CircRNA-DAPK1 promotes vascular cell pyroptosis in diabetes by regulating the circ-DAPK1/miR-4454/TXNIP axis.

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Abstract

Background

Circular RNAs have been demonstrated to play an important role in the development of vascular diseases. However, little is known about the role of circ-021774, also named circ-DAPK1, in vascular cell pyroptosis.

Methods

Circ-DAPK1 was selected from circular RNA sequencing data of HUVECs treated with high glucose medium and normal medium. RT-qPCR was used to determine the expression of circ-DAPK1 *in vivo* and *in vitro*. Dual luciferase reporter assay, fluorescence in situ hybridization (FISH) and RNA immunoprecipitation (RIP) were performed to prove the interaction of circ-DAPK1, miRNA-4454 and thioredoxin-interacting protein (TXNIP). Adeno-associated virus (AAV) was injected intravenously to establish mouse models. PI staining, western-blot and transmission electron microscopy (TEM) analyses were performed to identify the role of circ-DAPK1 in promoting pyroptosis.

Results

We found that circ-DAPK1 was highly expressed in high glucose medium cultured HUVECs and db/db mice. *In vitro* and *in vivo* experiments demonstrated that circ-DAPK1 knockdown decreased the number of PI⁺ cells, the expression of ASC, NLRP3, GSDMD-N, cleaved caspase-1, IL-18 and IL-1 β . In a mechanistic study, the circ-DAPK1/miRNA-4454/TXNIP signaling axis was demonstrated to promote vascular cell pyroptosis in diabetes.

Conclusions

Circ-DAPK1 functions as a promoter of vascular cell pyroptosis in diabetes via the circ-DAPK1/miRNA-4454/TXNIP signaling axis.

Background

Pyroptosis is defined as a kind of programmed cell death that differs from necrosis and apoptosis[1]. Previous studies have demonstrated that canonical and noncanonical signaling pathways are involved in pyroptosis. In the canonical pathway, nucleotide-binding oligomerization domain-like receptor family 3 (NLRP3) senses external stimuli. Then, the adapter apoptosis-associated speck-like protein (ASC) assembles with NLRP3 to activate the inflammatory protease caspase-1. Subsequently, caspase-1 cleaves the proinflammatory cytokines interleukin-18 (IL-18) and pro-interleukin-1 β (IL-1 β) to initiate the downstream immune response[2]. In the noncanonical pathway, caspase-4/5/11 can sense bacterial

lipopolysaccharide (LPS) to form the inflammasome, which cleaves the N-terminus of GSDMD to cause the secretion of inflammatory mediators that induce inflammatory reactions[3]. Therefore, in transmission electron microscopy (TEM) imaging, pyroptotic cells can be identified by the presence of multiple holes in the membranes[4].

Circular RNAs (circRNAs) are a subclass of endogenous noncoding RNAs that are characterized by a closed-loop structure without a 3'-5' polyadenylated tail or polarity. CircRNAs are conserved among different species including in human vascular cells[5]. Several studies have reported their vital role in cancers, osteoarthritis, atherosclerosis, vascular dysfunction and many other diseases[3, 6–9].

Diabetes has been shown to accelerate the progression of atherosclerosis, which promotes the progression of coronary artery disease[10, 11] and arteriosclerosis obliterans [12]. However, our understanding of the roles of insulin resistance and hyperglycemia in atherosclerosis is relatively incomplete[13, 14]. Recently, some studies have discovered the factors involved in pyroptosis-induced atherosclerosis, such as shear stress, microRNAs (miRNAs) and nicotine[15–17]. However, the underlying molecular mechanism and cellular pathways involved in the link between circRNAs and vascular cell pyroptosis in diabetes remain to be clarified.

In this study, we identified a new circRNA, circRNA-021774 (also named circ-DAPK1) and discovered its contributions to vascular cell pyroptosis in diabetes *in vitro* and *in vivo*. We also aimed to demonstrate the underlying mechanisms of circ-DAPK1 in promoting pyroptosis.

