



m⁶A 'writer' KIAA1429 regulates the proliferation and migration of endothelial cells in atherosclerosis

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Abstract

Increasing evidences have illustrated the important role of N⁶-methyladenosine (m⁶A) in atherosclerosis (AS). However, the role of m⁶A modification in AS pathophysiological process is still unknown. Here, the present work tried to investigate the expression and function of m⁶A methyltransferase KIAA1429 in AS pathology and explored its undergoing m⁶A-dependent molecular mechanism. Results indicated that KIAA1429 remarkably up-regulated in oxidative low-density lipoprotein (ox-LDL)-treated human umbilical vein endothelial cells (HUVECs). KIAA1429 over-expression inhibited the proliferation/migration in ox-LDL-treated HUVECs, while, KIAA1429 knockdown up-regulated the proliferation and migration. Mechanistically, via m⁶A modification sites binding, ROCK2 mRNA was post-transcriptionally upregulated by KIAA1429 in response to Actinomycin D. Collectively, our study demonstrated the regulation of KIAA1429 on ox-LDL-induced HUVECs via m⁶A/ROCK2 pathway. These findings provide new insights for m⁶A-mediated epigenetics in AS.

Keywords N⁶-methyladenosine · KIAA1429 · HUVECs · Endothelial cells · Atherosclerosis

Introduction

Atherosclerosis (AS) acts as chronic inflammatory vascular disorders with increasing morbidity worldwide, which is a deposition of plaque accumulation and multifactorial inflammatory process in vascular walls [1, 2]. AS is responsible for occurrence of various clinical manifestation, e.g., coronary heart diseases, peripheral arterial disease and myocardial infarction and stroke [3, 4]. Low density lipoprotein (LDL) functions as a basic factor of AS. LDL enters into the vascular wall through vascular endothelium, and the retained LDL is modified into ox-LDL. Ox-LDL is phagocytosed by macrophages to form foam cells, which continuously increases and fuse to form the lipid core of AS plaque [5, 6]. Endothelial dysfunctions, especially endothelial cells damage, function as critical pathology for AS [7, 8].

N⁶-methyladenosine (m⁶A) acts as the most abundant posttranscriptional modification for mRNAs, which partially determines the fate of RNA, including RNA stabilization, splicing, and nuclear export [9, 10]. m⁶A methylations have been shown to regulate series of inflammatory processes, including the inflammatory vascular disorder of endothelial cells in AS [11]. For example, FTO-mediated m⁶A demethylations regulate the expression of lipid-related genes and regulates lipid metabolism to lead to occurrence of diabetic hyperlipidemia [12]. m⁶A methyltransferase methyltransferase-like 3 (METTL3) is highly expressed in ox-LDL-induced HUVECs and METTL3 knockdown inhibits HUVECs' proliferation and tube formation in ox-LDL-treated HUVECs. Besides, METTL3 positively regulates JAK2/STAT3 pathway in m⁶A-dependent manner in HUVECs [13]. Therefore, the emerging findings indicate the critical roles of m⁶A in AS.

Given that the potential function of m⁶A in abnormal lipids metabolism-related AS pathology, we focused on the undergoing regulation of m⁶A in ox-LDL-induced HUVECs. In the initial screening, we detected several m⁶A key enzymes (i.e., KIAA1429, METTL3, WTAP) to discover the up-regulated or down-regulated elements in ox-LDL-induced HUVECs. Results showed that the novel m⁶A writer KIAA1429 up-regulated upon ox-LDL administered.

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Finally, we focused on KIAA1429 and then explored its functions. Our cellular assays' results showed that the expression of KIAA1429 (also known as VIRMA) increased upon ox-LDL administered and the response showed a dosage-dependent manner. KIAA1429 could regulate the proliferation and migration of HUVECs in ox-LDL administered. Moreover, KIAA1429 bound to the m⁶A modification sites of ROCK2 mRNA and then enhanced its mRNA stability. In conclusion, m⁶A writer KIAA1429 targeted m⁶A/ROCK2 axis to regulate the proliferation/migration of endothelial cells.

Materials and Methods

Cell Culture

HUVECs (Human umbilical vein endothelial cells) cell lines were provided by the Institute of the Chinese Academy of Sciences (Shanghai, China). Then, the cells were cultured in endothelial cell medium (Catalog #1001, ScienCell Research Laboratories, San Diego, CA, United States) supplemented with 10–12% fetal bovine serum (FBS, Catalog #0500, ScienCell Research Laboratories), 1% endothelial cell growth supplement (ECGS, Catalog #1052, ScienCell Research Laboratories), and 1% penicillin/streptomycin solution (Catalog #0513, ScienCell Research Laboratories). Passages 2–4 of cells were used for this project. All cells were cultured in humidified air in 37 °C at 5% CO₂. ox-LDL (Yesen, Shanghai, China) was added to HUVECs (0–100 µg/mL concentration, 24 h) to construct endothelial cell injury. This study was approved by the ethics committee of Tianjin Hospital.

