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Circular RNA TOP2A Promotes Apoptosis and Suppresses Cell Viability of Ox-LDL-induced HAEC Cells via Sponging MicroRNA-27a-3p

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ABSTRACT This study explored impacts of circular RNA TOP2A and miR-27a-3p on HAECs after ox-LDL treatment. RNA expressions of circRNA TOP2A and miR-27a-3p were assessed by RT-qPCR in normal and ox-LDL-treated HAECs, showing circRNA TOP2A was inhibited and miR-27a-3p was upregulated after ox-LDL treatment. HAECs viabilities were promoted by ox-LDL treatment but overexpressed circRNA TOP2A suppressed the cell viability. Moreover, Ki-67 protein expression was downregulated while cleaved caspase-3 was inhibited. MiR-27a-3p was then confirmed to be sponged and suppressed by circRNA TOP2A while miR-27a-3p mimics promoted its expression. Additionally, decreased cell viability caused by overexpressed circRNA TOP2A was also reversed by miR-27a-3p overexpression. Furthermore, downregulated Ki-67 and increased cleaved caspase-3 protein expressions caused by circRNA TOP2A upregulation were also restored by overexpressed miR-27a-3p, resulting in promoted Ki-67 and decreased cleaved caspase-3.

INTRODUCTION

Atherosclerosis, a disease induced by chronic inflammation, is an underlying cause of cardiovascular diseases (Gisterå and Hansson 2017). Normal endothelial cells maintain vascular homeostasis and suppress atherosclerosis through modulating tension of blood vessels and inflammation (Duan et al. 2021). Evidence has reported that endothelial cell apoptosis is the foundation of the atherosclerosis (Wang et al. 2018). Oxidative stress promotes oxidation of low-density lipoprotein to produce oxidised low-density lipoprotein (ox-LDL), causing inflammatory response, endothelial cells death and endothelial injury (Kattoor et al. 2017; Zhao et al. 2020). However, endothelial damage is a reversible step in progressions of atherosclerosis, reminding that prevention of endothelial injury can be a promising treatment method (Zhu et al. 2019).

Recently, increasing evidence has illustrated participations of noncoding RNAs in atherosclerosis progressions. Circular RNAs (circRNAs) are noncoding RNAs having loop forms with no 5'- 3' divergences and polyadenylated ends (Gong et al. 2019; Li et al. 2019). Majority of the circular RNAs are endogenous noncoding RNAs, which preserve among diverse species and present a sophisticated extent of constancy compared to linear mRNAs (Memczak et al. 2013; Salzman et al. 2013). Functions of circRNAs have also been revealed that they can sponge miRNA or act as competing endogenous RNAs (Chen et al. 2021). Recently, studies about circular RNAs in atherosclerosis have been increased rapidly, suggesting that they participate in progressions of atherosclerosis by modulating endothelial cells, macrophages and vascular smooth muscle cells (Cao et al. 2020). CircRNA -102541 was upregulated in atherosclerosis samples and promoted polo like kinase 1 (PLK1) through sponging miR-296-5p, resulting in increased cell viability and restrained apoptosis of human umbilical vein endothelial cells (HUVECs) (Du et al. 2021). CircRNA -0044073 was upregulated and promoted human vascular smooth muscle cells (HUVSMCs) and HUVEC proliferation, invasion and migratory ability through sponging miR-107, which participated

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in progressions of atherosclerosis by activating JAK/STAT signalling pathway and inflammation (Shen et al. 2019). Hence, research about circRNAs might be a potential way to treat atherosclerosis. However, studies about effects of circRNAs are far from enough, which urge one to explore new circRNAs to enrich research of this kind of noncoding RNAs.

MicroRNA (miRNA) is a kind of small noncoding RNA with about 19-23 nucleotides (Zhang et al. 2020), which has been well known to cause mRNAs degradation by binding them directly. MiRNAs have also been reported to participate in atherosclerosis progressions via regulating dysregulations of endothelial cells, macrophages and VSMCs (Lu et al. 2018). MiR-146b-5p inhibited mouse aortic smooth muscle cells proliferation through downregulating BCL2-associatedathanogene 1 (Bag1), which also inhibited migration by binding matrix metalloproteinase 16 (MMP 16) (Sun et al. 2020). MiR-27a-3p was sponged by lncRNA TUG1, resulting in accelerated HUVECs apoptosis, autophagy and inflammation by upregulating slit guidance ligand 2 (Dong et al. 2020). This previous study indicated that miR-27a-3p might be a promoter of atherosclerosis. Hence, new discovered circRNAs that sponge miR-27a-3p might be a new biomarker for treating atherosclerosis. By the prediction of StarBase (https://starbase.sysu.edu.cn/starbase2/index.php), circRNA TOP2A was predicted to have binding spots with miR-27a-3p. The researchers hypothesised that circRNA TOP2A might sponge miR-27a-3p in human aortic endothelial cells (HAECs) to modulate progressions of atherosclerosis.

