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Research Article

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CircRNA.0007127 triggers apoptosis through the miR-513a-5p/CASP8 axis in K-562 cells

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Abstract: Background: Circular RNAs (circRNAs) are covalently closed single-stranded RNAs with multiple biological functions. CircRNA.0007127 is derived from the carbon catabolite repression 4-negative on TATA-less (*CCR4-NOT*) complex subunit 2 (*CNOT2*), which was found to regulate tumor cell apoptosis through caspase pathway. Methods: Potential circRNA.0007127 target microRNAs (miRNAs) were analyzed by miRanda, TargetScan, and RNAhybrid software, and the miRNAs with binding sites for apoptosis-related genes were screened. The roles of circRNA.0007127 and its downstream target, microRNA (miR)-513a-5p, were validated by quantitative real-time polymerase chain reaction (qPCR), flow cytometry, mitochondrial membrane potential, immunofluorescence, western blot, and caspase-8 (CASP8) protein activity in vitro in H_2O_2-induced K-562 cells. The circRNA.0007127–miR-513a-5p and *CASP8*–miR-513a-5p interactions were verified by luciferase reporter assays. Results: Silencing circRNA.0007127 decreased cell apoptosis by inhibiting *CASP8* pathway activation in K-562 cells. Compared with the control group, the expression of *CASP8* was reduced by 50% and the 43-kD fragment of CASP8 protein was significantly reduced ($P\leqslant0.05$). The luciferase reporting assay showed that circRNA.0007127 combined with miR-513a-5p or *CASP8*, with extremely significant differences ($P\leqslant0.001$). The overexpression of miR-513a-5p inhibited the gene expression level of *CASP8* in a human myeloid leukemia cell model (75% change) and the level of a 43-kD fragment of CASP8 protein ($P\leqslant0.01$). The rescue experiment showed that cotransfection with circRNA.0007127 small-interfering RNA (siRNA) and the miR-513a-5p inhibitor increased *CASP8* gene expression and the apoptosis rate, suggesting that the miR-513a-5p inhibitor is a circRNA.0007127 siRNA antagonist. Conclusions: CircRNA.0007127 regulates K-562 cell apoptosis through the miR-513a-5p/*CASP8* axis, which can serve as a novel powerful molecular target for K-562 cells.

Key words: CircRNA.0007127; miR-513a-5p; Caspase-8 (CASP8); Apoptosis; K-562 cells

1 Introduction

Apoptosis means programmed cell death regulated by genes via different signaling pathways, such as the endogenous pathway (mitochondrial pathway) and the exogenous pathway (death receptor pathway) (Taylor et al., 2008; Obeng, 2021). In 1972, the word "apoptosis" was first used to describe different cell death patterns (Kerr et al., 1972). Apoptosis exhibits typical morphological characteristics, such as chromatin hydrolysis into fragments, nuclear pyknosis, hydrolysis of various organelles, formation of apoptotic bodies, and cell shrinkage (Taylor et al., 2008). It occurs at various life stages of human cells. When the regulation of apoptosis is not controlled, it damages the stability or defense mechanism of the body, leading to the occurrence of disease.

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Two protease families are involved in cell apoptosis, namely caspase and B-cell lymphoma-2 (Bcl-2) (Taylor et al., 2008; Grilo and Mantalaris, 2019). Caspases are mainly located in the cytoplasm and play key roles in the amplification of the apoptotic cascade of cells (Grilo and Mantalaris, 2019). To date, 14 kinds of caspases have been identified, among which caspase-8 (CASP8) is mainly involved in exogenous apoptotic pathways and can further induce apoptosis through two parallel cascades (Mandal et al., 2020). In addition, once cells receive apoptotic stimulation, reactive oxygen species (ROS) will induce the activation of CASP8 in the form of proenzyme, thus leading to the activation of downstream apoptotic effector molecules (Hung et al., 2016; Šrámek et al., 2021). Enhanced ROS production in neutrophils can cleavage CASP8 and CASP3, further reducing the mitochondrial membrane potential and ultimately leading to cell apoptosis (Dubey et al., 2016).