Methods

Ethics statement

All animal studies were approved by the Ethical Review Committee of the First Affiliated Hospital, Zhejiang University School of Medicine.

Cell culture and transfection

Human umbilical vein endothelial cells (HUVECs) were cultured in DMEM medium (Biological Industries, Israel) with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Biological Industries, Israel) at 37°C under 5% CO₂ atmosphere. HUVECs were treated with 33 mM glucose in the high glucose (HG) group and 5 mM glucose in the control normal glucose (NG) group.

RT-qPCR

Total RNA was extracted with TRIzol reagent (Ambion, USA). The HiScript Reverse Transcriptase kit (Vazyme, Nanjing, China) and Bio-Rad QX100 Droplet Digital PCR system were used to reverse transcribe RNA. Circ-DAPK1, miRNA-4454 and TXNIP were amplified using primers that target the splice junctions. All primer sequence are listed as follows: GAPDH (F)5'-TCAAGAAGGTGGTGAAGCAGG-3'; (R)5'-TCAAAGGTGGAGGAGTGGGT-3'. TXNIP (F)5'-TGAAGGCTTTTCTTGACCGC-3'; (R)5'-

TTCGAGCAGAGACAGACACC-3'; miRNA-4454 (F) 5'-TGCGCGGATCCGAGTCACGG-3'; (R) 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC TGGTGCCG-3'; circ-DAPK1 (F) 5'-ATAAGGGCGGGTCCAATG-3'; (R) 5'-AGTAATAGCCGTGCCAAGC-3'. All primers were obtained from Tsingke Technology Ltd.(Beijing, China).

Western-blot

The cells were lysed on ice for 60 min in RIPA buffer (Beyotime, China) supplemented with phosphatase and protease inhibitors. The supernatant was collected with centrifugation (12,000 ×g, 4°C). The concentrations were analyzed with a Pierce BCA protein assay kit (Thermo Scientific, USA). 20µg protein was separated by 10% SDS-PAGE (GenScript, USA) and then transferred to 0.45 µm PVDF membranes (Millipore, USA). The Thioredoxin-interacting protein (TXNIP) (Abcam, UK), NLRP3 (NOVUS, USA), and caspase-1 (Santa Cruz, USA) were detected by western-blot. GAPDH (Goodhere Biological Technology Group, China) was used as a loading control.

Fluorescence in situ hybridization (FISH)

In situ hybridization was performed using probes for circRNA-DAPK1 and miRNA-4454. All probe sequences (RiboBio, China) are listed as follows: miRNA-4454 probe: 5'-CY3-TGGTGCCGTGACTCGGATCC-3'; circ-DAPK1 probe: 5'-FAM-ACCTCCTGCAACACTTGTGAGAAGC-3'. The signals of circ-DAPK1 and miRNA-4454 were detected by an in situ hybridization kit. The procedures were conducted following the manufacturer's instructions.

RNA immunoprecipitation assay (RIP)

The RIP assay was performed using an Imprint RNA Immunoprecipitation Kit (Merck, USA). AGO2 and vector plasmids were transfected into HEK293 cells. Then, 1.5×10^7 cells were harvested and lysed in 100 µL RIP lysis buffer containing an RNase and protease inhibitor cocktail. Then, the cell lysates were incubated with 4 µL AGO2 antibody (Abcam, UK) or IgG-coated beads. After incubation, the cell lysates were rotated at 4°C for 24 h. Immunoprecipitated RNA was extracted with an RNeasy MinElute Cleanup Kit (Qiagen, USA) and reverse transcribed using HiScript Reverse Transcriptase (Vazyme, China). The expression of circRNA-DAPK1 and miRNA-4454 was detected by PCR.

Enzyme-linked immunosorbent assay (ELISA)

Mouse serum concentrations of interleukin-1β and IL-18 were determined by ELISA kits (Neobioscience, China) according to the manufacturer's instructions.

