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Original Research Article

Abnormal expression of PRKAG2-AS1 in endothelial cells induced inflammation and apoptosis by reducing PRKAG2 expression

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ABSTRACT

PRKAG2 is required for the maintenance of cellular energy balance. PRKAG2-AS1, a long non-coding RNA (lncRNA), was found within the promoter region of PRKAG2. Despite the extensive expression of PRKAG2-AS1 in endothelial cells, the precise function and mechanism of this gene in endothelial cells have yet to be elucidated. The localization of PRKAG2-AS1 was predominantly observed in the nucleus, as revealed using nuclear and cytoplasmic fractionation and fluorescence in situ hybridization. The manipulation of PRKAG2-AS1 by knockdown and overexpression within the nucleus significantly altered PRKAG2 expression in a *cis*-regulatory manner. The expression of PRKAG2-AS1 and its target genes, PRKAG2b and PRKAG2d, was down-regulated in endothelial cells subjected to oxLDL and Hcy-induced injury. This finding suggests that PRKAG2-AS1 may be involved in the mechanism behind endothelial injury. The suppression of PRKAG2-AS1 specifically in the nucleus led to an upregulation of inflammatory molecules such as cytokines, adhesion molecules, and chemokines in endothelial cells. Additionally, this nuclear suppression of PRKAG2-AS1 facilitated the adherence of THP1 cells to endothelial cells. We confirmed the role of nuclear knockdown PRKAG2-AS1 in the induction of apoptosis and inhibition of cell proliferation, migration, and lumen formation through flow cytometry, TUNEL test, CCK8 assay, and cell scratching. Finally, it was determined that PRKAG2-AS1 exerts direct control over the transcription of PRKAG2 by its binding to their promoters. In conclusion, downregulation of PRKAG2-AS1 suppressed the proliferation and migration, promoted inflammation and apoptosis of endothelial cells, and thus contributed to the development of atherosclerosis resulting from endothelial cell injury.

1. Introduction

Endothelial cells are a specialized type of cells that line the inner surface of blood vessels [1]. This layer of cells forms a continuous barrier separating the bloodstream from the surrounding tissues [2]. Endothelial cells are essential for maintaining blood vessels healthy and functioning properly [3]. They are responsible for the regulation of nutrients and waste exchange between the bloodstream and tissues, as well as the control of blood flow, and the prevention of blood clot formation [4]. Endothelial cells also produce and release substances that facilitate intercellular communication inside the body [5]. These compounds play a crucial role in the coordination of numerous physiological processes, such as the regulation of blood pressure and immune response [6]. The presence and proper functioning of endothelial cells play a vital role in maintaining the structural and functional integrity of blood vessels [7]. Dysfunction of endothelial cells leads to the development of cardiovascular disorders such as atherosclerosis, hypertension, and stroke [8,9]. Atherosclerosis serves as a primary etiological factor for the development of both coronary and ischemic cardiomyopathy [10]. The strong association between atherosclerosis and arterial endothelial inflammation is generally accepted as the major pathogenesis of atherosclerosis [6]. Extensive research is currently being conducted to investigate the function and underlying mechanisms of endothelial cells in the pathogenesis of atherosclerosis, with the aim of developing novel therapeutic

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interventions for vascular disorders [9].

Long non-coding RNAs (lncRNAs) are a class of RNA molecules characterized by their length exceeding 200 nucleotides and their lack of protein-coding capacity [11]. LncRNAs were formerly perceived as non-functional or inconsequential elements inside the genome. In contrast, these entities fulfill a diverse range of regulatory roles within cellular systems, encompassing the regulation of gene expression through both transcriptional and post-transcriptional mechanisms [12]. The utilization of genome-wide approaches in lncRNA identification and characterization has provided new opportunities for investigating hitherto undiscovered facets of gene regulation [13]. They are currently recognized as significant contributors to biological mechanisms, such as chromatin remodeling, transcriptional regulation, and alternative splicing [11,14,15]. LncRNAs execute their regulatory functions in diverse ways. Some lncRNAs, for example, serve as molecular scaffolds, facilitating the assembly of diverse proteins or RNAs into bigger complexes to execute specialized cellular functions [15]. Some lncRNAs exhibit a decoy-like behavior by engaging in molecular interactions with entities such as transcription factors or miRNAs, hence impeding their ability to bind to their intended target genes. One specific example is the lncRNA called H19. H19 has been found to exhibit decoy-like behavior by interacting with the transcription factor insulin-like growth factor 2 (IGF2). In this case, H19 acts as a molecular decoy, sequestering IGF2 and preventing it from binding to its target genes [16]. Certain lncRNAs serve as molecular agents that facilitate the recruitment of particular proteins to specific regions within the genome [17]. The significance of lncRNAs in numerous biological processes and pathological conditions, such as cancer, neurodegenerative diseases, and cardiovascular disease, is increasingly acknowledged [18]. Extensive research is currently underway to investigate the involvement of lncRNAs in diverse pathological conditions and their potential as therapeutic targets [16]. Certain lncRNAs have been identified as biomarkers, hence signifying their potential utility as diagnostic modalities or therapeutic targets for cardiovascular disease [18]. Although understanding the function and mechanism of lncRNAs may facilitate the identification of novel targets for pharmacological interventions or gene therapy approaches, it is important to note that there is currently a scarcity of research that has presented evidence showcasing the crucial roles of lncRNA in vascular endothelial cells [19]. The investigation of the function and mechanism of lncRNAs in endothelial cells provides an avenue for comprehending the molecular mechanisms behind both normal and abnormal endothelial cell behavior. We postulate that the dysregulation of lncRNAs might contribute to a variety of pathophysiological processes, such as vascular inflammation, and abnormality in angiogenesis and metabolism.

AMP-activated protein kinase (AMPK) is a pivotal regulator of cellular energy homeostasis, functioning as a sensor that responds to alterations in the AMP to ATP ratio. Upon activation through phosphorylation, AMPK orchestrates metabolic adaptations to ensure energy balance. It enhances catabolic pathways, including fatty acid oxidation and glycolysis, while inhibiting anabolic processes like gluconeogenesis and protein synthesis. AMPK also influences glucose homeostasis, promotes lipid metabolism by reducing synthesis and increasing oxidation, and regulates mitochondrial biogenesis. Additionally, AMPK inhibits protein synthesis through the mTOR pathway, influences autophagy, and exhibits anti-inflammatory effects. These multifaceted functions underscore AMPK's central role in cellular responses to energy status, oxidative stress, and metabolic challenges [20,21]. The protein kinase AMP-activated non-catalytic subunit gamma 2 (PRKAG2) functions as a regulatory subunit of AMPK [22,23]. Recent studies have revealed that PRKAG2 may also play a role in cardiovascular diseases, such as hypertrophic cardiomyopathy and arrhythmias [24–26]. However, there is currently a lack of known information regarding the function and mechanism of PRKAG2 in endothelial cells. The investigation of PRKAG2 in endothelial cells has the potential to yield valuable insights into the maintenance of endothelial function and the prevention of endothelial dysfunction and cardiovascular disease. The regulatory mechanism of PRKAG2 in endothelial cells is also a subject of great interest. The presence of a lncRNA called PRKAG2-AS1 has been discovered in the promoter region of the PRKAG2 gene. Nevertheless, there exists a dearth of documented information on its biological function and mechanism in endothelial systems. This manuscript demonstrates that PRKAG2-AS1 contributes to the pathophysiological processes of the vascular endothelium through its regulation of PRKAG2 expression in the endothelial system.

