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LncRNA MEG3 inhibits the inflammatory response of ankylosing spondylitis by targeting miR-146a

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Abstract

Ankylosing spondylitis (AS) is a progressive systemic disease characterized by chronic inflammation response of the sacroiliac joint and spine. Long non-coding RNAs (lncRNAs) are widely involved in the regulation of various diseases. However, the role of lncRNA maternally expressed gene 3 (MEG3) in the inflammatory response of AS has not been studied. Enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of inflammatory cytokines interleukin-1β (IL-1β) and interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in tissues and cells. The expression levels of MEG3, microRNA-146a (miR-146a), and inflammatory cytokines were measured by quantitative real-time PCR (qRT-PCR). Correlation between MEG3 or miR-146a and inflammatory cytokines was analyzed by Pearson analysis. Dual-luciferase reporter and RNA immunoprecipitation (RIP) assays were used to clarify the interaction between MEG3 and miR-146a. MEG3 was downregulated in AS patients, negatively correlated with the levels of IL-1β, IL-6, and TNF-α, and blocked the inflammatory response of AS. MiR-146a was upregulated in AS patients and could interact with MEG3. The expression of miR-146a was positively correlated with IL-1β, IL-6, and TNF-α levels. Overexpression of miR-146a reversed the inhibitory effect of abnormal MEG3 expression on inflammatory cytokines. LncRNA MEG3 plays an anti-inflammatory role in AS partially through targeting miR-146a, which provides a potential new means for the treatment of AS patients.

Keywords Ankylosing spondylitis · MEG3 · miR-146a · Inflammatory response · Cytokines

Introduction

Ankylosing spondylitis (AS) is a common chronic inflammatory disease, which belongs to the category of rheumatoid arthritis [1, 2]. It could lead to damage of the structure and function of patients' joints and reduce the quality of life in patients [3]. The early stage treatment of AS mainly focuses on controlling inflammation and preventing the malformation of the lesion site, and surgical correction can be carried out in the later stage [4, 5]. Although considerable progress has been made in the treatment of AS, the mortality rate of AS is still very high [6]. Therefore, it is urgent to explore new treatment strategies for AS.

Long non-coding RNAs (lncRNAs, > 200 nucleotides) have been proved to regulate gene expression at multiple levels and are considered as crucial regulatory factors for the development and progress of human diseases [7, 8]. It mainly acts as a competitive endogenous RNA (ceRNAs) interacting with microRNAs (miRNAs) to inhibit the expression of target genes [9]. Microarray analysis showed that 520 lncRNAs were differentially expressed after osteogenic induction of mesenchymal stem cells in AS patients [10]. Researches indicated that lncRNA AK001085 could function as an independent marker in the diagnosis of AS [11]. LncRNA LINC00311 was associated with predicted outcomes and recurrence of AS [12]. Besides, lncRNA TUG1 was related to disease activity, course of treatment, and rehospitalization rate [13]. Therefore, the functional analysis

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of lncRNAs may contribute to the treatment and prediction of many diseases.

Maternally expressed gene 3 (MEG3) is an imprinted gene located at 14q32, which was originally found to be involved in the occurrence and development of meningioma as a tumor suppressor gene [14, 15]. Later, with further research, it was found that lncRNA MEG3 was expressed in many diseases and widely involved in the regulation of various cancers [16], including glioma migration and invasion [17], vascular smooth muscle cells proliferation and apoptosis [18], proliferation and apoptosis of laryngeal cancer cells [19], and so on. In AS, Liu et al. believed that the expression of MEG3 was related to disease activity, hospitalization time, and the course of disease in patients [20]. However, so far, there have been no reports on the influence of MEG3 in the inflammatory response of AS.

This study aimed to explore the role and mechanism of lncRNA MEG3 in the inflammatory response of AS. The finding indicated that MEG3 was downregulated in AS. Through bioinformatics analysis and experimental verification, we confirmed that miR-146a could interact with MEG3. This study clarified that MEG3 suppressed the inflammatory response caused by AS through sponging miR-146a, which might provide an idea for the treatment of AS.