Circular RNAs (circRNAs) are loop structures closed by covalent bonds, which lack terminal 5' caps and 3' poly-A tails (Memczak et al., 2013). Since there is no free poly-A end, circRNA has certain resistance to RNA exonucleases and debranching enzymes, and it exists stably in the nucleus or cytoplasm (Guarnerio et al., 2016). CircRNAs have been reported to have various physiological functions: they can act as microRNA (miRNA) sponges, interact with proteins, influence translation, and so on (Kristensen et al., 2019). CircRNAs regulate cell behavioral phenotypes through different molecular mechanisms, such as differentiation and development, proliferation and apoptosis, or migration and invasion (Panni et al., 2020).

A large number of circRNAs have been observed to have elevated expression levels in hematologic tumors, glioma, gastrointestinal tumors, bladder cancer, and other diseases, and these circRNAs could inhibit the apoptosis of tumor cells and promote cell proliferation and metastasis (Hao et al., 2019; Jamal et al., 2019; Mei et al., 2019; Yuan et al., 2019). In the regulation of the apoptosis phenotype, circRNAs can act as apoptosis-related molecules. Evidence has shown that circRNAs, such as hsa_circ_0055538, hsa_circ_0007059, hsa_circ_0002483, hsa_circ_0001588, and circ-homer scaffold protein 1 (HOMER1), bind to miRNAs, regulate the expression of caspases, and participate in the regulation of cell apoptosis (Gao et al., 2019; Su et al., 2019; Du et al., 2020; Xu et al., 2020; Zhu et al., 2020; Xiao et al., 2021).

Carbon catabolite repression 4-negative on TATA-less (CCR4-NOT) is a highly conserved multifunctional complex composed of multiple subunits, that plays important roles in messenger RNA (mRNA) degradation, transcriptional inhibition, posttranscriptional regulation, and other signaling processes. The CCR4-NOT complex subunit 2 (CNOT2) has been shown to be involved in the regulation of apoptosis. The loss of CNOT2 can upregulate C/EBP homologous protein (*CHOP*) mRNA expression in cells and stimulate the activation of caspase-dependent apoptosis pathways (Ito et al., 2011). Furthermore, the downregulation of *CNOT2* expression reduced the protein expression level of homeodomain-interacting protein kinase 2 (HIPK2), an intermediate of cellular oxidative stress (Rodriguez-Gil et al., 2016). The CNOT2 signaling pathway has an essential function in the caspase-related apoptosis pathway in non-small cell lung cancer cells and liver cancer cells (Lee et al., 2019; Jung et al., 2020). CircRNA.0007127 is formed by the reverse splicing of exon 2 and exon 3 of the host gene *CNOT2*. Bioinformatics and functional analyses predicted that circRNA.0007127 is involved in the apoptosis regulation pathway. K-562 cells have been indicated to express circRNA.0007127 at relatively high levels (Salzman et al., 2013). In this study, a cell model of H_2O_2-induced K-562 cell apoptosis was constructed to explore the regulatory role of circRNA.0007127 in the apoptosis pathway.

2 Materials and methods

2.1 Culture, H_2O_2 treatment, and transfection of K-562 cells

The human myeloid leukemia cell line K-562 was purchased from Procell Life Science & Technology (Wuhan, China). The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo, Waltham, USA) supplemented with 10% (volume fraction) fetal bovine serum (Gibco, California, USA) at 37 ℃ and 5% CO_2. Then, cells were washed with D-Hanks's buffer (Solarbio, Beijing, China) and incubated at room temperature at 1.76 mmol/L H_2O_2 for 15 min. After washing again with D-Hanks's buffer, the cells were further cultured or transfected with small RNAs. Small-interfering RNAs (siRNAs), the microRNA (miR)-513a-5p mimic and inhibitor, were

purchased from RIBOBIO (Guangzhou, China) and transfected into K-562 cells with riboFECT™ CP (RIBOBIO) reagent.