Dual luciferase reporter assay

Luciferase reporters containing wild-type (WT) or mutated circ-DAPK1 plasmid (Mut) were constructed using the psi-CHECK2 vector (Promega, USA). The luciferase vector (2 µg) containing the pcDNA3.1 circ-DAPK1 plasmid was cotransfected with miR-4454 mimics, miR-4454 inhibitor or negative control

(scramble sequence of miR-4454) into human embryonic kidney 293 (HEK293) cells using Lipofectamine™ 2000 (Invitrogen, USA) with 10 ng of a Renilla luciferase reporter used as an internal control. After 48 h, the cells were collected and lysed. Then, luciferase activities were analyzed by the Dual-Luciferase Reporter Assay System (Promega, USA) following the manufacturer's instructions.

Establishment of the animal model

C57BL/KsJ-db/db mice (Cavens Lab Animal Co.,Ltd, China) were used to construct the animal models. A total of 7×10^7 VG adeno-associated virus (AAV) was injected intravenously in each group. The animals were sacrificed 4 weeks after AAV injection, serum and aorta of mice were harvested for further detection (Fig. 7A).

Hoechst 33342/PI fluorescent staining

Cell pyroptosis was assessed with a Hoechst 33342/PI double fluorescent staining kit. HUVECs (5×10^3) were cultured in 24-well plates (Nunc, USA). The cells were transfected with lentivirus before incubation. The stably infected cells were stained with 5 μ L Hoechst 33342 at 37°C in the dark for 10 min, and then incubated with 3 μ L PI solution at room temperature for 15 min. Finally, the images were captured by fluorescence microscopy (Olympus BX53, Japan).

Transmission electron microscopy (TEM)

TEM imaging procedures were performed according to our previous work[18]. HUVEC cell lines were used to construct the cell model.

Immunofluorescence (IF) staining

For immunofluorescence analysis, 4×10^4 HUVECs were plated in a confocal slide and cultured overnight. The cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.5% Triton X-100 for 20min. After permeabilization, the cells were blocked with goat serum albumin for 30 min at room temperature. Subsequently, the primary antibodies against caspase-1 (1:100, Abcam) and ASC (1:100, Affbiotech) were added to the slides for 24 h incubation at 4°C. The next day, the cells were incubated with the secondary antibodies for 1 h. DAPI was utilized to stain the nuclei for 5 min at room temperature. Finally, images were captured by fluorescence microscopy (Olympus BX53, Japan).

RNase R resistance assay

Total RNA (5 μ g) from HUVECs were treated with RNase R(3U/ μ g, Lucigen) previously and incubated at 37°C for 60 min. The RNA was reverse transcribed with primers and analyzed by PCR.

Statistical analysis

All data of this study are presented as the mean $\pm$ SD. Differences between two groups were analyzed by Student's t-test unless indicated otherwise. *P* values less than 0.05 were considered statistically significant.

Results

Circ-DAPK1 expression is relatively high in HUVECs cultured in HG medium and is located in the cytoplasm.

For a circRNA sequencing, we constructed a cell model using HUVECs. Three samples cultured with HG medium (HG group) for 72 h and their controls (NG group) were prepared to conduct RNA-seq. The results showed that 46 circRNAs were upregulated while 37 circRNAs were downregulated in the HG group (Fig. 1A, B). Among them, circ-DAPK1 was significantly overexpressed in the HG group (Fig. 1B). To confirm the expression of circ-DAPK1 under HG condition, we used RT-qPCR to determine its abundance in the HG and NG groups. Consistent with the results of the RNA-seq analysis, the expression of circ-DAPK1 was higher in the HG group (Fig. 1C). As shown in previous reports, circRNAs are formed by trans-splicing[19]. Subsequently, we identified the loop structure of circ-DAPK1. According to the circBase database, circ-DAPK1 is produced from the DAPK1 gene locus and contains exon 23–24 (Fig. 1E). As head-to-tail splicing could be the result of trans-splicing and genomic rearrangements, convergent primers for DAPK1 mRNA and divergent primers for circ-DAPK1 were utilized to distinguish these two molecules. PCR results indicated that circ-DAPK1 was amplified from cDNA but not gDNA (Fig. 1F). In the presence of RNase R, the linear form of DAPK1 decreased sharply (the right two lanes), while circ-DAPK1 remained stable (the left two lanes) in the Northern-blot analysis (Fig. 1G). Furthermore, FISH experiments revealed that circ-DAPK1 was located mainly in the cytoplasm (Fig. 1D).