Materials and methods

Serum samples collection

Blood samples from 33 AS patients and 16 non-AS normal peoples were collected from Jining No.1 People's Hospital (Jining First People's Hospital Affiliated to Jining Medical University), and serum was extracted for experimental study. All subjects in this study signed informed consent, and this experiment was approved by the Ethics Committee of Jining No.1 People's Hospital (Jining First People's Hospital Affiliated to Jining Medical University).

Cell extraction and culture

Human fibroblast-like synovial (HFLS) cells were extracted from AS patients for in vitro experiments. Synovial tissues of AS patients were cleaned with PBS, cut into pieces, and then collected into digestion bottles. Tissues were digested with collagenase (Gibco, Waltham, MA, USA) for 1 h, and the supernatant was discarded after centrifugation. After re-suspended with Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 20% fetal bovine serum (FBS, Gibco), the cells were placed in culture dishes at 37 °C and 5% CO_2 incubator for 24 h. After the cells attachment, they were washed with PBS and then added into DMEM containing 10% FBS for further culture to carry out the later experiment.

Cell transfection

MEG3 overexpression plasmid and small interference (si-MEG3) or their negative control (pcDNA and si-con) and miR-146a mimic and inhibitor (in-miR-146a) or their negative control (miR-con and in-miR-con) were provided by Vigene Biosciences (Shandong, China). HFLS cell transfection was executed by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

The levels of inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were detected by ELISA kit (Hengyuan, Shanghai, China). In short, the samples to be tested and the standard samples were added to the corresponding enzyme plate and cultured at 37 ℃ for 1 h. After washing with PBS, the chromogenic antibody was added to the enzyme plates and continued incubation for 30 min. Finally, the termination solution was added to terminate the reaction. OD value at 450 nm was measured by a microplate reader (Thermo Fisher Scientific, Rockford, IL, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from serum and HFLS cells using TRIzol reagent (Invitrogen) and reversely transcribed into cDNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China). QRT-PCR was executed using SYBR Green (Takara). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were expressed as internal controls. The primer sequences were listed as follows: MEG3: F, 5′-CTGCCCATCTACACC TCACG-3′, R, 5′-CTCTCCGCCGTCTGCGCTAGGGGC T-3′. IL-1β: F, 5′-ATAAGCCCACTCTACACCTCTGA-3′, R, 5′-ATTGGCCCTGAAAGGAGAGAGA-3′. IL-6: F, 5′-GCTTCTTAGCGCTAGCCTCAATG-3′, R, 5′-TGG GGCTGATTGGAAACCTTATT-3′. TNF-α: F, 5′-TCC TGCATCCTGTCTGGAAG-3′, R, 5′-GTCTTCTGGGCC ACTGACTG-3′. GAPDH: F, 5′-ACCAGGTATCTGCTG GTTG-3′, R, 5′-TAACCATGATGTCAGCGTGGT-3′. MiR-146a: 5′-GAACTGAATTCCATGGGTTGTGT-3′, R, 5′-GCCCACGATGACAGAGAGATCC-3′. U6: F, 5′-GTA GATACTGCAGTACG-3′, R, 5′-ATCGCATGACGTACC TGAGC-3′. The relative expression was processed using $2^{-\Delta\Delta C_T}$ method.

Dual-luciferase reporter assay

MEG3 containing miR-146a binding sites and mutant binding sites were cloned into the pmirGLO vector to generate MEG3-WT and MEG3-MUT reporters (General Biosystems, Anhui, China). Lipofectamine 2000 (Invitrogen) was used to co-transfect luciferase reporters and miR-146a mimic or inhibitor (in-miR-146a) into HFLS cells. Luciferase activities were measured at 48 h post-transfection using dual-luciferase reporter assay kit (Genomeditech, Shanghai, China).

RNA immunoprecipitation (RIP) assay

Binding degree of MEG3 and miR-146a with argonaute2 antibody (Anti-Ago2) protein was detected by Magna RIP kit (Merck, Darmstadt, Germany). After HFLS cell lysis, supernatant was obtained by centrifugation. Partial supernatant was taken as Input, which did not participate in the incubation of magnetic beads. Another part of supernatant was incubated with magnetic beads conjugated with Anti-Ago2 (1:50, Abcam, Cambridge, MA, USA) or IgG antibody (Anti-IgG, 1:100, Abcam) at 4℃ overnight. The enrichment of MEG3 and miR-146a in Anti-Ago2, IgG, and Input was detected by qRT-PCR.