2.2 RNA extraction

K-562 cells cultured as above were collected for RNA extraction. Briefly, TRIzol reagent (TaKaRa, Osaka, Japan) was used to extract RNA from K-562 cells by following the manufacturer's instructions. After adding chloroform and centrifuging each sample at 12 000*g* for 10 min, the precipitate was discarded, and 75% (volume fraction) ethanol was further added to the supernatant, which was gently mixed and incubated at room temperature for 10 min, followed by spinning at 7500*g* for 5 min. The supernatant was discarded, the precipitate was air-dried, and total RNA was obtained by resuspending the precipitate in ribonuclease (RNase)-free water.

2.3 Quantitative real-time polymerase chain reaction

The reverse transcription of mRNA was performed using PrimeScript RT Master Mix (TaKaRa), and the reverse transcription of circRNA or miRNA was performed using the PrimeScript RT Reagent Kit (TaKaRa) with random primers or specific stem-loop primers. The complementary DNA (cDNA) amplification was performed using PowerUp™ SYBR™ Green Master Mix (Thermo), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *U6* were used as reference genes. Each sample was assessed three times. All operations were performed under sterile RNase-free conditions. The reverse transcription primer for *U6* was AAAATATGGAACGCTTCACGAATTTG, and the primers for hsa-miR-513a-5p were purchased from RIBOBIO. The primer sequences were listed in Table S1.

2.4 RNase R treatment

Total RNA (10 μg) was incubated at 37 °C with or without 5 U/μg RNase R (Epicenter Technologies, Missouri, USA) for 30 min, followed by reverse transcription-polymerase chain reaction (RT-PCR) analysis and nucleic acid electrophoresis. Subsequently, 3% (0.03 g/mL) agarose gel electrophoresis with Tris-acetate-ethylenediamine tetraacetic acid (EDTA) (TAE) running buffer was performed to analyze the cDNA and genomic DNA (gDNA) PCR products. The DNA was separated by electrophoresis at 120 V for 15 min, and the DNA marker used was Marker L (50–500 bp; Sango Biotech, Shanghai, China).

2.5 Flow cytometry analysis

K-562 cells were washed with phosphate-buffered saline (PBS) once and then stained with Annexin V-PE (BioLegend, California, USA) and 7-amino-actinomycin (7AAD; BD Pharmingen™, Santiago, USA). The pellets were resuspended in 0.3 mL PBS, and the percentage of apoptotic cells was assessed by flow cytometry (BD FACSCelesta, Santiago, USA). In this assay, apoptotic cells were $7AAD^+$ (dead) or $7AAD^-$ (alive) and Annexin V^+ (phosphatidylserine$^+$). The data were analyzed by FlowJo software (FlowJo, Ashland, USA).

2.6 Western blotting

The protein content was measured by western blot analysis. First, cells were lysed for 1 h at 4 °C in 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF) buffer, which was disrupted by centrifugation at 12 000*g* for 20 min at 4 °C. Equal amounts of the proteins (30–50 μg) were applied to sodium dodecyl sulfate (SDS) polyacrylamide gels for electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Merck, USA). After blocking with 5% (volume fraction) skimmed milk in Tris-buffered saline with Tween-20 (TBST) buffer for 1 h at room temperature, the membranes were incubated with antibodies overnight at 4 °C. Then, the membranes were treated with the required secondary antibodies for 1 h at room temperature. Enhanced chemiluminescence detection reagent (Millipore) was used for visual signal detection, and GAPDH was applied as a control for normalization. The primary antibodies used were as follows: CASP8 (1:1000, volume ratio, the same below; Bioss, Beijing, China, bsm-33190M), CASP3 (1:1000; Cell Signaling Technology, Boston, USA, #9662), CASP6 (1:1000; Cell Signaling Technology, #9762), phospho Fas-associated protein with death domain (FADD) (p-FADD, 1:1000; Cell Signaling Technology, #2781), and GAPDH (1:1000; Cell Signaling Technology, #8884). The experiments were repeated three times.

