

Myricetin Ameliorates Ox-LDL-induced HUVECs Apoptosis and Inflammation via lncRNA GAS5 Upregulating the Expression of miR-29a-3p

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Research

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Abstract

Background

Oxidized low-density lipoprotein (ox-LDL)-induced an endothelial cell dysfunction is a significant event in the progression of atherosclerosis[1]. Even myricetin (Myr) has been exhibited strong antioxidant potency, the effect on atherosclerosis is still elusive.

Methods

HUVECs were subjected to ox-LDL, before which cells were preconditioned with Myr. Cell Counting Kit-8 (CCK-8) assay, flow cytometry, quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot were carried out to assess the impacts of ox-LDL and Myr on HUVECs. The expression of EndMT markers was determined by Western blot analysis and immunocytochemistry. In addition, the relationship of GAS5 and miR-29a-3p was evaluated by RNA Fluorescent in Situ Hybridization (FISH) and RNA immunoprecipitation (RIP) assay.

Results

Myr preconditioning prevented ox-LDL-induced apoptosis, inflammatory response, and EndMT. GAS5 was upregulated in response to ox-LDL while it was down-regulated by Myr preconditioning. GAS5 over-expression attenuates Myr protective effects against ox-LDL-mediated HUVEC injury. Besides, miR-29a-3p is a target of GAS5 and down-regulated miR-29a-3p could further reduced the effects of GAS5 in ox-LDL-mediated HUVEC. Furthermore, Myr inactivated the TLR4/NF- κ B signaling pathway in ox-LDL-treated HUVEC by down-regulating GAS5 or upregulating miR-26a-5p.

Conclusion

Myr possessed an anti-inflammatory and anti-EndMT function against ox-LDL-induced HUVEC injury by regulating the GAS5/miR-29a-3p, indicating that Myr may have an important therapeutic function for atherosclerosis.

1. Introduction

Atherosclerosis (AS) is a major cause of morbidity and mortality among cardiovascular diseases, which is initially triggered by endothelial dysfunction and characterized by an influx of atherogenic lipoprotein components. It is the main potential factor of most fatal diseases, such as myocardial infarction, unstable angina, sudden cardiac death, and stroke [2]. Although the etiology of AS is complex, the damage response theory that vascular endothelial cell (EC) injury is the initiating mechanism of AS is still widely adopted [3, 4]. Oxidative low-density lipoprotein (ox-LDL) has been shown to be involved in the formation of atherosclerosis, and its induced vascular endothelial injury is one of the early pathogenesis of atherosclerosis [5, 6]. So far, controlling the development of AS remains a major challenge because the

molecular mechanisms of AS are not fully understood. Therefore, reducing ox-LDL induced EC injury and further explaining its mechanism may be a potential strategy for the prevention and treatment of AS.

Myricetin (Myr) is a common natural flavonoid found in many fruits, vegetables, and herbs. Myricetin plays a substantial role in treating and preventing some diseases, including diabetic osteoporosis[7], hepatocellular carcinoma[8], alcoholic liver[9], and skin cancer[10], due to its powerful iron-chelating capability, antioxidant and free-radical scavenging activities. Recently, the cardioprotective role of Myr has attracted attention from the research community. Myr showed a protective effect on lipopolysaccharide (LPS) induced inflammatory myocardial injury[11], cardiac hypertrophy[12], and ischemia/reperfusion (I/R)-induced myocardial injury[13]. However, the effect of Myr on the lipid metabolism and atherosclerosis is not fully understood.

Long non-coding RNAs (lncRNAs) refer to a class of RNA transcripts that are more than 200 nucleotides in length but do not encode proteins[14]. Increasing evidence has demonstrated that lncRNAs are involved in the onset and development of various cardiocerebrovascular diseases[15], including hypertension, coronary artery disease, stroke, atrial fibrillation, myocardial infarction, heart failure, and AS. Widely expressed in various tissues and cells of human and mice, lncRNA growth arrest-specific 5 (GAS5) has been shown to be associated with cardiac diseases. For example, GAS5 can modulate fibroblast and fibrogenesis through TGF- β /Smad3 signaling[16]. Add Wu[17] et al. proved that down-regulation of GAS5 ameliorates myocardial I/R injury via the miR-335/ROCK1/AKT/GSK-3 β axis. In the present study, we aimed to explore whether GAS5 was involved in the role of Myr in ox-LDL-induced HUVECs apoptosis and further investigated the underlying mechanism in HUVECs.

2. Materials And Methods

2.1 Cell culture, ox-LDL-induced injury model, and proliferation

Human Umbilical Vein Endothelial Cells (HUVECs) were cultured in DMEM supplemented with 10% FBS under normoxic conditions at 37 °C. The HUVECs were treated with different concentrations (50, 100, 200 μ g/ml) of ox-LDL (Union-Bio Technology, China) and were collected at 24 h for further measurements. The proliferation rates of the cells were measured with CCK8 assay (Solarbio, China) according to the manufacturer's instructions.

2.2 Flow cytometry analysis of apoptosis with PI and annexin V double staining

Cell apoptosis was evaluated by flow cytometry using PI and annexin V double-staining apoptosis assay kit (Solarbio, China) following the manufacturer's instructions.

2.3 RNA immunoprecipitation (RIP) assay

RIP was performed using Magna RIP Kit (Millipore, USA) following to the manufacturer's protocol[18]. Briefly, HUVEC cells were collected and lysed in a complete RIP lysis buffer. The whole cell protein extract was then incubated with RIP buffer containing magnetic beads conjugated with human anti-AGO2 antibody or negative control normal mouse IgG. The retrieved RNA was then detected by qRT-PCR.

2.4 RNA Fluorescent in Situ Hybridization (FISH) assay

Specific probes to GAS5 sequence and miR-29a-3p were designed and synthesized by RiboBio. A FISH Kit (RiboBio) was used to detect probe signals according to the manufacturer's instructions. Nuclei were stained with DAPI.

2.5 Dual-luciferase reporter assay

Dual-luciferase reporter assay was used to detect the interaction between GAS5 and miR-29a-3p promoter region. The wild-type and mutation sequences of the binding sites (WT-GAS5 and MUT-GAS5) were designed and synthesized. HUVEC cells were transfected in combination with WT (Mut) GAS5 vector and miR-NC or miR-29a-3p using Lipofectamine 2000 Transfection Reagent (Invitrogen). After 48 hours of transfection, Dual-Luciferase reporter assay system (Promega) was used to conduct the luciferase assay.