Statistical analysis

All experiments were analyzed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA), and the data were expressed as mean ± standard deviation (SD). Student's *t* test or one-way ANOVA were used for comparison analysis between different groups. Pearson analysis was used to reveal correlations. $P < 0.05$ was deemed statistically significant.

Results

Expression of inflammatory cytokines and MEG3 in AS

In AS, inflammatory response is an important indicator to determine the course of disease. To assess the levels of inflammatory and lncRNA MEG3 in AS patients, ELISA and qRT-PCR analyses were performed in the study. ELISA results showed that the levels of IL-1β, IL-6, and TNF-α were significantly increased in the serum of AS patients ($n = 33$) when compared with non-AS normal people ($n = 16$) (Fig. 1a–c). Also, as qRT-PCR showed, the level of MEG3 in the serum of AS patients was markedly lower than that in non-AS normal people (Fig. 1d). These results indicated that inflammatory cytokines were highly expressed and MEG3 was poorly expressed in AS patients.

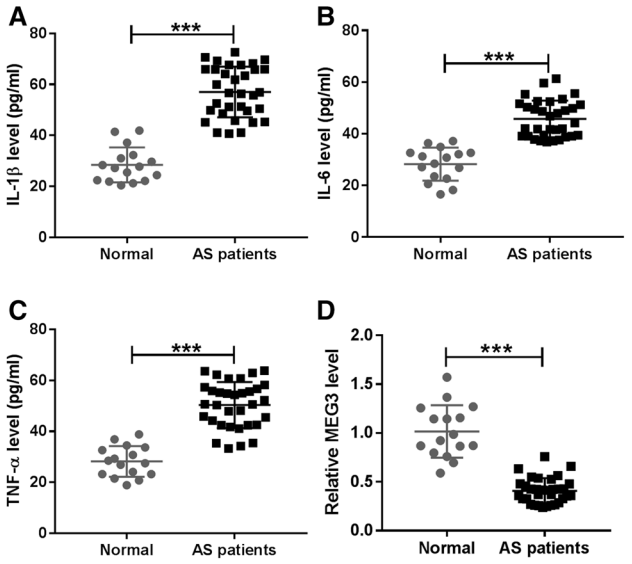

Fig. 1 Detection of inflammatory cytokines and MEG3 levels in AS. **a–c** The levels of IL-1β, IL-6, and TNF-α in the serum of AS patients and healthy normal people (Normal) were measured by ELISA. **d** The expression of lncRNA MEG3 in the serum of AS patients and healthy normal people (Normal) was detected by qRT-PCR. ***$P < 0.001$

MEG3 was negatively correlated with inflammatory cytokine levels

Given the contrary relationship between inflammatory cytokines and lncRNA MEG3 expression trend, we conducted Pearson correlation analysis between them. The results showed that the expression of MEG3 was remarkably negatively correlated with the contents of inflammatory cytokine IL-1β, IL-6, and TNF-α (Fig. 2a–c), indicating that MEG3 expression was related to the inflammatory response in AS patients.

MEG3 inhibited the inflammatory response in HFLS cells

To explore the influence of MEG3 expression on the inflammatory response, we verified it at the cellular level. The efficiency of HLFS cells transfected with MEG3 overexpressed plasmid and small interference (si-MEG3) was detected. As qRT-PCR observed, compared to pcDNA or si-con group, MEG3 was highly expressed in the MEG3-overexpressed plasmid group, while it was poorly expressed in the si-MEG3 group (Fig. 3a). The expression levels of inflammatory cytokines were detected by ELISA and qRT-PCR, and the results showed that MEG3 overexpression obviously reduced IL-1β, IL-6, and TNF-α levels, while silenced MEG3 markedly promoted IL-1β, IL-6, and TNF-α levels in HFLS cells (Fig. 3b–g). These suggested that lncRNA

