

Triptolide's anti-inflammatory effects on ARDS by down-regulating miR-9-5p and up-regulating LRG1 and CLDN5

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Research Article

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Abstract

Background

Acute respiratory distress syndrome (ARDS) is a life-threatening respiratory disease and its treatment is not fully established. Triptolide, one of *Tripterygium wilfordii*'s main active components, has been proved to alleviate Lipopolysaccharide (LPS)-induced ARDS. Imbalance of MicroRNAs (miRNAs) is recognized as the pathogenic mechanism of various diseases, including ARDS. However, the specific miRNAs that play a key regulatory role in the anti-inflammatory effect of triptolide in ARDS remain elusive.

Methods

In this study, we administered triptolide in a mouse model of ARDS, and whole transcriptome sequencing was applied to identify meaningful miRNAs and validate them in vitro.

Results

The results showed that triptolide may reduce the inflammatory response in ARDS by regulating miR-9-5p. The data further proved that LRG1 and CLDN5 expression are regulated by miR-9-5p, and triptolide can down-regulate the expression of miR-9-5p by regulating negatively the expression of LRG1 and CLDN5.

Conclusion

Our study revealed that miR-9-5p was the specific miRNAs that plays key role in triptolide's alleviation of ARDS inflammation by regulating target genes, and its inhibitory effect on LRG1 and CLDN5 expression was verified.

Background

ARDS is a serious respiratory disease characterized by uncontrolled oxidative stress, pulmonary edema, inflammation, and neutrophilic infiltration [1]. Despite decades of clinical efforts, the mortality rate of ARDS patients remains high (over 50%) [2]. At the same time, ARDS patients (including patients with severe Coronavirus Disease 2019 (COVID-19) [3]) present with Cytokine storm, showing typical inflammatory states such as elevated levels of interleukin-6 (IL-6) and interleukin-10 (IL-10), increased Th17 cells, and CD8+T cell overactivation [4].

Triptolide, a diterpene lactone in *Tripterygium wilfordii*, has anti-inflammatory, immunosuppressive, anti-tumor effects. Triptolide-treated mice exhibited significantly reduced leukocyte, myeloperoxidase (MPO) activity, edema of the lung, as well as TNF- α and IL-6 production in the bronchoalveolar lavage fluid,

thereby attenuating an LPS-induced inflammatory response [5–6]. Triptolide is considered to be a promising potential therapeutic reagent for ARDS treatment.

Moreover, miRNAs are short single-stranded RNAs that regulate post-transcriptional mRNA expression by binding to complementary mRNA sequences, leading to translation suppression and gene silencing [7]. These miRNAs play critical roles in various physiological processes such as tissue development and differentiation, cell proliferation and tissue repair [8–9]. It is believed that miRNAs may be alternative therapies for many intractable diseases [10–11]. However, whether there are miRNAs that play a vital role in the secretion of proinflammatory cytokines has not been investigated.

The aim of this study was to identify potential members of this subset that could serve as a new possible mechanism by which triptolide alleviates ARDS inflammation. Here, we report the identification of miR-9-5p, a miRNA previously unrelated to the phenomenon of ARDS, targeting two key mRNAs involved in ARDS: LRG1 and CLDN5.

Materials And Methods

Mice and Drugs

C57BL/6 mice (female, 8 weeks old) were purchased from Institute of Animal Modeling, Nanjing University. Mice were intraperitoneally injected with 10mg/kg LPS(Sigma). The triptolide (Provided by Fujian Academy of Medical Sciences) was dissolved in 0.1% dimethyl sulfoxide (DMSO). The concentrations of triptolide L, M and H were 2.5ng/mL, 5ng/mL and 10ng/mL, respectively. The corresponding concentrations of L, M and H were 0.1mg/kg, 0.2mg/kg and 0.4mg/kg when intraperitoneally injected into mice.

Cell culture and Drug toxicity

Human normal lung epithelial cell line BEAS-2B was purchased from Shanghai Cell Bank. BEAS-2B cells were cultured in specific medium for BEAS-2B cells (CM-0496(ProCell)), with 10% fetal bovine serum (FBS) and 5% CO₂ at 37°C. Take BEAS-2B cells (1×10^4 cells/well) and add 2.5ng/mL, 5ng/mL, 10ng/mL of triptolide, respectively. The effects of triptolide on the CELL Viability of BEAS-2B at 24, 48 and 72 hours were analyzed with the VI-CELL XRVI-CELL(Beckman Coulter).

