

# Propofol regulates the expression of TLR4 through miR-21 in human umbilical vein endothelial cells

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**Abstract.** Propofol (2,6-diisopropylphenol) is one of the most commonly used intravenous anesthetics. Anesthetics can regulate the inflammatory process; however, the mechanism remains to be fully elucidated. The present study aimed to investigate whether and how propofol affects the inflammatory reaction in human umbilical vein endothelial cells (HUVECs). The expression levels of Toll-like receptor 4 (TLR4) and cluster of differentiation 14 (CD14) were determined in HUVECs treated with propofol and lipopolysaccharide (LPS) using western blot and reverse transcription-quantitative polymerase chain reaction analyses. In addition, whether propofol regulated the expression of TLR4 through microRNA (miR)-21 was examined. The results showed that LPS promoted the expression levels of TLR4, CD14 and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and suppressed the expression of miR-21 in HUVECs. Propofol suppressed the expression levels of TLR4, CD14 and TNF $\alpha$ , and upregulated the expression of miR-21 in a concentration-dependent manner. miR-21 downregulated the expression of TLR4 at the mRNA and protein levels, whereas the miR-21 mimic reversed the effect of LPS on the expression of TLR4. In addition, the miR-21 inhibitor inhibited the downregulatory effect of propofol on the expression of TLR4. TargetScan analysis showed that TLR4 was included in the list of targets of miR-21. Fluorescent reporter assays showed that the miR-21 mimic and propofol treatment reduced the fluorescence intensity in cells transfected with a reporter vector containing the wild-type TLR4 3'-untranslated region. Taken together, the results of the present study demonstrated that propofol regulated the expression of TLR4 in HUVECs through miR-21.

## Introduction

Propofol (2,6-diisopropylphenol) belongs to phenol derivatives and its biosynthesis dates back to the 1970s (1). Propofol is one of the most commonly used intravenous anesthetics at present (2-4). However, several reports have shown that anesthetics, including propofol, can inhibit the stress response during surgery and exert adverse effects on the immune system (5-7). Toll-like receptors (TLRs) are a type of innate immune receptor, which are widely distributed in mononuclear cells, polymorphonuclear cells, macrophages, lymphocytes, dendritic cells and natural killer cells (8,9). TLRs are vital in the cell immune defense process (9,10). Firstly, TLR identifies and combines the specific highly conserved sequence of several pathogens or pathogenic products. A series of cell signal transduction pathways are induced and inflammatory mediators are released, and the adaptive immune system is activated (11-14). TLR4 was found to be a major receptor mediating the course of the lipopolysaccharide (LPS)-induced immune response (15-17). The identification and development of drugs, which inhibit the TLR4 signaling pathway, has been a focus of investigations.

MicroRNAs (miRNAs) are a class of small, non-coding RNAs (~20-25 nucleotides in length), which regulate gene expression post-transcriptionally (18,19). MiRNAs are involved in several aspects of growth and development, depending on their target genes (20-22). The present study aimed to investigate whether miRNAs are involved in the inflammatory response induced by propofol.

## Materials and methods

**Cell culture and transfection.** The human umbilical vein endothelial cell (HUVEC) line was purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, CA, USA) containing 10% fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were grown on sterilized culture dishes (37°C, 5% CO<sub>2</sub>) and passaged every 48 h with 0.25% trypsin (Invitrogen; Thermo Fisher Scientific, Inc.). A mimic negative control, miR-21 mimic, inhibitor negative control, and miR-221 inhibitor were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The miR-221 mimic and inhibitor were transfected into HUVECs using Dharmafect1 Transfection Reagent

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(GE Healthcare Dharmacon, Inc., Lafayette, CO, USA). LPS was dissolved in DMSO and added into the culture medium at a final concentration of 100  $\mu\text{g/ml}$  for 12 h. Propofol was added to the medium to a concentration of 25, 50 and 100  $\mu\text{M}$  for 24 h at 37°C.