2. Methods

2.1. Cell culture and transient cell transfection

The embryonic kidney cell line (293A cells) used in the experiment was cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and Penicillin-Streptomycin. The human umbilical vein endothelial cells (HUVEC) cell line, derived from the human umbilical vein, was grown in ECM medium. A cellular model of endothelial atherosclerosis was established by utilizing oxidized low-density lipoprotein (oxLDL) and homocysteine (Hcy). The transfection of small interfering RNAs (siRNAs) and antisense oligonucleotides was performed using FuGENE® HD Transfection Reagent (Promega). The siRNAs and antisense oligonucleotides shared the same targeting sequences. SiRNA1 and oligo1 target gaaccagtaagcccgttcgt, siRNA2 and oligo2 target tggatccgcgaatgaagcca, and siRNA2 and oligo2 target cagaataaaccagctcggag. The oligos were chemical modified by 2′-*O*-Methoxyethyl modifications. HUVEC was transfected with adenovirus at a multiplicity of infection (MOI) of 50.

RNA extraction, reverse transcription reaction, and quantitative polymerase chain reaction (qRT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, China, Shanghai). The isolated RNA samples were subsequently subject to reverse transcription using random primers by M-MLV. Quantitative PCR (qPCR) was performed to determine the amounts of target genes using SYBR Green-based methods on LightCycler-480 equipment. GAPDH was used as an endogenous control in the $2^{\Delta\Delta}$ Ct analysis to normalize gene expression data. The primers used to detect PRKAG2-AS1 was gggaatctggaaccagtaagc and gaagacccagaagtcccaca, to detect PRKAG2b was gatcgctgtcctcctcctc and gtcctcgaactccagcttct, and to detect PRKAG2d was ccagtcggagcctcatgg and gtcctcgaactccagcttct.

2.2. Nucleus and cytoplasm fractionation

The PARIS Kit (Life Technologies, AM1921) was employed to isolate the nuclear and cytoplasmic fractions from HUVEC in accordance with the instructions provided by the manufacturer. HUVEC cells were cultured on 10 cm diameter dishes and subsequently harvested using 0.25% trypsin solution. The cells were then lysed by incubation with 250 μl of Cell Disruption Buffer, followed by an additional 250 μl of Cell Fractionation Buffer for further lysis. After centrifuging the lysate at 500 g for 3 min at 4 °C, the resulting supernatant was utilized to extract cytoplasmic RNA. The pellet was then washed with 250 μl of Cell Fractionation Buffer before centrifuged again. The residual pellet was utilized for the extraction of nuclear RNA.

2.3. Fluorescence in-situ hybridization (FISH)

HUVEC were seeded and cultured on tablets that were placed in 24-well cell culture plates and coated with polylysine. After being fixed with 4% paraformaldehyde, the cells were treated with 0.5% Tritonx-100. A denatured probe for hybridization was applied to each well. After being washed with formamide and SSC buffer, the tablets were incubated with a biotin-labeled dye and DAPI counterstaining. Finally, the tablets were rinsed again with DEPC-treated phosphate-buffered saline (PBS), and the xylene and gum were mixed and prepared for

imaging with a laser confocal microscope.

2.4. Western blot

Samples were lysed with RIPA buffer (Beyotime, Hangzhou, China) and treated with ultrasonography on ice. After boiling for 10 min to denature the protein, a total of 40 μg protein/well was electrophoresed in a 10% SDS-PAGE gel and transferred to the PVDF membrane (Bio-Rad, America). After blocking with 5% skim milk for 2 h, the membranes were incubated with primary antibodies overnight and HRP-conjugated secondary antibodies for 2 h. Protein signals were visualized using ECL detection reagent (XinSaiMei, Suzhou, China) with the automatic chemiluminescence image analysis system. GAPDH acted as a reference. The information of antibodies was as follows: anti-*PRKAG2* (CST, #2536), anti-*PPARG* (A19676, Abclonal, Wuhan, China), and anti-*GAPDH* rabbit mAb (ABways, Ab0037).

2.5. Apoptosis detection by flow cytometry

The rate of cellular death was determined by an Annexin V-FITC Apoptosis Detection Kit (Beyotime, C1062) according to the manufacturer's instructions. HUVEC or human coronary artery endothelial cells (HCAEC) were digested with trypsin, washed, and double-stained with acridine orange (AV) and propidium iodide (PI). Flow cytometry was then employed to assess the fluorescence intensity of the individual cells present in the suspension.

2.6. TUNEL assay to detect apoptosis

HUVEC was grown on tablets that had been pretreated with polylysine. After knocking down PRKAG2-AS1 by antisense oligonucleotides for 36 h, the cells were subsequently fixed with a 4% paraformaldehyde solution. The Roche kit was used for TUNEL staining, and fluorescence signals were observed with a confocal microscope.

2.7. CCK8 to detect cell viability

HUVEC cells were transfected with antisense oligonucleotides or negative controls. After transfection for 24 h, the cells were trypsinized, collected, and planted at a certain density within 96-well plates. After 12 h, the CCK8 reagent was added to each well, and regular culture was subsequently resumed. The OD value at a wavelength of 450 nm was measured in each well using a microplate reader after a duration of 4 h.

2.7.1. Tubular formation

The matrix gel was subjected to a melting process within a refrigeration unit maintained at a temperature of 4 °C. The well plates were coated with a volume of 40 μl matrigel and then subjected to incubation for 30 min at 37 °C until the matrix gel solidified. Cells were digested and collected in ECM complete culture medium, counted using a cytometer technique, and planted in each well. Finally, the cells were observed, with photographs taken every hour for 3 h.

2.8. HUVEC adherence of monocytes

THP1 cells were labeled with green fluorescent protein (GFP) via adenovirus transfection. After 24 h, each well containing endothelial cells was incubated with 1 ml suspension of monocyte for 1 h at 37 °C. During incubation, the plate underwent regular shaking at 15-min intervals in order to achieve a uniform distribution of monocytes on the surface of the endothelial cell. Following incubation, each well was washed twice with PBS to remove unattached monocytes.

2.9. CHIRP [27]

HUVEC cells were digested, collected, and resuspended in PBS. Formaldehyde was used to crosslink the cells at room temperature for 30 min. The cross-linking reaction was stopped by adding 1/10 volume of 1.25 M glycine and incubating for 5 min at room temperature. Per 100 mg of cell sample was mixed with 1 ml of lysis buffer. The DNA fragments were interrupted by ultrasound. After hybridization with streptavidin magnetic beads, the samples were eluted with biotin-solution to obtain RNA binding complex. After the magnetic beads were removed, protein and nucleic acid were extracted for further investigation.

2.10. Statistical analysis

The data were presented as mean ± standard deviation. Sigmaplot and GraphPad Prism 8 softwares were used to identify statistical differences. One-way ANOVA was used to determine the existence of significant difference among several group means. The null hypothesis is that all the group means are the same. If it was different among them, the differences between the experimental and control groups were analyzed by *t*-test. A difference was considered significant when $p < 0.05$.

3. Results

3.1. The predominant localization of PRKAG2-AS1 is intranuclear with distribution observed in both the cytosol and nucleus

The promoter region of PRKAG2 contains a lncRNA called PRKAG2-AS1 (Fig. 1A). PRKAG2-AS1 is highly expressed in endothelial cells (Fig. 1B). The distribution of PRKAG2-AS1 in HCAEC and HUVEC cells was detected by nucleoplasmic RNA isolation. The presence of PRKAG2-AS1 was found in both the cytosol and nucleus, with a predominant localization within the nucleus (Fig. 1C and D). The distribution of PRKAG2-AS1 in HUVEC was confirmed by RNA in situ hybridization with biotin-labeled specific probes. As depicted in Fig. 1E, the expression of PRKAG2-AS1 was predominantly observed within the nucleus.

3.2. PRKAG2 expression was regulated by lncRNA PRKAG2-AS1

To explore the biological significance of PRKAG2-AS1 in regulating PRKAG2 expression in endothelial cells, we employed three siRNAs to knock down the cytoplasmic PRKAG2-AS1. We subsequently performed qRT-PCR to validate the decreased expression of PRKAG2-AS1 in HUVEC in comparison to the NC control group (Fig. 2A). Down-regulation of PRKAG2-AS1 by siRNAs had little impact on the expression level of PRKAG2b and PRKAG2d (Fig. 2B). After knocking down PRKAG2-AS1 with a siRNA cocktail (Fig. 2C), nucleoplasmic isolation of HUVEC RNA showed PRKAG2-AS1 was reduced mainly in the cytoplasm (Fig. 2D). The cytoplasmic knockdown of PRKAG2-AS1 has no effect on the expression of PRKAG2b and PRKAG2d (Fig. 2E).

