MicroRNA-223 negatively regulates LPS-induced inflammatory responses by targeting NLRP3 in human dental pulp fibroblasts

Diya Wang[1,2#], Shukai Sun[3#], Ying Xue[2#], Jun Qiu[1], Tao Ye[1], Rong Zhang[1,4], Bing Song[1,5], Wenxi He[1], Yaqing Zhang[1], Wenkai Jiang[1,5]

[1]State Key Laboratory of Military Stomatology & National Clinical Research Center for Oral Diseases & Shaanxi Key Laboratory of Stomatology, Department of Operative Dentistry & Endodontics, School of Stomatology, Fourth Military Medical University, Xi'an, Shaanxi, PR China. [2]Department of Occupational and Environmental Health and the Ministry of Education Key Lab of Hazard Assessment and Control in Special Operational Environment, School of Public Health, Fourth Military Medical University, Xi'an, Shaanxi, PR China. [3]State Key Laboratory of Military Stomatology & National Clinical Research Center for Oral Diseases & Shaanxi Clinical Research Center for Oral Diseases, Department of Pediatric Dentistry, School of Stomatology, Fourth Military Medical University, Xi'an, Shaanxi, PR China. [4]Department of Stomatology, First Medical Center, Chinese PLA General Hospital, Beijing, China. [5]School of Dentistry, College of Biomedical and Life Sciences, Cardiff University, Cardiff, UK.

# These authors contributed equally to the paper.

Key words: NLRP3, human dental pulp fibroblasts, miR-223, IL-1β, pulpitis

Running Head: MiR-223 negatively regulates NLRP3 in HDPFs

*Corresponding authors: Wenkai Jiang. State Key Laboratory of Military Stomatology & National Clinical Research Center for Oral Diseases & Shaanxi Key Laboratory of Stomatology, Department of Operative Dentistry & Endodontics, School of Stomatology, Fourth Military Medical University, Xi'an, Shaanxi, PR China. (tel.: +86-29-84776476; Fax: +86-29-84776476) ; e-mails: JiangW6@cardiff.ac.uk

Abstract

Aim To investigate the effect of miR-223 on NLRP3, subsequently regulating the production of the NLRP3/CASP1 inflammasome pathway-mediated proinflammatory cytokines IL-1β and IL-18 in human dental pulp fibroblasts (HDPFs).

Methodology Human dental pulp tissue (HDPT) and HDPFs were obtained from impacted third molars. The miR-223 mimics and inhibitor or NLRP3 plasmid were used to upregulate or downregulate miR-223 or NLRP3 in HDPFs, respectively. Computational prediction via TargetScan 5.1 and a luciferase reporter assay were conducted to confirm target association. The mRNA and protein expression of NLRP3, caspase-1, IL-1β and IL-18 were determined by qRT-PCR and western blotting, respectively. The release of IL-1β and IL-18 was analyzed by ELISA. The significance of the differences between the experimental and the control groups was determined by using one-way analysis of variance, $P<0.05$ indicated statistical significance.

Results A decrease in miR-223 and an increase in NLRP3 in HDPT occurred during the transformation of reversible pulpitis into irreversible pulpitis compared to that in healthy pulp tissue ($p<0.05$). The computational prediction and luciferase reporter assay confirmed that NLRP3 was a direct target of miR-223 in HDPFs. The miR-223 inhibitor further promoted ATP plus LPS-induced NLRP3/CASP1 inflammasome pathway activation compared to the ATP plus LPS-induced group ($p<0.05$). In contrast, the miR-223 mimic significantly inhibited the NLRP3/CASP1 inflammasome pathway activation induced by ATP plus LPS compared to the ATP plus LPS-induced group ($p<0.05$).

Conclusion MiR-223 serves as a negative regulator involved in the control of the production and secretion of proinflammatory cytokines mediated by the NLRP3/CASP1 inflammasome pathway by targeting NLRP3. These data provide insight into the potential regulatory effects of miRNAs on the NLRP3 inflammasome, thus opening up novel potential therapeutic avenues for future endodontic treatment.

Introduction

Pulpitis induced by dental caries is one of the most prevalent infectious diseases in the world (Cooper et al. 2017). Following dental tissue infection, the inflammatory response within the pulp is the first line of defense against bacterial invasion (Hahn & Liewehr 2007). It has been confirmed that dental pulp inflammation functions as a double-edged sword (Cooper et al. 2014). Moderate inflammatory responses not only effectively eliminate invading pathogens but also promote pulp tissue healing and dentine repair. However, excessive inflammatory responses lead to pulp tissue necrosis (Cooper et al. 2014). Therefore, how to control the process of pulpal inflammatory responses becomes of vital importance to protect pulp tissue from damage and to promote dentine regeneration.

Inflammatory responses are initiated via activation of pattern recognition receptors (PRRs) through recognition of pathogen-associated molecular patterns (PAMPs), which are derived from pathogenic microbes and danger-associated molecular patterns (DAMPs) (Rathinam et al. 2019). A wide range of PRRs expressed by cells within dental pulp tissue function to recognize PAMPs, thereby subsequently orchestrating the inflammatory cascade aimed at containing and ultimately eradicating the infection (Cooper et al. 2017). It has been reported that multiple PRRs, including nucleotide-binding oligomerization domain NOD-like receptors (NLRs) and Toll-like receptors (TLRs), are involved in pulpal immune/inflammatory responses (Veerayutthwilai et al. 2007, Farges et al. 2009, Hirao et al. 2009, Lin et al. 2009, Cooper et al. 2010, Farges et al. 2011, Horst et al. 2011, Lee et al. 2011, Staquet et al. 2011). Belonging to the NLR family, the NLRP3 inflammasome is a critical molecular platform of the innate immune system that regulates the maturation of the proinflammatory cytokines IL-1β and IL-18 through the activation of inflammatory caspase-1 in response to microbial infection and cellular damage (Schroder & Tschopp 2010). The NLRP3 inflammasome can be activated in a number of ways; one way is through the action of adenosine triphosphate (ATP) on the $P2X_7$ receptor (Schroder & Tschopp 2010). In pathological conditions, high level of ATP released from dying and stressed cells acts as an important extracellular signal for the immune system, particularly during an inflammatory

response (Miller et al. 2011). A previous study demonstrated that the NLRP3/CASP1 inflammasome pathway in HDPFs can be activated by LPS through a process involving the ATP activated $P2X_7$ receptor ATP-gated ion channel, which is critical for the pulpal inflammatory phase (Jiang et al. 2015). However, the mechanism of NLRP3/CASP1 inflammasome pathway activation from reversible pulpitis into irreversible pulpitis remains unclear.

MicroRNAs (miRNAs, miRs) are small, endogenously initiated, noncoding RNAs that bind to their target mRNAs, leading to the degradation or translational suppression of their respective mRNAs (Acuna et al. 2020). They have been reported to be expressed in various tissues and cells that play key roles in physiological processes such as inflammation, differentiation, cellular proliferation, development, and apoptosis (Acuna et al. 2020). Among them, miR-223 has been shown to be important in immune system regulation and to be associated with the development of cancers and inflammatory and autoimmune diseases (Taibi et al. 2014). Concerning innate immune responses, recent reports state that miR-223 has an important function as a gene expression regulator in innate immune responses, thus impacting the outcome of a variety of diseases (Zhou et al. 2018). However, the role of miR-223 during the pulpitis phase remains unknown. Therefore, this study aims to investigate the effect of miR-223 on NLRP3, subsequently regulating the production of the NLRP3/CASP1 inflammasome pathway-mediated proinflammatory cytokines IL-1β and IL-18 in HDPFs.

Material and Methods

Sample collection and preparation

The subjects in this study were recruited from the Department of Oral and Maxillofacial Surgery at the School of Stomatology, Fourth Military Medical University, Xi'an, China. Thirty human third molars, including ten free from caries, ten carious teeth with irreversible pulpitis and ten carious teeth with reversible pulpitis, were collected for the preparation of dental pulp specimens, as described previously (Jiang et al. 2015). Clinically, teeth with irreversible pulpitis are sensitive to heat and had spontaneous lingering pain, and teeth with reversible pulpitis had instant pain in cold sensitivity tests.

