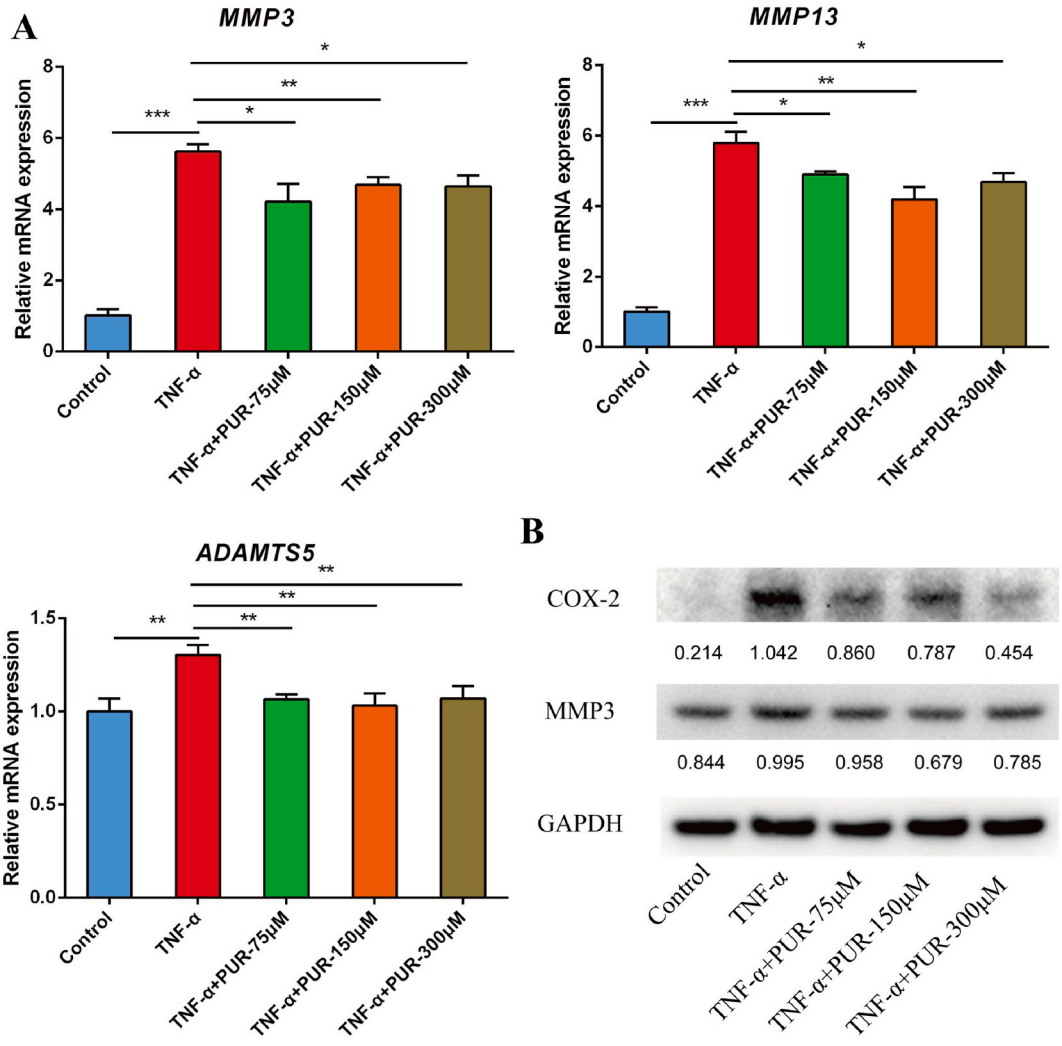

Fig. 4. Puerarin suppresses ECM degradation induced by TNF-α in human NPMSCs. (A) The mRNA expression levels of *MMP3*, *MMP13* and *ADAMTS5* measured by qRT-PCR. (B) The protein expression levels of COX-2 and MMP3 measured by Western blot analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 3$. PUR: puerarin.

3.4. Puerarin suppresses ECM degradation induced by TNF-α in human NPMSCs

ECM degradation plays an important role in IVDD. Therefore, we examined the effects of puerarin on ECM-degrading enzymes, including MMP3, MMP13 and ADAMTS5, in human NPMSCs. qRT-PCR analysis revealed that compared with expression levels in the Control group, the mRNA expression levels of *MMP3*, *MMP13* and *ADAMTS5* were significantly increased in the TNF-α group (Fig. 4A). The mRNA expression levels of *MMP3*, *MMP13* and *ADAMTS5* in the TNF-α + PUR-75 μM, TNF-α + PUR-150 μM and TNF-α + PUR-300 μM groups were significantly lower compared with those in the TNF-α group (Fig. 4A).