Three antisense oligonucleotides were utilized to knock down PRKAG2-AS1 in the nucleus. PRKAG2-AS1 expressions were significantly reduced by oligonucleotide 1 and oligonucleotide 3 compared to NC control (Fig. 2F). Interestingly, oligonucleotides down-regulated the expression of PRKAG2b and PRKAG2d (Fig. 2G). Following the knockdown of nuclear PRKAG2-AS1 using oligonucleotide mixes (Fig. 2H), nucleoplasmic RNA was isolated from HUVEC. The expression of PRKAG2-AS1 was primarily reduced in the nucleus (Fig. 2I). As a result, the downregulation of nuclear PRKAG2-AS1 affected the expression of PRKAG2b and PRKAG2d isoforms (Fig. 2J).

Overexpression of PRKAG2-AS1 by adenovirus was confirmed by Real-time PCR (Fig. 2K). The subcellular localization of PRKAG2-AS1 was further detected by nucleoplasmic fractionation and Real-time PCR analysis. Adenovirus predominantly increased PRKAG2-AS1 expression in the nucleus as compared to the GFP control group. The expression levels of the PRKAG2b and PRKAG2d were found to be elevated in HUVEC cells that overexpressed PRKAG2-AS1 (Fig. 2L).

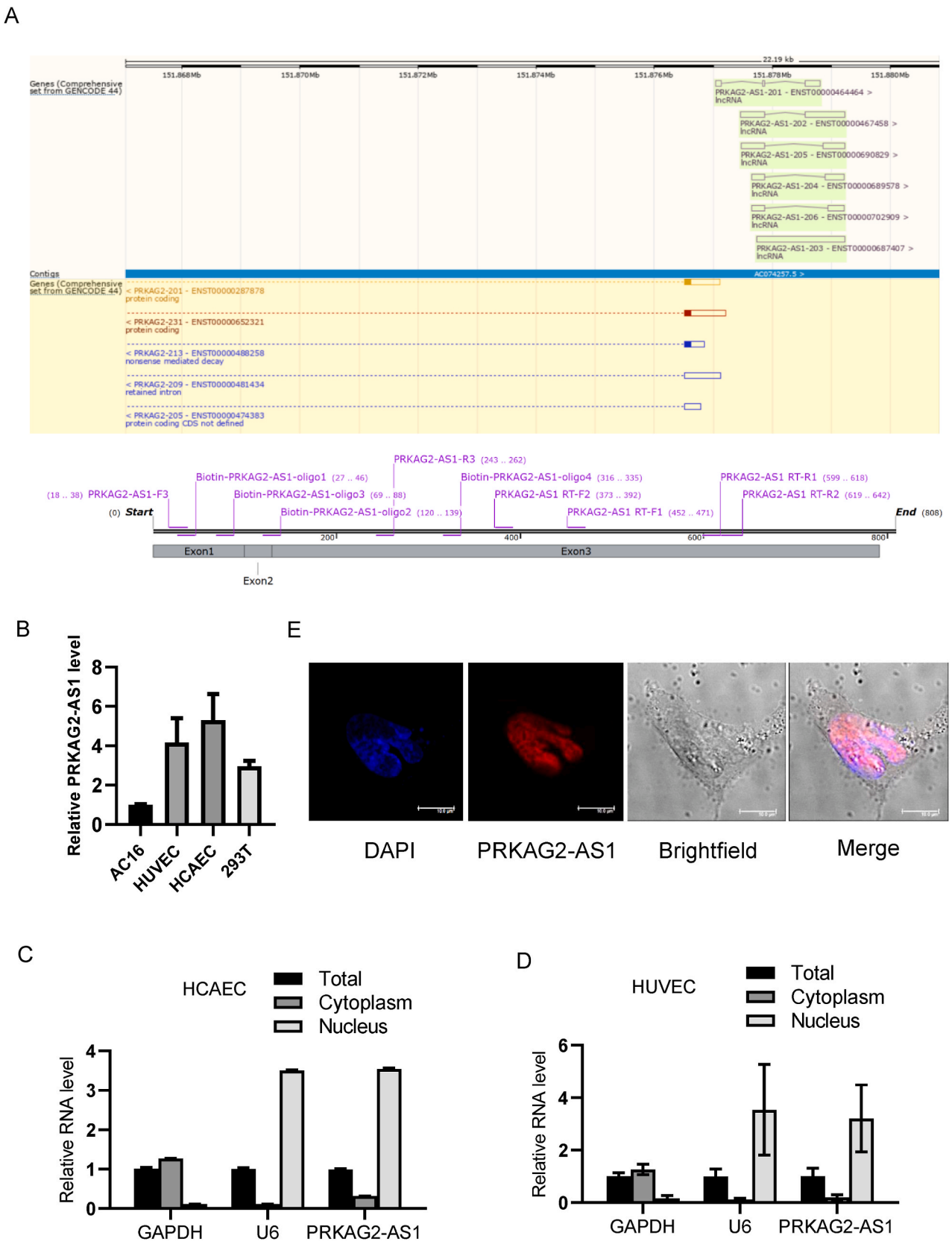

Fig. 1. The promoter region of PRKAG2 contains a lncRNA called PRKAG2-AS1 that was primarily localized in the nucleus. (A) PRKAG2-AS1 is located at the promoter region of PRKAG2. (B) PRKAG2-AS1 is highly expressed in endothelial cells. (C-D) The subcellular localization of PRKAG2-AS1 was observed in both HCAEC and HUVEC cells. PRKAG2-AS1 was distributed in both the cytosol and nucleus but was predominantly distributed within the nucleus. (E) RNA in situ hybridization with biotin-labeled specific probes revealed that PRKAG2-AS1 was primarily expressed in the nucleus.