Transfection

For the silencing of KIAA1429 in vitro studies, the shRNA targeting KIAA1429 (sh-KIAA1429) and negative control siRNA (sh-NC) were synthesized (RiboBio, Guangzhou, China) based on the manufacturer's suggestion. As regarding to overexpression of KIAA1429, pcDNA based overexpression plasmid specific to KIAA1429 or corresponding scrambled oligonucleotide sequences as a negative control were transfected.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from HUVECs samples using modified TRIzol co-purification technique (Invitrogen, Hemel Hempstead, UK) as previously reported [14]. In brief, 500 µl of cell suspension was washed by 75–78% ethanol before solubilization using nuclease-free water (50 µl). To identify the level of KIAA1429 mRNA, RNA (1 µg) was

reversely transcribed to cDNA by PrimeScript™ RT Reagent Kit (TaKaRa). Then, RT-qPCR was performed using One Step TB Green® PrimeScript™ PCR Kit (TaKaRa, Cat.#RR086B) as recommended by manufacturers' protocols. The quantification of mRNA reference gene was calculated using the 2^{-ΔΔCt} method. The primers used in PCR amplification were showed in Table S1.

Western Blot

Total proteins were collected using RIPA buffer (Thermo Scientific, CA, USA) and then quantified for protein quantification by using Pierce™ BCA Protein Quantification Kit (Thermo Scientific) [15]. Protein (20 µg) was used for electrophoresis loading SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk and incubated with primary antibodies, including anti-KIAA1429 (Cell Signaling Technology, 1:1000, #88,358), anti-ROCK2 (Cell Signaling Technology, 1:1000, #47,012), and beta-actin (CWBio, Beijing, China). After incubation by primary antibodies or their corresponding secondary antibodies, blots were developed using SuperSignal West Dura Persistence Substrate (Thermo Scientific).

Proliferation CCK-8 Assay

For the proliferation of HUVECs, CCK-8 assay was performed. HUVECs cells were plated onto 96-well plates. 10 µL CCK-8 reagent (Dojindo Japan) was added into each well at 24, 48, 72, and 96 h. Then, optical density (OD) value of wells was detected at 450 nm was recorded by automatic enzyme-mark reader (Multiskan FC, Thermo Fisher Scientific, Waltham, MA, USA) using a microplate reader.

Migration Assay

For the migration of HUVECs, wound healing assay was performed. In brief, 2 × 10⁴ HUVECs about 90% confluence were plated 6-well plates. Medium was removed and then cell monolayers were manually wounded with 200 µl pipette tip. After twice washing with PBS, cells were incubated at 37 °C. The wound closure was evaluated with an inverted microscope. The migration rate was quantified according to distance. The migration rate was calculated: migration rate = migration distance/original distance.

m⁶A Quantification

The m⁶A quantification of global mRNA was measured by EpiQuik m⁶A RNA Methylation Quantification Kit (Colorimetric) (Epigentek) following the manufacturer's protocol. 200 ng Poly-A-purified RNA was coated on assay wells and

capture antibody solution was separately added to suitable concentration following the manufacturer's instructions. The m⁶A level was colorimetrically quantified by absorbance at a wavelength of 450 nm.

RNA Immunoprecipitation (RIP) assay

The interaction within RNAs was identified by RIP-PCR. RIP assay was performed by Magna RIP-RNA-Binding Protein Immunoprecipitation Kit (Magna RIP Kit; Millipore, Bedford, MA, USA). Cell was lysed in lysis buffer with protease inhibitor/RNase inhibitor, and the cell extract was incubated with protein A/G agarose beads conjugated with antibody/IgG at 4 °C for 2 h. Magnetic dynabeads (Life Technologies, USA) were washed or incubated with Proteinase K. Finally, purified RNA was subjected for RT-PCR analysis.

m⁶A-RNA Immunoprecipitation PCR (MeRIP-PCR)

To quantify the m⁶A-modified ROCK2 mRNA, MeRIP-PCR was performed. In brief, anti-m⁶A antibody (Millipore, cat. #ABE572) was conjugated to protein A/G magnetic beads in IP buffer (20 mM Tris pH 7.5, 1% NP-40, 140 mM NaCl, 2 mM EDTA). Total RNA was isolated from HUVECs and incubated with the antibody in IP buffer supplemented with RNase inhibitor and protease inhibitor. The precipitated RNA was eluted with elution buffer and detected using further qRT-PCR assay.

RNA Stability

To analyze the ROCK2 mRNA stability, HUVECs were administrated with actinomycin D (Act D, 2 µg/mL, Sigma) and then collected in different time-point (0 h, 3 h, 6 h). ROCK2 mRNAs were extracted using Trizol reagent. Finally, mRNA levels were detected by qRT-PCR and normalized to the values measured in the mock treatment group (the 0 h group) using the primers: forward, 5'- TCAGAG GTCTACAGATGAAGGC-3', reverse, 5'- CCAGGGGCT ATTGGCAAAGG-3'.