Caries-free teeth from volunteers who had no clinical medical history and who were taking no medications were collected as controls. Clinical and radiographic examinations were used to exclude teeth with a diagnosis of pulp necrosis, periapical pathosis, periodontal diseases or other injuries, with the exception of crown fractures or any restoration in teeth with normal dental pulps. Written informed consent was obtained from all volunteers and routine surgical procedures were used. The ethics committee of the Fourth Military Medical University School of Stomatology approved the experimental protocols (permission number IRB-REV-2017-007). The specimens were used for real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR; n=10).

Cell cultures

The pulp tissues were obtained from third molars (donors aged from 17 to 20 years, n=3) with the patient's informed consent and ethical approval by the ethics committee of the School of Stomatology, Fourth Military Medical University (permission number IRB-REV-2017-007). All the methods in the study were carried out in accordance with the approved guidelines. HDPFs were cultured and characterized as previously reported (Jiang et al. 2015). Pulp tissues were digested with a 4 mg/mL solution of collagenase/dispase for 1 h at 37 °C. Following centrifugation and resuspension in alpha modification of Eagle's medium (α-MEM) supplemented with 100 units/mL penicillin, 100 mg/mL streptomycin, 20% foetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, USA). The clonal populations of HDPFs were isolated using a limiting dilution protocol, and cells at the third or fourth passages were used. For the ATP plus LPS induction experiments, HDPFs were incubated by ATP (5 mM) for 2 h, and then exposed to LPS (10 μg/ml) for 6h as previously reported (Jiang Lv et al. 2015).

Reverse Transcriptase PCR (RT-PCR) and qRT-PCR.

Total RNA was extracted from the cells using an RNeasy Mini Kit (QIAGEN, Crawley, UK) according to manufacturer's directions. The total yield of RNA per extraction was calculated using a Nanovue spectrophotometer (GE Healthcare, Amersham, UK) to measure the absorbance at 260 nm. A260/A280 ratios of 1.9-2.1 indicated extraction of

good quality RNA. CDNA was synthesised from 2000 ng RNA using MMLV reverse transcriptase (Promega, Southampton, UK). PCR reactions were performed on HDPFs using GoTaq Polymerase (Promega) and the product specific primers-CD146, CD29, CD90, CD105, CD34 and CD45 listed in Table 1 under the following cycling conditions: 1 minutes denaturation at 95°C followed by 1 minutes annealing at 60°C and 1.5 minutes elongation at 72°C for 30 cycles. The housekeeping gene D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as positive control. PCR products were visualised under UV light following electrophoresing in 1.4% (w/v) agarose/TAE gel. For qRT-PCR readings, three separate cDNA samples were used and each measured in triplicate. Target-specific primers (Table 1) were added to each cDNA sample together with Precision MasterMix with ROX and SYBRgreen (PrimerDesign, Southampton, UK). Readings were taken using an ABI Prism fast 7500 qPCR machine (Advanced Biosystems, Birmingham, UK) under the following cycling conditions: an initial denaturation step of 95°C for 2 minutes followed by 40 cycles of 15 seconds denaturation (95°C) and 1 minute annealing/elongation at 60°C. The relative amount or fold change of the target gene expression was normalized relative to the level of GAPDH and relative to a control (non-induced cells).

Immunocytochemistry

HDPFs at the third passage were fixed with 4 % paraformaldehyde (PFA) for 30 min at room temperature and then incubated in PBS containing 0.4 % Triton X-100 for 10 min on ice and then blocked with 2 % bovine serum albumin (BSA) for 60 min at 37 °C. After the blocking step, the cells were incubated with primary antibody-anti-vimentin (1:100, Boster, Wuhan, China), anti-keratin (1:100, Boster) at 4 °C overnight; PBS was used as the negative control. The cells were then washed with PBS and incubated for 1 h with the secondary antibodies, namely anti-mouse IgG Alexa Fluor-488 or at anti-rabbit IgG Alexa Fluor-594 1:1000 at room temperature (Life Technologies, Paisley, UK). Glass cover slips were mounted using mounting media supplemented with DAPI stain (VectorLabs, Peterborough, UK) and preparations imaged under a fluorescent microscope (Olympus, Tokyo, Japan).

Western blot analysis

The total protein content was extracted from the cells by using lysis buffer containing protease inhibitors (Sigma-Aldrich, St Louis, MO, USA). The protein concentration was measured by using a BCA-200 protein assay kit (Pierce, San Francisco, CA, USA). Equal amounts of protein were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (PVDF). The membrane was blocked in TRIS-buffered saline with Tween (TBST) containing 5 % non-fat dry milk for 2 h and probed with primary antibodies-NLRP3 (1:500; CST, London, UK), caspase-1 (1:400; CST), IL-1β (1:1000; CST), IL-18 (1:500; CST) and β-actin (1:1000; CST) overnight at 4 °C and then incubated for 2h with a horseradish-peroxidase-conjugated anti-mouse IgG antibody or anti-rabbit IgG diluted 1:2000 (CST). Protein bands were visualized on X-ray film by using an enhance chemiluminescence system (GE Healthcare). The relative protein expression intensities were quantified by densitometry by using Quantity One analysis software.

DNA construction and cell transfection

For NLRP3 over-expression, the full-length human NLRP3 coding region was amplified by PCR and cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). Empty vectors (Up NC) were used as control of NLRP3 over-expression. siRNAs of NLRP3 were obtained from Ribo Biotechnology Company (Guangzhou, China) and were transfected into cells using Lipofectamine 2000 reagent (Invitrogen). Empty vectors (Down NC, Ribo) were used as control of NLRP3 down-expression.

The miR-223 mimic, mimic negative controls (mimic NC), miR-223 inhibitor and inhibitor negative controls (inhibitor NC) were obtained from Ribo Biotechnology Company (Guangzhou, China). HDPFs were transfected with miR-223 mimic (100 nM), mimic NC (100 nM), miR-223 inhibitor (100 nM) and inhibitor NC (100 nM) using Lipofectamine RNAiMAX transfection reagent (Life Technologies). Cells were collected at 48 h after transfection. The effect of miR-223 mimic and miR-223 inhibitor were determined by qRT-PCR.

Luciferase Reporter Assay

The wild type human NLRP3 3'UTR luciferase reporter vectors were constructed by amplifying the human NLRP3 mRNA 3'UTR and cloning it into the pMIR-REPORT™

Luciferase vector. Constructs with the CTGAC to GACTG NLRP3 mutation at the pupative binding site was also generated and used as the control. HDPFs were cotransfected with 80 ng luciferase reporter plasmid, 40 ng thymidine kinase promoter-Renilla luciferase reporter plasmid, and the miRNA-223 mimics or controls (final concentration, 10 nM). After 24 h, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions.

ELISA analysis

Upon termination of the treatments, the cell culture supernatants were isolated and stored at -80°C until use. The amount of IL-1β and IL-18 protein in the culture medium was quantified by using a ELISA kit (R&D, Minneapolis, MN, USA) following the manufacturer's protocol.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Each experiment was performed at least three times, unless otherwise indicated. Data are reported as the mean ± SE (standard error) deviation from three independent experiments. The significance of the differences between the experimental and the control groups was determined by using one-way analysis of variance (AVOVA) with Tukey-Kramer. Values of P<0.05 were considered statistically significant.

Results

Isolation and characterization of human dental pulp fibroblasts (HDPFs)

HDPFs were successfully isolated from the pulp tissue of the extracted third molars as described previously (Jiang et al. 2015). HDPFs obtained from the cell clones were characterized by immunocytochemical staining and RT-PCR. Immunocytochemical staining of HDPFs revealed that the cells positively expressed vimentin (Fig 1A) and were negative for keratin expression (Fig 1B). The results of RT-PCR revealed that HDPFs expressed a range of mesenchymal cell markers, including CD146, CD29, CD90 and CD105, but did not express the markers CD34 and CD45 (Fig 1C).