Circ-DAPK1 promotes pyroptosis of HUVECs under HG conditions.

To explore the role of circ-DAPK1 in regulating pyroptosis, we transfected the HUVECs with circ-DAPK1-specific siRNA (circ-KD) or overexpression plasmid (circ-OE) respectively and determined the efficiencies of circ-DAPK1 knockdown and overexpression by RT-qPCR (Fig. 2A). PI staining showed that knockdown of circ-DAPK1 significantly decreased the pyroptosis of HUVECs cultured in HG medium, and circ-DAPK1 overexpression increased the PI uptake in NG medium (Fig. 2B, C). Western-blot analysis showed that circ-DAPK1 suppressed the expression of NLRP3, ASC, cleaved caspase-1 (c-caspase-1), GSDMD, IL-18, and IL-1 β . In contrast, circ-DAPK1 downregulated the expression of the above pyroptosis-related proteins (Fig. 2D). Moreover, TEM imaging revealed that many pores formed in the cell membranes of the HG-treated group and circ-OE group (Fig. 2E). Since ASC and c-caspase-1 are key regulators in the process of pyroptosis, we performed immunofluorescence staining in HUVECs. In the HG groups, circ-KD inhibited the fluorescence intensity, and consistent with this finding, circ-OE enhanced the fluorescence intensity in the NG groups (Fig. 2F, G, H, I).

CircRNA-DAPK1 acts as a sponge for miRNA-4454, and miRNA-4454 attenuates pyroptosis in HUVECs.

Because circ-DAPK1 is stable in the cytoplasm, we further investigated the capability of circ-DAPK1 to bind miRNAs. We performed a miRNA expression profile analysis and compared the results with two databases (miRanda and TargetScan). Ten miRNAs were predicted as the potential targets of circ-DAPK1 (Fig. 3A). RT-qPCR showed that only miRNA-4454 increased significantly after circ-DAPK1 knockdown (Fig. 3B). To prove the correlation between miRNA-4454 and circ-DAPK1, HEK293 cells were transfected with an AGO2 overexpression plasmid or empty vector and subjected to a RIP assay. By PCR analysis, we found that circ-DAPK1 and miRNA-4454 pulled down by the anti-AGO2 antibody were enriched in the AGO2 overexpression group (Fig. 3C). Furthermore, in the HG-treated group, miRNA-4454 expression was decreased (Fig. 3D). And immunofluorescence staining showed that miRNA-4454 was predominately located in the cytoplasm (Fig. 3E).

We next explored the function of miRNA-4454 in HUVECs via a PI staining assay. As shown in Fig. 3F and 3G, the inhibition of miRNA-4454 resulted in higher pyroptosis rates of HUVECs in the HG group. However, miRNA-4454 overexpression reduced the percentage of PI⁺ cells in the NG group (Fig. 3G). TEM revealed that miRNA-4454 could attenuate the pyroptosis of HUVECs (Fig. 3H). Furthermore, miRNA-4454 overexpression decreased the levels of NLRP3, ASC, c-caspase-1, N-GSDMD, IL-18 and IL-1 β (Fig. 3I).

TXNIP is a target gene of miRNA-4454.