Measurement of cytokines

Levels of IL-6, TNF- α and MCP-1 in the supernatant were analyzed using ELISA kits, and optical density (OD) values were determined using a multifunctional enzyme plate analyzer (Synergy 2, USA, Bio-Tek, Inc.).

Lung Wet to Dry Weight Ratio and Pathological score of lung

The lower lobe of the right lung was excised, and the wet weight (W) was measured after removal of the liquid on the surface of the collected lung tissue. Then, the lung tissue was placed in an oven at 80 °C for 24 h to obtain the dry weight, and the lung tissue W/D ratio was calculated by dividing the wet weight by the dry weight. Lung injury scores were determined based on the following histological features, as described previously [12].

RT-qPCR and Complete transcriptome sequencing

The expression of miR-9-5p was determined using the TaqMan microRNA assay kit and SYBR green real-time polymerase chain reaction (ABI 7500 system). RT-qPCR primers are shown in Table 1. The complete transcriptomes were sequenced with RNA sequencing (RNA-Seq) and miRNA sequencing (miRNA-Seq) (Beijing Aovesen Gene Technology Co., Ltd).

Table 1. Primers of RT-qPCR

Mouse	Forward Primers	Reverse Primers
MPO	5'-GCAGATGACGGTTATGGTGTTC-3'	5'-CCGTCTCAGGACTGGAGAACTT-3'
Cu/Zn-SOD	5'-GTCGTCTGGCTTGTGGAGTG-3'	5'-TGTCAGCGGGCTAGTGCTT-3'
Mn-Sod	5'-CCGGACTATGTTAAGGCCATCT-3'	5'-ACACTCGGTTGCTCTCTTTTCTCT-3'
β-actin	5'-CTTGGGTATGGAATCTTGCG-3'	5'-AGCATTTGCGGTGGACGAT-3'
miR-9-5p	5'-ACACTCCAGCTGGGTCTTTGGTTATGTAGCT-3'	5'-TGGTGTCTGGAGTCG-3'

Cell transfection

MiR-9-5p-NC(5'-AUUGUCCUUAUUCAUUGUCAU-3'), miR-9-5p-mimic(5'-UCUUUGGUUAUCUAGCUGUAUGA-3'), and the miR-9-5p-inhibitor(5'-ACAAACCAUAGAUCGACAUACU-3') were synthesized by Sangon Biotech Company (Shanghai, China). Subsequently, 30 nmol miRNAs were directly transfected into 10⁶ cells using Lipofectamine[®]2000 (Invitrogen, USA).

Luciferase report analysis

According to the manufacturer's instructions, the miR-9-5p binding sequence of LRG1 and CLDN5 3'UTR fragments was mutated using a gene-customized site-directed mutagenetic system (PM CLDN5(5'-AGAAGAACCTACTGAACCATTGG-3'), PM LRG1(5'-TGTTTCAG-GGGTCACCATTGC-3')). The wild-type and mutant 3'UTR of LRG1 and CLDN5 were inserted into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Inc.). Subsequently, WT and MUT constructs were transfected into BEAS-2B cells with miR-9-5p mimics or miR-NC using Lipofectamine 2000. At 48 hours after transfection, relative luciferase activity was measured using a Infinite M1000 enzyme plate instrument (Tecan).

Western blot

LRG1 and CLDN5 primary antibodies (1:1000, Abcam) and β -Actin (1:10000, Abcam) were incubated overnight at 4°C, followed by horseradish peroxidase conjugated secondary antibodies (1:10000, Abcam). The target protein bands were visualized using Automatic chemiluminescence imaging system (Bio-Rad Lab) and quantified using Image-Pro Plus 6.0 software.

Statistical analysis

All data were analyzed using GraphPad Prism 8.0 software and represented as the mean SD of at least three experiments. The relationship between miR-9-5p and LRG1 and CLDN5 gene levels was determined by Spearman correlation analysis. $p < 0.05$ was considered statistically significant.

Results

Triptolide alleviates pulmonary inflammation in ARDS

The concentration of triptolide less than 10ng/mL had no significant cytotoxic effects on the BEAS-2B, and the amount of triptolide did not affect the survival of the mice (Fig.S1). As shown in Fig.1A, pretreatment with triptolide (L, M, and H) significantly decreased the lung W/D ratio compared with LPS group ($p < 0.01$). Then, compared with the LPS group, the lung injury scores were reduced significantly in the triptolide group (Fig.1B) ($P < 0.01$). In addition, Fig.1C shows that triptolide decreased the expression of myeloperoxidase (MPO) mRNA in lung, while superoxide dismutase (SOD) mRNA expression increased after administration of triptolide. The levels of cytokines IL-6, TNF- α and MPC-1 were reduced in the bronchoalveolar lavage fluid (Fig.1D-F, $P < 0.01$). Based on these results, triptolide can help reduce the inflammatory response in ARDS.