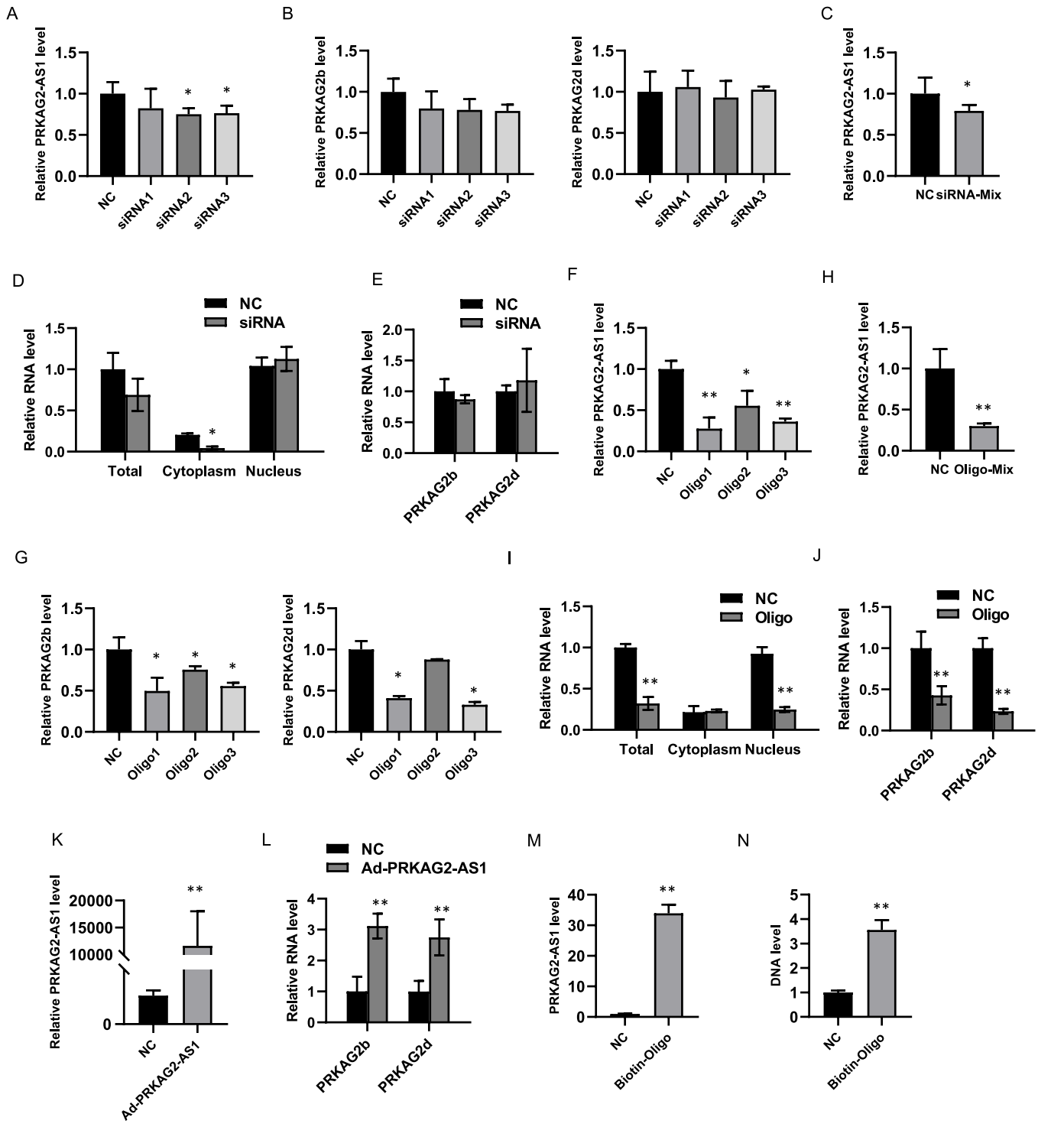

Fig. 2. Nuclear PRKAG2-AS1 promoted PRKAG2 expression. (A) siRNAs were used to knock down PRKAG2-AS1 in the cytosol. (B) The expression of PRKAG2b and PRKAG2d did not change after knocking down PRKAG2-AS1 in the cytoplasm. (C-D) A mixture of siRNAs was used to knock down PRKAG2-AS1 in the cytosol and confirmed by nucleoplasmic RNA isolation. (E) The expression of PRKAG2b and PRKAG2d was not affected by siRNA mix-mediated cytoplasmic PRKAG2-AS1 knockdown. (F) Antisense oligonucleotide was used to knock down PRKAG2-AS1 in the nucleus. (G) knockdown of intranuclear PRKAG2-AS1 led to a decrease in the expression of PRKAG2b and PRKAG2d. (H-I) Antisense oligonucleotide mix decreased the expression of PRKAG2-AS1 in the nucleus. (J) The expression of the PRKAG2b and PRKAG2d was downregulated by nuclear PRKAG2-AS1 knockdown. (M) Adenovirus overexpressed PRKAG2-AS1. (L) Overexpression of PRKAG2-AS1 increased the expression of PRKAG2b and PRKAG2d. (M) PRKAG2-AS1 could be enriched by antisense oligonucleotide mixture in vitro. (N) PRKAG2-AS1 binds to the promoter region of PRKAG2. $^{*}P < 0.05$, $^{**}P < 0.01$.

The CHIRP experiment was performed with the aim of investigating the potential binding of PRKAG2-AS1 to the promoters of PRKAG2b and PRKAG2d. The levels of PRKAG2-AS1 were detected in the input, flow, and probe groups, and it was shown that PRKAG2-AS1 exhibited enrichment when exposed to biotin-antisense oligonucleotides (Fig. 2M). Importantly, the level of the PRKAG2 gene was shown to be significantly higher in the probe group that utilized specific biotin oligonucleotides as probes, as compared to the group that did not employ any probes (Fig. 2N). Therefore, the mechanism of PRKAG2-AS1 in regulating PRKAG2 was through its direct interaction with the promoters of PRKAG2.

3.3. Down-regulation of PRKAG2-AS1 led to endothelial cell inflammation

We examined the expression of PRKAG2-AS1 in endothelial injury models to explore whether PRKAG2-AS1 is involved in atherosclerosis. Following a 24-h exposure to oxLDL, the expression of PRKAG2-AS1 was significantly reduced ($p < 0.05$) in comparison to the control group (Fig. 3A). Furthermore, oxLDL treatment significantly decreased the expression level of PRKAG2b and PRKAG2d in both HUVEC and HCAEC cells (Fig. 3B and C). The expression of PRKAG2-AS1 as well as PRKAG2b and PRKAG2d was decreased in Hcy-treated HUVEC and HCAEC (Fig. 3D–F). This observation is consistent with the decrease in PRKAG2-AS1 expression observed in endothelial cells treated with oxLDL, indicating a potential direct regulatory role of PRKAG2-AS1 in the expression of PRKAG2 during atherosclerosis. Furthermore, it suggests that PRKAG2-AS1 may be involved in the mechanism of endothelial damage induced by Hcy or oxLDL.

The impact of OxLDL and Hcy on endothelial cells is mediated by an inflammatory response, wherein it induces the upregulation of various inflammatory molecules, such as cytokines, adhesion molecules, and chemokines [8–10,28,29]. These molecules play a crucial role in recruiting immune cells to the site of inflammation. Following a 24-h exposure to oxLDL, the expression levels of inflammatory factors interleukin-6 (IL6), monocyte chemoattractant protein-1 (MCP1), C-reactive protein (CRP), and adhesion molecule intercellular adhesion molecule-1 (ICAM1) in HUVEC were detected by qRT-PCR. The group that was treated with oxLDL exhibited greater expression levels of IL6, MCP1, CRP, and ICAM1 in comparison to the control group (Fig. 4A). Hcy is an amino acid that is synthesized as a byproduct of methionine metabolism [30]. High levels of Hcy have been found to play a significant role in the development of atherosclerotic lesions [31]. This is mostly attributed to the detrimental effects of Hcy on endothelial cells, which include increased cell death and enhanced expression of adhesion molecules and chemokines. Hcy additionally facilitates the process of leukocyte recruitment and contributes to the initiation and progression of inflammation inside the vascular wall. Hcy treatment up-regulated the expression of IL6, MCP1, CRP, and ICAM1 in endothelial cells (Fig. 4B). These abnormal molecular expressions might result from