2.6 Determination of VCAM-1, IL-6 and MCP-1

The levels of supernatant monocyte chemo-attractant protein-1 (MCP-1), interleukin-6 (IL-6), and vascular cell adhesion molecule 1 (VCAM-1) were determined by ELISA kits. IL-6 ELISA Kit (ab178013), MCP-1 ELISA kit (ab179886), and VCAM-1 ELISA kit (ab223591) were used according to manufacturer's instructions.

2.7 Real-Time Quantified PCR (qRT-PCR)

Total RNA was isolated using Trizol reagent (Takara Bio Inc., Japan) according to the manufacturer's protocol. The cDNA was generated from the total RNA (2 µg) with a cDNA Reverse Transcription Kit (Applied Biosystems, USA), and SYBR-based real-time PCR was performed to detect the total mRNA transcripts on an Applied Biosystems (ABI) 7300 rapid real-time PCR system.

2.8 Western blot assay

Protein expression of Bax, Bcl-2, caspase3, CD31, SM22a, p-p65, p65, p-IkBa, IkBa and TLR4, was measured by Western blotting. Protein samples isolated from HUVEC cells (25 µg) were resolved using SDS-PAGE and then moved to polyvinylidene fluoride (PVDF) membrane (Millipore, USA) with being probed with GAPDH (1:5000, Cell Signaling), Bax (1:1000, Cell Signaling), Bcl-2 (1:1000, Cell Signaling), caspase3 (1:1000, Cell Signaling), CD31 (1:1000, Abcam), SM22a (1:1000, Abcam), p-p65 (1:1000, Proteintech), p65(1:1000, Proteintech), p-IkBa (1:1000, Proteintech), IkBa (1:1000, Proteintech) and TLR4 (1:1000, Proteintech) antibody followed by appropriate secondary antibody. All Western blots were repeated at least 3 times. Finally, the protein expression was visualized with enhanced chemiluminescence (ECL) system.

2.9 Immunofluorescence assay

Cells grown on slides were fixed, permeabilized, blocked, and immunostained overnight with antibodies of CD31 and SM22a (1:200, Abcam). After washing, specific second FITC-labeled IgG antibodies were incubated with cells continuously. Finally, the images were obtained under a microscope (Olympus, Japan) with appropriate magnification.

2.10 Statistical analysis

All the results are shown as the means \pm standard error of mean (SEM). Statistically significant results were determined using one-way ANOVA, followed by the Student–Newman–Keuls (S–N–K) *q* test. A *p*-value < 0.05 was considered to be significant.

3. Results

3.1 Myricetin attenuates ox-LDL-induced apoptosis in HUVECs

First of all, 100 $\mu\text{g/ml}$ ox-LDL incubation for 24 h led to a significant dose-dependent decrease in cell viability. Pretreatment with 1 μM Myr for 2 h had no significant influence on cell viability. However, 2.5 and 5 μM Myr caused a significant decrease in cell viability (Fig. 1A and B). We also examined the effect of Myr on apoptosis. Moreover, flow cytometry and apoptosis-related proteins results also revealed that the 100 $\mu\text{g/ml}$ ox-LDL-induced HUVEC apoptosis was dramatically dose-dependently reversed MYR-pretreated cells (Fig. 1C and D).

3.2 Myricetin inhibits the inflammatory response and EndMT in ox-LDL-induced HUVEC cells

Immunofluorescence staining showed that incubation of HUVECs with ox-LDL decreased expression of the endothelial marker CD31 and increased expression of the mesenchymal marker SM22 α were mitigated by MYR. Consistently, western blot showed that Myr attenuated the upregulated SM22 α protein level in HUVECs induced by ox-LDL (Fig. 2A and B). In the presence of ox-LDL, the mRNA levels of IL-6, MCP-1, VCAM-1, ICAM-1 were obviously upregulated in HUVECs, while Myr treatment reversed these aberrant changes (Fig. 2C). Likewise, similar results were further confirmed by ELISA (Fig. 2D).

3.3 GAS5 over-expression attenuates myricetin protective effects against ox-LDL-mediated HUVEC injury

We further detected the expression of GAS5 in HUVECs with qRT-PCR. As shown in Fig. 3A, a significantly higher level of GAS5 was observed in ox-LDL group than that in the control. In contrast, the mRNA level of GAS5 was reduced in the Myr-treated group in a dose-dependent manner. These data indicated that GAS5 might involve in the effect of Myr on endothelial cells. To understand the mechanisms by which GAS5 might involve in the protective effect of Myr, we employed pcDNA-GAS5 to overexpress the expression of

GAS5 in HUVECs. The expression of GAS5 was enhanced by pcDNA-GAS5, which was originally decreased by Myr in ox-LDL-induced HUVECs (Fig. 3B). Further experiments revealed that the viability of HUVEC cells with GAS5 overexpression was significantly decreased, and apoptosis was markedly elevated under the treatment of Myr (Fig. 3C and D). These data suggest that upregulating the expression of GAS5 attenuates myricetin protective effects against ox-LDL-mediated HUVEC injury.

3.4 miR-29a-3p is a target of GAS5 in HUVEC

The target GAS5 was identified via RegRNA 2.0 and starBase, the search results were shown in Fig. 4A. Among these miRNAs, miR-29a-3p was the most significant decrease in ox-LDL-mediated HUVEC injury (Fig. 4B). Bioinformatics analyses were carried to predict the binding regions between GAS5 and miR-29a-3p (Fig. 4C). To validate this bioinformatics prediction, dual-luciferase reporter gene assays indicated that GAS5-WT presented lower luciferase activity than the corresponding MUT group, confirming the binding relationship of GAS5 and miR-29a-3p (Fig. 4D). Meanwhile, the level of GAS5 enriched by Ago2 RIP was higher in the cells transfected with miR-29a-3p than that in the miR-NC group (Fig. 4E). Meanwhile, FISH analysis in HUVEC cells showed that GAS5 was co-localized with miR-29a-3p mainly in the nucleus (Fig. 4F). These findings suggested that GAS5 targeting miR-29a-3p.