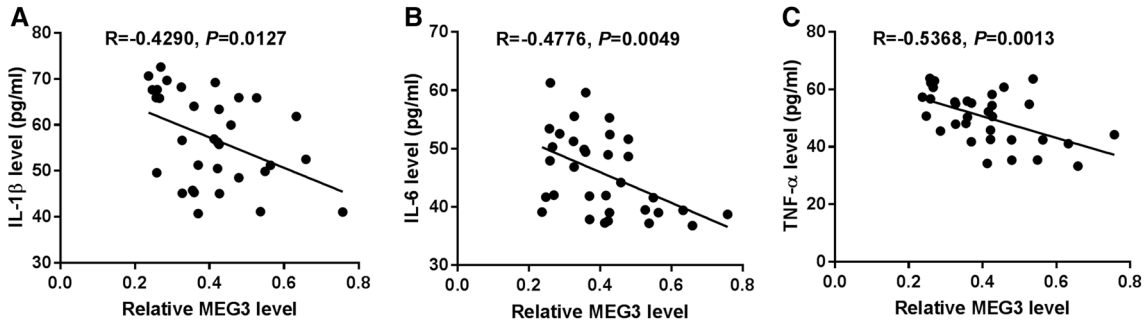

Fig. 2 Correlation analysis of MEG3 and inflammatory cytokines. **a**–**c** The correlation analysis between MEG3 and IL-1β, IL-6, or TNF-α level was determined by Pearson analysis

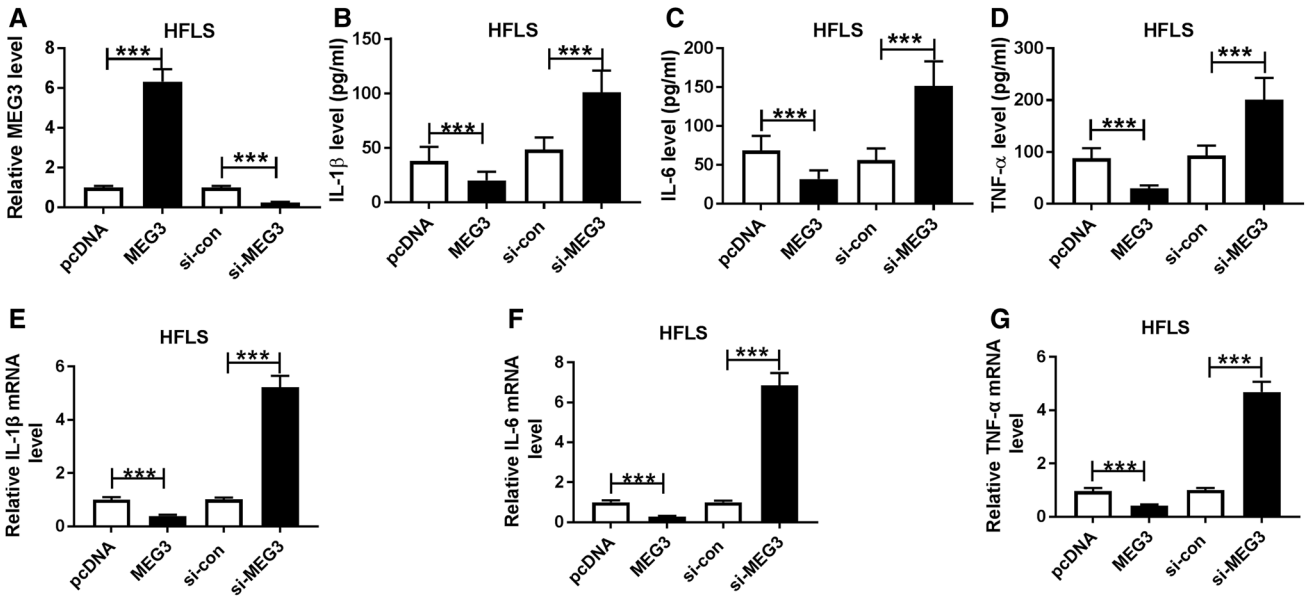

Fig. 3 Effect of MEG3 on inflammatory response in HFLS cells. HFLS cells were transfected with MEG3 overexpression plasmid (MEG3) and small interference (si-MEG3) or their negative control pcDNA and si-con, respectively. **a** The level of MEG3 was performed by qRT-PCR. **b**–**d** ELISA assay was used to assess the contents of IL-1β, IL-6, and TNF-α in HFLS cells. **e**–**g** The levels of IL-1β, IL-6, and TNF-α were measured by qRT-PCR. ***$P < 0.001$

MEG3 could block the inflammatory response in HFLS cells.