Objectives

This study aimed at exploring expressions of circRNA TOP2A and miR-27a-3p and their effects on modulating cell viability and apoptosis factors caspase-3 and Ki67 in HAECs after ox-LDL induction.

MATERIAL AND METHODS

Main Reagents

The main reagents used for the research included human aortic endothelial cells (Procell, Wuhan, China), ox-LDL (Solarbio, Beijing, China), RPMI 1640 (Procell), foetal bovine serum (FBS, Sigma Aldrich, USA), penicillin and streptomycin (Sigma Aldrich), Lipofectamine 3000 (Invitrogen, US), TRIzol reagent (Invitrogen, USA), RNase R (Abcam, UK), cDNA synthesis kit (Beyotime, Beijing, China), SYBR qPCR mix (Beyotime), CCK-8 kit (Beyotime), radioimmunoprecipitation assay (RIPA) lysate (Thermo Scientific, US), anti-Ki67 (1:1000, ab ab92742, Abcam), anti-cleaved caspase-3 (1:1000, ab2302), and anti-GAPDH (1:2000, ab9484).

Cell Culture

Normal human aortic endothelial cells (HAECs) were bought from Procell (Wuhan, China). Then, cells were infected with ox-LDL (10, 20 and 30μM, Solarbio, Beijing, China). All cells were preserved in RPMI 1640 solution (Procell) mixed with ten percent foetal bovine serum (FBS, Sigma Aldrich, USA), 50 U/ml penicillin and 50 g/ml streptomycin (Sigma Aldrich). All the cells were cultivated in an incubator with five percent CO_2 at 37 centigrade.

Transfection

CircRNA TOP2A was cloned into pcDNA3.1 (Thermo Fisher Scientific USA). MiR-27a-3p mimics and miR-NC were acquired from GenePharma (Shanghai, China). The DNA sequencing was used for confirmation of the recombinant plasmids sequence. Confirmation of circRNAs was using convergent and divergent primers. Cells were planted into 6-well plates for exactly 1 day. When the cells achieved the confluence of eighty percent, Lipofectamine 3000 (Invitrogen, US) was used to perform the transfections following the prescriptions of the manufacturer. Afterwards, transfected cells were cultured for 1 to 3 days using five percent CO_2 at 37 centigrade prior to any following experiments.

RT-qPCR

TRIzol reagent (Invitrogen, USA) was applied for segregating total RNA. For separating circRNA TOP2A, the isolated total RNA was treated with RNase R (Abcam, UK) and processed at 37 centigrade for half an hour. Then,

the RNA was quantified by a Nano Drop 2000 (Thermo Fisher Scientific, New Jersey, US) and the cDNA synthesis kit (Beyotime, Beijing, China) was for reverse transcription. The Thunderbird SYBR qPCR mix (Beyotime) was used to perform the RT-qPCR analysis. The RT-qPCR conditions were denaturation at 94 centigrade for 2 minutes, then an additional 30 cycles of denaturation at 94 centigrade for half a minute and strengthening at 56 centigrade for half a minute. All responses were done in four individual experiments. GAPDH and U6 were used for normalisation. The comparative circRNA TOP2A and miR-27a-3p variations were determined using the $2^{-\Delta\Delta CT}$ system.

CCK8 Assay

Cells were planted into the wells of a 96-well plate and incubated over the night. At 24 hours, 48 hours and 72 hours, cell viability was determined using a CCK-8 kit (Beyotime) following the instructions of the manufacturer. Absorbance was determined at 450nm by Spectra Max M5 (Molecular Devices). Tests were performed with six duplicates and done for three different times.