We measured the protein expression levels of MMP3 in human NPMSCs by western blotting. MMP3 protein levels were higher in the TNF-α group than in the Control group (Fig. 4B). Compared with protein expression in the TNF-α group, the protein expression levels of MMP3 were significantly decreased in the TNF-α + PUR-75 μM, TNF-α + PUR-150 μM and TNF-α + PUR-300 μM groups (Fig. 4B). These results show that puerarin suppresses ECM degradation induced by TNF-α in human NPMSCs.

3.5. Puerarin suppresses ferroptosis induced by TNF-α and upregulates LINC01535 in human NPMSCs

We next investigated the effect of puerarin on ferroptosis induced by TNF-α in human NPMSCs. Higher mRNA expression levels of acyl-CoA synthetase long-chain family (ACSL4) and prostaglandin endoperoxide synthase 2 (PTGS2) were observed in the TNF-α group than in the Control group (Fig. 5A). The mRNA expression levels of glutathione peroxidase 4 (GPX4) were decreased in the TNF-α group compared with those in the Control group (Fig. 5A). Compared with the TNF-α group, the TNF-α + PUR-75 μM, TNF-α + PUR-150 μM and TNF-α + PUR-300 μM groups showed reduced mRNA expression levels of *ACSL4* and *PTGS2* and higher mRNA expression