MiR-146a directly interacted with MEG3

To understand the anti-inflammatory mechanism of MEG3, we used LncBase v.2 tool to make bioinformatics prediction, and the results disclosed that miR-146a has a binding site with MEG3. MEG3-WT and MEG3-MUT luciferase reporters were generated containing miR-146a binding sites and mutant binding sites, respectively (Fig. 4a). Dual-luciferase reporter assay showed that the overexpression of miR-146a markedly decreased the luciferase activity of MEG3-WT reporter, while miR-146a inhibitor (in-miR-146a) significantly enhanced its luciferase activity, but they had no effect on MEG3-MUT reporter (Fig. 4b). Also, RIP assay results indicated that MEG3 and miR-146a were remarkably enriched in Anti-Ago2 complex compared with IgG (Fig. 4c). Finally, through qRT-PCR, we found that overexpression of MEG3 significantly inhibited the level of miR-146a, and the expression of miR-146a obviously increased after MEG3 knockdown (Fig. 4d). These results showed that lncRNA MEG3 could directly bind with miR-146a.

MiR-146a was highly expressed in AS patients and positively correlated with inflammatory cytokines

To confirm our results, we detected the expression of miR-146a in the serum of AS patients and analyzed

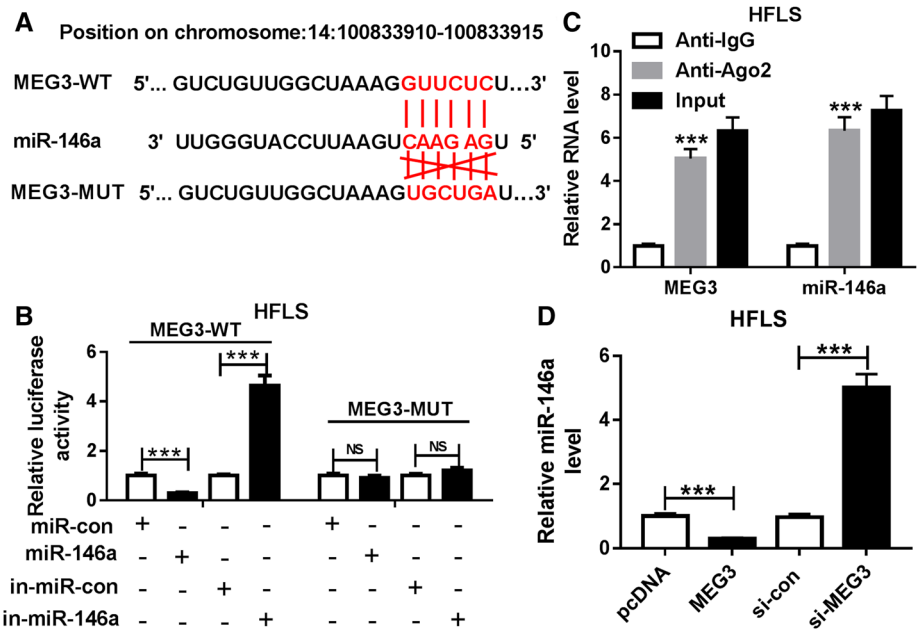

Fig. 4 The targeted regulatory relationship between MEG3 and miR-146a. **a** The binding sites between MEG3 and miR-146a were shown. **b** After HFLS cells were transfected with miR-146a mimic and inhibitor (in-miR-146a), MEG3-WT/MUT luciferase activity was detected by dual-luciferase reporter assay. **c** RIP assay was performed on the HFLS cells transfected with miR-146a or MEG3, followed by qRT-PCR to detect MEG3 or miR-146a associated with Anti-Ago2. **d** After transfection with MEG3 overexpression plasmid (MEG3) and small interference (si-MEG3) or their negative control pcDNA and si-con, respectively, the level of miR-146a was measured by qRT-PCR. ***$P < 0.001$