Luciferase Reporter Assay

The wild and mutated types of circRNA TOP2A were created using psiCHECK (Promega, USA). Diverse recombinant plasmids were co-transfected into infected HAECs with miR-27a-3p mimics or miR-NC. The luciferase activity of the respective transfection groups was determined by the luciferase assay system (Promega).

Western Blot

Protein extraction was performed with radioimmunoprecipitation assay (RIPA) lysate (Thermo Scientific, US) added with protease inhibitor cocktail (ABCAM, Cambridge, United Kingdom), parted on SDSPAGE, and moved to PVDF membranes. After cultivation with anti-Ki67 (1:1000, ab ab92742, Abcam), anti-cleaved caspase-3 (1:1000, ab2302) and anti-GAPDH (1:2000, ab9484) at 4 centigrade over the night, horseradish peroxidase (HRP) labelled secondary antibody (Abcam) was for incubating membranes at 23 centigrade for 60 minutes. Lastly, improved chemiluminescence (Thermo Fisher Scientific, New Jersey, US) was for protein signal finding, and Lab Works 4.5 was for capturing images and ImageJ for comparative measurement to analyse the mean grey value.

Statistical Analysis

All data are signified as mean ± standard deviations (SD). $P<0.05$ was statistically meaningful. Variances between two groups were evaluated by the student's t-test. One-way ANOVA and two-way ANOVA were used to evaluate the variances in numerous groups. Data analyses were evaluated with SPSS 19.0 and GraphPad Prism 9.

RESULTS

CircRNA TOP2A was Downregulated in Ox-LDL-induced HAECs

The RT-qPCR analysis was first executed to evaluate circRNA TOP2A expressions in HAECs with ox-LDL (10μM, 20μM and 30μM) treatment, which indicated that circRNA TOP2A expression was downregulated dose-dependently (Fig. 1A). The CCK-8 analysis was to determine the HAECs viability with ox-LDL induction, revealing that cell viabilities were decreased dose-dependently as well (Fig. 1B). Meanwhile, factors related to apoptosis were validated and results of Western blot revealed that cleaved caspase-3 protein expression was increased with ox-LDL (30¼M) treatment while the Ki67 protein expression was inhibited (Figs. 1C, D, E). Based on these results, ox-LDL treatment reduced circRNA TOP2A expression and HAEC viability but facilitated cell apoptosis.

Overexpressed circRNA TOP2A Accelerated HAEC Viability and Inhibited Cell Apoptosis

To further explore effects of circRNA TOP2A on ox-LDL induction, it was overexpressed, and results of RT-qPCR indicated that circRNA TOP2A overexpression elevated its expression in normal and ox-LDL-induced HAECs (Fig.2A). CCK-8 results revealed that overexpressed cir-