**Western blot analysis.** Total protein was extracted using lysis buffer purchased from Pierce; Thermo Fisher Scientific, Inc. Total proteins were quantified according to the Bradford method, following which 30  $\mu\text{g}$  samples were separated using 10% SDS-PAGE. The proteins were transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) and incubated overnight at 4°C with antibodies against TLR4 (1:800; cat. no. ab22048; Abcam, Cambridge, MA, USA), CD14 (1:800; cat. no. ab182032; Abcam), TNF $\alpha$  (1:2,000; cat. no. ab6671; Abcam) and GAPDH (1:2,000; cat. no. 60004-1-Ig; ProteinTech Group, Inc., Chicago, IL, USA). The membranes were then incubated with peroxidase-coupled anti-mouse (cat. no. 5127)/rabbit (cat. no. 58802) IgG (1:2,000; Cell Signaling Technology, Inc., Boston, MA, USA) at 37°C for 2 h. Finally, the proteins were visualized using electrochemiluminescence (Pierce; Thermo Fisher Scientific, Inc.) and detected using a DNR Bio-Imaging system (DNR Bio-Imaging Systems, Ltd., Jerusalem, Israel). ImageJ version 1.48 u software (National Institutes of Health, Bethesda, MA, USA) was used to quantify the relative protein levels.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of miR-21 using the SYBR-Green method.** Total RNA was extracted from cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The total RNA was then quantified using a Multiskan FC microplate reader (Thermo Fisher Scientific, Inc.). The quantification of miRNA from the extracted RNA was performed according to the SYBR-Green method. The qPCR reaction volume was 20  $\mu\text{l}$  and consisted of the following: cDNA (0.01  $\mu\text{g}/\mu\text{l}$ ), 5  $\mu\text{l}$ ; forward primer (5  $\mu\text{M}$ ), 1  $\mu\text{l}$ ; reverse primer (5  $\mu\text{M}$ ), 1  $\mu\text{l}$ ; H<sub>2</sub>O, 3  $\mu\text{l}$ ; SYBR-Green Master mix, 10  $\mu\text{l}$  (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling steps were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Primers for miR-21 (Bulge-Loop™ miRNA qRT-PCR primer set for has-mir-2) and U6 (snRNA qRT-PCR primer set; Guangzhou RiboBio Co., Ltd.) were used for qPCR analysis using an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences were as follows: Has-miR-21-5p forward primer, GGC GTAGCTTATCAGACTGATG and reverse primer, GTGCAG GTCCGAGGTATTC; U6 forward primer, CTCGCTTCG GCAGCACA and reverse primer, AACGCTTCACGAATT TGGCT. Experiments were performed in triplicate. The relative levels of gene expression were determined as:  $\Delta\text{Cq} = \text{Cq gene} - \text{Cq reference}$ . The fold change of gene expression was calculated using the  $2^{-\Delta\Delta\text{Cq}}$  method (23).

**RT-qPCR of target genes using the SYBR Green method.** RT-qPCR analysis was performed using SYBR Green PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on the 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The total volume of the PCR reaction system was 20  $\mu\text{l}$  and consisted of the following:

cDNA (0.02  $\mu\text{g}/\mu\text{l}$ ), 5  $\mu\text{l}$ ; forward primer (10  $\mu\text{M}$ ), 0.5  $\mu\text{l}$ ; reverse primer (10  $\mu\text{M}$ ), 0.5  $\mu\text{l}$ ; H<sub>2</sub>O, 4  $\mu\text{l}$ ; SYBR-Green Master mix, 10  $\mu\text{l}$  (Applied Biosystems, Thermo Fisher Scientific, Inc.) and the reaction process was as follows: 95°C for 30 sec, 40 cycles of 95°C for 5 sec, 60°C for 30 sec. A dissociation step was performed to generate a melting curve to confirm the specificity of the amplification.  $\beta$ -actin was used as the reference gene. Each PCR analysis was performed in triplicate. The relative levels of gene expression were determined as:  $\Delta\text{Cq} = \text{Cq gene} - \text{Cq reference}$ . The fold change of gene expression was calculated using the  $2^{-\Delta\Delta\text{Cq}}$  method (23). The primer sequences were as follows: TLR4 forward, 5'-CGAATGGAATGTGCAACACCT-3' and reverse, 5'-ACAAGCACACTGAGGACCGAC-3'; TNF $\alpha$  forward, 5'-CCACGCTCTTCTGCCTGCT-3' and reverse, 5'-GCCAGA GGGCTGATTAGAGAGA-3';  $\beta$ -actin forward, 5'-GATAGC ACAGCCTGGATAGCAAC-3' and reverse, 5'-CCTGAACCC CAAGGCCAAC-3'.