Gene expression of miR-223 and NLRP3 in human dental pulp tissues (HDPTs)

and HDPFs

The mRNA expression of miR-223 and NLRP3 in normal pulp and pulp with reversible pulpitis or irreversible pulpitis was evaluated by qRT-PCR. The mRNA expression of NLRP3 appeared to increase while the mRNA expression of miR-223 appeared to decrease with the transformation of pulpitis from reversible to irreversible compared to that in healthy pulp tissue (Fig 2A). Furthermore, the expression of miR-223 mRNA in HDPFs was examined in response to stimulation with ATP only, LPS only and ATP plus LPS by qRT-PCR. miR-223 mRNA was found to be decreased significantly in the ATP plus LPS-induced group and LPS-induced group compared to the control group (Fig 2B).

NLRP3 is a direct target of miR-223 in HDPFs.

Computational prediction via TargetScan 5.1 revealed that miR-223 was one of the nonconserved miRNAs that putatively target the human NLRP3 3'-UTR (Fig 3A). Wild-type NLRP3 or NLRP3 with a mutant 3'UTR was cloned into luciferase reporters, and then luciferase reporter assays were performed in HDPFs. Cells were transfected with a wild-type or 3'UTR-mutant NLRP3 luciferase reporter plasmid and a miR-223 mimic. The results revealed that the group with overexpression of miR-223 had markedly decreased luciferase activity compared to the control group. However, no change in luciferase activity was observed in cells transfected with the 3'-UTR-mutant NLRP3 construct (Fig 3B).

Effects of miR-223 on NLRP3 and ATP plus LPS-induced NLRP3/CASP1 inflammasome pathway activation in HDPFs.

Based on the present finding that miR-223 directly targets NLRP3, the effects of miR-223 on ATP plus LPS-induced NLRP3/CASP1 inflammasome pathway activation in HDPFs were examined. Increased and decreased miR-223 mRNA levels were noted in the HDPFs transfected with miR-223 mimic and miR-223 inhibitor compared to mimic NC and inhibitor NC, respectively (Fig 4A). The mRNA and protein expression of NLRP3, IL-1β and IL-18 in HDPFs were examined in response to ATP and LPS by qRT-PCR and western blotting. The protein levels of caspase-1 were analyzed by western blotting, and caspase-1 activation was assessed by measuring caspase-1 p20.

The release of IL-1β and IL-18 was analyzed by ELISA. Cells stimulated with ATP plus LPS had upregulated NLRP3, IL-1β and IL-18 mRNA (Fig 4B-D) and protein expression compared with untreated cells, as well as upregulated caspase-1 p20 protein expression (Fig 4E-L), and featured increased secretion of IL-1β and IL-18 (Fig 4M, N). In contrast, the miR-223 mimic significantly inhibited the increased levels of NLRP3, caspase-1 p20, IL-1β and IL-18 induced by ATP plus LPS, as well as the secretion of IL-1β and IL-18 (Fig 4B-N). The miR-223 inhibitor further increased ATP plus LPS-induced expression of NLRP3, caspase-1 p20, IL-1β and IL-18, as well as the secretion of IL-1β and IL-18 (Fig 4B-N).

Effects of NLRP3 on ATP plus LPS-induced NLRP3/CASP1 inflammasome pathway activation in HDPFs.

Cells stimulated with ATP plus LPS had upregulated NLRP3, IL-1β and IL-18 mRNA expression compared with untreated cells (Fig 5A-C), as well as NLRP3 and caspase-1 p20 protein expression (Fig 5D-G), and featured increased secretion of IL-1β and IL-18 (Fig 5H, I). Overexpression of NLRP3 further promoted ATP plus LPS-induced expression of NLRP3, caspase-1 p20, IL-1β and IL-18, as well as the secretion of IL-1β and IL-18 (Fig 5A-I). In contrast, knockdown of NLRP3 inhibited ATP plus LPS-induced levels of NLRP3, caspase-1 p20, IL-1β and IL-18, as well as the secretion of IL-1β and IL-18 (Fig 5A-I).

Overexpression of miR-223 prohibited the activation of the NLRP3/CASP1 inflammasome pathway induced by ATP plus LPS, which was alleviated by upregulating NLRP3 in HDPFs.

The miR-223 mimic significantly inhibited ATP plus LPS-induced levels of NLRP3, caspase-1 p20, IL-1β and IL-18 (Fig 6A-G), as well as the secretion of IL-1β and IL-18 (Fig 6H, I). Overexpression of NLRP3 promoted ATP plus LPS-induced expression of NLRP3, caspase-1 p20, IL-1β and IL-18, as well as the secretion of IL-1β and IL-18 (Fig 6A-I). Additionally, the miR-223 mimic decreased the expression of NLRP3, caspase-1 p20, IL-1β and IL-18 induced by ATP plus LPS, which was reversed by upregulating NLRP3 in HDPFs (Fig 6A-I).

Knockdown of miR-223 promoted the activation of the NLRP3/CASP1

inflammasome pathway induced by ATP plus LPS, which was attenuated by downregulating NLRP3 in HDPFs.

The miR-223 inhibitor significantly enhanced ATP plus LPS-induced levels of NLRP3, caspase-1 p20, IL-1β and IL-18 (Fig 7A-G), as well as the secretion of IL-1β and IL-18 (Fig 7H-I). Downregulation of NLRP3 inhibited ATP plus LPS-induced expression of NLRP3, caspase-1 p20, IL-1β and IL-18, as well as the secretion of IL-1β and IL-18 (Fig 7A-I). Additionally, the miR-223 inhibitor increased the expression of NLRP3, caspase-1 p20, IL-1β and IL-18 induced by ATP plus LPS, which was attenuated by downregulating NLRP3 in HDPFs (Fig 7A-I).

Discussion

Cariogenic bacteria invade dentine and pulp tissues, triggering tissue immune/inflammatory responses, as well as regenerative responses (Farges et al. 2015). Frequently, the inflammatory and regenerative processes within the diseased pulp appear to be both distinct and antagonistic (Cooper et al. 2014). However, increasing evidence is now emerging that indicates that these two processes have a significant interrelationship (Cooper et al. 2014). Low-grade inflammation may promote regenerative mechanisms, including angiogenic and stem/progenitor cell processes, whereas more intense inflammation may impede regeneration and further lead to extracellular matrix breakdown, as well as the death of resident cells (Farges et al. 2015). Therefore, maintaining a fine balance between these two processes is of vital importance to determine the fate of dentine and pulp tissue. A wide range of immune and nonimmune cells within pulp tissue have been demonstrated to be capable of detecting bacterial components via PRRs, thus orchestrating an inflammatory response (Renard et al. 2016, Cooper et al. 2017). Odontoblasts located at the periphery of the pulp are the first cells to encounter the infectious process (Durand et al. 2006, Veerayutthwilai et al. 2007). As the infection advances, cells deeper in the pulp core, including pulp fibroblasts, endothelial cells, and stem cells, are also involved in defense responses (Gaudin et al. 2015, Renard et al. 2016). It has been reported that several TLRs (including TLR-1-TLR-6 and TLR-9) and NLRs (including NOD1, NOD2, and NLRP3) are upregulated during pulpal inflammation (Chang et al. 2005, Zampetaki et

al. 2006, Veerayutthwilai et al. 2007, Farges et al. 2009, Hirao et al. 2009, Farges et al. 2011, Staquet et al. 2011, Song et al. 2012). Our previous study showed that HDPFs in inflamed pulp tissue contain more functional NLRP3/CASP1 inflammasome than those in normal tissue and that the NLRP3/CASP1 inflammasome pathway is activated in irreversible pulpitis in vivo (Jiang et al. 2015). Additionally, ATP activates the $P2X_7$ receptor on the cell membrane triggering K^+ efflux and inducing the gradual recruitment of the membrane pore pannexin-1 in HDPFs. Extracellular LPS is able to penetrate the cytosol and upregulate NLRP3 and pro-IL-1β expression via TLR4/MyD88/NF-κB pathway. ATP promotes ROS production which further activates NLRP3 inflammasome. The activation of NLRP3/CASP1 inflammasome processes pro-IL-1β into its mature form (Jiang et al. 2015, Zhang et al. 2015). These findings indicate that the activation of the NLRP3/CASP1 inflammasome pathway plays an important role in the progression of pulpitis. Therefore, regulatory strategies targeting NLRP3 provide a basis for the development of novel molecular therapeutics to control the level of pulpal inflammation.