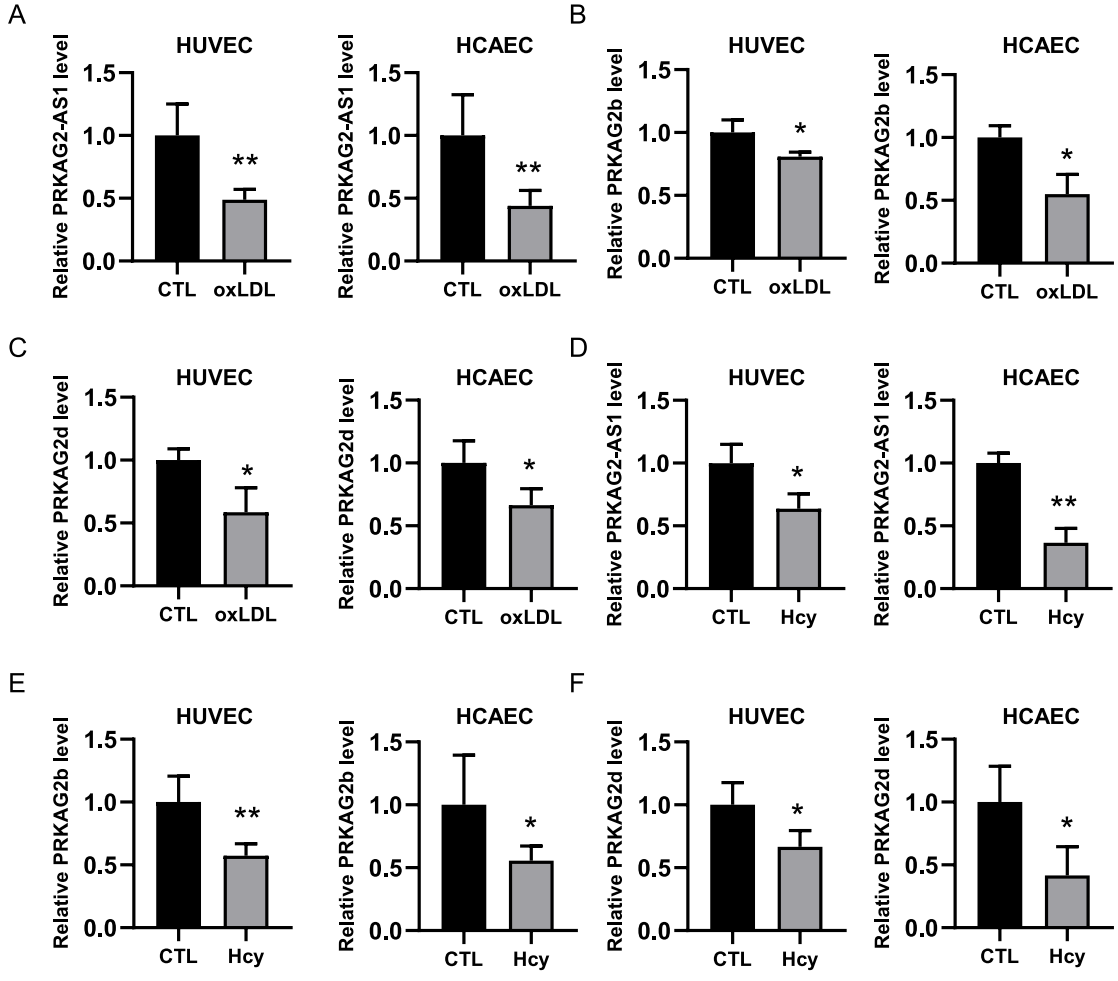

Fig. 3. Down-regulation of PRKAG2AS and PRKAG2 in Endothelial injury model. (A) The expression of PRKAG2-AS1 was downregulated in oxLDL-treated HUVEC and HCAEC. (B-C) The expression of PRKAG2b and PRKAG2d was downregulated in oxLDL-treated HUVEC and HCAEC. (D) PRKAG2-AS1 was decreased in Hcy-treated HUVEC and HCAEC. (E-F) PRKAG2b and PRKAG2d were decreased in HUVEC and HCAEC treated with Hcy. $^{*}P < 0.05$, $^{**}P < 0.01$.

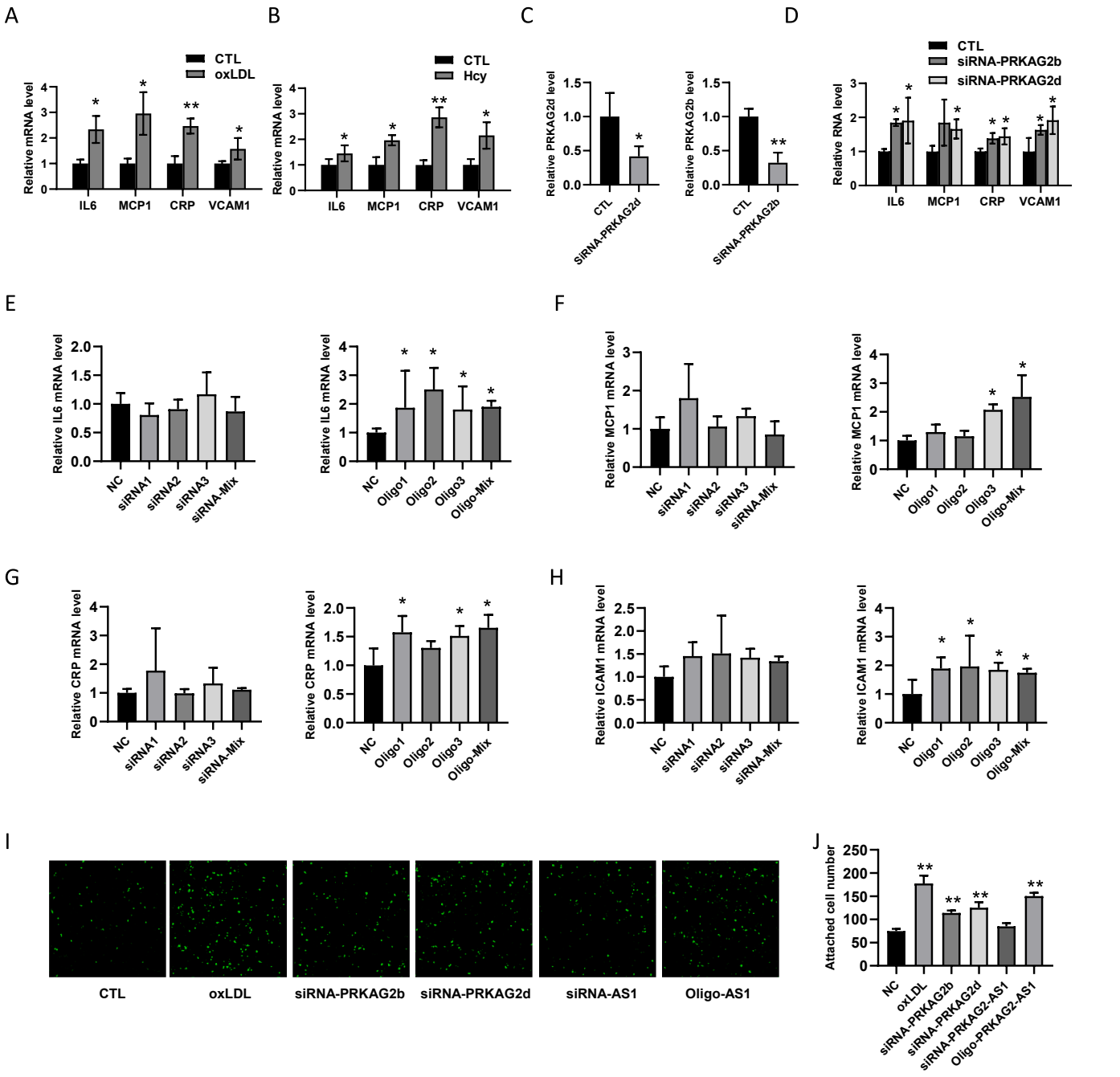

Fig. 4. Knockdown PRKAG2AS induced inflammation and adhesion of macrophage to endothelial cells. (A-B) The expression of IL6, CRP, MCP1, and VCAM1 was up-regulated in HUVEC treated with oxLDL or Hcy. (C) siRNA knocked down PRKAG2b and PRKAG2d in HUVEC. (D) Knockdown of PRKAG2b and PRKAG2d increased the expression levels of IL6, MCP1, CRP, and VCAM1. (E-H) Knockdown of PRKAG2-AS1 in the cytoplasm by siRNAs did not, while knockdown of PRKAG2-AS1 in the nucleus by antisense oligonucleotides, increase the expression of IL6 (E), MCP1 (F), CRP (G) and VCAM1 (H). (I-J) Knockdown of PRKAG2-AS1 in the cytoplasm by siRNAs did not, while knockdown of PRKAG2-AS1 in the nucleus by antisense oligonucleotides, affected the adhesin of THP1 to HUVEC. *$P < 0.05$, **$P < 0.01$.