**Confirmation of the interaction between miR-21 and target genes using luciferase reporter assays.** A pmiR-Reporter vector, which was obtained from Addgene (Cambridge, MA, USA), was used for the reporter assays to detect interactions between miR-21 and TLR4. The wild-type miR-21 target site in the TLR4 3'-untranslated region (3'-UTR) was AUAAGCUA, which was predicted using TargetScan (<http://targetscan.org/>). The mutant miR-21 target site was AUGGGGUA. Luciferase activity was examined using the luciferase reporter gene assay kit from Promega Corporation (Madison, WI, USA).

**Statistical analysis.** SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Student's t-test was performed to compare all data.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Propofol downregulates the LPS-induced expression of TLR4 in HUVECs.** The expression levels of TLR4 in HUVECs treated with LPS and propofol were detected using western blot and RT-qPCR analyses. The results indicated that (Fig. 1) LPS upregulated the protein expression levels of TLR4 and CD14 in the HUVECs. Propofol inhibited the protein expression levels of TLR4 and CD14 in a concentration-dependent manner at propofol concentrations of 25, 50 and 100  $\mu\text{M}$ . The results of the RT-qPCR analysis showed the same trend (Fig. 1B). LPS upregulated the mRNA level of TLR4 in the HUVECs, and propofol suppressed the mRNA level of TLR4 in a concentration-dependent manner in the HUVECs ( $P < 0.05$  in control, vs. LPS;  $P < 0.05$  in LPS, vs. LPS+25  $\mu\text{M}$  propofol). In addition, LPS treatment significantly upregulated the mRNA and protein levels of TNF $\alpha$ , whereas propofol treatment downregulated its expression (Fig. 1A and B).

**Propofol upregulates miR-21 in HUVECs.** The present study analyzed changes in the expression of miR-21 in HUVECs using RT-qPCR analysis. As shown in Fig. 1B, LPS treatment decreased the expression of miR-21 ( $P < 0.05$ , control vs. LPS), whereas propofol treatment increased the expression of miR-21 in a concentration-dependent manner in the HUVECs ( $P < 0.05$  in LPS, vs. LPS+25  $\mu\text{M}$  propofol).