Increasing evidence has reported that miRNAs play key roles in modulating immune responses and that their dysregulation contributes to inflammatory diseases (Acuna et al. 2020). Zhong et al. (2012) reported that thirty-three miRNAs were down-regulated in inflamed pulps as compared with normal pulps, which highlights miRNAs play intricate and specific roles in pulpal inflammation and immunity. However, due to the limitations of small sample size and low RNA yields in that study, several important immuno-miRNAs remain unclear. Belonging to the class of immuno-miRs, miR-223 was demonstrated to be involved in the regulation of hematopoiesis, immune responses and different types of inflammation disorders (Aziz 2016, Yuan et al. 2018). Located on the X chromosome and transcribed independently from any known gene, miR-223 is preferentially expressed by hematopoietic cells, especially cells of bone marrow and myeloid origin, but absent in T and B cells (Chen et al. 2004). Li et al. (2010) demonstrated that overexpression of miR-223 decreased the expression of IκB kinase (IKKa) by targeting its mRNA and enhancing p52 protein production, thus promoting NF-κB signaling-mediated inflammation during monocyte-macrophage differentiation

in innate immune responses. Additionally, knockdown of miR-223 promoted TLR-triggered IL-6 and IL-1β production in macrophages by targeting STAT3 (Chen et al. 2012). Upregulation of NLRP3 and downregulation of miR-223 occurred during the transformation of reversible pulpitis into irreversible pulpitis in vivo. In addition, ATP plus LPS stimulation reduced miR-223 expression in HDPFs. This finding is consistent with the data reported by Haneklaus et al. (2012), in which miR-223 expression was inversely correlated with NLRP3 during macrophage differentiation. However, it is inconsistent with the findings presented by Bauernfeind et al. (2012), in which miR-223 was highly expressed in the myeloid cell lineage and its expression was not significantly changed by inflammasome-priming stimuli, such as LPS or proinflammatory cytokines. Computational prediction via TargetScan 5.1 and luciferase reporter assays further confirmed that the 3'UTR of NLRP3 was a direct target site of miR-223 in HDPFs. Experiments using a miR-223 mimic and inhibitor revealed that miR-223 could regulate the ATP plus LPS-induced proinflammatory cytokine expression mediated by the NLRP3/CASP1 inflammasome pathway by targeting NLRP3 in HDPFs. Previous findings from Wan et al. (2018) demonstrated that overexpression of miR-223 inhibited hepatocellular carcinoma (HCC) cell proliferation and promoted apoptosis by directly targeting NLRP3, which provided evidence of the association between innate immune responses and the genesis of HCC. The present study has provided insight into the negative regulatory effect of miR-223 on NLRP3/CASP1 inflammasome-mediated innate immune responses in HDPFs during the development of pulpal inflammation.

Proinflammatory cytokines and chemokines, which are produced and secreted by immune and nonimmune cells within pulp, generate an intricate signaling network and are thus involved in regulating the processes of pulpal inflammation and regeneration (Khorasani et al. 2020). To date, a large number of proinflammatory cytokines and chemokines, such as IL-8, IL-6, IL-1, CCL2, and CCL5, have been identified in diseased dental tissue and exhibit regulatory functions, including lymphocyte recruitment, angiogenesis, stem cell proliferation, migration, and differentiation (Khorasani et al. 2020). As a well-recognized key regulator in inflammation of the

dental pulp, IL-1β can regulate angiogenesis, activate neutrophils and macrophages to defend against infection, and mediate periapical bony destruction (Morsani et al. 2011, Nakanishi et al. 2015, Chang et al. 2016). Additionally, IL-1β has also been shown to stimulate collagen synthesis and inhibit the proliferation of pulp cells in a COX-dependent manner, which suggests its role in pulpal repair (Lertchirakarn et al. 1998). IL-1β is synthesized as an inactive cytoplasmic precursor (called pro-IL-1β) that is cleaved into the biologically active form (called mature IL-1β) in response to proinflammatory stimuli (Schroder & Tschopp 2010). The maturation and release of IL-1β require certain cysteine proteases (Schroder & Tschopp 2010). The NLRP3/CASP1 inflammasome is one of the primary pathways that can regulate the maturation of IL-1β (Schroder & Tschopp 2010). In the present study, miR-223 functioned as an efficient regulator to control the ATP plus LPS-induced production and secretion of the proinflammatory cytokines IL-1β and IL-18 mediated by the NLRP3/CASP1 inflammasome pathway by targeting NLRP3 in HDPFs. Overexpression of NLRP3 amplified the secretion of IL-1β and IL-18, which could be reversed by upregulation of miR-223. Knockdown of NLRP3 decreased the secretion of IL-1β and IL-18, which could be restored by downregulation of miR-223. The findings contribute to expanding the knowledge of immuno-miR-223, which serves as an effective regulator in the control of pulpal immune/inflammatory responses to confer protection against excess inflammation and tissue damage as well as to promote pulp tissue healing and dentine repair. miRNAs research provides a unique angle for studying the interrelationship and interaction between the immune/inflammatory and regenerative responses in the infected dental pulp. The role of miRNAs in bacterial pathogen infection has greatly enhanced understanding of cellular physiology and immunology. However, due to the complexity of gene regulatory networks, one miRNA may regulate several different targets in different stages during bacterial infection. Hence, the elucidation of the precise mechanism underling the regulatory function of miRNAs on host–pathogen interactions may provide novel, effective and rational therapeutic strategies.

Conclusions

This study demonstrated the upregulation of NLRP3 and downregulation of miR-223 during the transformation of reversible pulpitis into irreversible pulpitis in vivo. Additionally, miR-223, as a negative regulator, was involved in the control of the ATP plus LPS-induced production and secretion of the proinflammatory cytokines IL-1β and IL-18 mediated by the NLRP3/CASP1 inflammasome pathway by targeting NLRP3 in HDPFs. The finding of a novel functional and mechanical miR-223-NLRP3 axis provides insight into the potential regulatory effects of miRNAs on the NLRP3 inflammasome and opens up novel potential therapeutic avenues for future endodontic treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

Acuna SM, Floeter-Winter LM, Muxel SM (2020) MicroRNAs: Biological Regulators in Pathogen-Host Interactions. *Cells* **9**(1): 113.

Aziz F (2016) The emerging role of miR-223 as novel potential diagnostic and therapeutic target for inflammatory disorders. *Cellular Immunology* **303,** 1-6.

Bauernfeind F, Rieger A, Schildberg FA, Knolle PA, Schmid-Burgk JL, Hornung V (2012) NLRP3 inflammasome activity is negatively controlled by miR-223. *Journal of Immunology* **189**(8)**,** 4175-81.

Chang J, Zhang C, Tani-Ishii N, Shi S, Wang CY (2005) NF-kappaB activation in human dental pulp stem cells by TNF and LPS. *Journal of Dental Research* **84**(11)**,** 994-8.

Chang MC, Tsai YL, Chang HH *et al.* (2016) IL-1beta-induced MCP-1 expression and secretion of human dental pulp cells is related to TAK1, MEK/ERK, and PI3K/Akt signaling pathways. *Archives of Oral Biology* **61,** 16-22.

Chen CZ, Li L, Lodish HF, Bartel DP (2004) MicroRNAs modulate hematopoietic lineage differentiation. *Science* **303**(5654)**,** 83-6.