Triptolide regulates miR-9-5p in ARDS

Through complete transcriptome resequencing, the Gene Ontology (GO) and Pathway enrichment analysis of differential genes after triptolide intervention was mainly involved in substance exchange between membranes and the tight connection between cells, as is shown in Fig. 2A-B. Furthermore, 102 miRNAs were altered after treatment with triptolide (Fig. 2C), and these statistically significant changes could be visualized by using a fire map (Fig.2D). Finally, differential miRNAs clustering analysis were used to determine the clustering patterns of differential miRNAs expression (7 miRNAs, including miR-9-5p) (Fig.2E).

LRG1 and CLDN5 expression are regulated by miR-9-5p

As is shown in Fig.3A, the mRNA expression of miR-9-5p was significantly decreased after administration of triptolide in lung, compared with the LPS group. The MiRanda prediction software identified the putative binding sites (BSs) for miR-9-5p in the 3' untranslated regions (3'UTRs) of the LRG1 and CLDN5

mRNA, respectively (Fig. 3F). This prediction was functionally validated by over-expressing miR-9-5p in BEAS-2B cells, which resulted in a decrease of LRG1 and CLDN5 mRNA and protein levels (Fig. 2B-E).

To determine whether miR-9-5p directly regulates LRG1 and CLDN5 expression by binding to their 3'UTR, putative WT and mutated 3'UTR were cloned into the luciferase reporter vector pmirGLO. Transfection with pre-miR-5p significantly decreased the luciferase activity of the reporter vector containing WT 3'UTRs while this reduction was reversed in the presence of miRNA inhibitor-9 (Fig.3G-H). The decrease in the luciferase activity was completely reversed after point mutations (PM) in both miR-9-5p BSs in the 3'UTR of LRG1 and CLDN5 (Fig.3G-H). These observations indicated that BSs were critical for the regulation of LRG1 and CLDN5 3'UTRs, respectively. These data are consistent with a direct regulation of LRG1 and CLDN5 by miR-9-5p.

MiR-9-5p affected the changes of inflammation in vitro

To determine whether miR-9-5p was involved in the pro-inflammatory cytokines of ARDS by triptolide, BEAS-2B cells were transfected with pre-miR-9-5p and treated with triptolide. The transfection effect was verified by detecting the mRNA expression level of miRNA through RT-PCR (Fig. 4A). Increasing miR-9-5p levels significantly elevated the level of IL-6, TNF- α and MCP-1 (Fig.4B-D). Similarly, over-expression of miR-9-5p strongly reduced LRG1 and CLDN5 protein abundance (Fig.4E). Importantly, the reduction in the expression of LRG1 and CLDN5 observed in the presence of miR-9-5p was significantly restored after administration of triptolide (Fig.4E). These data suggested that triptolide can exert anti-inflammatory effects by targeting LRG1 and CLDN5 through miR-9-5p.

Discussion

In alleviating pulmonary inflammation of ARDS, miR-9-5p played a communication role in connecting triptolide, LRG1 and CLDN5. In other words, our study indicated that triptolide can down-regulate the expression of miR-9-5p, and miR-9-5p can regulate negatively the expression of LRG1 and CLDN5. More importantly, triptolide can reverse the negative regulatory effect of miR-9-5p, and then the expression level of LRG1 and CLDN5 tends to be normal.

ARDS is a common and serious clinical complication with high morbidity and mortality. The initiation and amplification of pro-inflammatory cytokines or mediators, such as IL-6, TNF- α and MCP-1 [13–14], are one of the factors that induce ARDS. Therefore, reducing the secretion of these inflammatory cytokines will significantly improve the clinical symptoms and prognosis of ARDS. Our study revealed that triptolide has a powerful anti-inflammatory effect, which significantly inhibits the expression of pro-inflammatory cytokines. MiRNAs have also been involved in the studies of triptolide, for example in the anti-tumor [15], anti-inflammatory [16] and immunosuppressive activities [17]. The quantitatively important change observed in miR-9-5p and the absence of information on this miRNA in the context of triptolide encouraged us to focus on miR-9-5p as a potential candidate susceptible of regulating LPS-mediated ARDS. Therefore, we screened out miR-9-5p and found that overexpression of miR-9-5p could increase the

secretion of inflammatory cytokines such as IL-6, which further prompted us to explore target genes of miR-9-5p.