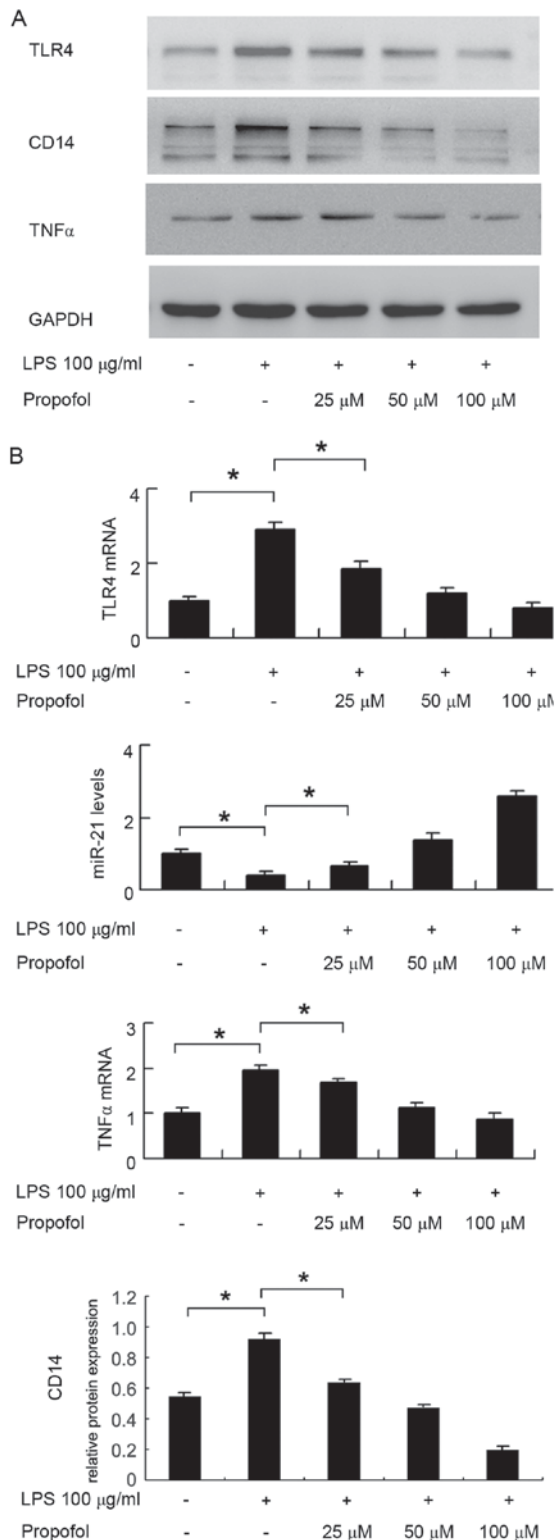


Figure 1. Expression pattern of TLR4, CD14 and miR-21 in HUVECs treated with LPS and propofol. (A) Western blots show the expression levels of TLR4, CD14 and TNF $\alpha$  were upregulated in HUVECs treated with LPS. Propofol inhibited the expression levels of TLR4, CD14 and TNF $\alpha$  in HUVECs in a concentration-dependent manner at propofol concentrations of 25, 50 and 100  $\mu$ M. (B) Reverse transcription-quantitative polymerase chain reaction analysis showed that LPS upregulated the expression levels of TLR4 and TNF $\alpha$ , and propofol inhibited the expression of TLR4 and TNF $\alpha$  in HUVECs, in a concentration-dependent manner. LPS downregulated the expression of miR-21, whereas propofol upregulated the expression of miR-21 in HUVECs in a concentration-dependent manner (LPS, 100  $\mu$ g/ml; propofol, 25, 50 and 100  $\mu$ M). \* $P$ <0.05. HUVECs, human umbilical vein endothelial cells; TLR4, Toll-like receptor 4; CD14, cluster of differentiation 14; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; LPS, lipopolysaccharide; miR, microRNA.

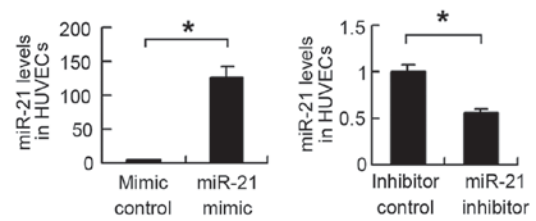


Figure 2. Transfection efficiency of miR-21. miR-21 mimic and miR-21 inhibitor were transfected into HUVECs. Reverse transcription-quantitative polymerase chain reaction analysis showed that the miR-21 mimic upregulated its expression and the miR-21 inhibitor downregulated its expression. \* $P$ <0.05. HUVECs, human umbilical vein endothelial cells; miR, microRNA.