Chen Q, Wang H, Liu Y *et al.* (2012) Inducible microRNA-223 down-regulation promotes TLR-triggered IL-6 and IL-1beta production in macrophages by targeting STAT3. *Plos One* **7**(8)**,** e42971.

Cooper PR, Chicca IJ, Holder MJ, Milward MR (2017) Inflammation and Regeneration in the Dentin-

pulp Complex: Net Gain or Net Loss? *Journal of Endodontics* **43**(9S)**,** S87-S94.

Cooper PR, Holder MJ, Smith AJ (2014) Inflammation and regeneration in the dentin-pulp complex: a double-edged sword. *Journal of Endodontics* **40**(4 Suppl)**,** S46-51.

Cooper PR, Takahashi Y, Graham LW, Simon S, Imazato S, Smith AJ (2010) Inflammation-regeneration interplay in the dentine-pulp complex. *Journal of Dentistry* **38**(9)**,** 687-97.

Durand SH, Flacher V, Romeas A *et al.* (2006) Lipoteichoic acid increases TLR and functional chemokine expression while reducing dentin formation in in vitro differentiated human odontoblasts. *Journal of Immunology* **176**(5)**,** 2880-7.

Farges JC, Alliot-Licht B, Renard E *et al.* (2015) Dental Pulp Defence and Repair Mechanisms in Dental Caries. *Mediators of Inflammation* **2015,** 230251.

Farges JC, Carrouel F, Keller JF *et al.* (2011) Cytokine production by human odontoblast-like cells upon Toll-like receptor-2 engagement. *Immunobiology* **216**(4)**,** 513-7.

Farges JC, Keller JF, Carrouel F *et al.* (2009) Odontoblasts in the dental pulp immune response. *Journal of Experimental Zoology part B Molecular and Developmental Evolution* **312B**(5)**,** 425-36.

Gaudin A, Renard E, Hill M *et al.* (2015) Phenotypic analysis of immunocompetent cells in healthy human dental pulp. *Journal of Endodontics* **41**(5)**,** 621-27.

Hahn CL, Liewehr FR (2007) Innate immune responses of the dental pulp to caries. *Journal of Endodontics* **33**(6)**,** 643-51.

Haneklaus M, Gerlic M, Kurowska-Stolarska M *et al.* (2012) Cutting edge: miR-223 and EBV miR-BART15 regulate the NLRP3 inflammasome and IL-1beta production. *Journal of Immunology* **189**(8)**,** 3795-9.

Hirao K, Yumoto H, Takahashi K, Mukai K, Nakanishi T, Matsuo T (2009) Roles of TLR2, TLR4, NOD2, and NOD1 in pulp fibroblasts. *Journal of Dental Research* **88**(8)**,** 762-7.

Horst OV, Horst JA, Samudrala R, Dale BA (2011) Caries induced cytokine network in the odontoblast layer of human teeth. *BMC Immunology* **12,** 9.

Jiang W, Lv H, Wang H *et al.* (2015) Activation of the NLRP3/caspase-1 inflammasome in human dental pulp tissue and human dental pulp fibroblasts. *Cell and Tissue Research* **361**(2)**,** 541-55.

Khorasani MMY, Hassanshahi G, Brodzikowska A, Khorramdelazad H (2020) Role(s) of cytokines in pulpitis: Latest evidence and therapeutic approaches. *Cytokine* **126,** 154896.

Lee YY, Chan CH, Hung SL, Chen YC, Lee YH, Yang SF (2011) Up-regulation of nucleotide-binding

oligomerization domain 1 in inflamed human dental pulp. *Journal of Endodontics* **37**(10)**,** 1370-5.

Lertchirakarn V, Birner R, Messer HH (1998) Effects of interleukin-1 beta on human pulpal fibroblast proliferation and collagen synthesis. *Journal of Endodontics* **24**(6)**,** 409-13.

Li T, Morgan MJ, Choksi S, Zhang Y, Kim YS, Liu ZG (2010) MicroRNAs modulate the noncanonical transcription factor NF-kappaB pathway by regulating expression of the kinase IKKalpha during macrophage differentiation. *Nature Immunology* **11**(9)**,** 799-805.

Lin ZM, Song Z, Qin W *et al.* (2009) Expression of nucleotide-binding oligomerization domain 2 in normal human dental pulp cells and dental pulp tissues. *Journal of Endodontics* **35**(6)**,** 838-42.

Miller CM, Boulter NR, Fuller SJ *et al.* (2011) The role of the P2X(7) receptor in infectious diseases. *Plos Pathogens* **7**(11)**,** e1002212.

Morsani JM, Aminoshariae A, Han YW, Montagnese TA, Mickel A (2011) Genetic predisposition to persistent apical periodontitis. *Journal of Endodontics* **37**(4)**,** 455-59.

Nakanishi T, Mukai K, Hosokawa Y, Takegawa D, Matsuo T (2015) Catechins inhibit vascular endothelial growth factor production and cyclooxygenase-2 expression in human dental pulp cells. *International Endodontic Journal* **48**(3)**,** 277-82.

Rathinam VAK, Zhao Y, Shao F (2019) Innate immunity to intracellular LPS. *Nature Immunology* **20**(5)**,** 527-33.

Renard E, Gaudin A, Bienvenu G *et al.* (2016) Immune Cells and Molecular Networks in Experimentally Induced Pulpitis. *Journal of Dental Research* **95**(2)**,** 196-205.

Schroder K, Tschopp J (2010) The inflammasomes. *Cell* **140**(6)**,** 821-32.

Song Z, Lin Z, He F *et al.* (2012) NLRP3 is expressed in human dental pulp cells and tissues. *Journal of Endodontics* **38**(12)**,** 1592-97.

Staquet MJ, Carrouel F, Keller JF *et al.* (2011) Pattern-recognition receptors in pulp defense. *Advances in Dental Research* **23**(3)**,** 296-301.

Taibi F, Metzinger-Le Meuth V, Massy ZA, Metzinger L (2014) miR-223: An inflammatory oncomiR enters the cardiovascular field. *Biochimica et Biophysica Acta* **1842**(7)**,** 1001-9.

Veerayutthwilai O, Byers MR, Pham TT, Darveau RP, Dale BA (2007) Differential regulation of immune responses by odontoblasts. *Oral Microbiology and Immunology* **22**(1)**,** 5-13.

Wan L, Yuan X, Liu M, Xue B (2018) miRNA-223-3p regulates NLRP3 to promote apoptosis and inhibit proliferation of hep3B cells. *Experimental and Therapeutic Medicine* **15**(3)**,** 2429-35.

Yuan X, Berg N, Lee JW *et al.* (2018) MicroRNA miR-223 as regulator of innate immunity. *Journal Leukocyte Biology* **104**(3)**,** 515-24.

Zampetaki A, Xiao Q, Zeng L, Hu Y, Xu Q (2006) TLR4 expression in mouse embryonic stem cells and in stem cell-derived vascular cells is regulated by epigenetic modifications. *Biochemical and Biophysical Research Communications* **347**(1)**,** 89-99.

Zhang A, Wang P, Ma X *et al.* (2015) Mechanisms that lead to the regulation of NLRP3 inflammasome expression and activation in human dental pulp fibroblasts. *Molecular Immunology* **66**(2)**,** 253-62.

Zhong S, Zhang S, Bair E, Nares S, Khan AA (2012) Differential expression of microRNAs in normal and inflamed human pulps. *Journal of Endodontics* **38**(6)**,** 746-52.

Zhou X, Li X, Wu M (2018) miRNAs reshape immunity and inflammatory responses in bacterial infection. *Signal Transduction and Targeted Therapy* **3,** 14.

Table 1 Primer sequences.