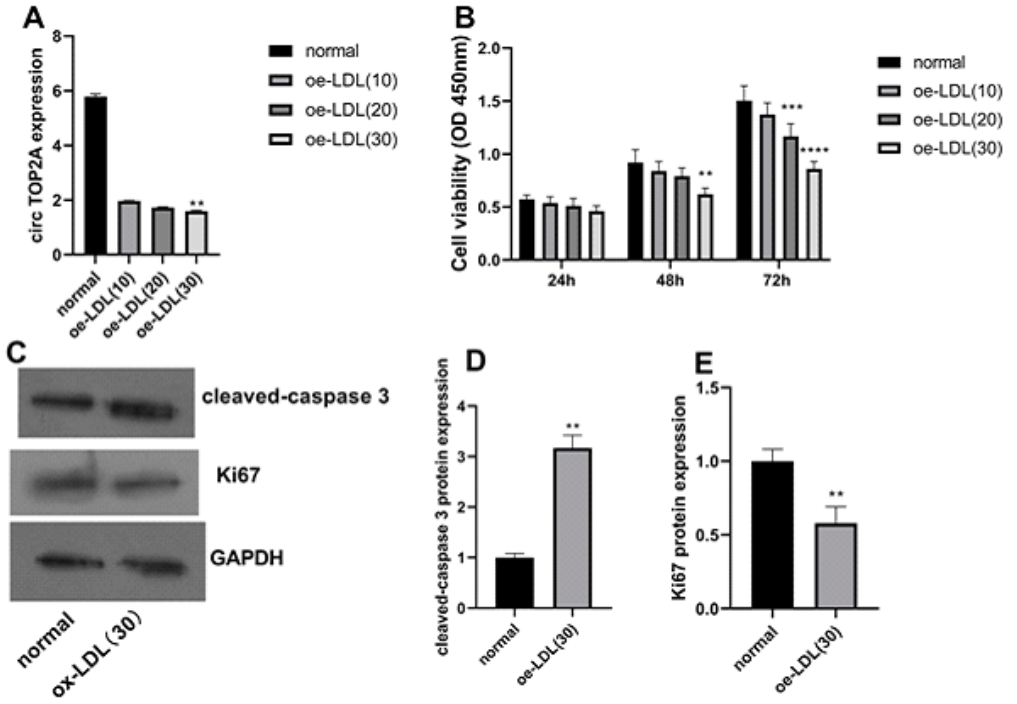

Fig. 1. Ox-LDL induction inhibited circRNA TOP2A expression
A: CircRNA TOP2A expressions in HAECs with ox-LDL induction (10, 20 and 30μM) were assessed by RT-qPCR, **P<0.05 in ox-LDL (30μM) vs. normal.
B: CCK-8 validated HAEC viability after induced by ox-LDL (10, 20 and 30μM), **P<0.05 in ox-LDL (30μM, 48h) vs. normal, ***P<0.001 in ox-LDL (20μM, 72h) vs. normal, ****P<0.0001 in ox-LDL (30μμM,72h) vs. normal.
C, D, E: Western blot examined cleaved caspase-3 protein expression and Ki67 protein expression after ox-LDL induction (30μM), **P<0.05 in ox-LDL (30μM) vs. normal.

Tables

Figure 1	
RT-qPCR for circRNA TOP2A	**P=0.0067 in ox-LDL (30μM) vs. normal
CCK-8 for HAEC viability with ox-LDL	**P=0.0012 in ox-LDL (30μM, 48h) vs. normal.
***P=0.0003 in ox-LDL (20μM, 72h) vs. normal,	****P<0.0001 in ox-LDL (30μM,72h) vs. normal.
Western blot for cleaved caspase-3	**P=0.0022 in ox-LDL (30μM) vs. normal
Western blot for Ki67	**P=0.0076 in ox-LDL (30μM) vs. normal

cRNA TOP2A accelerated HAEC viability after induced by ox-LDL (Fig. 2B). Additionally, cleaved caspase-3 protein expression was down-regulated with circRNA TOP2A overexpression while Ki67 protein expression was promoted (Fig. 2C). Hence, circRNA TOP2A overexpression accelerated HAEC viability and restrained cell apoptosis.

Upregulated miR-27a-3p Expression by Ox-LDL Induction was Sponged by circRNA TOP2A

Afterwards, miR-27a-3p expression was validated, revealing that its expression was upregulated dose dependently in HAECs with ox-LDL treatment (Fig. 3A). Thereafter, predicted binding spots between circRNA TOP2A and miR-