Statistical Analysis

Data was expressed as the mean ± standard deviation (SD) in independent replicate. Student's *t*-test or variance (ANOVA) analysis was used to compare difference of independent groups. Assays were performed in triplicate. Data were exhibited as Mean ± Standard Deviation (SD). ***p* < 0.01, **p* < 0.05 were considered a statistical difference.

Results

KIAA1429 up-Regulated in the ox-LDL-Induced HUVECs

To construct the cellular AS model, human endothelial cells (HUVECs) were administrated with ascending ox-LDL (0–100 µg/mL) concentration. With the ascending dosage of ox-LDL, m⁶A quantitative analysis was performed and results demonstrated that m⁶A modification level increased upon ox-LDL administered (Fig. 1A). Besides, the level of m⁶A writer KIAA1429 in the ox-LDL-induced HUVECs was detected and results indicated that KIAA1429 mRNA (Fig. 1B) and protein levels (Fig. 1C) were upregulated in ox-LDL treated HUVECs. Moreover, the proliferation of HUVECs was detected and results illustrated that proliferative ability of HUVECs was repressed in ox-LDL administered in dosage-dependent manner (Fig. 1D). In conclusion, these results and findings suggested that KIAA1429 upregulated in ox-LDL-induced HUVECs.

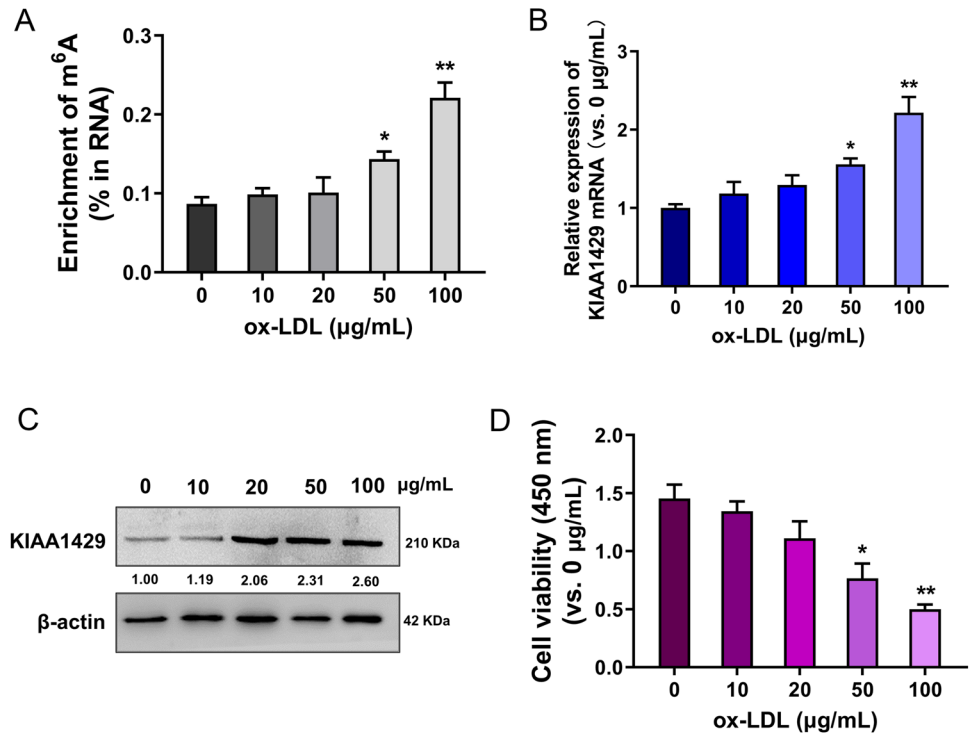
KIAA1429 Inhibited the Proliferation/Migration for ox-LDL Administrated HUVECs

Given that KIAA1429 showed an overexpression in ox-LDL administrated human endothelial cells (HUVECs), we subsequently performed the bio-functional assays to confirm the role of KIAA1429 in AS. Firstly, KIAA1429 knockdown (Fig. 2A) and overexpression (Fig. 2B) transfection were respectively constructed in HUVECs. The proliferation of HUVECs was detected and results illustrated that proliferative ability of HUVECs was up-regulated in KIAA1429 knockdown, while KIAA1429 overexpression repressed proliferation (Fig. 2C). Wound healing assay was performed and results indicated that KIAA1429 knockdown promoted the migration of HUVECs, while KIAA1429 overexpression reduced it (Fig. 2D). In conclusion, these results and findings suggested that KIAA1429 inhibited the proliferation/migration for ox-LDL administrated HUVECs.