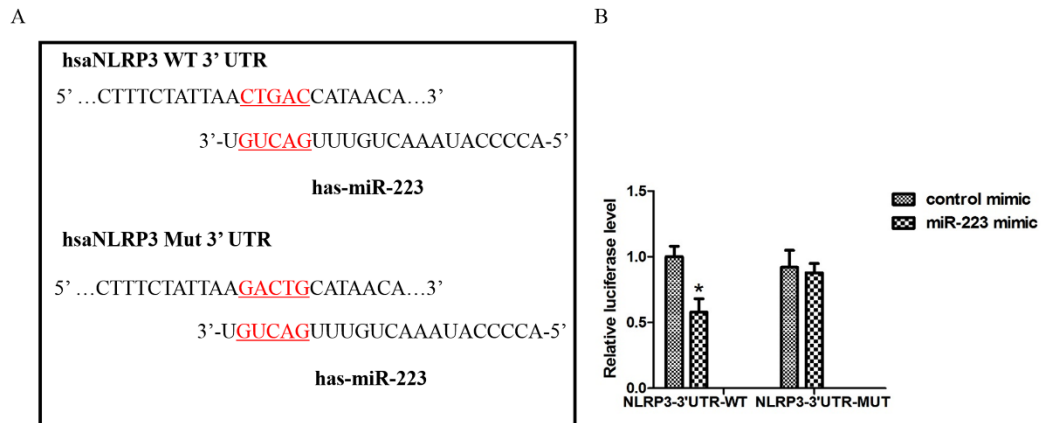| Genes	Forward and reverse primers	Accession number
GAPDH	5'-GCACCGTCAAGGCTGAGAAC-3'	NM_002046.3
	5'-TGGTGAAGACGCCAGTGGA-3'	
CD146	5'-CATCGTGGCTGTGATTGTG-3'	NM_006500.3
	5'-TTCTGGGAGCTTATCTGACTTA-3'	
CD29	5'-AATGTAACCAACCGTAGC-3'	NM_002211.4
	5'-CAGGTCCATAAGGTAGTAGAG-3'	
CD90	5'-TAGTGGACCAGAGCCTTCG-3'	NM_006288.3
	5'-TTCGGGAGCGGTATGTG-3'	
CD105	5'-CACTAGCCAGGTCTCGAAGGG-3'	NM_001114753.2
	5'-CTGTTTACACTGAGGACCAGAAGC-3'	
CD34	5'-GTCTTCCACTCGGTGCGTCTC-3'	NM_001025109.1
	5'-GTTCCCTGGGTAGGTAACTCT-3'	
CD45	5'-ATTGCGATTTCCGTGTAA-3'	NM_001267798.2
	5'-CAAGCAGGGCTATTGATG-3'	
miR-223	5'-ACACTCCAGCTGGGTGTCAGTTTGTCAAAT-3'	NC_000023.11
	5'- TGGTGTCGTGGAGTCG-3'	
NLRP3	5'- ATTCGGAGATTGTGGTTGGG-3'	XM_017000183.1
	5'- GAGGGCGTTGTCACTCAGGT-3'	
IL-1β	5'- GAATCTCCGACCACCACTAC-3'	NM_000576.3
	5'- CACATAAGCCTCGTTATCCC-3'	
IL-18	5'- AGATAGCCAGCCTAGAGGTA-3'	NM_001243211.2
	5'- TTATCAGGAGGATTCATTTC-3'	

Figure Legends
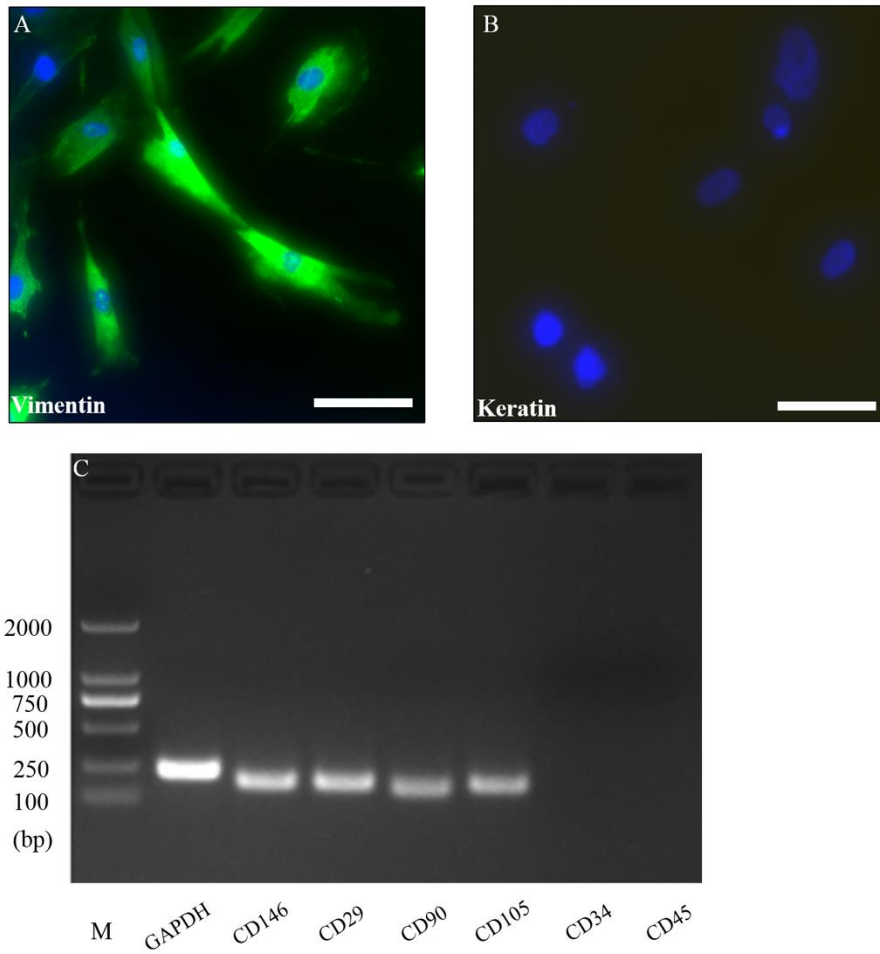
Figure 1. Isolation and characterization of human dental pulp fibroblasts (HDPFs)

The characterization of HDPFs by immunocytochemical staining and RT-PCR; positive immunostaining for vimentin (A); negative immunostaining for keratin (B); positive for the markers CD146, CD29, CD90, CD105; and negative for the markers CD34 and CD45 (C). Each experiment was performed three times. Bar: 50 μm.
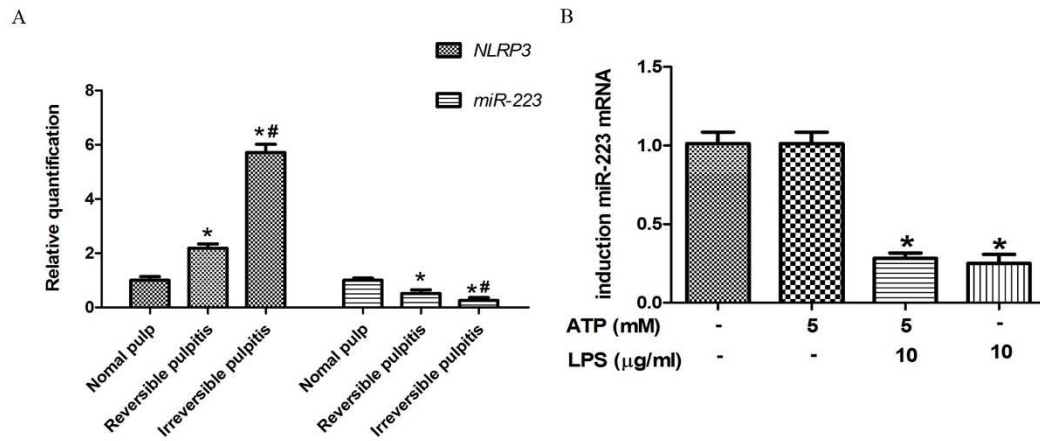
Figure 2. The expression of miR-223 and NLRP3 in HDPFs and HDPTs.