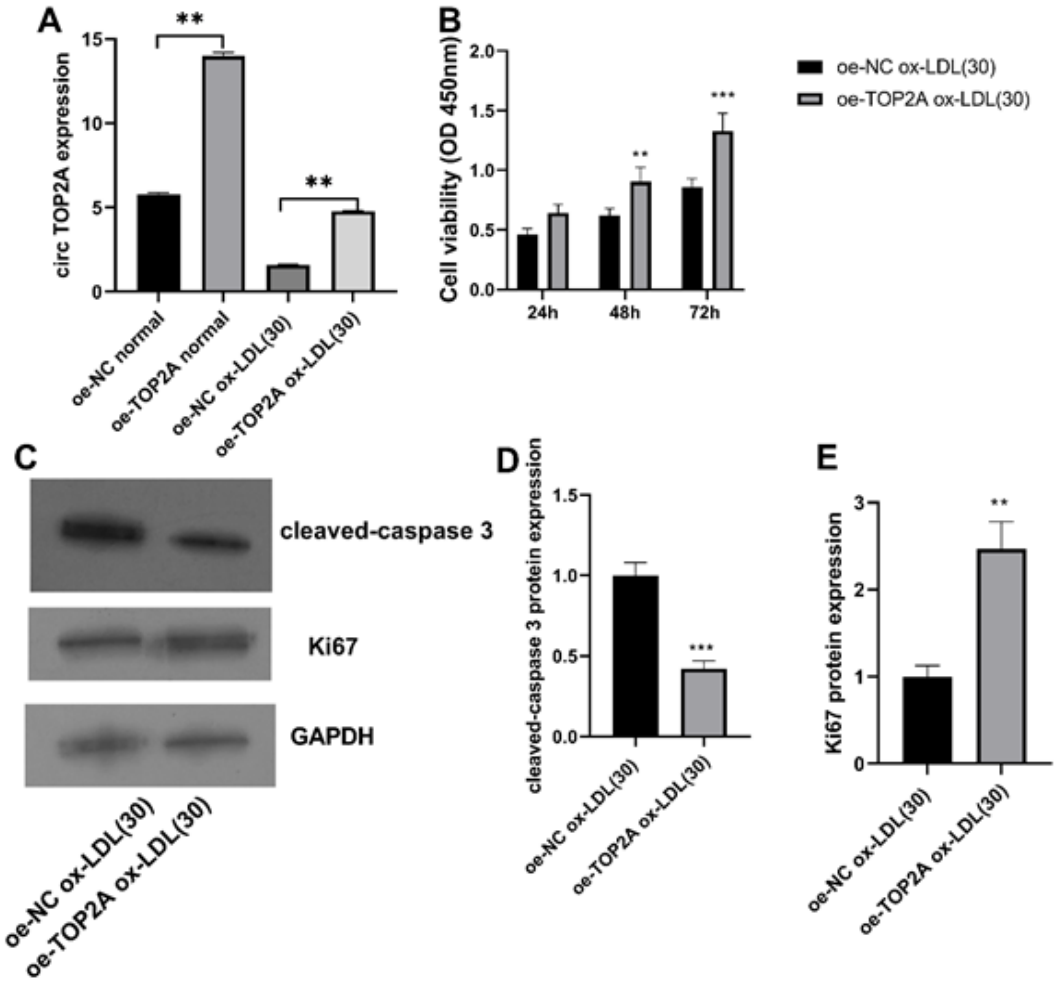

Fig. 2. CircRNA TOP2A overexpression promoted HAEC cell viability and hampered apoptosis
A: CircRNA TOP2A expression with circRNA TOP2A overexpression were examined by RT-qPCR in normal HAEC and ox-LDL (30μM)-induced HAECs, **P<0.05 vs. normal.
B: CKK-8 evaluated cell viability of ox-LDL-induced HAECs after circRNA TOP2A overexpression, **P<0.05, ***P<0.001.
C, D, E: Western blot examined Ki67 and cleaved caspase-3 protein expressions after circRNA TOP2A upregulation, **P<.0.05, ***P<0.001.

Tables

Figure 2	
RT-qPCR for circRNA TOP2A	**P<0.05 in oe-TOP2A normal vs. oe-NC normal **P<0.05 in oe-TOP2A ox-LDL vs. oe-NC ox-LDL
CCK-8 for HAEC viability with ox-LDL	**P=0.009 in oe-TOP2A ox-LDL (48h) vs. oe-NC ox-LDL (48h), ***P=0.0002 in oe-TOP2A ox-LDL (72h) vs. oe-NC ox-LDL (72h)
Western blot for cleaved caspase-3	***P=0.0004 in oe-TOP2A vs. oe-NC
Western blot for Ki67	**P=0.0016 in oe-TOP2A vs. oe-NC