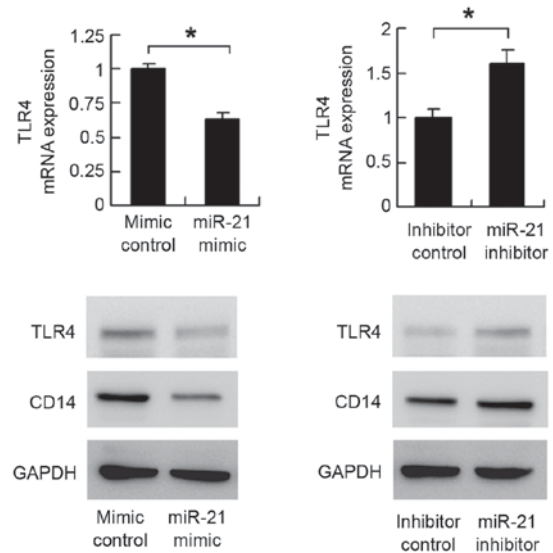


Figure 3. miR-21 downregulates the expression of TLR4 in human umbilical vein endothelial cells. The results of the reverse transcription-quantitative polymerase chain reaction analysis demonstrated that the mRNA expression of TLR4 was downregulated following miR-21 mimic transfection. miR-21 inhibitor upregulated the mRNA expression of TLR4 mRNA. Western blot results analysis revealed that the miR-21 mimic downregulated the protein expression of TLR4 and CD14. miR-21 inhibitor upregulated the protein expression of TLR4 and CD14. \* $P$ <0.05. TLR4, Toll-like receptor 4; CD14, cluster of differentiation 14; miR, microRNA.

**miR-21 downregulates TLR4 in HUVECs.** The present study examined the link between miR-21 and TLR4 in HUVECs, and found that there were binding sites between miR-21 and the 3'-UTR of TLR4. Therefore, TLR4 may be a target gene of miR-21 in HUVECs. In order to verify whether miR-21 targeted TLR4 in HUVECs, the miR-21 mimic and miR-21 inhibitor were transfected into HUVECs, and the transfection efficiency was confirmed using RT-qPCR analysis (Fig. 2). The expression of TLR4 was then examined. As shown in Fig. 3, the mRNA level of TLR4 decreased following miR-21 mimic transfection. The mRNA expression of TLR4 increased in the miR-21 inhibitor-transfected cells. The results of the western blot analysis showed that miR-21 mimic suppressed the protein expression of TLR4, whereas transfection with the miR-21 inhibitor upregulated the protein expression of TLR4. These results indicated that miR-21 regulated TLR4 at the mRNA and protein levels.

**Propofol regulates TLR4 through miR-21.** The above results indicated that propofol regulated the expression of TLR4

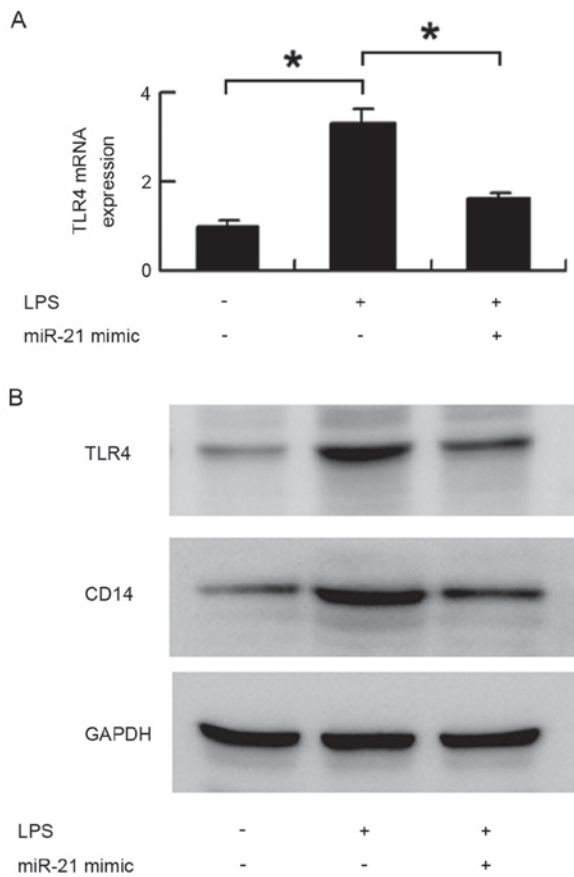


Figure 4. miR-21 reverses the effects of LPS on TLR4 and CD14. (A) Reverse transcription-quantitative polymerase chain reaction analysis demonstrated that LPS upregulated the mRNA expression of TLR4, whereas miR-21 mimic inhibited the effect of LPS. (B) miR-21 significantly inhibited the LPS-induced protein expression of TLR4 and CD14. \* $P < 0.05$ . Toll-like receptor 4; CD14, cluster of differentiation 14; LPS, lipopolysaccharide; miR, microRNA.

through miR-21 in the HUVECs. Subsequently, the present study examined whether the LPS-induced upregulation of TLR4 and CD14 were reversed by the miR-21 mimic. As shown in Fig. 4, miR-21 significantly inhibited the protein and mRNA expression of TLR4, which were upregulated by LPS treatment (Fig. 4A and B). miR-21 also downregulated the protein expression of CD14 induced by LPS (Fig. 4B).