ROCK2 Acted as the Target of KIAA1429

Using diverse screening methods, we carefully discovered the target of KIAA1429, e.g., bioinformatics predictive analysis and multiplex cell assays (RT-PCR, western blot et. al). Our pre-experiments data illustrated that ROCK2 (Rho associated coiled-coil containing protein kinase 2) had m⁶A modification sites in its 3'-UTR, which suggested that ROCK2 might act as the target of KIAA1429 (Fig. 3A). In the pathophysiology of AS, ROCK2 functions as an essential element in AS [16]. The m⁶A modification site on ROCK2 mRNA was 'GGACU' motif. In the ROCK2 gene, the m⁶A

Fig. 1 KIAA1429 up-regulated in the ox-LDL-induced HUVECs. **A** m⁶A quantitative analysis was performed to detect the m⁶A modification level in HUVECs with ox-LDL administered (0–100 µg/mL). **B** RT-PCR detected the level of KIAA1429 mRNA in HUVECs with ox-LDL administered (0–100 µg/mL). **C** Western blot was performed to show the KIAA1429 protein in HUVECs with ox-LDL administered (0–100 µg/mL). **D** The proliferation of HUVECs was detected using CCK-8 to illustrate the proliferative ability of HUVECs. Assays were performed in triplicate. Data were exhibited as Mean ± Standard Deviation (SD). ***p* < 0.01, **p* < 0.05



modification site distributed in 3'-UTR of ROCK2 (Fig. 3B). Using the SRAMP online tool (<http://www.cuilab.cn/sramp>), analysis demonstrated that there were m⁶A modification site on ROCK2 mRNA (Fig. 3C). In ox-LDL-treated HUVECs, m⁶A quantitative analysis indicated that KIAA1429 knockdown reduced the m⁶A modification level, while KIAA1429 overexpression up-regulated the m⁶A modification level (Fig. 3D). RIP-PCR analysis revealed that KIAA1429 remarkably interacted with ROCK2 mRNA in HUVECs (Fig. 3E). In conclusion, these results and findings suggested that ROCK2 acted as the target of KIAA1429.

KIAA1429 Enhanced the Stability of ROCK2 mRNA

Moreover, we tried to explore the function of KIAA1429 on ROCK2. Firstly, MeRIP-PCR analysis was performed to detect the m⁶A modification level on ROCK2 mRNA. Results indicated that KIAA1429 knockdown reduced the m⁶A modification on ROCK2 mRNA (Fig. 4A), while KIAA1429 overexpression up-regulated the m⁶A modification level (Fig. 4B). In the ox-LDL-induced HUVECs, ROCK2 mRNA increased upon ox-LDL administered in dosage-dependent manner (Fig. 4C). RIP-qPCR analysis found that KIAA1429 knockdown reduced the precipitated ROCK2 mRNA enrichment, while KIAA1429 overexpression up-regulated the precipitated ROCK2 mRNA enrichment (Fig. 4D). RNA stability analysis revealed that KIAA1429 knockdown decreased the ROCK2 mRNA upon Act D treatment, while KIAA1429 overexpression

up-regulated the ROCK2 mRNA upon Act D treatment (Fig. 4E). Moreover, KIAA1429 knockdown reduced the ROCK2 protein in ox-LDL-induced HUVECs, while KIAA1429 overexpression increased the ROCK2 protein (Fig. 4F). In conclusion, these results and findings suggested that KIAA1429 enhanced the stability of ROCK2 mRNA.

Discussion

In atherosclerosis (AS), the activity of vascular endothelial cells could reflect cells' metabolism and proliferation [17–19]. AS is a life-threatening vascular disease, and m⁶A modification level is dysregulated in its pathophysiologic processes of AS [20, 21]. Here, we found that m⁶A writer KIAA1429 upregulated in ox-LDL-induced HUVECs. Moreover, the function and corresponding molecular mechanism in AS progression are of great value for precision targeted therapy.

Here, this research constructed cellular AS model using ox-LDL-induced HUVECs, and results revealed that m⁶A level increased upon ox-LDL administered. Because the deregulated m⁶A level in ox-LDL-induced HUVECs, we assumed that m⁶A regulators might participate in HUVECs' pathophysiology.

In the initial screening, we detected several m⁶A key enzymes (i.e., KIAA1429, METTL3, WTAP) to discover the up-regulated or down-regulated elements. Finally, we focused on the novel m⁶A writer KIAA1429 and then