3.5 miR-29a-3p mimics rescued the effects of GAS5 in ox-LDL-induced HUVEC treated with myricetin

As shown in Fig. 5A, the expression of miR-29a-3p was significantly increased in the ox-LDL + Myr + pcDNA-GAS5 + miR-29a-3p mimics group compared with the ox-LDL + Myr + pcDNA-GAS5 + mimic control group (Fig. 5A). Moreover, upregulated of GAS5 and miR-29a-3p significantly increased the viability of ox-LDL induced HUVECs treated by Myr (Fig. 5B). Additionally, addition of GAS5 and miR-29a-3p remarkably suppressed apoptosis in ox-LDL induced HUVECs treated by Myr (Fig. 5C).

3.6 sh-GAS5 or miR-29a-3p mimics can accelerate the protective role of myricetin in ox-LDL-induced HUVECs cell

To explore whether GAS5 and miR-29a-3p were involved in Myr-mediated regulatory mechanism, HUVECs were transfected with NC, sh-GAS5 mimics control, miR-29a-3p mimics. The expression of GAS5 was significantly decreased in the sh-GAS5 group compared with the NC group (Fig. 6A). After the transfection, ox-LDL + Myr increased the viability when compared with the ox-LDL group, and inhibition of GAS5 or upregulated of miR-29a-3p increased the viability of ox-LDL induced HUVECs treated by Myr (Fig. 6B). Additionally, Myr restrained cell apoptosis caused by ox-LDL, which was subsequently altered by sh-GAS5 and miR-29a-3p mimics (Fig. 6C). Moreover, the protein level of CD31 and SM22a was increased in ox-LDL + Myr + sh-GAS5 and ox-LDL + Myr + miR-29a-3p mimics groups compared with the ox-LDL + Myr group (Fig. 6D). Likewise, similar results of IL-6, MCP-1, VCAM-1 were detected by ELISA (Fig. 6E).

3.7 Myricetin inactivated the TLR4/NF- κ B signaling pathway in ox-LDL-treated HUVEC by down-regulating GAS5 or upregulating miR-29a-3p

The TLR4/NF- κ B signaling pathway is closely involved in the process of AS. In order to explore whether Myr suppressed the TLR4/NF- κ B signaling pathway, the TLR4 and several pivotal downstream proteins, e.g., p-p65, p65, p-I κ Ba, I κ Ba, and TLR4, were identified in HUVEC cells via western blot analysis. Figure 6 shows that Myr downregulated the expression of p-p65, p-I κ Ba, and TLR4 as compared to the ox-LDL group, while the protein level of p-p65, p-I κ Ba, and TLR4 were decreased in ox-LDL + Myr + sh-GAS5 and ox-LDL + Myr + miR-29a-3p mimics groups compared with the ox-LDL + Myr group (Fig. 7). Taken together, these results suggest that GAS5/miR-29a-3p /TLR4/NF- κ B pathway might be one of the underlying mechanisms through which Myr reduced HUVEC cell inflammatory and EndMT.

4. Discussion

AS is a persistent inflammatory condition that is started by the deposition as well as accumulation of low-density lipoproteins in the artery wall[19]. In spite of excellent advances in both basic and scientific study, is still among the leading reasons for death in the world [20]. In this study, we explored the molecular mechanism of Myr on ox-LDL-induced damage of HUVECs, revealing the crucial role of lncRNA GAS5 in this event. Our results suggested that Myr regulates HUVECs cell viability, apoptosis, inflammation, and EndMT through the GAS5/miR-29a-3p/TLR4/NF- κ B pathway, clarifying a new mechanism of Myr on protecting against AS.

Ox-LDL has been shown to induce an enhanced generation of ROS, which play critical role in the progression of AS. Substantial evidence suggests that increased oxidative stress plays a prominent role in the pathogenesis of vascular endothelial dysfunction along with endothelial cell EndMT, inflammation, and apoptosis. We used ox-LDL stimulated HUVECs to simulate the occurrence of early atherosclerosis and the inflammatory response and EndMT of HUVECs was increased, and Myr significantly inhibited inflammatory response and EndMT to HUVECs stimulated with ox-LDL. Myr is known to exert antioxidative cytoprotective effects in various cells, including a HUVEC cell line [21]. A previous study found that Myr exhibited pro-proliferative and anti-apoptotic effects in LPS-induced cardiomyocytes H9c2 cells injury [22]. In this research study, we checked out the result of Myr on ox-LDL-induced atherosclerotic cell version. Our results suggested that 5 μ M Myr pretreatment can considerably turn around the ox-LDL-induced downregulation of cell viability in HUVECs. Our results demonstrated that ox-LDL therapy could advertise the inflammation and EndMT of HUVECs through regulating CD31, SM22a, and inflammatory cytokines (IL-6, MCP-1, VCAM-1), which can be reversed by Myr pretreatment. A number of elements established that inflammation and EndMT accompanies atherosclerosis [23]. A previous study found inhibition of the expression of VCAM-1 and ICAM-1 has been recognized as an important strategy against atherosclerosis[24]. Moreover, vaccarin suppressed ox-LDL induced endothelial EndMT through

downregulating endothelial marker CD31 and upregulating mesenchymal marker SM22 α [25]. Our findings are in substantial agreement with these researches.

The expression of GAS5 was highly expressed in both human and animal models [26, 27]. In this study, we found that GAS5 was upregulated and miR-29a-3p was downregulated in ox-LDL-induced HUVEC injury. Myr treatment can reverse these effects. A previous study showed that Myr inhibited the HMGB1, TLR4, and MyD88 expressions in the neurons, and it restored neuronal damage and inflammation caused by activation of NF- κ B and MAPK signal pathways [28]. In this study, up-regulation of lncRNA GAS5 and down-regulation of miR-29a-3p led to a decrease of cell apoptosis, inflammation, and EndMT, as well as reduction of activity of TLR4/NF- κ B signaling pathway. According to our findings, Myr reduces the level of p-p65, p-I κ B α , and TLR4 in HUVECs by regulating GAS5/miR-29a-3p, thus attenuating the inflammatory response and EndMT in ox-LDL-induced HUVEC injury.

Finally, our research study exposed that Myr is capable of ameliorating cell apoptosis, cell inflammation, and EndMT via GAS5/miR-29a-3p/TLR4/NF- κ B pathway (Figure 8), suggesting Myr as a potential therapeutic agent for AS. In addition, GAS5 was proposed to be a promising target molecule involving in the pathophysiological processes of AS.

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

We declared that there were no conflicts of interest in this paper.