It is important that the involvement of LRG1 and CLDN5 in the pathogenesis of ARDS has also been reported [18–19]. LRG1 protein is a highly conserved member of the leucine-rich (LRR) protein family. LRG1 can also inhibit the secretion of inflammatory cytokines and pro-fibrotic cytokines [20], participate in epithelial cell proliferation, affect airway remodeling [21]. On the other hand, Membrane protein Claudin 5 (CLDN5) is an important component of endothelial tight junctions, which can maintain the normal barrier function of endothelial cells [22] and the integrity of endothelial tight junctions, and determine vascular permeability as well [23]. What's more, the destruction of pulmonary epithelial and endothelial cell barriers is the key to pulmonary edema caused by ARDS.

The results of our Complete transcriptome sequencing also focused on the substance exchange between membranes and the tight intercellular connection. Therefore, it can be inferred that triptolide may exert an anti-inflammatory effect by regulating LRG1 and CLDN5. Then, CLDN5 and LRG1 were predicted and verified in vitro. When we upregulated miR-9-5p, the expression of LRG1 and CLDN5 was decreased. However, when we administered triptolide, the expression levels of LRG1 and CLDN5 were reversed, which verified that these two genes could play their protective role in ARDS and eventually reduce inflammation.

Although results revealed the regulatory effect between miR-9-5p and triptolide, Bioinformatics and other tools are still needed to synthesize the complex networks involved in triptolide's pharmacological activity. It is also necessary to further analyze the mechanism of triptolide's action on miR-9-5p in combination with the knowledge of drug structure analysis, and to study the specific signaling pathway in vivo. At the same time, X-box binding protein1 (XBP1) should also be considered in the regulation of LRG1 and CLDN5 by triptolide, so as to further explore its specific and detailed mechanism.

Conclusions

Triptolide plays an anti-inflammatory role in ARDS through down-regulation of miR-9-5p and up-regulation LRG1 and CLDN5.

Abbreviations

ARDS

Acute respiratory distress syndrome

LPS

lipopolysaccharide

miRNAs

MicroRNAs

COVID-19

Coronavirus Disease 2019

XBP1
X-box binding protein1
IL-6
interleukin-6
IL-10
interleukin-10
MPO
myeloperoxidase
SOD
superoxide dismutase
FBS
fetal bovine serum
DMSO
dimethyl sulfoxide
OD
optical density
GO
Gene Ontology.

Declarations

Ethics approval and consent to participate

This work was approved by the Local Animal Care and Use Committee, Fujian Academy of Medical Sciences.

Consent for publication

Not applicable.

Availability of data and material

All datasets and reagents are available from the corresponding author on reasonable request.

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Competing interests

The authors declare no conflicts of interest.

Authors' contributions:

JYH developed animal models, performed experiments and data analysis, contributed to manuscript writing. HQX and WL performed experiments and data analysis. SJX and SL supervised the research and interpretation of data. GC conceived the primary hypothesis, designed the research, analyzed and interpreted the data. All authors read and approved the final manuscript.