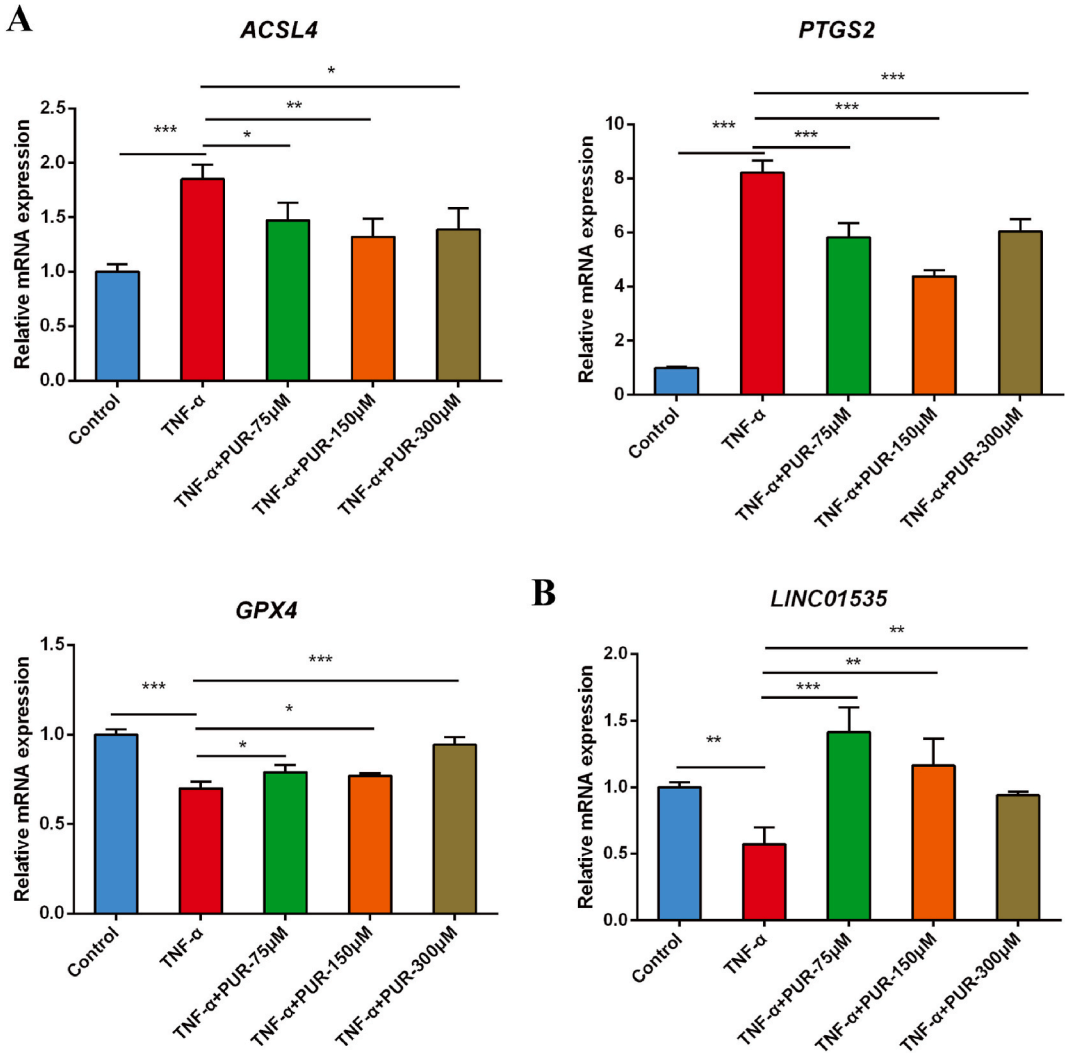

Fig. 5. Puerarin suppresses ferroptosis and upregulates *LINC01535* in human NPMSCs. (A) The mRNA expression levels of *ACSL4*, *PTGS2* and *GPX4* measured by qRT-PCR. (B) The mRNA expression of *LINC01535* measured by qRT-PCR. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, $n = 4$. PUR: puerarin.