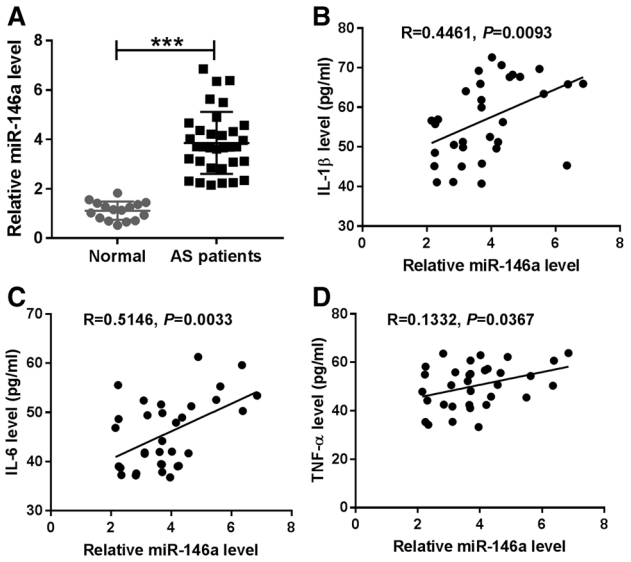

Fig. 5 The expression of miR-146a and its correlation analysis with inflammatory cytokines. **a** The expression of miR-146a in the serum of AS patients and healthy normal people (Normal) was detected by qRT-PCR. **b–d** The correlation analysis between miR-146a and IL-1β, IL-6, and TNF-α levels was determined by Pearson analysis. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$

the relationship between miR-146a and inflammatory cytokines. The results showed that the expression of miR-146a in AS patients was higher than that in the normal group (Fig. 5a), and the level of miR-146a was positively correlated with IL-1β, IL-6, and TNF-α levels (Fig. 5b–d).

MiR-146a promoted the inflammatory response in HFLS cells

Besides, the effect of miR-146a expression on inflammatory cytokines was detected. HFLS cells were treated with miR-146a mimic and inhibitor (in-miR-146a) and their efficiency was tested. The results showed that miR-146a mimic significantly promoted the level of miR-146a, while miR-146a inhibitor suppressed its expression (Fig. 6a). ELISA and qRT-PCR results revealed that overexpression of miR-146a obviously promoted the levels of IL-1β, IL-6, and TNF-α, while the inhibition of miR-146a showed the opposite inhibitory effect (Fig. 6b–g). It suggested that miR-146a had a pro-inflammatory effect in AS.

MEG3 suppressed inflammatory response by regulating the expression of miR-146a

To investigate whether MEG3 was involved in the regulation of inflammatory cytokines through miR-146a, we co-transfected MEG3-overexpressed plasmid with miR-146a mimic to detect the expression of miR-146a and inflammatory cytokines. The results showed that MEG3 markedly blocked the expression of miR-146a, while the addition of miR-146a mimic restored its expression (Fig. 7a). ELISA and qRT-PCR results indicated that aberrant expression of miR-146a reversed the suppression effect of overexpressed MEG3 on IL-1β, IL-6, and TNF-α levels in HFLS cells (Fig. 7b–g). These results confirmed that the anti-inflammatory effect of MEG3 was achieved by inhibiting the expression of miR-146a.