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Figures

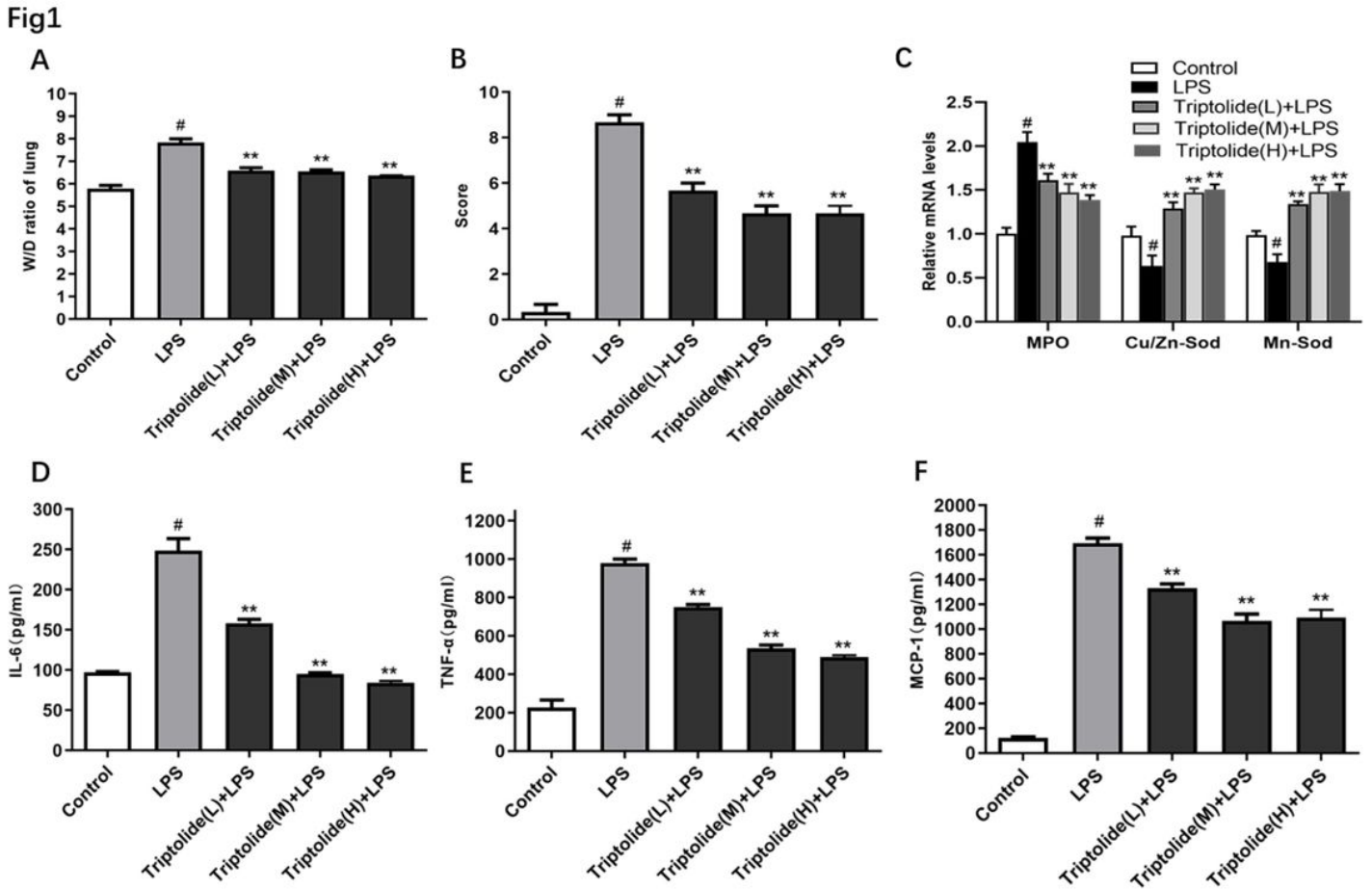


Figure 1

Triptolide alleviated pulmonary inflammation in ARDS. (A) Effects of triptolide on the lung W/D ratio of LPS-induced ARDS mice. (B) Lung injury scores were determined from four independent parameters. (C) The mRNA expressions of peroxidase (MPO) and superoxide dismutase (SOD) in lung tissue were detected by RT-PCR. (D-F) The contents of IL-6, TNF-A and MCP-1 in alveolar lavage fluid were determined by Elisa assay. Data information: Data are shown as mean \pm SEM. #P < 0.01 vs. control group; **p<0.01 vs. LPS group.