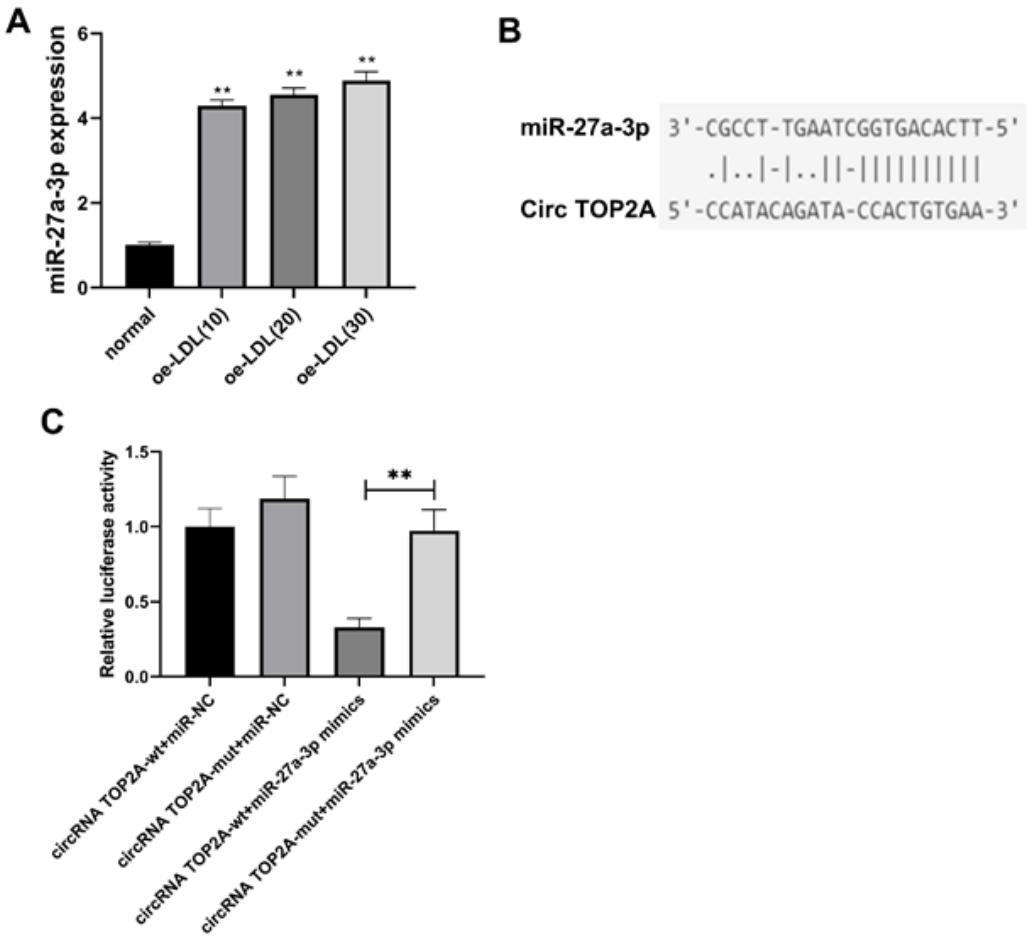

Fig. 3. MiR-27a was upregulated in HAECs after ox-LDL induction
A: MiR-27a-3p expressions were examined by RT-qPCR in HAECs after ox-LDL induction (10, 20 and 30μM), **P<0.05.
B: Binding spots were provided by StarBase v2.0.
C: Fluorescence was evaluated by luciferase reporter test, **P<0.05.

Tables

Figure 3	
RT-qPCR for miR-27a-3p	**P<0.05 vs. normal
fluorescence	**P<0.05

27a-3p were displayed by StarBase (https://starbase.sysu.edu.cn/starbase2/index.php) (Fig. 3B). Using luciferase reporter test, the fluorescence was the lowest in circRNA TOP2A-wt with miR-27a-3p mimics group, revealing that circRNA TOP2A sponged miR-27a-3p in HAECs (Fig.3C). According to these results, miR-27a-3p was upregulated in HAECs after ox-LDL induction, which was sponged by circRNA TOP2A.

MiR-27a-3p Overexpression Inhibited the Viability but Facilitated the Apoptosis of Oox-LDL-Induced HAECs

After the binding between them was examined, impacts of miR-27a-3p were evaluated. RT-qPCR results indicated that miR-27a-3p was downregulated by overexpressed circRNA TOP2A in both HAECs and ox-LDL-induced HAECs (Fig. 4A). Thereafter, functions of overexpressed miR-27a-3p were detected, showing that HAEC cell viability was suppressed by miR-27a-3p overexpression (Fig. 4B). Moreover, cleaved caspase-3 protein expression was upregulated by overexpressed miR-27a-3p while Ki67 protein expression was suppressed (Figs. 4C, D, E). These results showed that overexpressed miR-27a-3p prompted HAEC apoptosis and restrained cell viability.