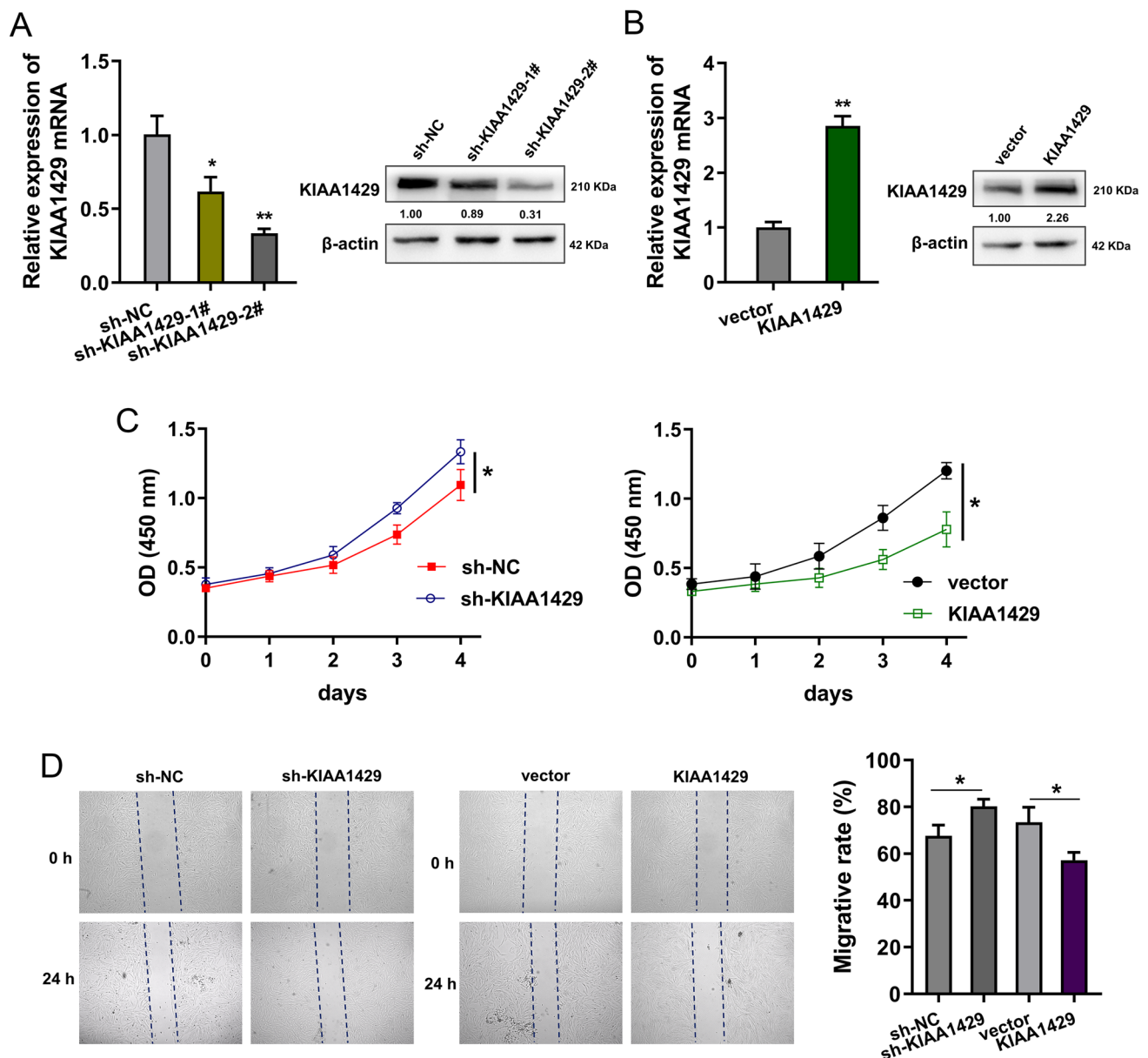


Fig. 2 KIAA1429 repressed the proliferation/migration for ox-LDL administrated HUVECs. **A**, **B** RT-qPCR and western blot for (A) KIAA1429 knockdown and (B) KIAA1429 overexpression were performed to detect the efficient of transfection. **C** CCK-8 assay for the proliferation of HUVECs was performed to illustrate the prolifera-

tive ability of HUVECs upon KIAA1429 knockdown and KIAA1429 overexpression. **D** Wound healing assay indicated the migration of HUVECs upon KIAA1429 knockdown and KIAA1429 overexpression. Assays were performed in triplicate. Data were exhibited as Mean \pm Standard Deviation (SD). ** $p < 0.01$, * $p < 0.05$

explored its functions. Our cellular assays' results showed that the expression of KIAA1429 increased upon ox-LDL administered and the response showed a dosage-dependent manner.

Functional assays illustrated that KIAA1429 overexpression repressed proliferation and migration of HUVECs, while KIAA1429 knockdown recovered the repression on HUVECs' proliferation and migration. As is known to all that the ability of proliferation/migration of vascular endothelial cell is very critical for the vascular bio-function.

Thus, in these findings, KIAA1429 regulated the proliferation and migration of HUVECs, which significantly showed the roles of KIAA1429 on HUVECs.