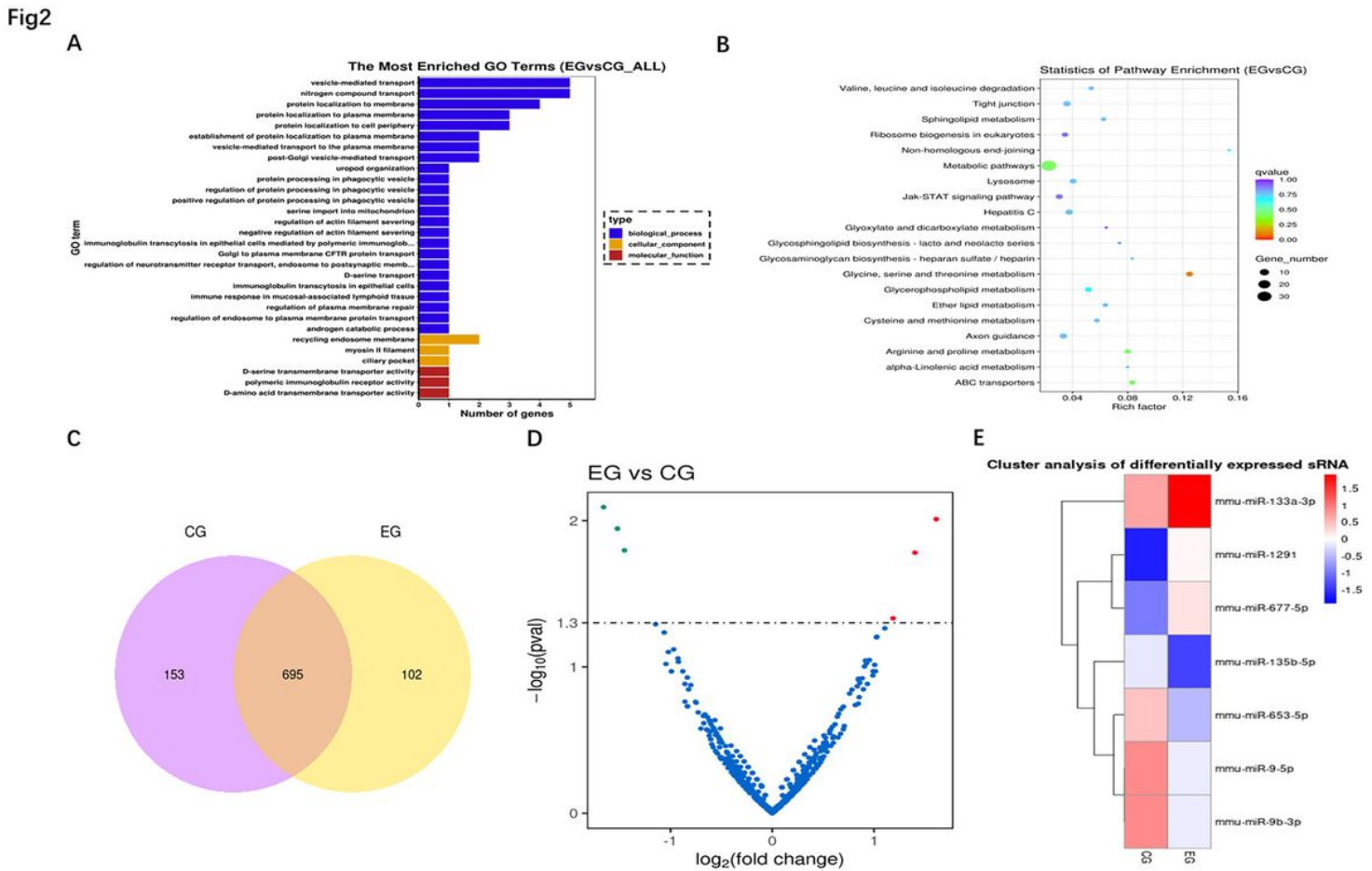


Figure 2

Triptolide regulates miR-9-5p in ARDS. (A) GO enrichment analysis of Illumina HiSeqTM2500/MiSeq sequencing target genes. (B) KEGG enrichment analysis showed the most important biochemical metabolic pathways involved in differentially expressed genes. (C) Differential miRNA Venn diagram shows the number of differential miRNA. (D) Volcano map showed the overall distribution of differential miRNA. (E) Cluster analysis of differential miRNA, overall hierarchical cluster diagram, clustering with Log10 (TPM+1) value, red represents high expression miRNA, blue represents low expression miRNA.