Author contributions

Research idea and study design: Yunpeng Bai, Zhigang Guo, Nan Jiang; data acquisition: Yunpeng Bai, Qingliang Chen; data analysis/interpretation: Yunpeng Bai, Qingliang Chen; statistical analysis: Qingliang Chen; supervision or mentorship: Zhigang Guo, Nan Jiang.

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Figures

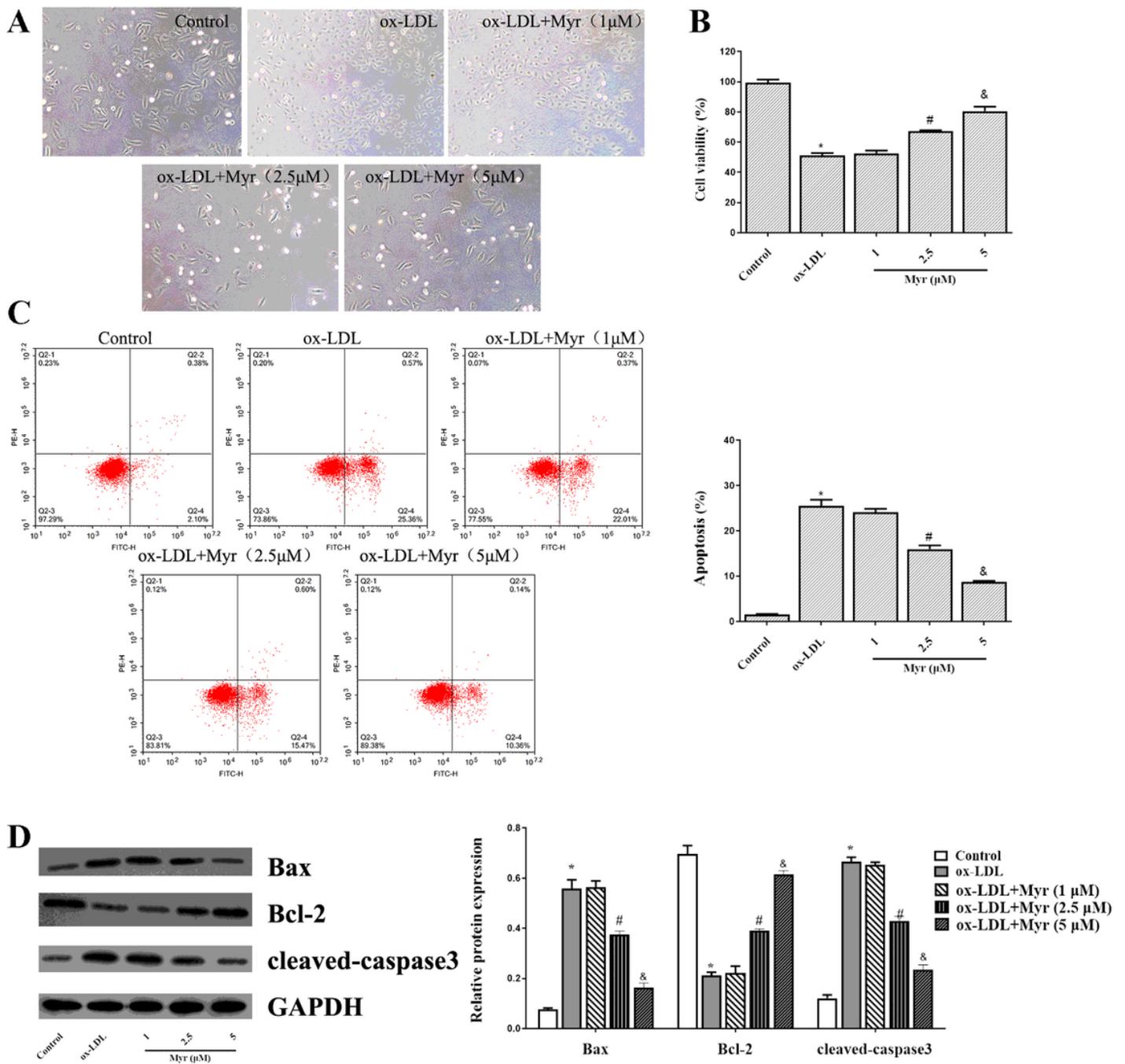


Figure 1

Myricetin attenuates ox-LDL-induced apoptosis in HUVECs. (A) Observed under a microscope and photographed for different groups. (B) CCK8 assay of different Myr concentration on ox-LDL-induced HUVECs cell viability. (C) Representative FACS plots of Annexin V fluorescein isothiocyanate (FITC)/propidium iodide (PI) stained endothelial cells treated with ox-LDL or cotreated with ox-LDL and Myr at different concentrations for 24 h. (D) The expression of apoptosis-associated proteins were

determined by western blotting. *P < 0.05 compared with control; #P < 0.05 compared with ox-LDL; &P < 0.05 compared with 2.5μM.