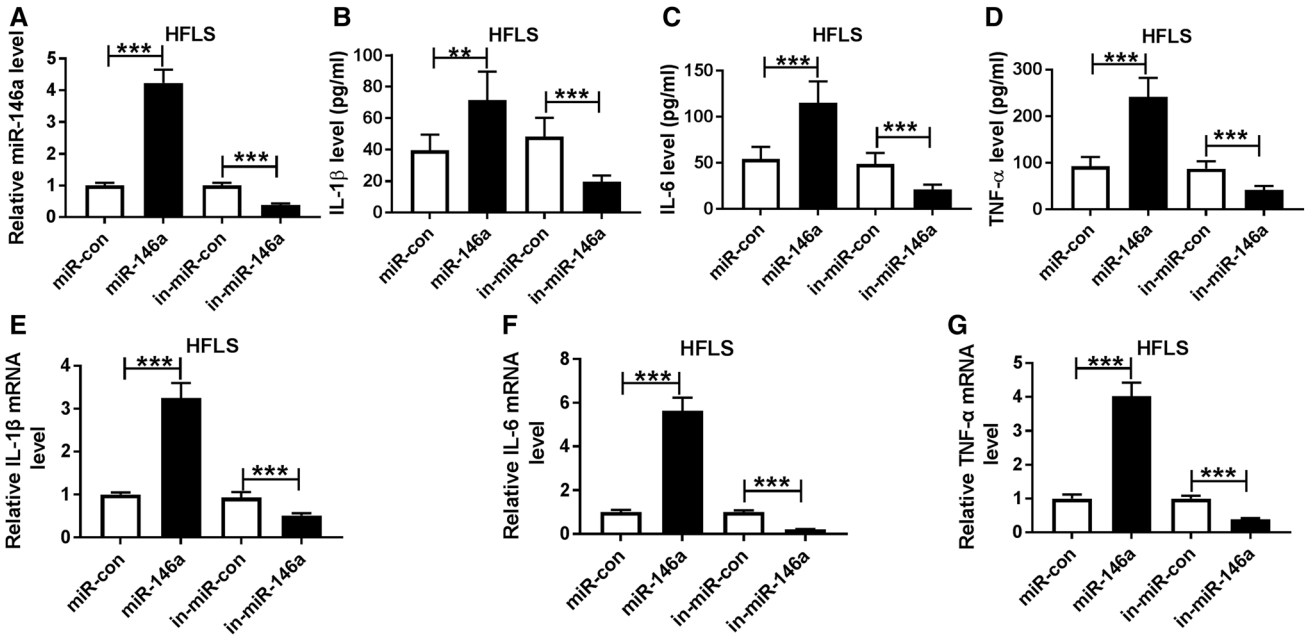

Fig. 6 MiR-146a affects the inflammatory response of HFLS cells. HFLS cells were transfected with miR-146a mimic and inhibitor (in-miR-146a) or their negative control (miR-con and in-miR-con), respectively. **a** The level of miR-146a was measured by qRT-PCR. **b**–**d** ELISA assay was used to evaluate the levels of IL-1β, IL-6, and TNF-α. **e**–**g** The levels of IL-1β, IL-6, and TNF-α were measured by qRT-PCR. $^{**}P < 0.01$ and $^{***}P < 0.001$