aberrant expression of PRKAG2. Knockdown of PRKAG2b and PRKAG2b by siRNAs (Fig. 4C) increased the expression of inflammatory and apoptosis-related factors (Fig. 4D). The impact of cytoplasmic knockdown of PRKAG2-AS1 using siRNAs on the expression levels of IL6, MCP1, CRP, and ICAM1 was shown to be insignificant when compared to the control group (Fig. 4E–H). It is consistent with the findings that downregulation of cytoplasmic PRKAG2-AS1 had no effect on PRKAG2 expression. We then used antisense oligonucleotides to knock down PRKAG2-AS1 in the nucleus and detected the expression levels of IL6, MCP1, CRP, and ICAM1. The results showed that IL6, MCP1, CRP, and ICAM1 were significantly increased by decreasing nuclear PRKAG2-AS1 (Fig. 4E–H). These results suggest that the downregulation of nuclear PRKAG2-AS1 contributes to monocyte or macrophage adherence to endothelial cells. The quantification of the adhesion rate assay between GFP-labeled THP1 cells to HUVEC cells was determined by counting the number of GFP-positive cells in each well. The representative images showed that knockdown PRKAG2b and PRKAG2d in HUVEC increased the THP1 adhesion to HUVEC. Conversely, knockdown PRKAG2-AS1 in the cytoplasm did not, but knockdown PRKAG2-AS1 in the nucleus significantly increased THP1 adhesion to HUVEC (Fig. 4I and J).

3.4. Knockdown PRKAG2AS induced apoptosis of endothelial cells

Apoptosis is a hallmark of endothelial cells during the development of atherosclerosis. We knocked down PRKAG2-AS1 in the cytoplasm by siRNAs and in the nucleus by antisense oligonucleotides. The changes in the amount of apoptotic factor Caspase3 were detected by qRT-PCR and Western Blot. The expression of Caspase3 mRNA did not change significantly when PRKAG2-AS1 was downregulated using siRNAs or antisense oligonucleotides (Fig. 5A). The protein level of cleaved-Caspase3 was significantly increased by nuclear PRKAG2-AS1 down-regulation as compared to the NC control group (Fig. 5B). To further explore the biological function of PRKAG2-AS1 in HCAEC, we used cell flow cytometry to measure apoptosis. Our findings revealed a significant increase in apoptosis within the group subjected to oligonucleotide knockdown of PRKAG2-AS1, as compared to the NC control group (Fig. 5C and D). Similar results were observed in HUVEC as depicted in Fig. 5E and F. Apoptosis was further detected using the TUNEL kit subsequent to the transfection of HUVEC and HCAEC cells with oligo-nucleotides. The results showed that the level of apoptosis in PRKAG2-AS1 knockdown cells was much higher than that in the NC control group (Fig. 5G and H). The observed phenomenon of SOD1 and SOD3 downregulation may be attributed to the influence of PRKAG2-AS1 (Fig. 5I).

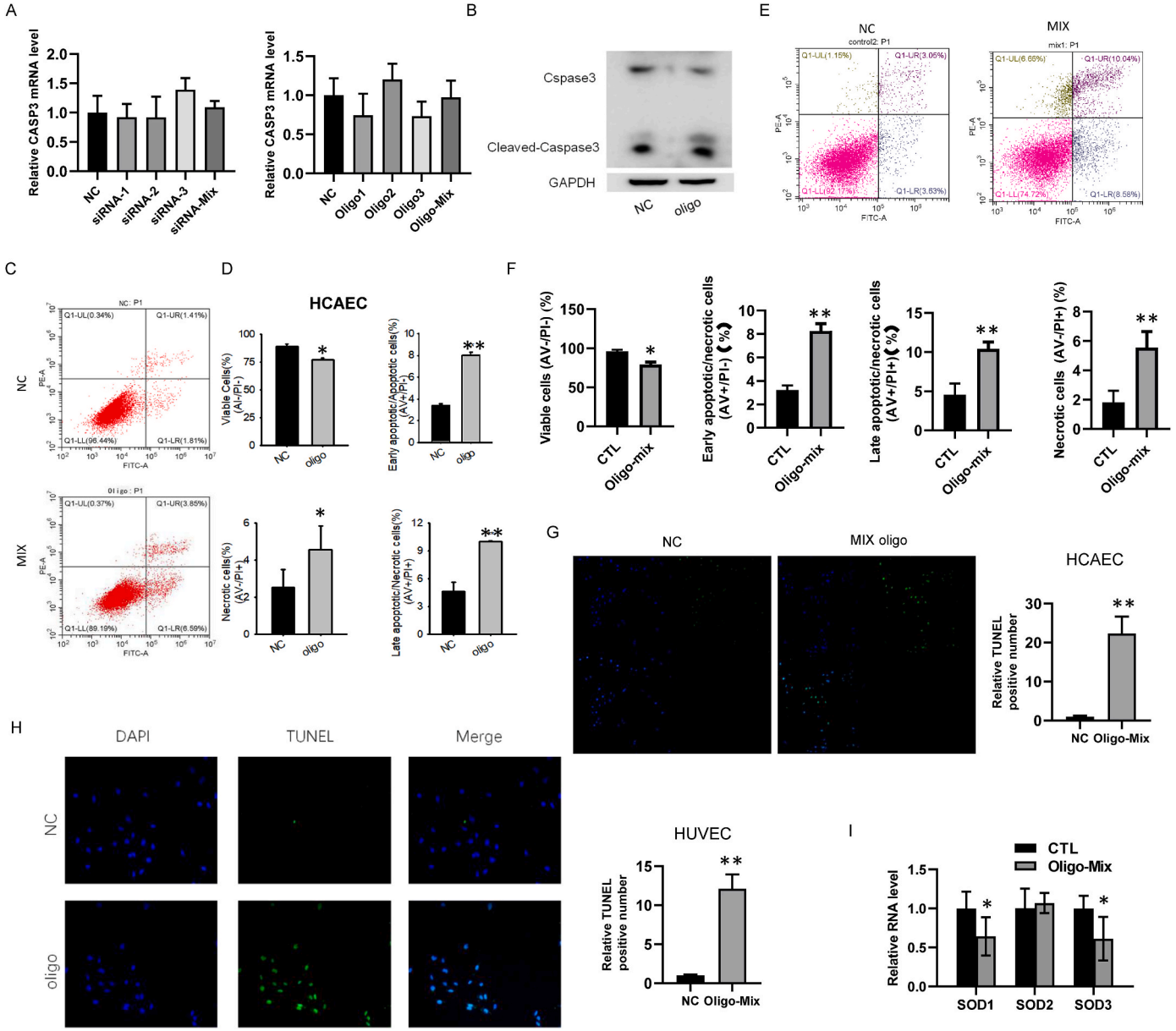

Fig. 5. Knockdown PRKAG2-AS1 led to apoptosis of endothelial cells. (A) The mRNA level of Caspase3 did not change by siRNA-mediated cytoplasmic or antisense oligonucleotide-mediated nuclear knockdown of PRKAG2-AS1. (B) The cleavage of Caspase3 was increased by oligonucleotide-mediated nuclear knock-down of PRKAG2-AS1. (C-D) Antisense oligonucleotides knocking down nuclear PRKAG2-AS1 promoted apoptosis of HCAEC. Compared with the NC control, the number of Annexin V+/PI- and Annexin V+/PI + stained cells in the oligonucleotide group was significantly increased, indicating early apoptosis and late apoptotic cells. (E-F) Reducing PRKAG2-AS1 in the nucleus of HUVEC increased apoptosis by Annexin V-FITC flow cytometry. (G-H) Apoptosis was detected using the TUNEL method in HUVEC and HCAEC. NC represented the control group, and oligonucleotide represented the experimental group using antisense oligonucleotides to knock down PRKAG2-AS1 in the nucleus. Compared with the NC control group, the green fluorescence signal stained by TUNEL in the oligonucleotide group was significantly higher than that in the NC control in HUVEC and HCAEC. (I) Reducing PRKAG2-AS1 in the nucleus of HUVEC decreased the expression of SOD1 and SOD3. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.5. The effect of PRKAG2-AS1 on the proliferation, migration, and lumen formation of endothelial cells