To determine which target genes were regulated by miRNA-4454 in HUVECs, we screened the transcriptomes of the HG and NG groups (Fig. 4A). Among the identified differentially expressed genes, TXNIP was higher in the HG groups. For validate its expression, RT-qPCR was performed to demonstrate that TXNIP expression was higher in the HG groups (Fig. 4B). Based on RT-qPCR and western-blot analysis, TXNIP expression substantially increased under miRNA-4454 inhibitor treatment (Fig. 4C), while miRNA-4454 overexpression reduced the expression of TXNIP (Fig. 4D). According to the competing endogenous RNA (ceRNA) mechanism, the expression of circ-DAPK1 should be positively correlated with the expression of target genes. The RT-qPCR results indicated that circ-DAPK1 silencing decreased the mRNA expression of TXNIP in the HG group, while circ-DAPK1 overexpression increased the abundance of TXNIP in the NG group (Fig. 4E). Luciferase assays confirmed that miRNA-4454 mimics inhibited luciferase activity in cells transfected with the wildtype(WT) 5'-UTR of TXNIP but not the mutant (Mut) 5'-UTR of TXNIP (Fig. 4F). In addition, FISH imaging revealed that both miRNA-4454 and TXNIP mainly were located in the cytoplasm (Fig. 4G). These results suggest that miRNA-4454 targets TXNIP and that TXNIP seems to be a downstream regulator of circ-DAPK1 and miRNA-4454.

Circ-DAPK1 promotes cell pyroptosis via the circ-DAPK1/miRNA-4454/TXNIP axis in vitro.

As TXNIP is a downstream factor of circ-DAPK1 and miRNA-4454, we next investigated the relationship of these three molecules. In the RT-qPCR assay, circ-DAPK1 inhibited the expression of miRNA-4454 (Fig. 5A). FISH assays revealed that under HG conditions, both circ-DAPK1 and miRNA-4454 were located in the cytoplasm (Fig. 5B). This result suggested that circ-DAPK1 might regulate miRNA-4454 by acting

as a ceRNA. As expected, a luciferase assay confirmed that circ-DAPK1 could bind to miRNA-4454 (Fig. 5C).

We next performed a ceRNA network analysis by circRNA-seq, miRNA profiling and RNA-seq. The bioinformatics analysis of the ceRNA network revealed that circ-DAPK1 could regulate miRNA-4454/TXNIP (Fig. 5D). In other words, the circ-DAPK1/miRNA-4454/TXNIP axis exists in the HUVECs. To verify the role of this signaling axis in the process of pyroptosis, we designed dual luciferase reporter and rescue assays. In Fig. 5E, comparing the TXNIP 5'-UTR WT + miRNA-4454 OE group with the TXNIP 5'-UTR WT + miRNA-4454 OE + circ-DAPK1 OE group, circ-DAPK1 overexpression enhanced the luciferase activity. By RT-qPCR analysis, miRNA-4454 suppression reversed the expression of TXNIP in the circ-KD + miRNA-4454 inhibitor group compared with the circ-KD group (Fig. 5F). Analogously, western-blot confirmed that the miRNA-4454 inhibitor restored the expression of NLRP3, ASC, c-caspase-1, N-GSDMD, IL-18 and IL-1 β (Fig. 5G). Consistent with the western-blot results, PI staining demonstrated that the miRNA-4454 inhibitor significantly increased the number of PI⁺ cells (Fig. 5H). Taken together, these results revealed that the circ-DAPK1/miRNA-4454/TXNIP axis plays a vital role in cell pyroptosis.

The circ-DAPK1-regulated mechanism exists in a diabetic mouse model.

To further investigate the role of circ-DAPK1 under HG conditions, we constructed a diabetes model with C57BL/KsJ-db/db mice. After the mice were fed a high-fat diet for 4 weeks, the tail vein blood was collected for blood glucose testing. In the db/db group, blood glucose was higher than that in the db/m group (Fig. 6A). By FISH, circ-DAPK1 was enriched in the db/db group. Contrarily, we found miRNA-4454 expressed higher in db/m group (Fig. 6B). In contrast, by RT-qPCR analysis, the expression of circ-DAPK1 and TXNIP was detected higher, while miRNA-4454 expression was lower, in the db/db group (Fig. 6C, D, E). Western-blot analysis revealed that c-caspase-1, NLRP3 and TXNIP were highly expressed in the db/db group (Fig. 6F). These results suggested that the circ-DAPK1/miRNA-4454/TXNIP axis may exist *in vivo*.

The circ-DAPK1/miRNA-4454/TXNIP axis promotes cell pyroptosis in vivo.