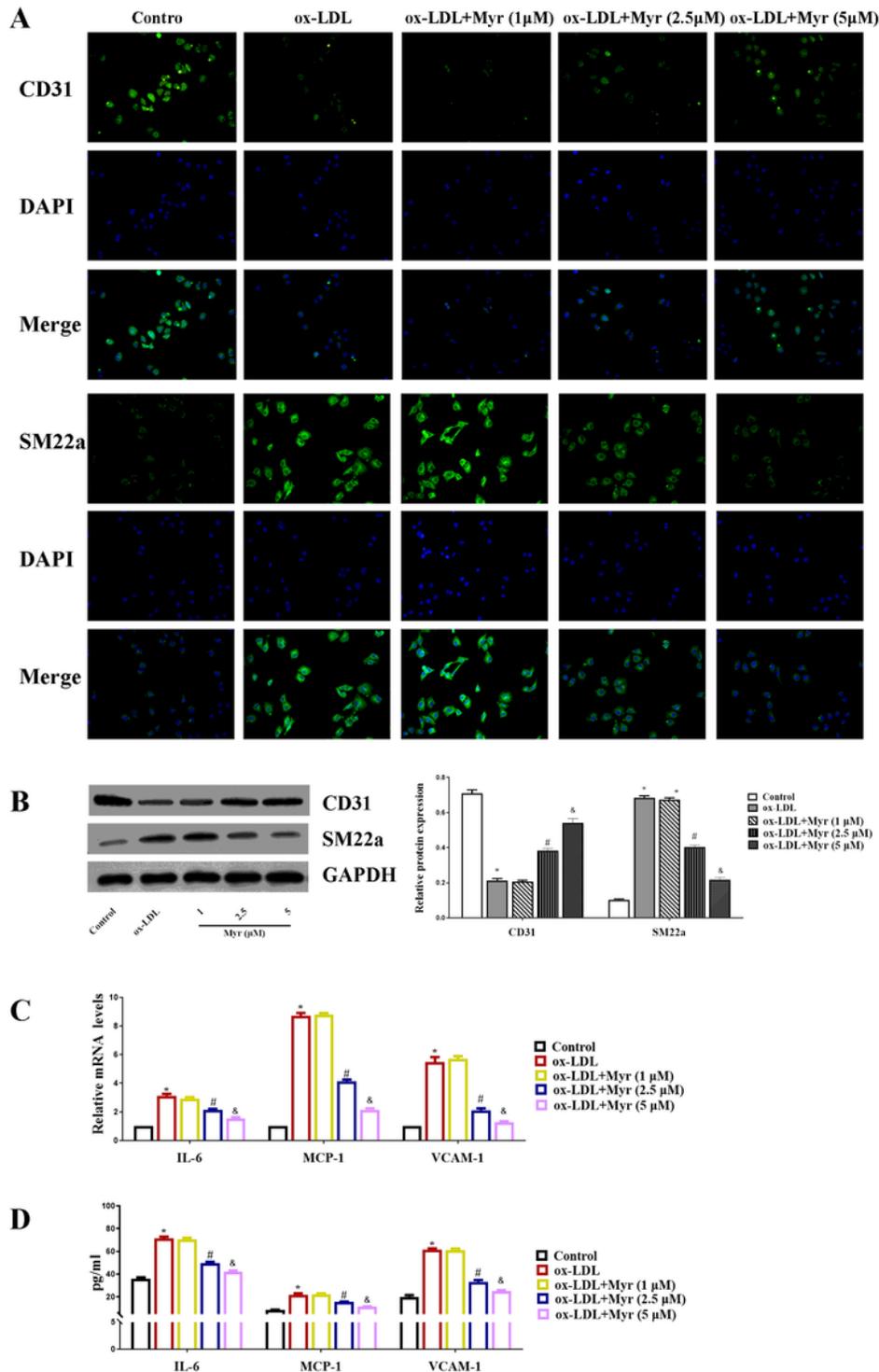


Figure 2

Myricetin inhibits the inflammatory response and EndMT in ox-LDL-induced HUVEC cells. (A) Representative immunofluorescence images of CD31 and SM22a expression in each group. (B) Western blot analysis of CD31 and SM22a expression in HUVEC cells. (C) The mRNA levels of IL-6, MCP-1 and

VCAM-1 in each group. (D) The protein levels of IL-6, MCP-1 and VCAM-1 were detected by ELISA assay. *P < 0.05 compared with control; #P < 0.05 compared with ox-LDL; &P < 0.05 compared with 2.5µM.