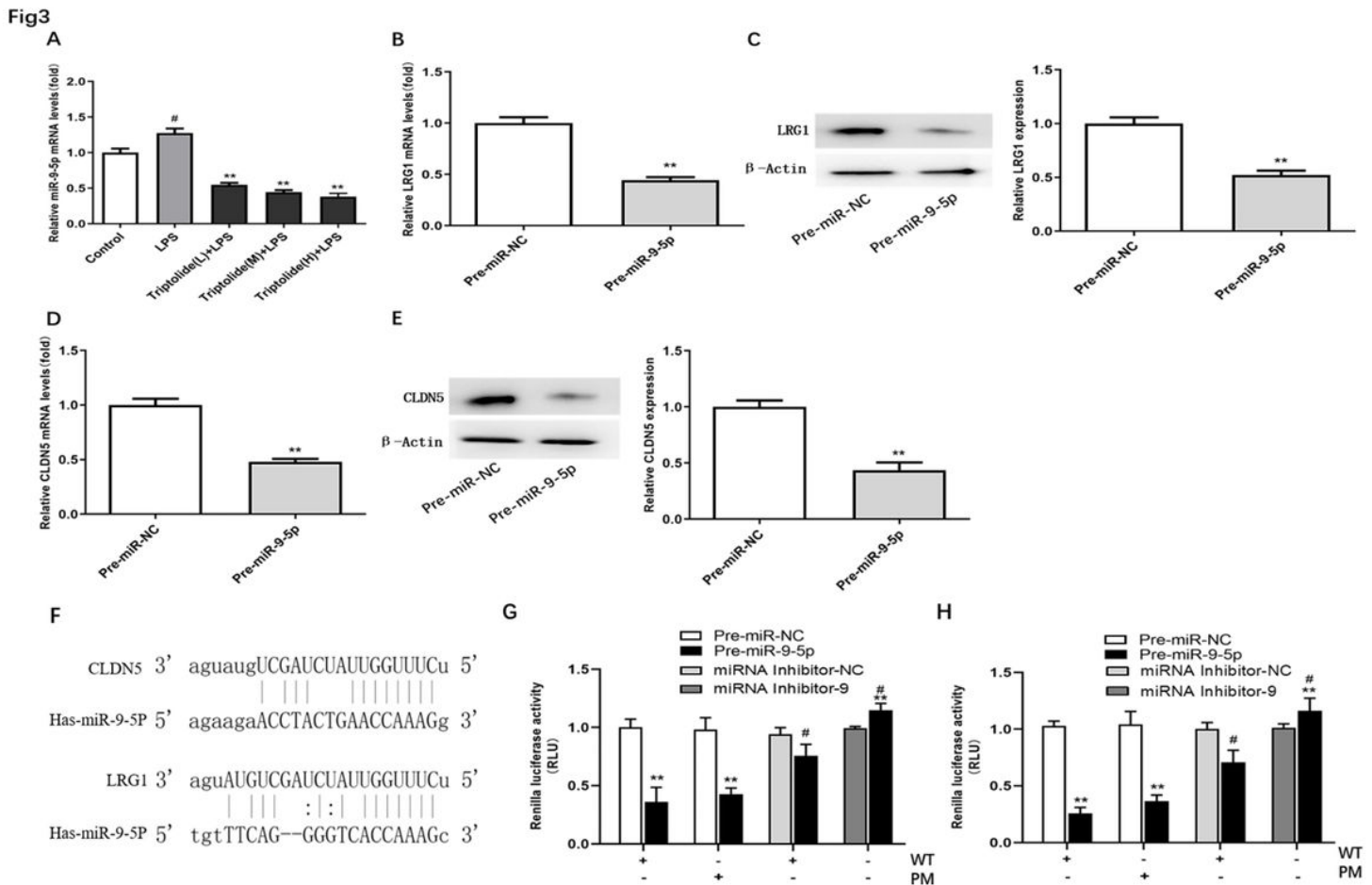


Figure 3

LRG1 and CLDN5 expression are regulated by MiR-9-5p (A) RT-qPCR confirmed that miR-9-5p was dose-dependent after treatment with triptolide. [#]P < 0.01 vs. control group; ^{**}p<0.01 vs. LPS group. (B-E) miR-NC and miR-9-5p analogs were transfected into BEAS-2B cells. CLDN5 and LRG1 mRNA and protein expression was observed by RT-qPCR and Western blot analysis. ^{**}P<0.01 vs. its corresponding negative control Group. (F) MIRANDA was used to predict miR-9-5p specific target genes. The binding sites of miR-9-5p were identified in 3'untranslated regions of CLDN5 and LRG1 genes respectively.(G-H) Luciferase activity in BEAS-2B cells co-transfected with pmirGLO containing WT or mutated 3'UTR sequences of human CLDN5 (or LRG1) and 40 nM of pre-miRs or miRNA inhibitors (n=3). Data are shown as mean ± SEM; and ^{**}P < 0.01 vs. control group, [#]P < 0.01 vs. WT 3'UTR sequence and pre-miR-9-5p co-transfected cells.