We next continued to investigate whether the circ-DAPK1/miRNA-4454/TXNIP axis aggravates pyroptosis *in vivo*. Therefore, we injected circ-DAPK1, miRNA-4454 or TXNIP AAV intravenously (Fig. 7A). As shown in Fig. 7B, AAV had no influence on the weight of mouse model. RT-qPCR was performed to determine the expression of the circ-DAPK1/miRNA-4454/TXNIP axis in db/db mice, and the results confirmed that circ-DAPK1 knockdown and miRNA-4454 overexpression transfection markedly decreased the level of TXNIP (Fig. 7C). This result suggested that the circ-DAPK1/miRNA-4454/TXNIP axis could function *in vivo*. Blood glucose tests illustrated that circ-DAPK1 knockdown, miRNA-4454 overexpression and TXNIP knockdown reduced the glucose levels of db/db mice (Fig. 7D). Then, immunofluorescence staining was performed to detect the ASC and caspase-1 in the arteries of each group. The results confirmed that in the circ-KD, miRNA-4454 OE and TXNIP-KD groups, the fluorescence intensity of ASC and caspase-1 dramatically declined (Fig. 7E, F). Moreover, the serum and aortas of db/db mice were collected for ELISA and western-blot analysis respectively. The experiments demonstrated that circ-DAPK1 knockdown,

miRNA-4454 overexpression and TXNIP knockdown reduced the expression of NLRP3, ASC, TXNIP, c-caspase-1, N-GSDMD, IL-18 and IL-1 β *in vivo* (Fig. 7G, H, I, J, K, L, M, N). These results verified that the circ-DAPK1/miRNA-4454/TXNIP axis promotes pyroptosis *in vivo*.

Discussion

At present, pyroptosis has been reported to induce atherosclerosis[20]. Many molecules and mechanisms, such as circRNAs and ceRNAs, are involved in the process of pyroptosis[21, 22]. CircRNAs are a type of noncoding RNA that has been reported to mediate different biological and pathological processes, including vascular diseases[23, 24]. However, their functions in the progression of vascular cell pyroptosis and roles in atherosclerosis remain to be clearly defined. Therefore, understanding their mechanisms in the regulation of pyroptosis may provide prospects for atherosclerosis. We identified a potential circRNA—circ-DAPK1 by circRNA sequencing, but the role of circ-DAPK1 in vascular cell pyroptosis in diabetes has not been clarified. In this study, we focused on the biological role and mechanism of circ-DAPK1.

According to frequent reports, circRNAs can modulate miRNAs by acting as sponges in what we called ceRNA networks[23]. Some well-known circRNAs can mediate biological functions by interacting with miRNAs. CircITCH acts as a sponge of miR-330-5p to upregulate SIRT6, Survivin and SERCA2a expression, thus alleviating doxorubicin-induced cardiomyocyte dysfunction[25]. In addition, circ-ZNF532 could induce vascular dysfunction through miR-29a-3p[26]. In our study, we identified circ-DAPK1 as a sponge of miRNA-4454 by dual luciferase reporter and RIP assays. Moreover, under HG conditions, circ-DAPK1 knockdown enhanced the expression of miRNA-4454. These results were mirrored by *in vivo* experiments. Therefore, we propose a mechanism by which circ-DAPK1 act as a sponge of miRNA-4454 to promote vascular cell pyroptosis.

TXNIP is an important factor in various diseases, such as cardiovascular diseases, cancer and diabetes[27]. In recent years, TXNIP has been reported to mediate the process of pyroptosis. According to previous studies, TXNIP directly interacts with NLRP3 to promote cell pyroptosis[28, 29]. In our study, we found that circ-DAPK1 acts as a sponge of miRNA-4454 to regulate the expression of TXNIP. Moreover, western-blot analysis revealed that circ-DAPK1 could activate the pyroptosis signaling pathway. In other words, the circ-DAPK1/miRNA-4454/TXNIP signaling axis could promote the development of vascular cell pyroptosis.

In this study, we designed *in vitro* and *in vivo* experiments to identify the role of circ-DAPK1 in vascular pyroptosis in diabetes. We will continue to explore its status in clinical specimens and its function in the procession of atherosclerosis in our future work.