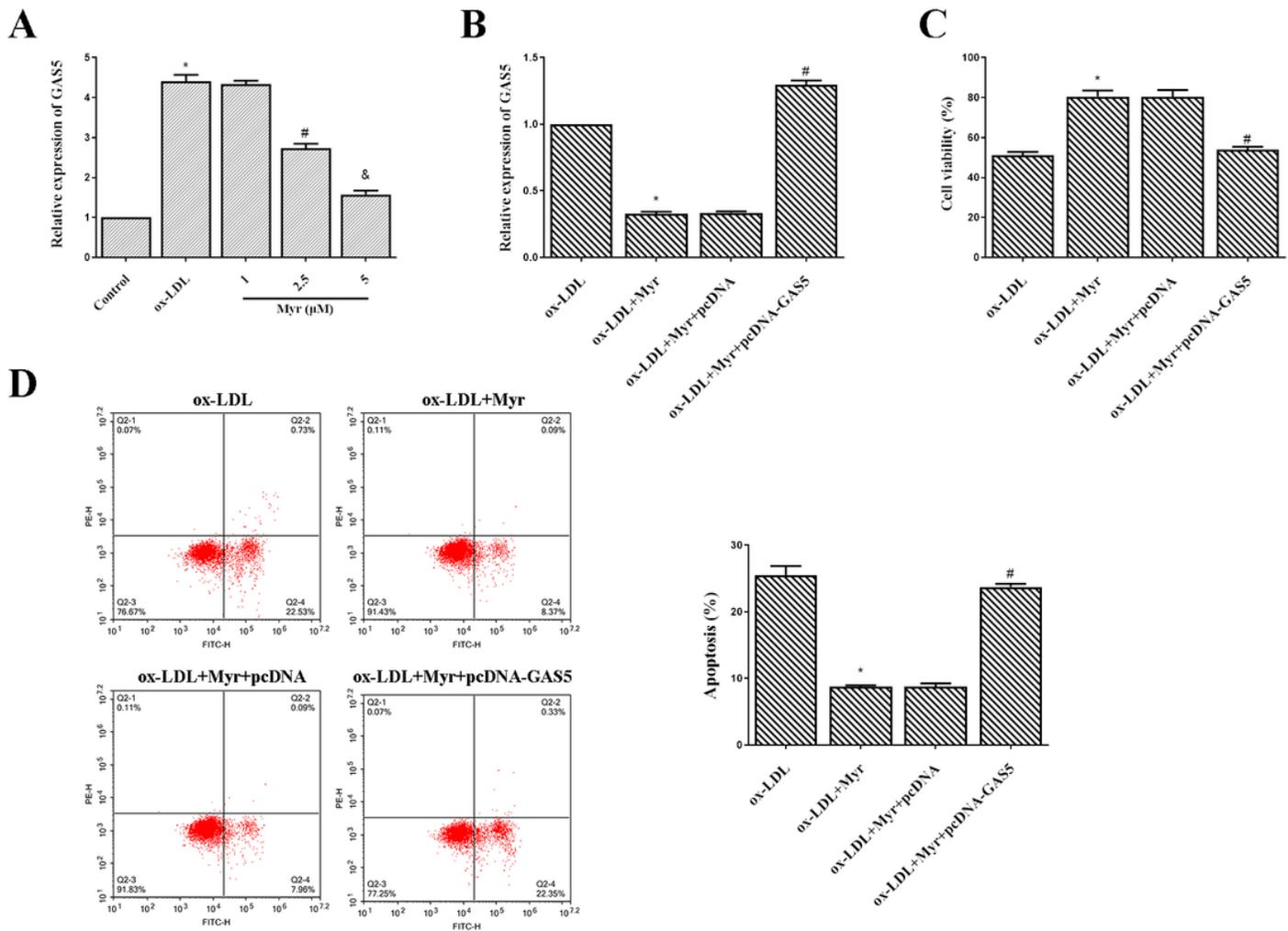


Figure 3

GAS5 over-expression attenuates myricetin protective effects against ox-LDL-mediated HUVEC injury. (A) GAS5 expression was determined with qRT-PCR. HUVEC cells were pre-incubated with 1/2.5/5 µM Myr for 2 h and then stimulated with or without 100 µg/mL ox-LDL for 24h. Representative immunofluorescence images of CD31 and SM22a expression in each group. (B) Western blot analysis of CD31 and SM22a expression in HUVEC cells. (C) The mRNA levels of IL-6, MCP-1 and VCAM-1 in each group. (D) The protein levels of IL-6, MCP-1 and VCAM-1 were detected by ELISA assay. *P < 0.05 compared with control; #P < 0.05 compared with ox-LDL; &P < 0.05 compared with 2.5µM.

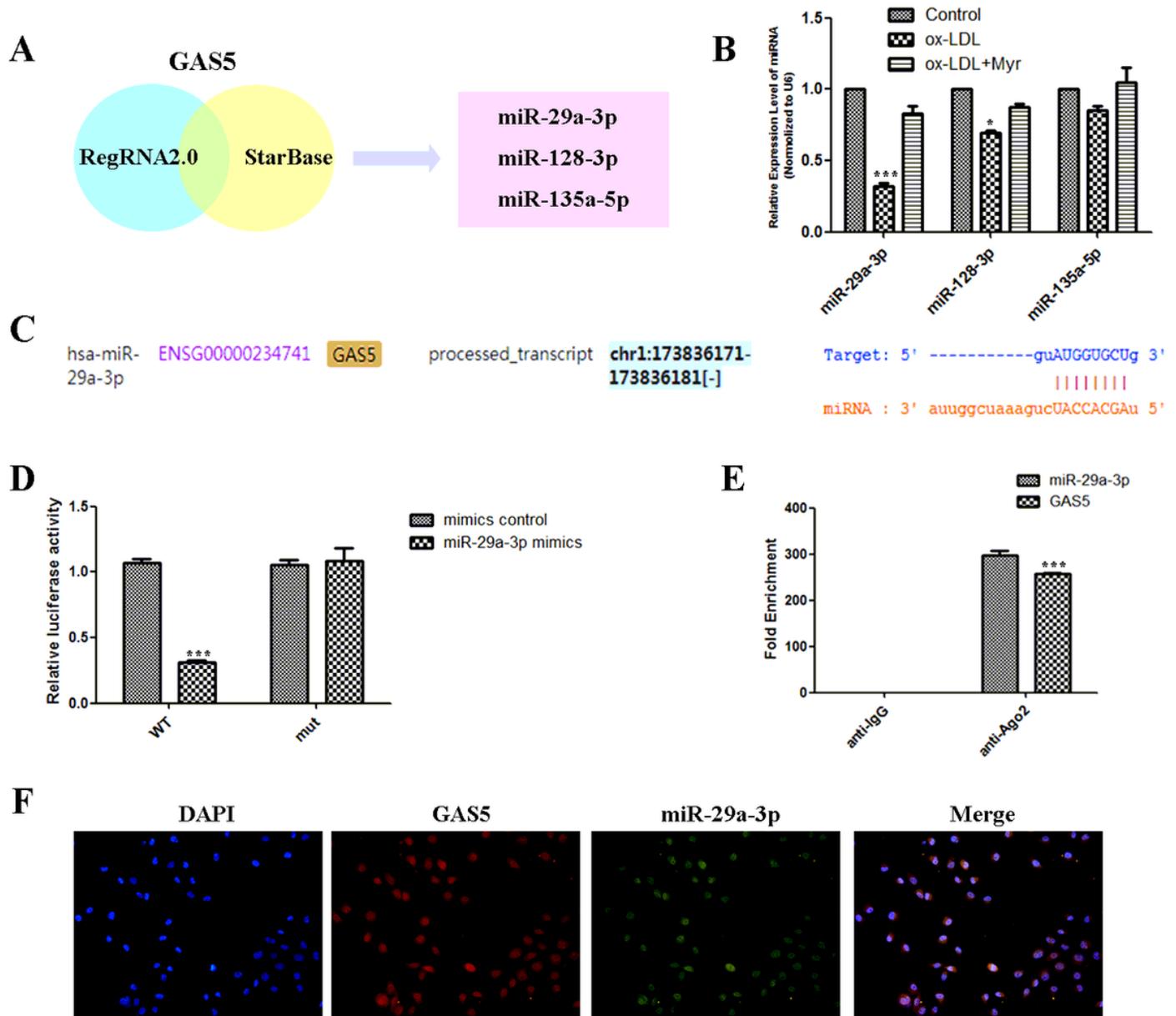


Figure 4

miR-29a-3p is a target of GAS5 in HUVEC. (A) Bioinformatics analyses including RegRNA2.0 and StarBase were used for prediction. (B) Three microRNAs were identified, including miR-29a-3p, miR-128-3p and miR-135a-5p. (C) The predicted binding between GAS5 and miR-29a-3p. (D) Dual-luciferase reporter assay validating the interaction between GAS5 and miR-29a-3p. (E) RIP assay and qRT-PCR were conducted to detect the enrichment of GAS5 and miR-29a-3p by using AGO2 antibody in HUVEC cells. (F) RNA FISH for GAS5 and miR-29a-3p was detected in HUVEC cells, miR-29a-3p was co-localized with GAS5 in nucleus (magnification, $\times 400$). Nuclei was stained blue (DAPI), GAS5 was stained red, miR-29a-3p was stained green. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