The expression of PRKAG2-AS1 was upregulated in vascular endothelial growth factor (VEGF) treated HUVEC (Fig. 6A). The CCK8 assay was used to investigate the effect of PRKAG2-AS1 on the proliferation of endothelial cells. The results showed that knockdown of nuclear PRKAG2-AS1 significantly suppressed HUVEC proliferation when compared to the NC control group. The optical density (OD) value of nuclear PRKAG2-AS1 knockdown cells exhibited a notably slower rise compared to the NC control group, suggesting a decrease in cell proliferation ability (Fig. 6B). The expression of proliferating cell nuclear antigen (PCNA) was significantly down-regulated (Fig. 6C). We investigated the effect of PRKAG2-AS1 on endothelial cell migration using cell scratch experiments. The migration was significantly hindered compared to the NC control group after PRKAG2-AS1 was knocked down in the nucleus using oligonucleotide (Fig. 6D and E). Using a transwell assay, we found that knockdown PRKAG2-AS1 by antisense oligonucleotides also markedly suppressed cell migration (Fig. 6F and G). The effect of PRKAG2-AS1 in the nucleus on the formation of tubules by endothelial cells showed that knocking down intranuclear PRKAG2-AS1 significantly inhibited the ability of HUVEC to form tubules. In contrast to the NC control group, where nearly all tubules were formed, the cells in the experimental group were dispersed and no lumenal formation was observed (Fig. 6H and I).

4. Discussion

As described in Fig. 7, endothelial cells, which play a crucial role in regulating vascular function, exhibit a high degree of sensitivity to alterations in their surroundings. Consequently, they modify the pattern of gene expression in response to these environmental changes. The work presented herein characterizes the dynamic regulation of PRKAG2-AS1 in endothelial cells in response to stimulation by VEGF, oxLDL, or Hcy. We found that VEGF increased the expression of PRKAG2-AS1 in HUVEC while oxLDL and Hcy were discovered to down-regulated its expression. Further inquiry is needed to understand the specific mechanism by which VEGF, oxLDL, or Hcy, regulate PRKAG2-AS1. However, this study focused on examining the function of PRKAG2-AS1 in VEGF-induced angiogenesis and oxLDL- and Hcy-induced atherosclerosis. The suppression of endothelial cell proliferation, migration, and tube formation was seen upon the knockdown of PRKAG2-AS1 within the nucleus. Based on scholarly investigation, the activation of AMPK plays a crucial role in the process of angiogenesis [20,24]. The potential role of PRKAG2-AS1 in regulating the activity of AMPK in endothelial cells could potentially play a part in the process of VEGF-induced angiogenesis [24]. The occurrence of endothelial cell dysfunction and the subsequent progression of atherosclerosis has been linked to high levels of oxLDL or Hcy. Our investigation revealed that knockdown of PRKAG2-AS1 in the nucleus results in the facilitation of apoptosis and the upregulation of inflammatory markers. The observed effects on the expression of IL6 and apoptosis-related factors suggested that

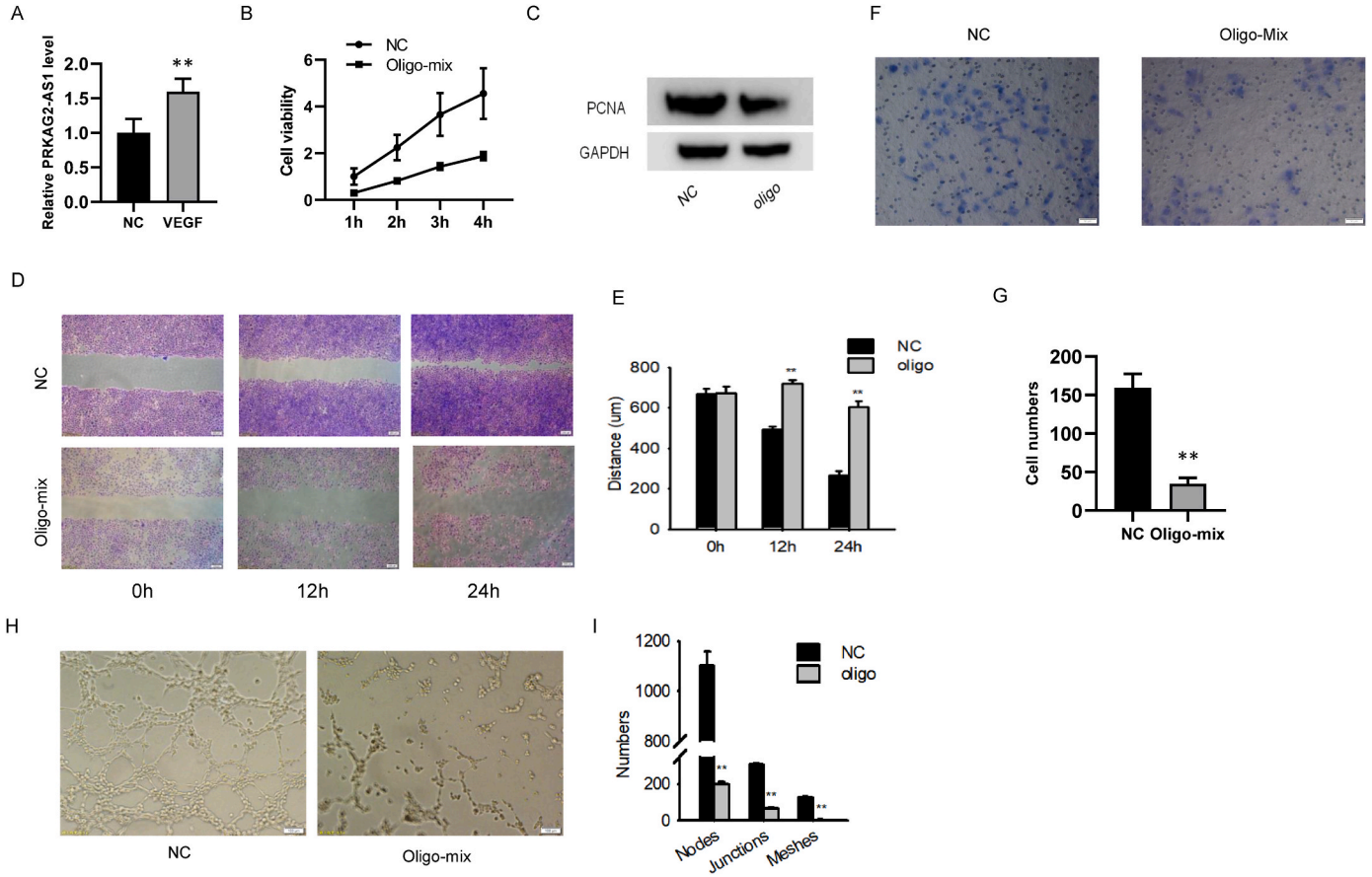

Fig. 6. Knockdown PRKAG2-AS1 suppressed proliferation, migration, and tube formation of endothelial cells. (A) The expression of PRKAG2-AS1 in HUVEC was upregulated by VEGF. (B) Knockdown intranuclear PRKAG2-AS1 in HUVEC suppressed cell proliferation and viability by CCK8 assay. (C) The expression of PCNA was suppressed by antisense oligonucleotides-mediated intra-nuclear PRKAG2-AS1 knockdown. (D-G) Knockdown intranuclear PRKAG2-AS1 inhibits endothelial cell migration by scratching assay (D-E) and transwell assay (F-G). A Crystal violet staining after light microscopy. (H-I) The NC control group was widely connected into tubules, the cells of the experimental group were scattered and distributed, and there was basically no lumen formation. Image J software analyzes statistical comparison of the number of nodes, number of intersections, and number of meshes between the two groups. $^{*}P < 0.05$, $^{**}P < 0.01$.