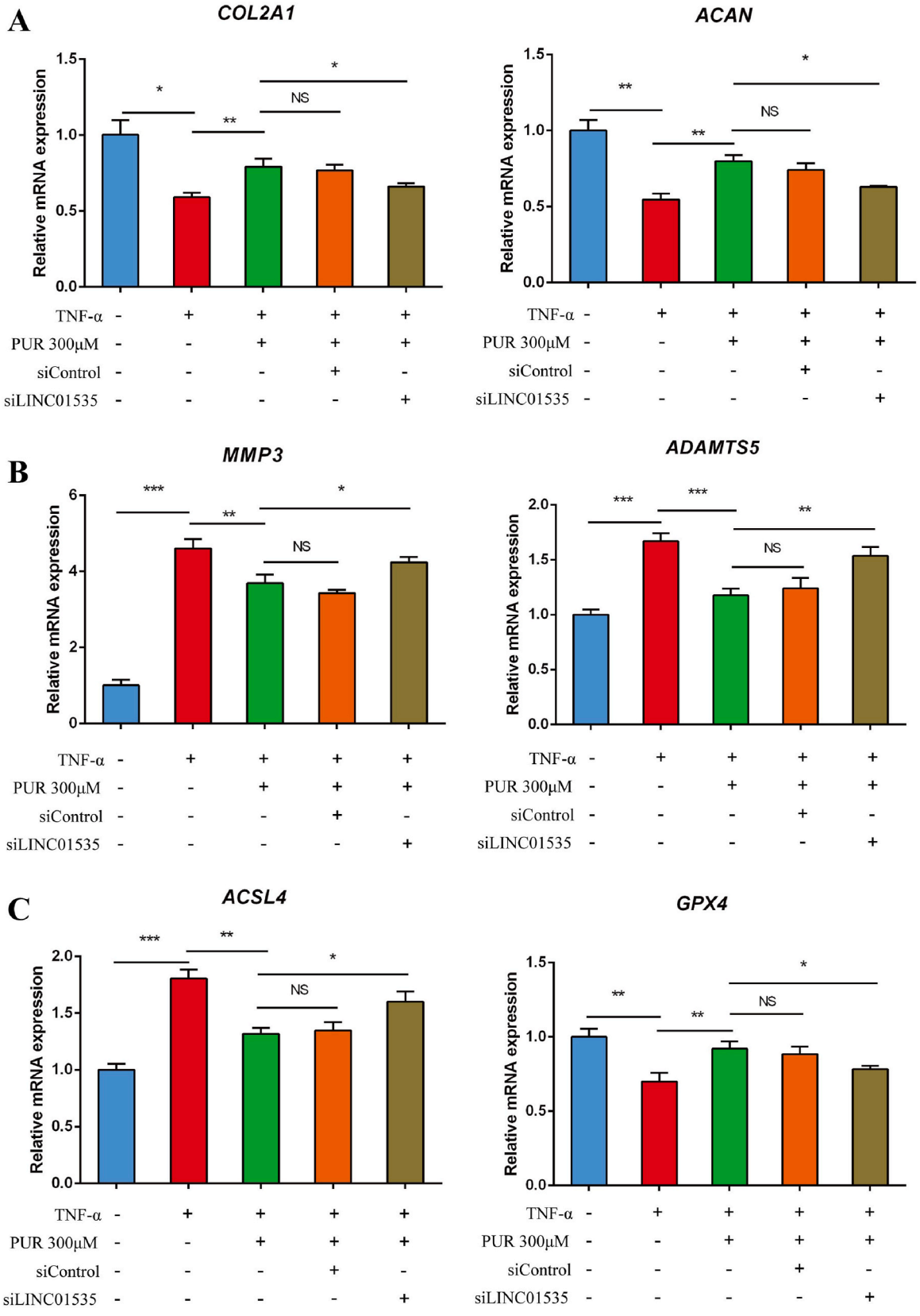

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Fig. 6. Puerarin suppresses ferroptosis by upregulating *LINC01535* in human NPMSCs. (A) The mRNA expression levels of *COL2A1* and *ACAN* measured by qRT-PCR. (B) The mRNA expression levels of *MMP3* and *ADAMTS5* measured by qRT-PCR. (C) The mRNA expression levels of *ACSL4* and *GPX4* measured by qRT-PCR. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $n = 4$. PUR: puerarin.

levels of *GPX4* (Fig. 5A). Compared with levels in the Control group, the mRNA expression levels of *LINC01535* were significantly decreased in the TNF-α group (Fig. 5B). Moreover, the mRNA expression levels of *LINC01535* in the TNF-α + PUR-75 μM, TNF-α + PUR-150 μM and TNF-α + PUR-300 μM groups were significantly higher compared with those in the TNF-α group (Fig. 5B). These results demonstrate that puerarin inhibits ferroptosis induced by TNF-α and upregulates LINC01535 in human NPMSCs.

3.6. Puerarin inhibits ferroptosis by upregulating LINC01535 in human NPMSCs

To study whether the effects of puerarin on NPMSCs were dependent on LINC01535, we used a siRNA, namely, siLINC01535, to downregulate LINC01535 in NPMSCs, with a scrambled interfering RNA (siControl) used as a control. Compared with expression in the Control group, the mRNA expression levels of *COL2A1*, *ACAN* and *GPX4* were reduced, while the mRNA expression levels of *MMP3*, *ADAMTS5* and *ACSL4* were increased in the TNF-α group (Fig. 6A and B). The *COL2A1*, *ACAN* and *GPX4* transcript levels were higher, while those of *MMP3*, *ADAMTS5* and *ACSL4* were reduced in the TNF-α + PUR-300 μM group compared with those in the TNF-α group (Fig. 6A and B). There was no significant difference between the TNF-α + PUR-300 μM group and the TNF-α + PUR-300 μM + siLINC01535 group (Fig. 6A and B). Compared with expression levels in the TNF-α + PUR-300 μM group, the mRNA expression levels of *COL2A1*, *ACAN* and *GPX4* were decreased, while those of *MMP3*, *ADAMTS5* and *ACSL4* were increased in the TNF-α + PUR-300 μM + siLINC01535 group (Fig. 6A and B). These results demonstrate that depletion of LINC01535 abrogates the effects of puerarin on the synthesis of ECM components, the degradation of ECM and ferroptosis in human NPMSCs (see Fig. 7).

4. Discussion

NP tissues in the IVD undergo severe pathological changes in the setting of IVDD [35], including a decrease in the number and function of NP cells [36]. The ECM secreted by NP cells is the major component of IVDs. A decrease in the number of NP cells (caused by NP cell apoptosis and a reduction in cell proliferation) and in ECM components (such as collagen 2A1 and aggrecan) can directly lead to various pathological changes in NP tissues [37]. In addition, degenerative NP tissues produce a variety of inflammatory factors and hydrolases that cause ECM degradation, including IL-1β, TNF-α and MMPs, ultimately resulting in IVDD [38]. Therefore, the key to treating IVDD is to increase the proliferative capacity of NP cells, enhance the synthesis of ECM, inhibit the degradation of ECM and