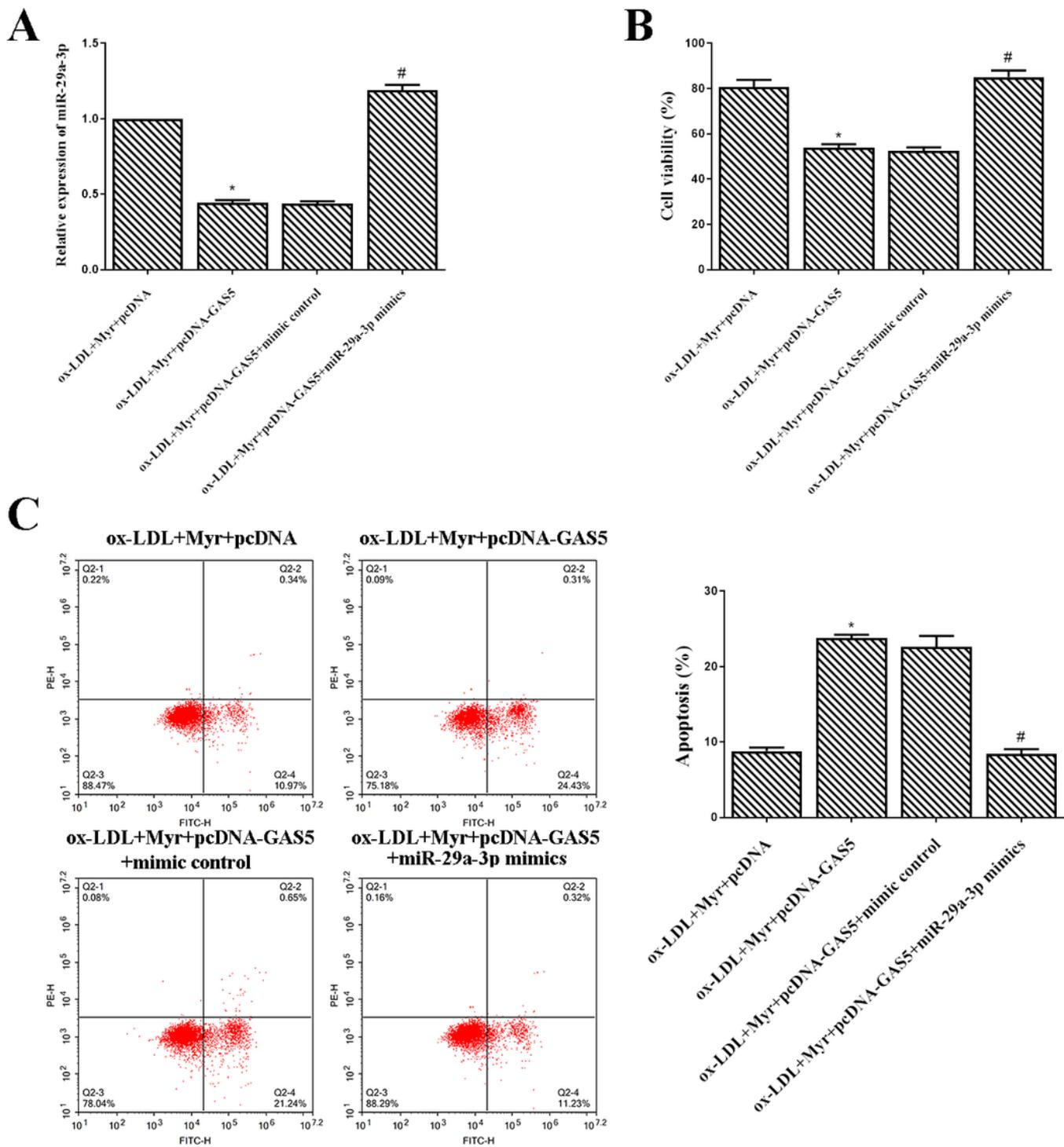


Figure 5

miR-29a-3p mimics resuscitated the effects of GAS5 in ox-LDL-mediated HUVECs. (A) . miR-29a-3p expression was detected using qRT-PCR with U6 as an internal control. (B) Cell viability was detected by MTT assay. (C) Apoptosis of HUVEC cells. Flow cytometry assay was used to test apoptosis analysis. *P < 0.05 compared with ox-LDL+Myr+pcDNA; #P < 0.05 compared with ox-LDL+Myr+ pcDNA-GAS5+mimic control.