Conclusions

In summary, we have demonstrated the vital role of the circ-DAPK1/miRNA-4454/TXNIP axis during the development of cell pyroptosis in diabetes. This signaling axis may serve as a therapeutic target for

diabetic vascular diseases.

Abbreviations

circRNAs: circular RNAs; FISH: fluorescence in situ hybridization; RIP: RNA immunoprecipitation; TXNIP: thioredoxin-interacting protein; AAV: Adeno-associated virus; TEM: transmission electron microscopy; NLRP3: nucleotide-binding oligomerization domain-like receptor family 3; ASC: apoptosis-associated speck-like protein; IL-18: interleukin-18; IL-1 β : interleukin-1 β ; miRNA: microRNA; HUVECs: Human umbilical vein endothelial cells; HG: high glucose; NG: normal glucose; ELISA: Enzyme-linked immunosorbent assay; WT: wild type; Mut: mutation; IF: Immunofluorescence; KD: knockdown; OE: overexpression.

Declarations

Ethics approval and consent to participate

All animal studies were approved by the Ethical Review Committee of the First Affiliated Hospital, Zhejiang University School of Medicine.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interests.

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Author contributions

T.c.C., X.t.Z., Y.y.H, H.k.Z, and Z.h.W. designed the study, conducted the experiments, wrote the paper. C.y.Q, X.h.W, and Y.l.X, edited the paper. Q.q.Z, and Q.l.Z., edited the figures. T.S, Y.j.H, and D.l.L performed the statistical analysis and checked the manuscript.

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Figures

Figure 1

Circ-DAPK1 expression and subcellular localization in HUVECs. (A) Heat map of the differentially expressed circRNAs in HG and NG medium cultured HUVECs. (B) Volcano plot of differential circRNAs expression. (C) The expression of circ-DAPK1 in HG medium and NG cultured HUVECs. (D) The subcellular localization of circ-DAPK1. Magnification: 400. (E) Schematic illustration of circ-DAPK1 showing DAPK1 exons 23-24 circularisation to form circ-DAPK1 splicing sites. (F) PCR analysis validated

the existence of circ-DAPK1 in HUVEC cells. Circ-DAPK1 was amplified by divergent primers in cDNA but not gDNA. (G) Northern blot analysis of circ-DAPK1 in the HUVECs treated with/without RNase R. GAPDH was used as negative control. (HG: high glucose; NG: normal glucose)

Figure 2

Circ-DAPK1 promotes pyroptosis of HUVECs. (A) The expression of circ-DAPK1 in HUVECs detected by RT-qPCR. (B) PI staining of HUVECs in the HG and NG group. (C) RT-qPCR analysis of circ-DAPK1 expression

after circ-DAPK1 inhibition or overexpression. (D) Western-blot analysis of NLRP3, caspase-1,ASC, GSDMD, IL-18 and IL-1 β . In the caspase-1 and GSDMD bands, cleaved caspase-1(c-caspase-1) and GSDMD-N terminal segments were the below one respectively. (E) TEM imaging of HUVECs in the HG and NG group. (F) Immunofluorescence staining of caspase-1 of HUVECs in the HG and NG group. Magnification:200. (G) The fluorescence intensity of capase-1. (H) Immunofluorescence staining of ASC of HUVECs in the HG and NG group. (I) The fluorescence intensity of ASC. Magnification:200.

Figure 3

CircRNA-DAPK1 acts as a sponge for miRNA-4454 and miRNA-4454 attenuates cell pyroptosis in HUVECs. (A) The overlapping of the target miRNAs of circ-DAPK1 predicted by TargetScan, miRanda and miRNA-seq profiling. (B) RT-qPCR analysis of miRNAs in circ-KD and NC group. (C) AGO2 RIP assay for circ-DAPK1 expression levels in HEK239T cells. (D) RT-qPCR analysis of miRNA-4454 in the HG and NG group. (E) The miRNA-4454 expression detected by miRNA-4454 probe. Magnification:200 (F) PI staining of HUVECs by miRNA inhibition or overexpression. (G) The percentage of PI+ cells in HUVECs. (H) TEM analysis of HUVECs after miRNA-4454 inhibition or overexpression. (I) Western-blot analysis of pyroptosis-related protein. (circ-KD: circ-DAPK1 knockdown; NC: Negative Control; OE: overexpression; Vector: empty vector).