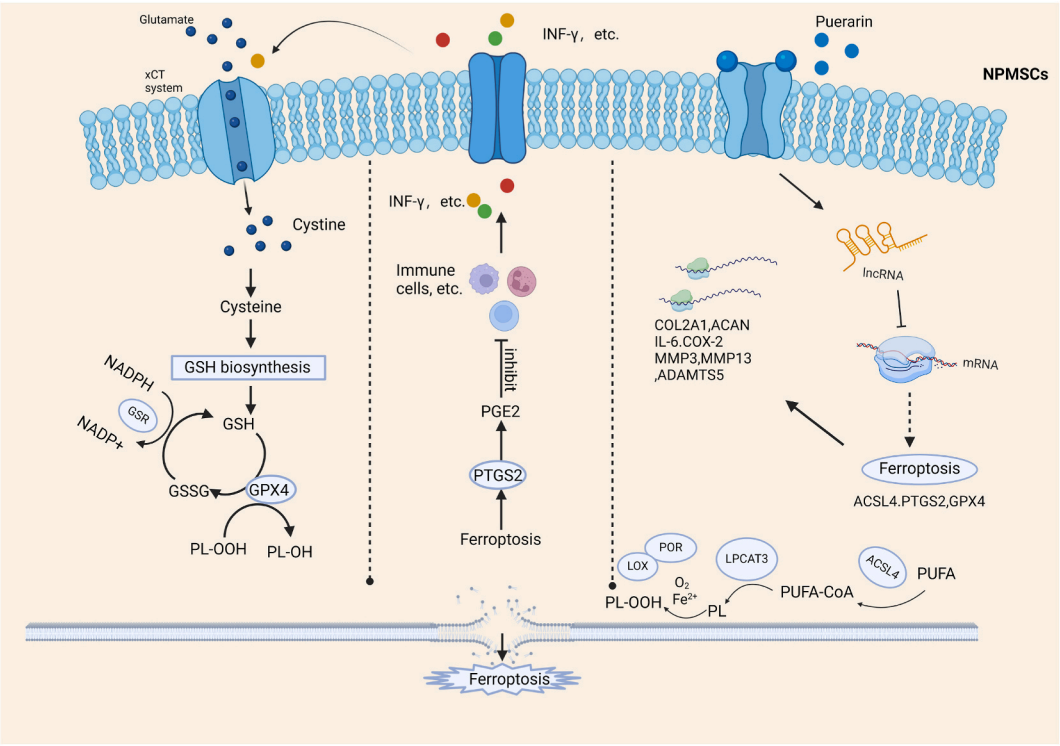

Fig. 7. Schematic illustration of puerarin modulating ferroptosis via lncRNA LINC01535 in human nucleus pulposus mesenchymal stem cells.

reduce the inflammatory response.

Puerarin, a natural isoflavone compound derived from the root of *Pueraria lobata*, has recently been clinically applied in the treatment of cardiovascular and cerebrovascular diseases because of its protective effects on the myocardium, vascular epithelium and neural cells. Puerarin has been reported to play a key role in modulating the proliferation of stem cells [39].

In the current study, puerarin significantly promoted the proliferation of NPMSCs and enhanced the colony formation ability of these cells. The increases in the viability and proliferative ability of NPMSCs in response to puerarin treatment suggest its potential in enhancing the regenerative ability of MSCs. Our findings suggest that puerarin may have potential applications in regenerative medicine and tissue engineering, where the proliferation and expansion of MSCs are critical for therapeutic application. However, further research is needed to fully understand the underlying mechanisms and to optimize the conditions for using puerarin to enhance MSC proliferation. Chen et al. showed that puerarin inhibits inflammation and ECM degradation induced by IL-1β by suppressing the NF-κB signaling pathway, thereby delaying the progression of osteoarthritis [14]. Puerarin also plays an important role in regulating the inflammatory response, and puerarin-containing hydrogels promoted wound healing by suppressing the inflammatory response in a mouse model of diabetes [40]. Moreover, puerarin exhibited anti-inflammatory and antioxidative properties in a mouse model of colitis [41]. Studies have shown that the inflammatory response plays a major role in IVDD progression. The ECM is mainly composed of collagen 2A1 and aggrecan. Inflammation can reduce collagen 2A1 and aggrecan synthesis by NP cells, thereby compromising ECM integrity and function. The enzymes mainly responsible for the destruction of the NP ECM include MMPs and ADAMTS [42,43]. MMP3 and ADAMTS5 degrade collagen 2A1, while MMP13 degrades both collagen 2A1 and aggrecan [44–46]. Downregulating MMP3, MMP13 and ADAMTS5 expression can effectively reduce ECM degradation and the inflammatory response and can thereby inhibit IVDD.