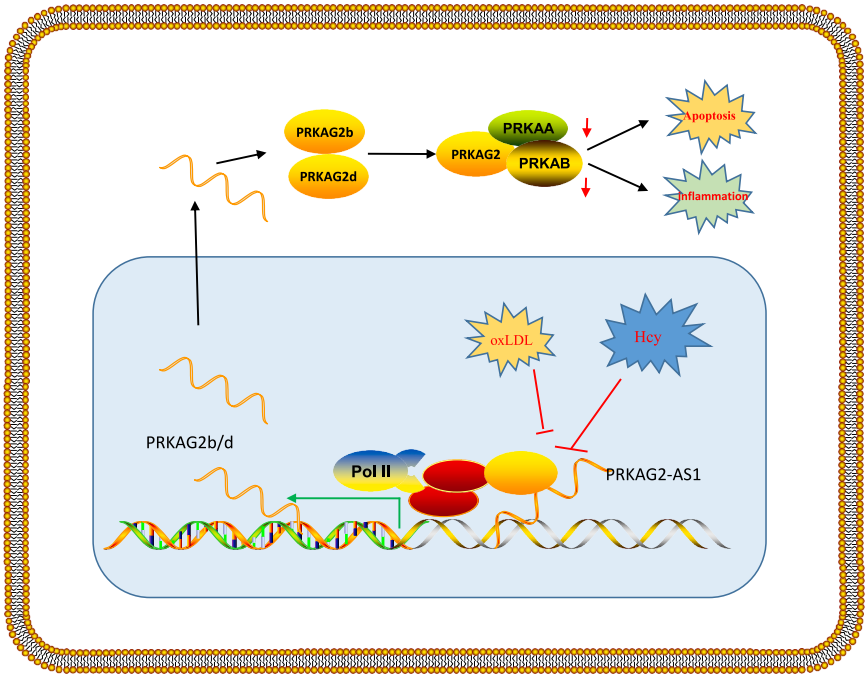

Fig. 7. The mechanism of PRKAG2-AS1 in endothelial cells. The expression of PRKAG2-AS1 promotes the expression of PRKAG2b and PRKAG2d. The proper function of PRKAG2b and PRKAG2d suppressed the effects of oxLDL or Hcy on apoptosis or inflammation and maintained the normal function of endothelial cells in proliferation, migration, and tubular formation.

PRKAG2-AS1 may have different functions in the cytoplasmic and nuclear compartments. These results suggest that PRKAG2-AS1 plays critical functions in endothelial cells and may contribute to the regulation of numerous processes that are essential for the maintenance of vascular health.

The function and mechanism of PRKAG2-AS1 in the context of atherosclerosis have not been reported in the literature. Here, we showed that oxLDL or Hcy could reduce the expression of PRKAG2-AS1 in endothelial cells. This study aimed to investigate the effects of PRKAG2-AS1 knockdown and overexpression in the nucleus on apoptosis and the production of inflammatory factors in endothelial cells. The production of reactive oxygen species (ROS) by oxLDL or Hcy is a mechanism by which endothelial cell inflammation is initiated, leading to enhanced adhesion of immune cells to the endothelium and the synthesis of molecules that contribute to the progression of atherosclerosis [32]. The modulation of endothelial genes and miRNAs, particularly in relation to endothelial nitric oxide synthase (eNOS), has been reported in studies including oxLDL or Hcy [19]. Further investigation is needed to comprehensively understand the molecular mechanisms underlying the impact of PRKAG2-AS1 on endothelial cells, as well as its potential as a therapeutic target for cardiovascular disease through regulating oxidative stress and nitric oxide generation. In this study, we explored the function and underlying mechanism of PRKAG2-AS1 in the regulation of apoptosis and the expression of inflammatory factors. Our findings suggest that PRKAG2-AS1 induces the expression of PRKAG2, hence influencing these cellular processes. The investigation of PRKAG2-AS1's functions in angiogenesis or atherosclerosis using the mouse model was hindered due to the lack of evolutionary conservation of PRKAG2-AS1. In mice, it has been observed that the promoter region of PRKAG2 contains another lncRNA, PRKAG2OS1. We believe that this lncRNA regulates the expression of PRKAG2 in a manner analogous to that of PRKAG2-AS1 in mice. In addition to the *cis*-regulatory mechanism of PRKAG2 regulation, it is worth investigating whether the involvement of PRKAG2-AS1 in endothelial proliferation, migration, or apoptosis occurs through regulating the expression of other genes via binding to additional DNA regions or by facilitating the transcriptional activity of other transcription factors.

To investigate the function of PRKAG2-AS1 in endothelial cells, this manuscript used two strategies, namely siRNAs and antisense oligonucleotides, to knock down PRKAG2-AS1. Our findings indicate that the primary impact of siRNAs was the reduction of cytoplasmic PRKAG2-AS1, while no discernible effect was observed on nuclear PRKAG2-AS1. The use of antisense oligonucleotides resulted in a significant reduction in the expression of nuclear PRKAG2-AS1. It is posited that the efficacy of the RNA-induced silencing complex (RISC) system utilizing siRNAs for the suppression of nuclear lncRNAs comparatively inferior to that of RNase H system employing antisense oligonucleotides. Therefore, it is imperative to take into account the localization of lncRNAs because different lncRNA localizations require distinct investigative strategies. Given that the majority of lncRNAs were found in the nucleus, it was critical to carefully choose the strategies for knocking them down. Additional methodologies for suppressing nuclear lncRNAs, such as those involving CAS13d-related approaches, could potentially be devised to investigate the function or underlying mechanism of lncRNAs [33,34]. The current investigation of these RNA-modulating approaches is focused on examining the efficacy and off-targets in the regulation of nuclear lncRNAs.

Our findings indicate that the expression of PRKAG2 is regulated by PRKAG2-AS1, which directly interacts with the promoter region of PRKAG2. This suggests that PRKAG2-AS1 exerts its regulatory effect on PRKAG2 expression through a *cis*-regulatory mechanism. It is consistent with the functional mechanism of the majority of lncRNAs [15]. The precise mechanism by which PRKAG2-AS1 regulated the expression of PRKAG2 has not yet been fully elucidated. Further investigation is warranted to examine the transcription factors or RNA-binding proteins (RBPs) responsible for facilitating these effects [35]. Studies have identified PRKAG2 as a key player in endothelial cells, with notable involvement in processes such as angiogenesis and modulation of vascular tone. Recent research has also shown a potential involvement of PRKAG2 in the cellular response of endothelial cells to exposure to

oxLDL. In this study, we demonstrated that the reduction of nuclear PRKAG2-AS1 decreased the expression of PRKAG2, increased apoptosis, and upregulated the expression of inflammatory markers in endothelial cells. These results suggest that the function of PRKAG2-AS1 in endothelial cells can be attributed to its *cis*-regulatory activities in regulating the expression of PRKAG2. According to additional studies, PRKAG2 may be involved in the modulation of autophagy in response to oxLDL [21]. The researchers found that autophagy induction in response to oxLDL requires both PRKAG2 expression and AMPK activation. The process of autophagy plays a vital role in protecting endothelial cells from the damaging effects of oxidative stress. These findings suggest that PRKAG2 might serve as a protective factor for endothelial cells against oxidative stress. This study showed that PRKAG2-AS1 plays an important function in the regulation of PRKAG2. Further investigation is required to determine the function of PRKAG2-AS1 in regulating oxLDL-induced autophagy.

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CRediT authorship contribution statement

Xiao-Wei Song: Writing – original draft. **Wen-Xia He:** Data curation. **Ting Su:** Writing – original draft. **Chang-Jin Li:** Writing – review & editing, Writing – original draft. **Li-Li Jiang:** Methodology. **Song-Qun Huang:** Writing – review & editing. **Song-Hua Li:** Writing – review & editing. **Zhi-Fu Guo:** Writing – review & editing. **Bi-Li Zhang:** Writing – review & editing.

Declaration of competing interest

All the authors have declared that no competing interest exists.

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