Figure 4

TXNIP is a target gene of miRNA-4454. (A) Heatmap of differentially expressed genes in the HG and NG group. (B) RT-qPCR analysis of TXNIP in the HG and NG group. (C) RT-qPCR analysis of TXNIP after miRNA-4454 inhibition. (D) Western-blot analysis of TXNIP with miRNA inhibition or overexpression. (E) The mRNA levels of TXNIP detected by RT-qPCR. (F) Dual luciferase reporter assay demonstrated miRNA-4454 interact with TXNIP. (G) FISH assays revealed that both miRNA-4454 and TXNIP mainly located in

the cytoplasm. Magnification:200. (circ-KD: circ-DAPK1 knockdown; NC: Negative Control; OE: overexpression; Vector: empty vector).

Figure 5

Circ-DAPK1 promotes cell pyroptosis via the circ-DAPK1/miRNA-4454/TXNIP axis in vitro. (A) Relative expression of miRNA-4454 with circ-DAPK1 knockdown or overexpression. (B) FISH analysis of circ-DAPK1 and miRNA-4454 in different groups. Magnification:400. (C) Dual luciferase reporter assay of circ-

DAPK1 and miRNA-4454. (D) The ceRNA network analysis of HUVECs, all data derived from circRNA sequence, miRNA profiling and RNA sequence. This analysis predicts the link among circ-DAPK1, miRNA-4454 and TXNIP. (E) Luciferase reporter assay demonstrated the correlation among circ-DAPK1, miRNA-4454 and TXNIP. (F) Relative expression of TXNIP. Comparing with circ-KD, miRNA-4454 inhibition reversed the expression levels of TXNIP. (G) miRNA-4454 inhibition reversed the expression of ASC, c-caspase-1, GSDMD-N, IL-18, IL-1 β and TXNIP in western-blot analysis. (H) and (I)PI staining of high glucose medium treated HUVECs in the rescue assay. Magnification:200. (J) TEM analysis of HUVECs in the rescue assay. Magnification: 8000. (circ-KD: circ-DAPK1 knockdown; NC: Negative Control; OE: overexpression; Vector: empty vector).

Figure 6

The circ-DAPK1-regulated mechanism exists in a diabetic mouse model. (A) The blood glucose level in the db/db and db/m group. (B) FISH analysis of circ-DAPK1 or miRNA-4454 in the aorta specimens of db/db and db/m group. Magnification: 100. (C) The expression of circ-DAPK1 in db/db and db/m group. (D) The expression of miRNA-4454 in db/db and db/m group. (E) The expression of TXNIP in db/db and db/m group. (F) Western-blot analysis of TXNIP, c-caspase-1 and NLRP3 in db/db and db/m group.

Figure 7

The circ-DAPK1/miRNA-4454/TXNIP axis promotes cell pyroptosis in vivo. (A) Protocol of establishment of mouse model. (B) The weight of mice injected with circ-DAPK knockdown, TXNIP knockdown, miRNA-4454 overexpression AAV and their control groups. (C) Relative expression of TXNIP in five groups of mice. (D) The blood glucose of five groups. Blood glucose were detected before sacrifice. (E) and (F) The immunofluorescence analysis of ASC and caspase-1 respectively. Magnification: 200. (G) and (H) ELISA

detection of IL-1 β and IL-18 of mice serum. (I) ASC, TXNIP, GSDMD, NLRP3 and caspase-1 of mice aorta specimens detected by western-blot. (J)(K)(L)(M) Quantification analysis of NLRP3, ASC, TXNIP, c-caspase-1 and GSDMD-N in western-blot. GAPDH was used as control. (KD: knockdown; NC: Negative Control).

Figure 8

Schematic model representing the role of circ-DAPK1 in vascular cells.