To confirm the role of miR-21 during propofol-induced downregulation of TLR4, the miR-21 inhibitor was transfected into HUVECs and the cells were treated with propofol. The expression of TLR4 was determined using western blot and RT-qPCR analyses. As shown in Fig. 5A and B, in the cells transfected with the miRNA inhibitor control, propofol significantly downregulated the mRNA and protein expression levels of TLR4. In cells transfected with the miR-21 inhibitor, propofol had no significant effect on TLR4. Similar to TLR4, the miR-21 inhibitor eliminated the downregulation of CD14 induced by propofol 4 (Fig. 5B). These results showed that propofol regulated the expression of TLR4/CD14 through miR-21.

TLR4 is a direct target of miR-21. To further determine whether TLR4 was a direct target of miR-21, fluorescent reporter assays were performed. The 3'-UTR of TLR4, containing wild-type (AUAAGCUA) or mutant (AUGGGGUA) binding sites for miR-21, was cloned into a reporter vector (Fig. 6A).

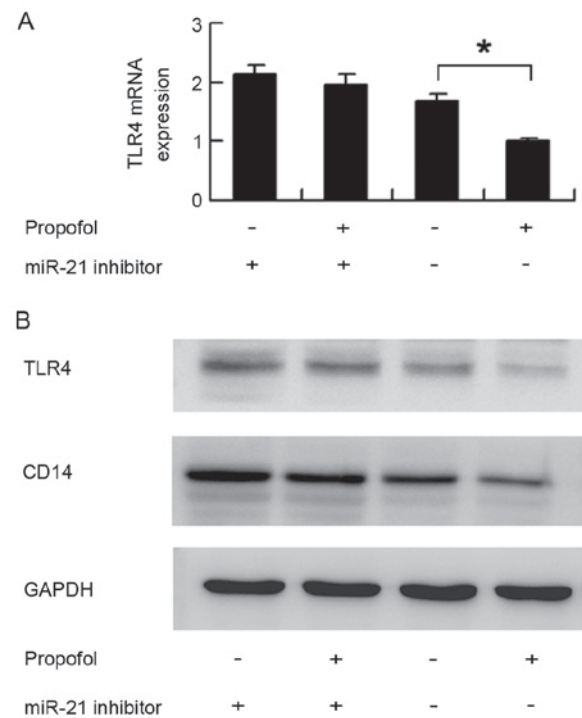


Figure 5. Propofol regulates the expression of TLR4/CD14 through miR-21. (A) Reverse transcription-quantitative polymerase chain reaction analysis demonstrated that propofol significantly downregulated the expression of TLR4. In HUVECs transfected with miR-21 inhibitor, propofol had no significant effect on the expression of TLR4. (B) Western blot analysis revealed that propofol significantly downregulated the protein expression of TLR4 and CD14 in cells transfected with the miRNA inhibitor control. In HUVECs transfected with miR-21 inhibitor, propofol had no significant effect on the expression of TLR4 or CD14. \* $P < 0.05$ . HUVECs, human umbilical vein endothelial cells; TLR4, Toll-like receptor 4; CD14, cluster of differentiation 14; LPS, lipopolysaccharide; miR, microRNA.

The ratio of fluorescence intensity for the wild-type and mutant binding sites was then calculated. As shown in Fig. 6B, the miR-21 mimic reduced the fluorescence intensity in cells transfected with the vector containing the wild-type TLR4 3'-UTR, compared with that in the controls, whereas no significant change was observed in the cells transfected with the vector containing the mutant binding site. In addition, a reporter assay was used to assess the effect of propofol. As shown in Fig. 6C, propofol treatment reduced the fluorescence intensity of cells transfected with the reporter vector containing the wild-type TLR4 3'-UTR. These results indicated that miR-21 binds to the TLR4 3'-UTR directly and downregulates the mRNA expression of TLR4.