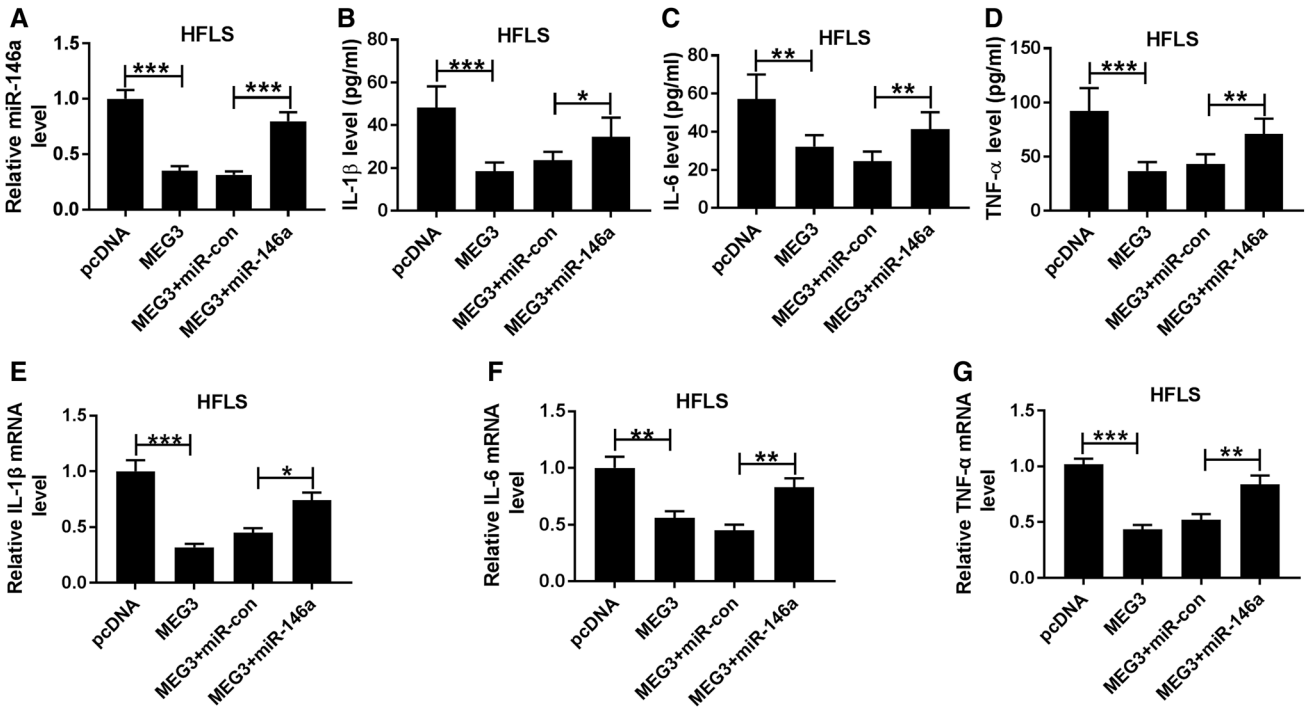

Fig. 7 Effect of MEG3 and miR-146a on the inflammatory response. HFLS cells were co-transfected with MEG3 overexpression plasmid and miR-146a mimic or their negative controls (pcDNA and miR-con), respectively. **a** The level of miR-146a was detected by qRT-PCR. **b**–**d** The levels of IL-1β, IL-6, and TNF-α were evaluated by ELISA assay. **e**–**g** QRT-PCR was used to measure the levels of IL-1β, IL-6, and TNF-α. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$

Discussion

AS is characterized by a persistent inflammatory response. Therefore, the elimination of inflammation is the primary goal of the treatment of AS [21, 22]. Currently, inhibitors of inflammatory cytokines TNF-α and IL-17A have been used in the clinical treatment of AS [23-25]. Therefore, exploring the mechanism which affects the inflammatory response of AS may provide more evidences for finding effective anti-inflammatory drugs.

The progression of AS usually changes the expression patterns of many genes, including lncRNAs [10, 26]. In this study, we found that the expression of lncRNA MEG3 was downregulated in AS patients, which was consistent with the study by Liu et al. [20]. Moreover, we concluded that MEG3 was negatively correlated with the level of inflammatory cytokines, and its overexpression could markedly reduce the level of IL-1β, IL-6, and TNF-α, suggesting that MEG3 was expected to become a new anti-AS drug.

To further understand the anti-inflammatory mechanism of MEG3, we conducted bioinformatics prediction and speculated that it might interact with miR-146a. MiR-146a is a key regulator of innate immune response which is related to the regulation of inflammatory response [27, 28]. Early studies have shown that polymorphism of miR-146a was associated with the risk of AS [29]. Qian et al. found that miR-146a has different expression in the AS group and control group through sequencing and verification, and it could be used as a biomarker to verify the occurrence of AS [30]. Di et al. studies pointed out that miR-146a was improved in AS, and its knockdown could inhibit the fibroblasts proliferation and osteogenic potential of AS [31]. Similarly, our results showed that miR-146a was significantly highly expressed in the serum of AS patients, and was positively correlated with the level of inflammatory cytokines. Loss- and gain-of-function experimental results confirmed that miR-146a promoted the expression of inflammatory cytokines. Besides, through co-transfection with MEG3 overexpression plasmids and miR-146a mimic, we also verified that MEG3 played an anti-inflammatory role by inhibiting the expression of miR-146a.

Of course, there is still much room for improvement in our research. Due to the limited number of samples, we only compared the samples of 33 AS patients and 16 non-AS normal people, which made our findings to have certain limitations. However, it is certain that the correlation between inflammatory cytokines and MEG3 or miR-146a expression does exist, and the results of MEG3-targeting miR-146a are also certain. In future studies, we will select a larger sample size to confirm our conclusions, so as to verify the feasibility of MEG3 as a target for the clinical treatment of AS.

Conclusion

In conclusion, our results indicated that MEG3, as a negative regulator, participated in regulating inflammatory responses in AS, which suppressed the levels of inflammatory cytokines by sponging miR-146a. Therefore, MEG3 could be used as a biomarker for AS, which is of great significance for the exploration of targeted therapy for AS.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no conflict of interest.

Ethical approval All authors have read the Journal's position on issues involved in ethical publication, and all authors have approved the final version of the manuscript.

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