In this study, puerarin significantly upregulated the expression of collagen 2A1 and aggrecan, key components of the NP ECM, in our *in vitro* model of IVDD. Moreover, puerarin significantly reduced the mRNA expression levels of *MMP3*, *MMP13* and *ADAMTS5*, and western blotting confirmed that puerarin decreased the protein expression levels of MMP3, indicating its potential to inhibit the progression of IVDD.

qRT-PCR analysis showed that puerarin effectively downregulated *IL-6* and *COX-2*, and western blotting confirmed the reduction in COX-2 protein levels, demonstrating its anti-inflammatory effects on NPMSCs. The anti-inflammatory effects of puerarin are particularly noteworthy, as chronic inflammation is a key contributor to IVDD progression. By suppressing the expression of proinflammatory cytokines, puerarin may help create a more favorable microenvironment for NPMSCs and support their regenerative functions, thereby promoting IVD repair and slowing the progression of IVDD.

Ferroptosis is a unique form of regulated cell death characterized by iron-dependent lipid peroxidation. It has been reported that ferroptosis contributes to the pathogenesis of IVDD [47]. Puerarin has gained attention for its potential therapeutic effects in various diseases. However, its role in regulating ferroptosis in NPMSCs remains poorly understood.

ACSL4, an enzyme involved in lipid metabolism, has been identified as a critical regulator of ferroptosis [26]. It promotes the incorporation of polyunsaturated fatty acids into phospholipids, rendering cells more susceptible to lipid peroxidation and subsequent ferroptosis. PTGS2 is an enzyme responsible for the production of prostaglandins and other lipid mediators [48] and has been implicated in the regulation of ferroptosis by promoting lipid peroxidation and oxidative stress. GPX4, a key enzyme that inhibits lipid peroxidation, plays a central role in suppressing ferroptosis [49]. GPX4 catalyzes the reduction of lipid hydroperoxides to their corresponding alcohols, thereby protecting cells from ferroptotic damage. TNF-α markedly upregulated ACSL4 and PTGS2 expression and decreased the expression of GPX4, which is essential for maintaining cellular redox homeostasis, demonstrating that TNF-α promotes NPMSC ferroptosis. Notably, puerarin significantly downregulated ACSL4 and PTGS2 and upregulated GPX4 expression, suggesting that puerarin suppresses ferroptosis induced by TNF-α in NPMSCs. Furthermore, puerarin upregulated LINC01535, and silencing LINC01535 partially suppressed the protective effects of puerarin on NPMSCs. These findings suggest that puerarin attenuates ferroptosis induced by TNF-α by upregulating LINC01535 in NPMSCs. The findings of this study highlight the potential of puerarin as a therapeutic agent for IVDD based on its ability to modulate ferroptosis in NPMSCs.

Together, the findings of this study highlight the therapeutic potential of puerarin in the treatment of IVDD. However, there are a number of limitations to this study. First, NPMSC degeneration was induced by TNF-α in our *in vitro* model, which cannot fully mimic the *in vivo* microenvironment in IVDD. Second, NPMSCs were cultured under normal oxygen conditions. This situation contrasts with the conditions of hypoxia, hypoglycemia and high osmolarity present in IVDD. Further *in vivo* study and clinical trials are necessary to validate the efficacy and safety of puerarin as a therapeutic agent for IVDD. Additionally, further research is needed to elucidate the molecular mechanisms underlying the actions of puerarin on NPMSCs. Nonetheless, our findings provide key insights into the phenotypic modulation of NPMSCs by puerarin and pave the way for future investigations on IVDD.

5. Conclusion

Our findings demonstrate that puerarin promotes proliferation and ECM synthesis and inhibits inflammation and ECM degradation in human NPMSCs by suppressing ferroptosis via LINC01535. Puerarin may have therapeutic potential in the treatment of IVDD.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

CRediT authorship contribution statement

Penglei Cui: Writing – original draft, Resources, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yueyang Sheng:** Writing – original draft, Project administration, Methodology, Investigation, Formal analysis. **Chengai Wu:** Writing – review & editing, Visualization, Resources, Project administration. **Da He:** Writing – review & editing, Supervision, Software, Resources, Project administration.

Declaration of competing interest

All authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33083.

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