## Discussion

TLR4 predominantly responds to LPS from Gram-negative bacteria through its co-receptor (24). TLR4 contains three protein domains (25): Extracellular, transmembrane and intracellular. The extracellular domain consists of a leucine-rich fragment and is involved in the identification of pathogen-associated molecular patterns through combining with CD14. The TLR4 signal transduction pathway is widespread and important in inflammatory molecular signaling pathways (26-28). The cascade of inflammatory signals triggered by TLR4 is crucial during the development of several diseases (29). In the present



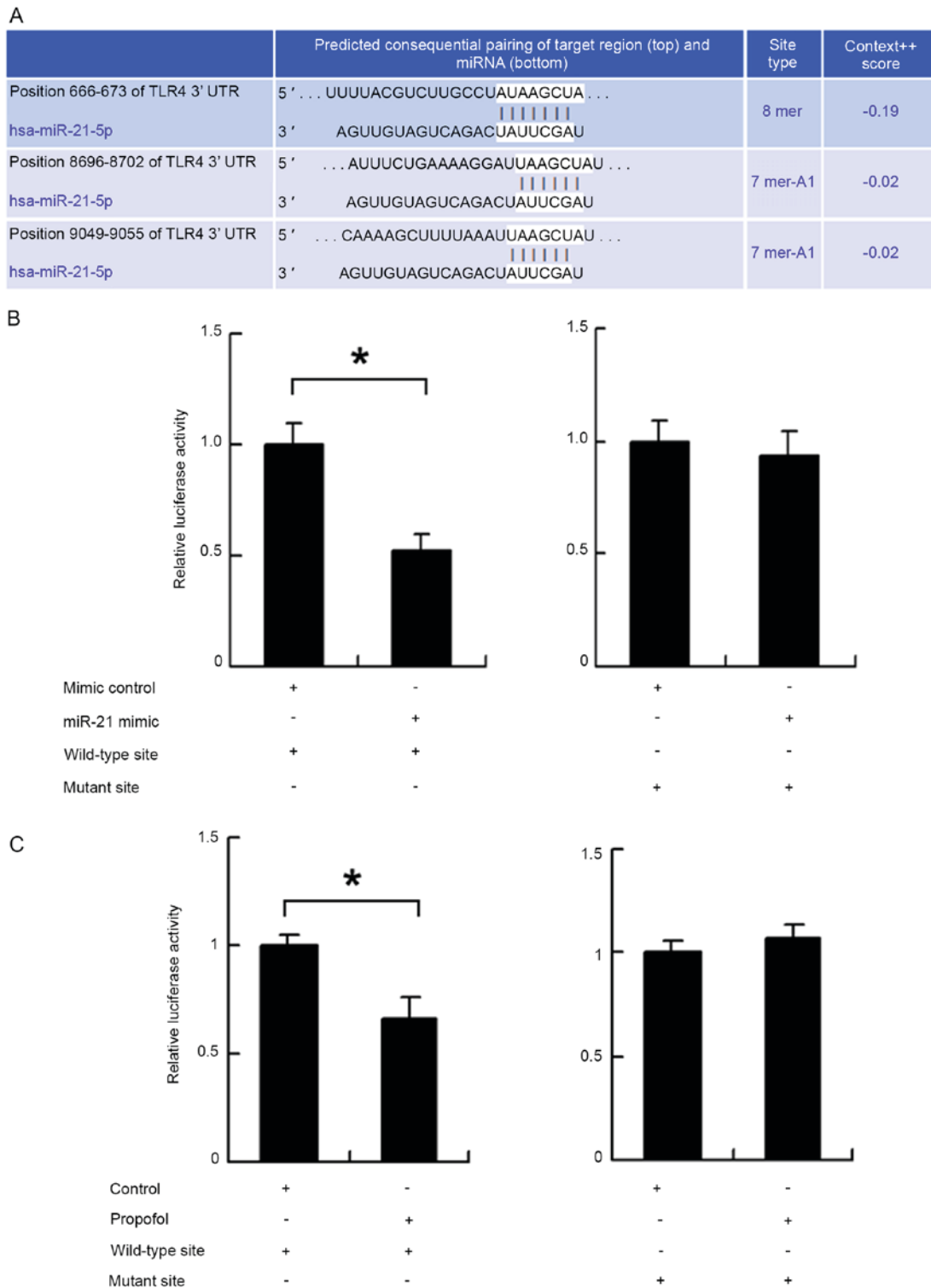


Figure 6. miR-21 targets TLR4 in human umbilical vein endothelial cells. (A) miR-21-TLR4 information from the TargetScan human database. (B) Wild-type and mutant miR-21 binding sites in the 3'-UTR of TLR4 were assessed using fluorescent reporter assays. The fluorescence activity of the miR-21 mimic/mimic control was calculated. In cells transfected with the reporter vector containing the wild-type binding site, luciferase activity was lower in the miR-21 mimic-transfected cells, compared with that in the control cells. (C) Propofol treatment reduced the fluorescence intensity in cells transfected with the reporter vector containing the wild-type TLR4 3'-UTR. \*P<0.05. TLR4, Toll-like receptor 4; miR, microRNA; 3'-UTR, 3'-untranslated region.

study, it was found that LPS promoted the expression of TLR4, CD14 and TNF $\alpha$ , suggesting that TLR4 may serve as a mediator of inflammatory responses in HUVEC cells. In addition, propofol suppressed the expression of TLR4, CD14 and TNF $\alpha$  in a concentration-dependent manner, suggesting that propofol reversed the inflammatory response induced by

LPS. The role of propofol on inflammation has been reported previously. Propofol inhibits the NOD-like receptor family, pyrin domain-containing 3 inflammasome and attenuates blast-induced traumatic brain injury (30). Propofol attenuates high glucose-induced superoxide anion accumulation in HUVECs (31), and inhibits pro-inflammatory cytokines in