The mRNA expression of miR-223, and NLRP3 in HDPFs and HDPTs was analyzed by qRT-PCR (A, B). Each experiment was performed three times. Statistical analysis was performed using one-way ANOVA. The data are shown as the mean ± SE. *P < 0.05 compared with the control group. #P < 0.05 compared with the reversible pulpitis group.

A

hsaNLRP3 WT 3' UTR

5'…CTTTCTATTAACTGACCATAACA…3'

3'-UGUCAGUUUGUCAAAUACCCCA-5'

has-miR-223

hsaNLRP3 Mut 3' UTR

5'…CTTTCTATTAAGACTGCATAACA…3'

3'-UGUCAGUUUGUCAAAUACCCCA-5'

has-miR-223

B

**Figure 3. miR-223 directly targets NLRP3**

The targeting site in the 3' UTR of NLRP3 and the corresponding mutant sequence (A). Relative luciferase activity of cells after cotransfection with wild-type (WT) or mutant (MUT) NLRP3 3' UTR luciferase reporter vector and the miR-223 mimic or control mimic (B). Each experiment was performed three times. Statistical analysis was performed using one-way ANOVA. The data are shown as the mean ± SE. *P < 0.05 when compared with the miR-con group.
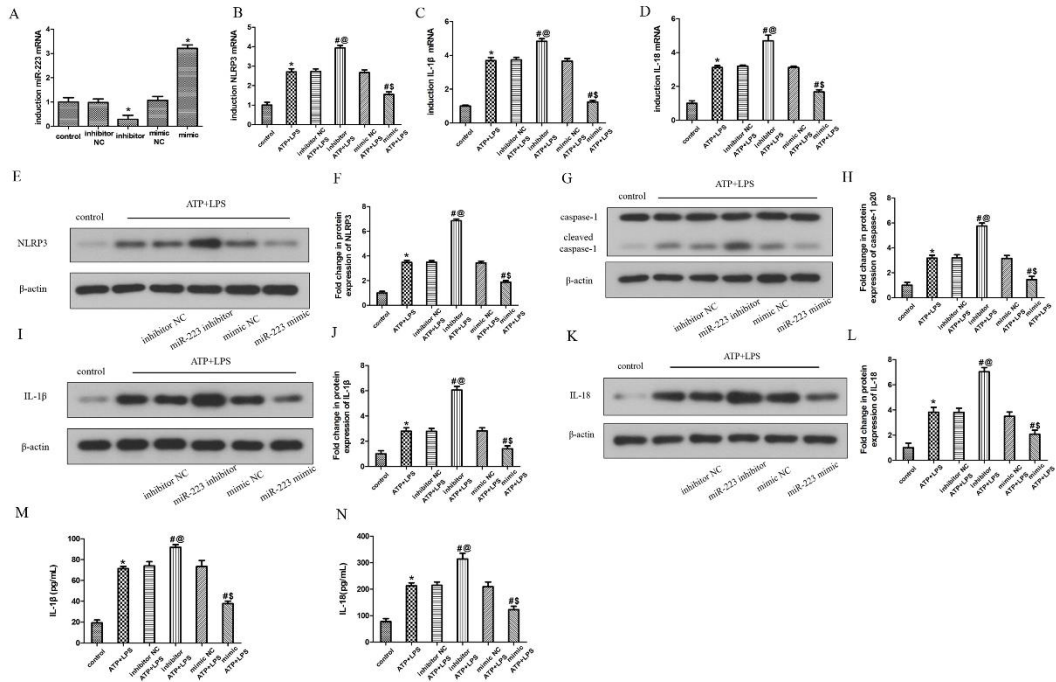
Figure 4. miR-223 is a negative regulator of ATP plus LPS-induced NLRP3/CASP1 inflammasome pathway activation in HDPFs.

HDPFs were induced with ATP plus LPS and transfected with or without the miR-223 mimic, mimic NC, miR-223 inhibitor, and inhibitor NC. The mRNA expression of miR-223, NLRP3, IL-1β and IL-18 was analyzed by qRT-PCR (A-D). The protein expression of NLRP3, caspase-1, IL-1β, IL-18 and β-actin was analyzed by western blotting, and the relative band intensities were determined by densitometry (E-L). The release of IL-1β and IL-18 was analyzed by ELISA (M, N). Each experiment was performed three times. Statistical analysis was performed using one-way ANOVA. The data are shown as the mean ± SE. *P < 0.05 compared with the control group. #P < 0.05 compared with the ATP plus LPS-induced group. @P < 0.05 compared with the inhibitor NC ATP plus LPS-induced group. $P < 0.05 compared with the mimic NC ATP plus LPS-induced group.
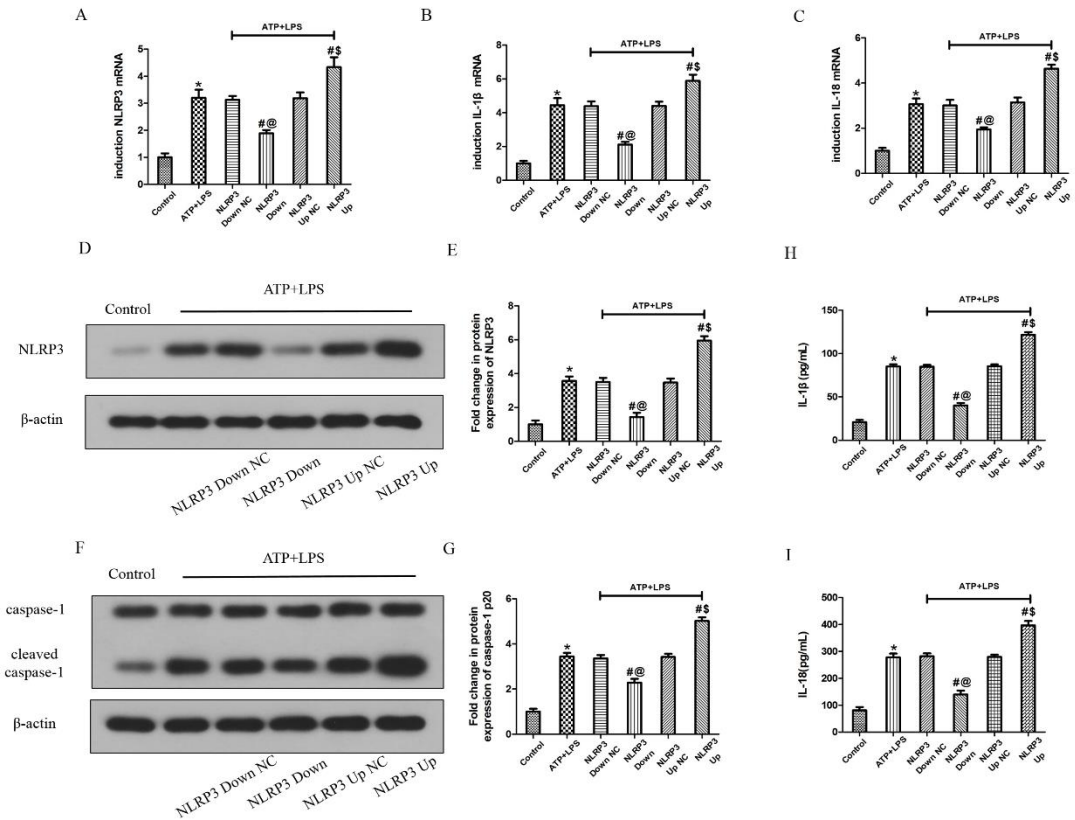
Figure 5. Effects of NLRP3 on ATP plus LPS induced NLRP3/CASP1 inflammaome pathway activation in HDPFs.