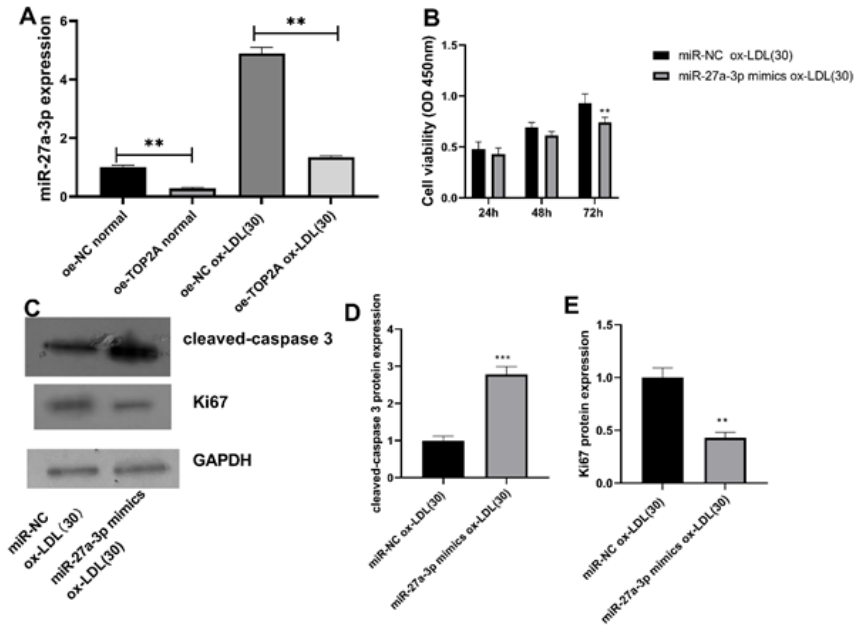

Fig. 4. MiR-27a-3p overexpression restrained HAEC viability and facilitated apoptosis
A: RT-qPCR examined miR-27a-3p in normal and ox-LDL-induced HAECs with circRNA TOP2A over expression, **P<0.05 vs. oeNC.
B: HAEC viability with miR-27a-3p were analyzed by CCK-8, **P<0.05.
C, D, E: Cleaved caspase-3 and ki67 protein expressions with miR-27a-3p overexpression were validated using western blot, **P<0.05.

Tables

Figure 4	
RT-qPCR for miR-27a-3p	**P<0.05 in oe-TOP2A normal vs. oe-NC normal, **P<0.05 in oe-TOP2A ox-LDL vs. oeNC ox-LDL
CCK-8 for cell viability	**P=0.0084 in miR-27a-3p mimics (72h) vs. miR-NC
Western blot for Ki67	**P=0.0019 in miR-37a-3p mimics vs. miR-NC
Western blot for cleaved caspase-3	***P=0.0008 in miR-37a-3p mimics vs. miR-NC

DISCUSSION

Atherosclerosis has been recognised as systemic disorder that mainly affects the aorta, carotid artery and coronary artery (Libby and Hansson 2015). Evidence has revealed that oxidation of LDL cholesterol is essential in progressions of atherosclerosis and low LDL level has been reported to decrease the risk of cardiovascular diseases (Herrington et al. 2016). Ox-LDL was produced when LDL was exposed to oxidation, resulting in damage to endothelium and triggered inflammation reactions (Miteva et al. 2018). Therefore, antioxidant therapy might be a promising approach to avoid injury of endothelial cells. In this study, circRNA TOP2A was downregulated in HAECs after ox-LDL induction, whose overexpression facilitated HAEC viability and decreased apoptosis through sponging miR-27a-3p.

With the development of biotechnology, discovering the molecular mechanism that is underlying the initiation of atherosclerosis is vital for establishing effective means of treatment. Circular RNA, having a covalently closed loop structure, has been reported to participate in progressions of atherosclerosis by modulating endothelial cells (Sun et al. 2021). CircRNA RSF1 overexpression promoted histone deacetylase 1 expression through sponging miR-135b-5p, resulting in increased cell viability and restrained apoptosis and inflammation in ox-LDL-induced HUVECs (Zhang et al. 2021). CircRNA USP36 was upregulated in endothelial cells after ox-LDL treatment, which promoted WNT4 to decrease cell viability and migratory ability of endothelial cells via sponging miR-637 (Huang et al. 2021). In this study, the researchers analysed the effects of circRNA TOP2A on HAECs with ox-LDL induction, showing that ox-LDL induction decreased expression of circRNA TOP2A and HAEC viability but accelerated HAEC apoptosis by upregulating cleaved caspase-3 and reducing Ki67 protein expressions. Furthermore, overexpression of circRNA TOP2A promoted HAEC viability and restrained apoptosis via downregulating cleaved caspase-3 and increasing Ki67 protein expressions. Therefore, the researchers have detected that circRNA TOP2A restrained progressions of HAECs with ox-LDL induction, suggesting that circRNA TOP2A might be a protector against atherosclerosis.