adult rats following traumatic brain injury (32). In accordance with these reports, the present study demonstrated that propofol was able to reduce the expression of TLR4, which serves as an important component of the inflammatory response. However, the exact mechanism underlying the regulation of inflammation and TLR4 by propofol remains to be fully elucidated. The present study aimed to determine how propofol downregulates the expression of TLR4, and found miR-21 was an important mediator.

miR-21, which is highly conserved, has been found in several vertebrates, including mammals, fish and birds (33-35). It has been reported that the expression pattern of miR-21 is closely associated with the development of several diseases (36-38). However, whether and how miR-21 is involved in the inflammatory response of cells regulated by propofol remain to be elucidated. In the present study, it was found that LPS treatment downregulated the expression of miR-21, whereas propofol treatment upregulated the expression of miR-21 in a concentration-dependent manner in HUVECs. In addition, the present study showed that the miR-21 mimic downregulated the expression of TLR4, whereas the miR-21 inhibitor upregulated the expression of TLR4, indicating that TLR4 is a target of miR-21.

These results suggested that propofol regulated the LPS-induced expression of TLR4 through miR-21 in HUVEC cells. To confirm this hypothesis, the present study demonstrated that miR-21 reverses the effect of LPS on TLR4. The cells were also transfected with miR-21 inhibitor and then exposed to propofol, and the results demonstrated that the miR-21 inhibitor eliminated the downregulatory effect of propofol on TLR4, suggesting that miR-21 is essential in the biological effect of propofol. To confirm TLR4 as a direct target of miR-21, the present study performed a fluorescent reporter assay, which demonstrated that miR-21 was able to bind directly to the TLR4 3'-UTR. Taken together, these results confirmed that propofol regulated the expression of TLR4 through miR-21.

In conclusion, the present study demonstrated that propofol regulated the expression of TLR4 through the upregulation of miR-21 in HUVECs, which may explain the protective effects of propofol against inflammation.

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