Increasing evidence synergistically indicates the potential vital roles of m^6A modification on AS [22]. For instance, ox-LDL remarkably stimulation promotes the m^6A modification level of macrophages in AS and METTL3 knockdown inhibits the oxLDL-induced inflammatory response and m^6A modification [23]. Besides, ALKBH5 low-expression significantly increases SPHK1 m^6A mRNA methylation,

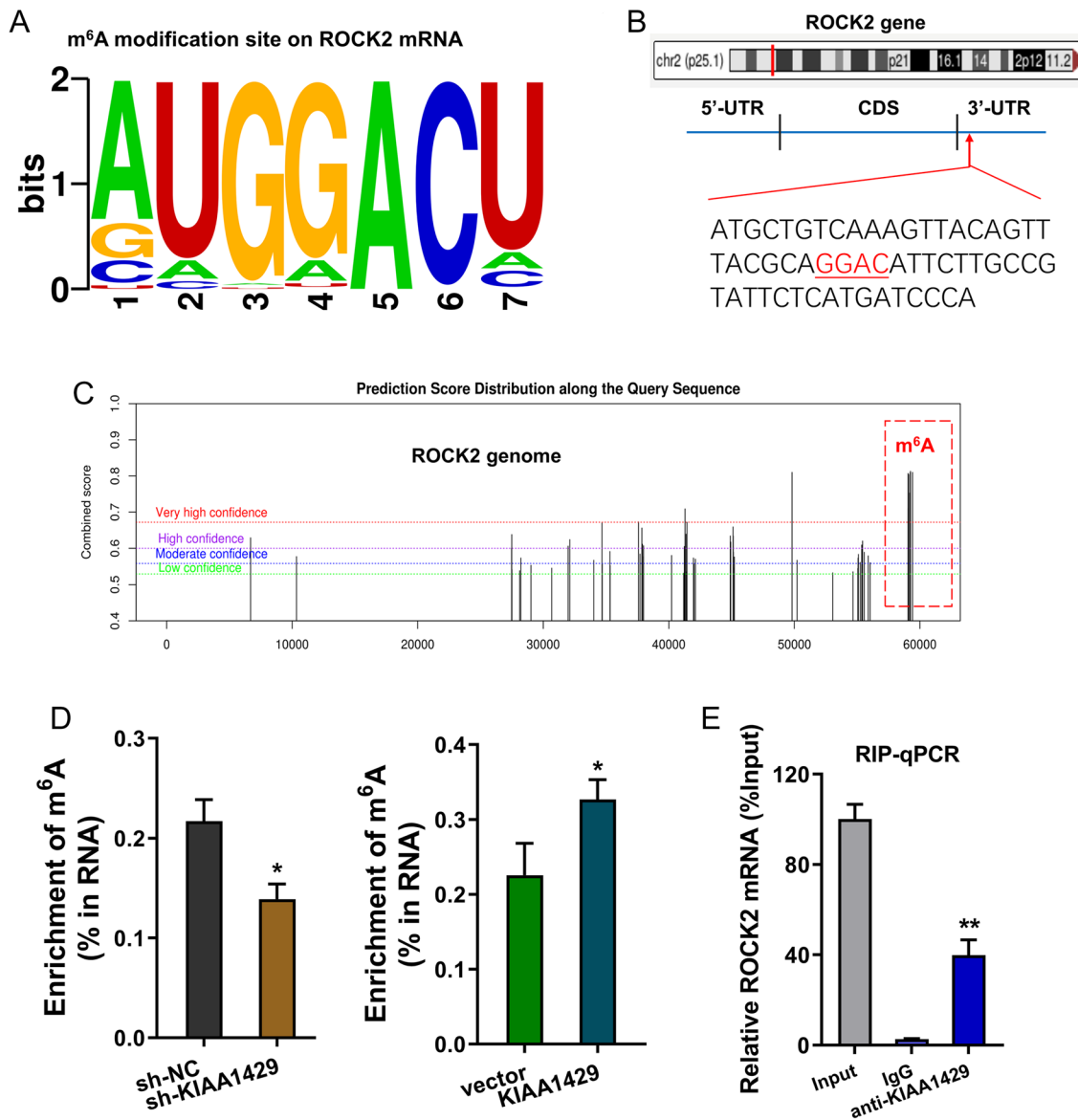


Fig. 3 ROCK2 acted as the target of KIAA1429. **A** The m⁶A modification site on ROCK2 (Rho associated coiled-coil containing protein kinase 2) mRNA was ‘GGACU’ motif. **B** In the ROCK2 gene, the m⁶A modification site distributed in 3'-UTR of ROCK2. **C** SRAMP online tool (<http://www.cuilab.cn/sramp>) demonstrated the m⁶A modification site on ROCK2 mRNA. **D** m⁶A quantitative analysis

was performed to detect the role of KIAA1429 knockdown/over-expression on the ox-LDL-treated HUVECs. **E** RIP-qPCR analysis indicated interaction within KIAA1429 and ROCK2 mRNA in HUVECs. Assays were performed in triplicate. Data were exhibited as Mean \pm Standard Deviation (SD). ** $p < 0.01$, * $p < 0.05$

in contrast, METTL3 overexpression reduces expression of SPHK1 mRNA [24]. In vascular endothelium of atherogenic inflammatory cascades, METTL3-mediated RNA hypermethylation up-regulates NLRP1 mRNA transcript and down-regulates KLF4 transcript via YTHDF1/YTHDF2 m⁶A reader proteins [25]. Thus, these findings suggest the vital functions of m⁶A on AS.