Particular circRNAs can bind and adversely induce microRNAs, which are significantly engaged in the competing endogenous RNA (ceRNA) system, thus controlling linear-RNA transcription as well as protein creation. A newly discovered circRNA 010567 enhanced myocardial fibrosis through repressing miR-141 (Zhou and Yu 2017). Accumulating information keep confirming that some circRNAs may control miRNA expression as miRNA sponges and have a substantial function in transcriptional regulation of the microRNAs (Rong et al. 2017). MiR-27a-3p has been detected to be upregulated in coronary artery diseases, which has positive correlation with ox-LDL level (Rafiei et al. 2021). MiR-27a-3p targeted growth factor receptor-bound protein 2 (Grb2) to decrease PI3K-AKT signalling pathway, resulting in enhanced oxidative stress and aggravated RI/R injury of mice (Zhao et al. 2020). In this study, miR-27a-3p was also elevated in HAECs after ox-LDL induction. Moreover, using the StarBase, binding spots of miR-27a-3p with circRNA TOP2A were displayed, which implied a potential interaction between them. Thereafter, results of the dual-luciferase reporter test showed that circRNA TOP2A-wt with miR-27a-3p mimics exhibited the lowest luciferase activity, which confirmed that miR-27a-3p was sponged directly by circRNA TOP2A. Furthermore, the researchers explored impacts of miR-27a-3p on ox-LDL-induced HAECs, revealing that miR-27a-3p overexpression restrained HAEC viability and accelerated apoptosis via promoting cleaved caspase-3 and decreasing Ki67 protein expressions. Therefore, miR-27a-3p was a promoter of oxidative stress, which could be sponged by circRNA TOP2A.

Variations in the expression and initialisation of chief apoptotic regulators lead to dysregulated cell death (Yanamadala et al. 2009; Zhang et al. 2013). The caspase family of cysteine proteases plays vital roles in connecting the occurrences of cell death. Caspases are classified into two groups according to their structures and functions. These are upstream establisher, such as caspase-8, -9, and -10, and the downstream executioner, such as caspase-3, -6, and-7 (Walsh et al. 2008). Antigen Ki-67 is a nuclear protein expressed in proliferating mammalian cells. It is

commonly used in various illnesses histopathology but its roles are still mysterious (Sobecki et al. 2016). In the present study, Ki67 was upregulated by circRNA TOP2A overexpression but inhibited by miR-27a-3p upregulation while cleaved caspase-3 protein had totally opposite expressions compared with Ki67. Compared to previous study, the researchers have found a new circRNA TOP2A that restrained ox-LDL inductions on HAECs via sponging miR-27a-3p.

CONCLUSION

CircRNA TOP2A was downregulated by ox-LDL induction while its overexpression promoted HAEC viability and restrained apoptosis via sponging miR-27a-3p, suggesting that circRNA TOP2A might be a promising circRNA for treating atherosclerosis. However, further studies in vivo and clinical stage are necessary to get more knowledge.

RECOMMENDATIONS

Ox-LDL induction decreased HAEC cell viability and accelerated apoptosis by promoting cleaved caspase-3 and reducing Ki67. CircRNA TOP2A was downregulated by ox-LDL induction but its overexpression facilitated HAEC cell viability and hampered apoptosis via inhibiting cleaved caspase-3 and upregulating Ki67. MiR-27a-3p was upregulated by ox-LDL induction, which was sponged and suppressed by circRNA TOP2A. Overexpression of miR-27a-3p hindered HAEC viability and enhanced apoptosis through upregulating cleaved caspase-3 and suppressing Ki67.

ABBREVIATION LIST

- CircRNA TOP2A: circular RNA DNA Topoisomerase II Alpha
- MiR-27a-3p: microRNA-27a-3p
- HUVECs: human umbilical vein endothelial cells
- HAEC: human aortic endothelial cell
- Ox-LDL: oxidized low-density lipoprotein
- PLK1: polo like kinase 1
- HUVSMCs: human vascular smooth muscle cells
- Bag1: BCL2-associatedathanogene 1
- MMP 16: matrix metalloproteinase 16

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