HDPFs were induced by ATP plus LPS for 6h with or without NLRP3 downregulation or upregulation. The mRNA expression of NLRP3, IL-1β and IL-18 was analyzed by qRT-PCR (A-C). The protein expression of NLRP3, caspase-1 and β-actin was analyzed by western blotting, and the relative band intensities were determined by densitometry (D-G). The release of IL-1β and IL-18 was analyzed by ELISA (H, I). Each experiment was performed three times. Statistical analysis was performed using one-way ANOVA. The data are shown as the mean ± SE. *$P < 0.05$ compared with the control group. #$P < 0.05$ compared with the ATP plus LPS-induced group. @$P < 0.05$ compared with the NLRP3 Down NC group. $$P < 0.05$ compared with the NLRP3 Up NC group.
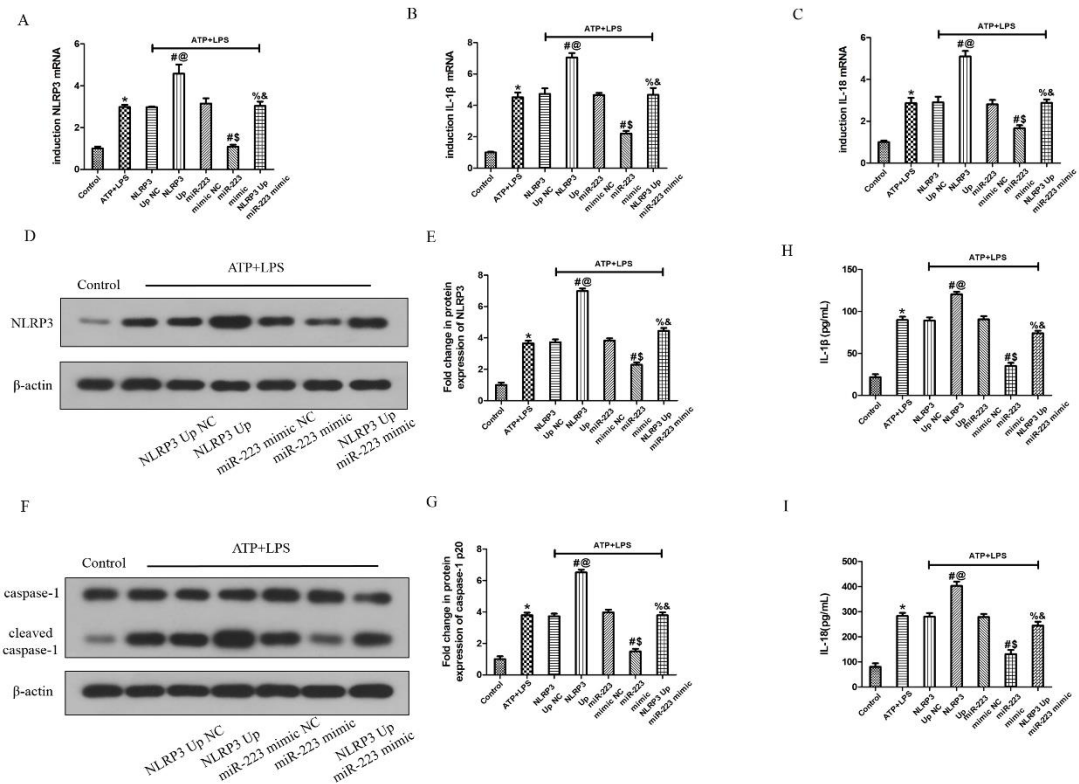
Figure 6. Overexpression of miR-223 prohibited the activation of the NLRP3/CASP1 inflammasome pathway induced by ATP plus LPS, which was alleviated by upregulating NLRP3 in HDPFs.

HDPFs were stimulated by ATP plus LPS for 6 h with or without miR-223 mimic or NLRP3 upregulation. The mRNA expression of NLRP3, IL-1β and IL-18 was analyzed by qRT-PCR (A-C). The protein expression of NLRP3, caspase-1 and β-actin was analyzed by western blotting, and the relative band intensities were determined by densitometry (D-G). The release of IL-1β and IL-18 was analyzed by ELISA (H, I). Each experiment was performed three times. Statistical analysis was performed using one-way ANOVA. The data are shown as the mean ± SE. *P < 0.05 compared with the control group. #P < 0.05 compared with the ATP plus LPS-induced group. @P < 0.05 compared with the NLRP3 Up NC group. $P < 0.05 compared with the miR-223 mimic NC group. %P < 0.05 compared with the NLRP3 Up group. &P < 0.05 compared with the miR-223 mimic group.
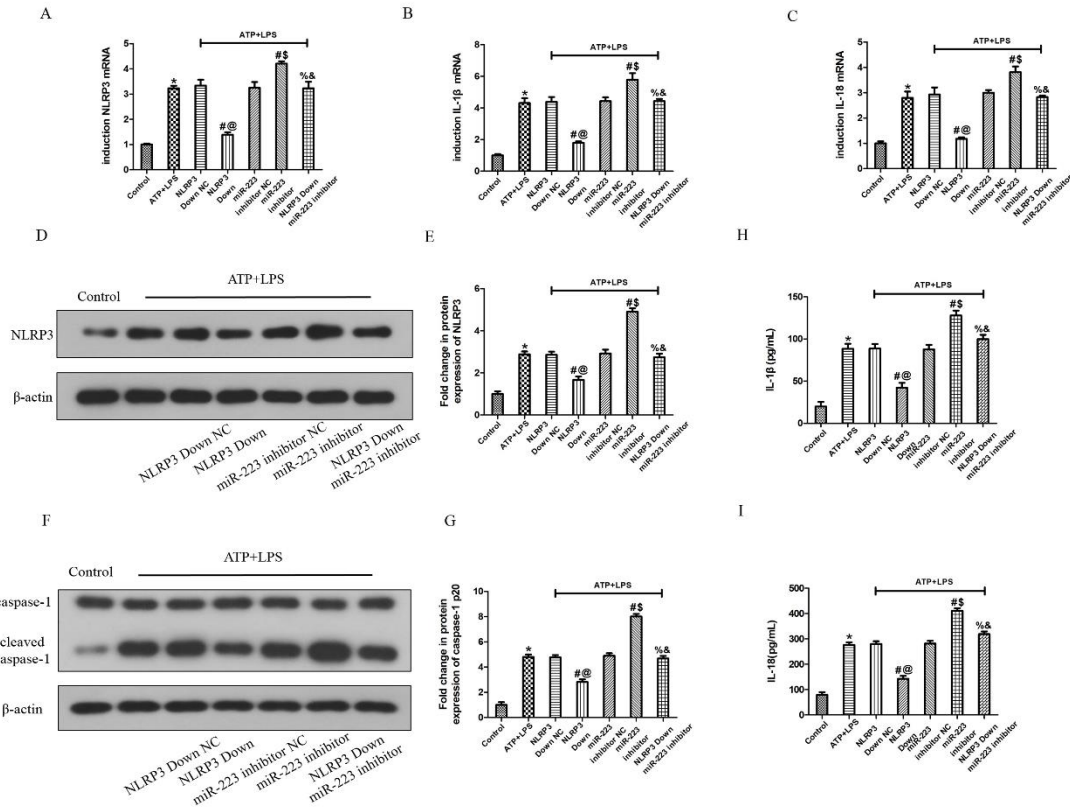
Figure 7. Knockdown of miR-223 stimulated the activation of the NLRP3/CASP1 inflammasome pathway induced by ATP plus LPS which was attenuated by downregulaing NLRP3 in HDPFs.

HDPFs were stimulated by ATP plus LPS for 6h with or without miR-223 inhibitor or NLRP3 downregulation. The mRNA expression of NLRP3, IL-1β and IL-18 was analyzed by qRT-PCR (A-C). The protein expression of NLRP3, caspase-1 and β-actin was analyzed by western blotting, and the relative band intensities were determined by densitometry (D-G). The release of IL-1β and IL-18 was analyzed by ELISA (H, I). Each experiment was performed three times. Statistical analysis was performed using one-way ANOVA. The data are shown as the mean ± SE. *P < 0.05 compared with the control group. #P < 0.05 compared with the ATP plus LPS-induced group. @P < 0.05 compared with the NLRP3 Down NC group. $P < 0.05 compared with the miR-223 inhibitor NC group. %P < 0.05 compared with the NLRP3 Down group. &P < 0.05 compared with the miR-223 inhibitor group.