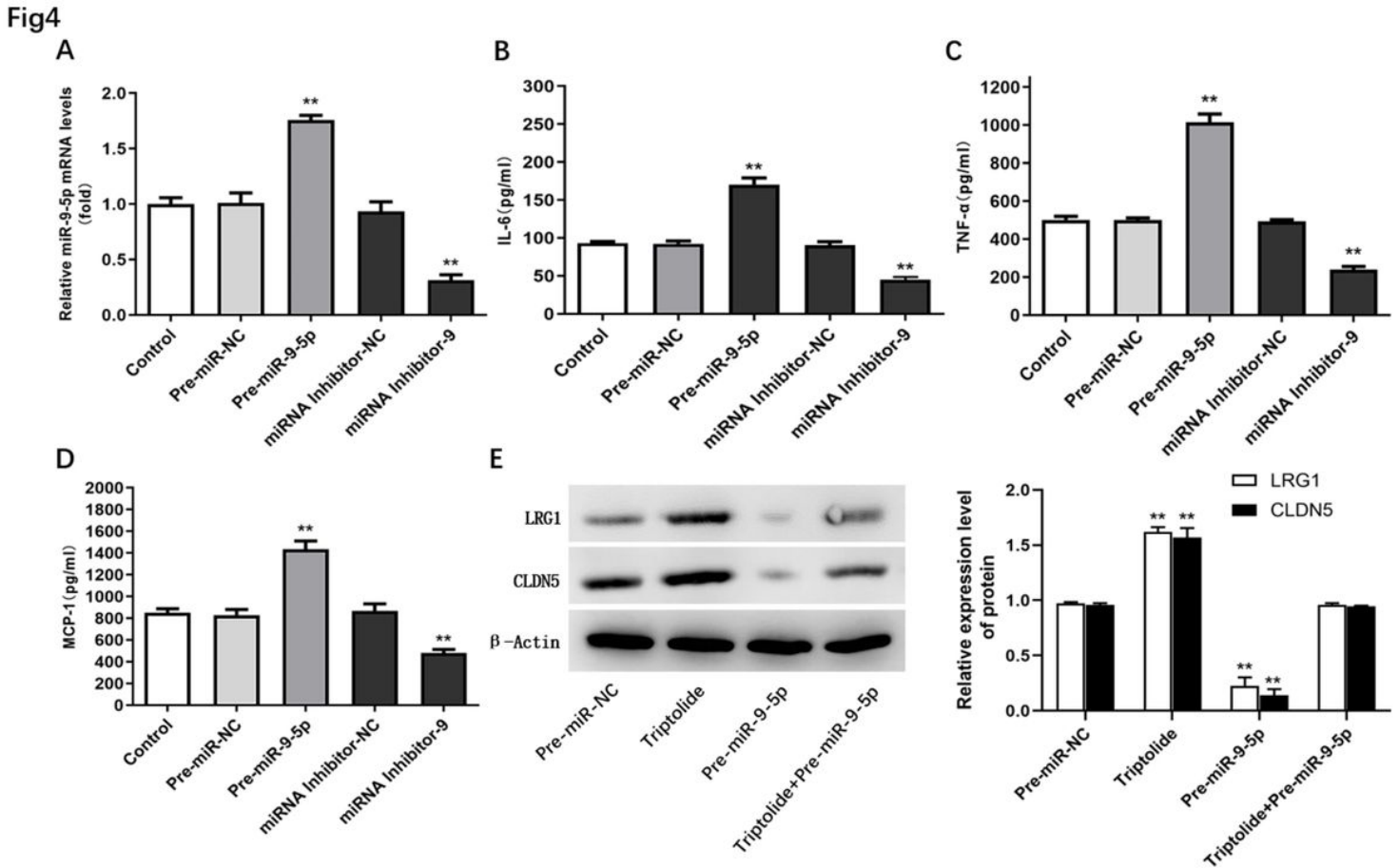


Figure 4

MiR-9-5p affected the changes of inflammation in vitro. (A) The transfection efficiency of miR-9-5p was tested by RT-PCR after transfection with BEAS-2B. (B-D) After the expression of miR-9-5p was changed in BEAS-2B, the contents of anti-inflammatory factors IL-6, TNF- α and MCP-1 were detected by ELISA. $**P < 0.01$ vs. Pre-miR-NC or miRNA Inhibitor-NC group. (E) After the expression of miR-9-5p was changed in BEAS-2B, Western blot analysis detected the expression levels of LRG1 and CLDN5 proteins. Data are shown as mean \pm SEM; and $**P < 0.01$ vs. Pre-miR-NC or Triptolide + Pre-miR-9-5p group.

Supplementary Files

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