The online tool suggested that there were m⁶A modification sites on ROCK2 mRNA (Fig. 3C). Moreover, we performed RIP-PCR (Fig. 3E, 4D) and MeRIP-PCR

(Fig. 4A, B) to identify the molecular interaction within KIAA1429 and ROCK2 mRNA. In the pathophysiology of AS, ROCK2 functions as an essential element in AS [16]. For instance, circCHMP5/ROCK2 axis regulates cell cycle, proliferation, angiogenesis and inflammation in ox-LDL-induced HUVECs [26]. CircUSP36/ROCK2 axis regulates cell apoptosis and inflammatory responses, and promotes cell migration and invasion in ox-LDL-induced injury for HUVECs [27]. Here, we found that ROCK2 up-regulated in the ox-LDL-induced HUVECs. KIAA1429 targeted the m⁶A

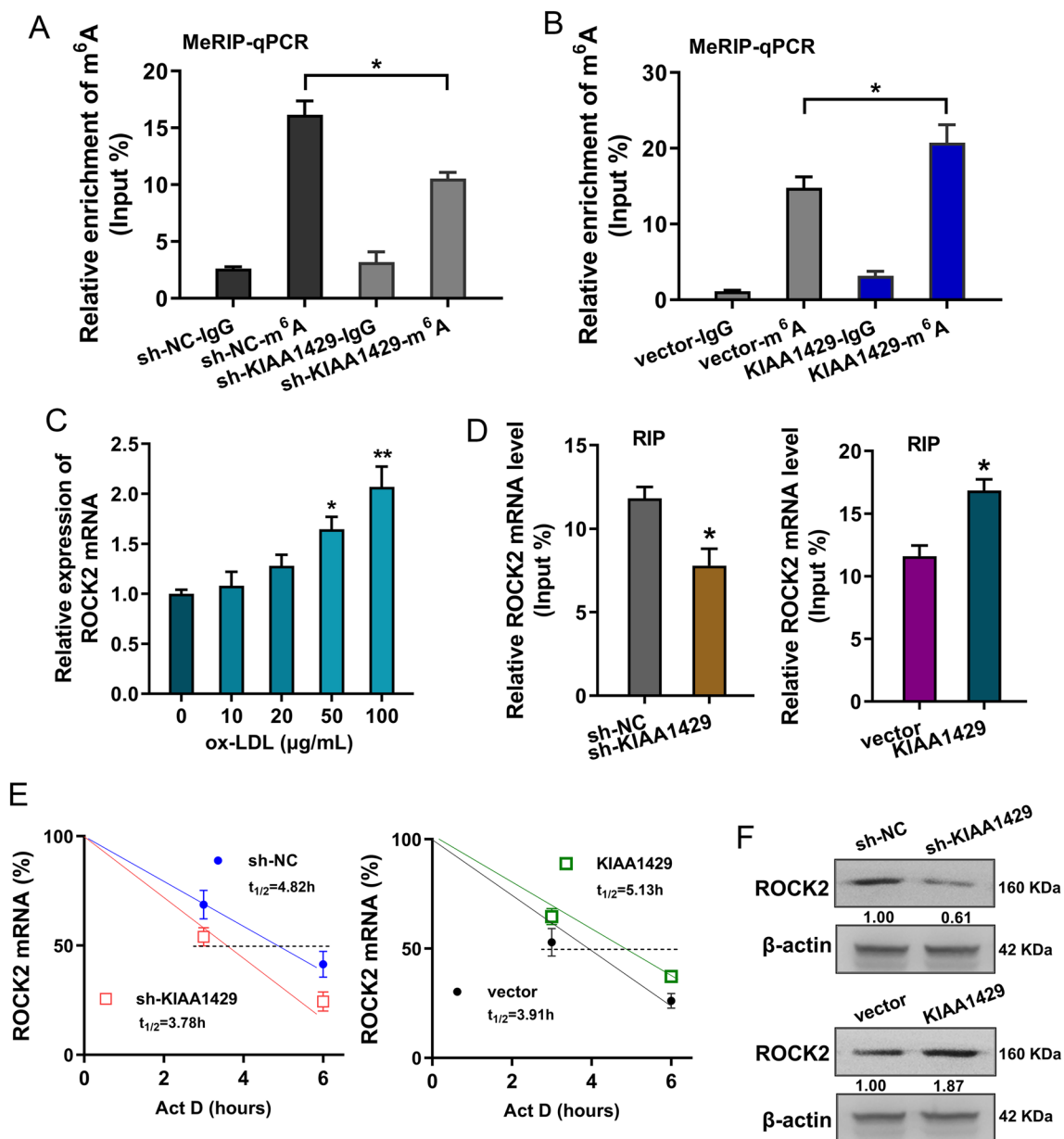


Fig. 4 KIAA1429 enhanced the stability of ROCK2 mRNA. **A** MeRIP-PCR analysis was performed using the anti-m⁶A antibody to detect the m⁶A modification level on ROCK2 mRNA. Ox-LDL-induced HUVECs were transfected with KIAA1429 knockdown (sh-NC-IgG, sh-NC-m⁶A, sh-KIAA1429-IgG, sh-KIAA1429-m⁶A). **B** Ox-LDL-induced HUVECs were transfected with KIAA1429 over-expression (vector-IgG, vector-m⁶A, KIAA1429-IgG, KIAA1429-m⁶A). **C** RT-PCR was performed to detect the ROCK2 mRNA level

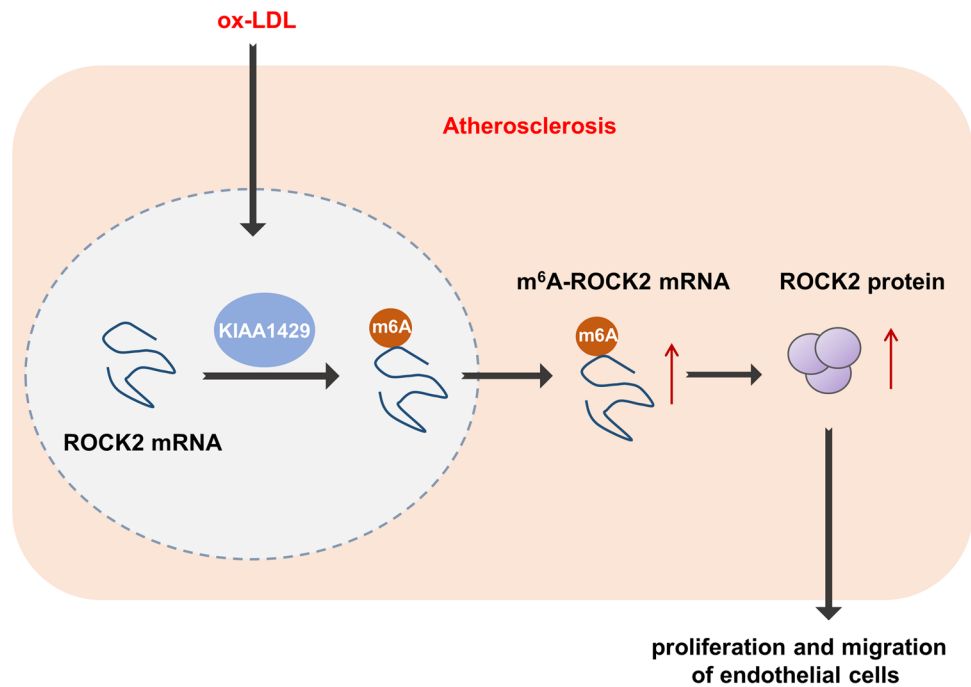
in the ox-LDL-induced HUVECs. **D** RIP-qPCR analysis was performed using anti-m⁶A antibody. The precipitated ROCK2 mRNA enrichment was analyzed as compared to controls. **E** RNA stability analysis was performed to detect the ROCK2 mRNA upon Act D treatment in ox-LDL-induced HUVECs. **F** Western blot analysis was performed to detect the ROCK2 protein in ox-LDL-induced HUVECs. Assays were performed in triplicate. Data were exhibited as Mean ± Standard Deviation (SD). ***p* < 0.01, **p* < 0.05

modification site of ROCK2 mRNA and then up-regulated the mRNA stability of ROCK2. Therefore, in this results, KIAA1429/ROCK2 axis accelerated the proliferation and migration of HUVECs.

For the deficiencies and defects, we talk briefly about what we've found in this research. Firstly, being limited by the laboratory external conditions, in vivo assays were

unable to proceed as we initially assumed. Moreover, clinical sample research was difficult to put into practice due to the COVID-19. Furthermore, our understanding of m⁶A is still in its infancy because of the absence of more research. With the great development of m⁶A and Epigenomics, it would be greatly help to the contribution of our work to the field of AS.

Fig. 5 KIAA1429/m⁶A/ROCK2 axis regulates the proliferation and migration of endothelial cells in atherosclerosis



Conclusion

In conclusion, the present research found a novel manner in AS by which KIAA1429 negatively regulated the proliferation and migration of HUVECs. Mechanistically, KIAA1429 targeted the m⁶A modification site of ROCK2 mRNA to install m⁶A modification of ROCK2, thereby enhancing ROCK2 mRNA stability (Fig. 5). These new findings provide novel insight for vascular endothelial cells injury for AS.

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Data Availability No research data shared.

Declarations

Conflict of interest All authors declare no conflicts of interest.

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