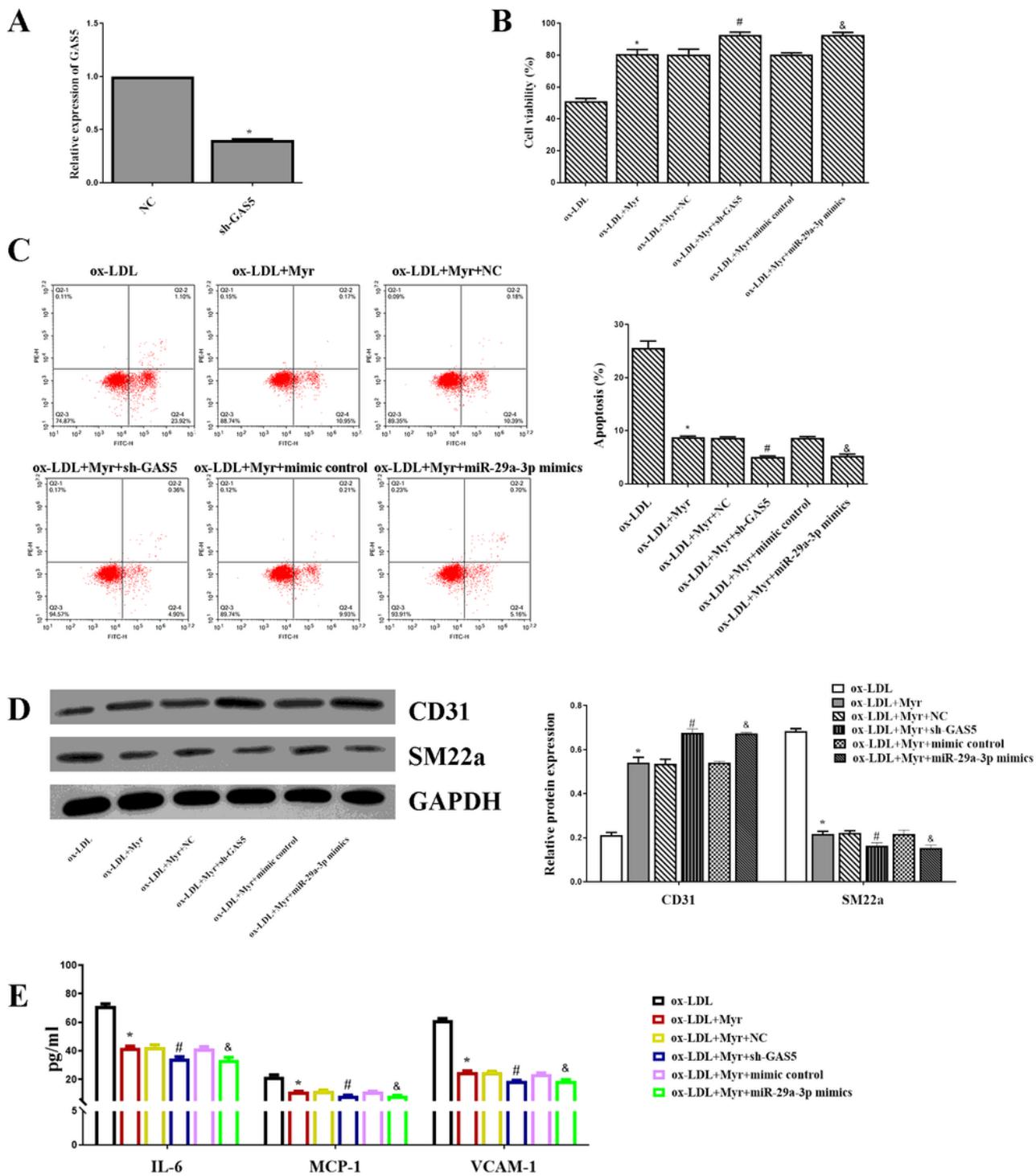


Figure 6

sh-GAS5 or miR-29a-3p mimics can accelerate the protective role of myricetin in ox-LDL-induced HUVECs cell apoptosis. (A) GAS5 expression in HUVEC cells infected with shRNA-GAS5 or negative controls for 48 hr. (B) Cell viability was detected by MTT assay. (C) Apoptosis of HUVEC cells. Flow cytometry assay was used to test apoptosis analysis. (D) Western blot analysis of CD31 and SM22a at the protein level. (E)

ELISA analysis of IL-6, MCP-1 and VCAM-1. *P < 0.05 compared with ox-LDL; #P < 0.05 compared with ox-LDL+Myr+NC; &P < 0.05 compared with ox-LDL+Myr+mimic control.

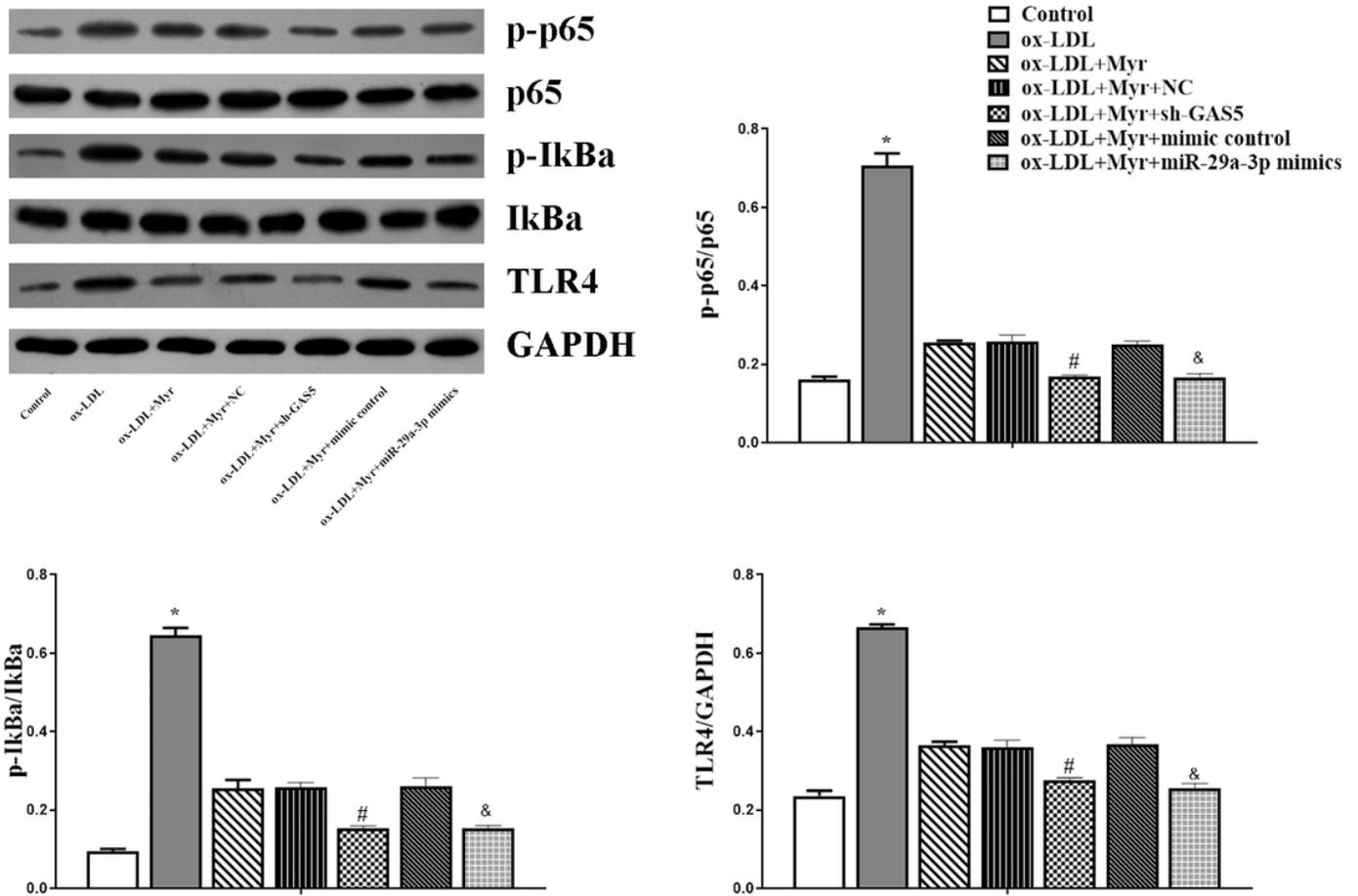


Figure 7

Myricetin inactivated the TLR4/NF- κ B signaling pathway in ox-LDL-treated HUVEC by down-regulating GAS5 or upregulating miR-29a-3p. The expressions of p-p65, p65, p-IkBa, IkBa and TLR4 in the HUVEC cells were determined by Western blot analysis. *P < 0.05 compared with control; #P < 0.05 compared with ox-LDL+Myr+NC; &P < 0.05 compared with ox-LDL+Myr+mimic control.