2.7 Immunofluorescence assay

K-562 cells were fixed with 4% (volume fraction) paraformaldehyde for 15 min and then permeabilized

with 0.5% (volume fraction) Triton X-100 (SolarBio, Beijing, China) for 20 min. After blocking with 3% (0.03 g/mL) goat serum for 30 min at room temperature, the cells were incubated with primary antibodies against CASP8 and CASP3 at 4 ℃ overnight. After the sections were washed and incubated with secondary antibody (Cell Signaling Technology) at 37 ℃ in the dark for 1 h, the cell nuclei were counterstained with 4,6-diamidinos-2-phenylindole (DAPI; SolarBio).

2.8 JC-1 fluorescence measurement

The mitochondrial membrane potential was detected with a JC-1 fluorescent probe (MedchemExpress, New Jersey, USA). K-562 cells were incubated with JC-1 solution for 30 min at 37 ℃. After the cells were washed with PBS buffer, images were taken immediately using a single-photon confocal microscope (Nikon, Tokyo, Japan). The decline of mitochondrial membrane potential is a landmark event of the early stage of apoptosis. When the mitochondrial membrane potential is high, JC-1 aggregates in the matrix form a polymer, which produces red fluorescence. On the contrary, when the mitochondrial membrane potential is low, JC-1 exists as a monomer and emits green fluorescence. Therefore, the ratio of red to green fluorescence can indicate early apoptosis (Perelman et al., 2012; de Biasi et al., 2015; Marcondes et al., 2019).

2.9 Detection of CASP8 protein activity

According to the instructions of the CASP8 protein activity assay kit (SolarBio), the cell lysis supernatants from different groups were collected, and detection reagents were added in the recommended amounts. A blank tube and a standard tube were used as the negative control (NC) and positive control, respectively. After the reaction solution was mixed, the 96-well plate was closed with a sealing membrane and incubated at 37 ℃ for 60–120 min. When the color change was obvious, the absorption value was measured at the 405-nm wavelength. The concentration of free *p*-nitroaniline (pNA) in each sample was calculated according to the standard equation and the measured wavelength values. pNA is a free fragment produced by the specific hydrolysis of the CASP8 polypeptide substrate; thus, the hydrolytic activity of CASP8 can be measured by the absorption photometric value of pNA.

2.10 Luciferase reporter assay

The potential binding sites of miR-513a-5p and circRNA.0007127 or *CASP8* were predicted by TargetScan (https://www.targetscan.org). Then, the sequences were mutated and cloned into a dual-luciferase (Renilla luciferase (Rluc) and firefly luciferase (Fluc)) vector, psiCHECK-2 (Geneseed, Guangzhou, China), and the products were termed as circRNA.0007127-wild type (WT), circRNA.0007127-mutant (MUT), CASP8-WT, and CASP8-MUT. All of these plasmids were co-transfected with the miR-513a-5p mimic or inhibitor into 293T cells. The luciferase values were normalized to the corresponding Rluc values, and the fold changes were calculated.

2.11 Quantification and statistical analysis

Each experiment was repeated at least three times to determine the biological significance. The data were shown as mean±standard error of the mean (SEM), and statistical significance was confirmed by a *t*-test analysis of the two groups of data. Differences were considered statistically significant when $P<0.05$. GraphPad Prism software (Version 8; GraphPad Software Inc., CA, USA) was used for statistical analysis.

3 Results

3.1 Pathway prediction and identification of circRNA.0007127

The miRanda (https://www.miranda.org), TargetScan, and RNAhybrid database (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) were employed to predict the target miRNAs of circRNA.0007127. The bioinformatics analysis showed that 26 miRNAs had binding sites for circRNA.0007127, including miR-513a-5p (Fig. 1a). Meanwhile, miRNAs with binding sites for apoptotic genes were screened, and the regulatory pathway of circRNA.0007127/miR-513a-5p/*CASP8* was identified (Fig. 1b). The circRNA.0007127 is spliced from the *CNOT2* gene on chr12:70 671 911–70 704 797 (Fig. 1c and Table S2). Therefore, a set of specific divergent primers for circRNA.0007127 was designed, and the back-splice junction of circRNA.0007127 was confirmed by Sanger sequencing in the cDNA of K-562 cells (Fig. 1d). To determine whether the head-to-tail splicing of circRNA.0007127 was generated by trans-splicing

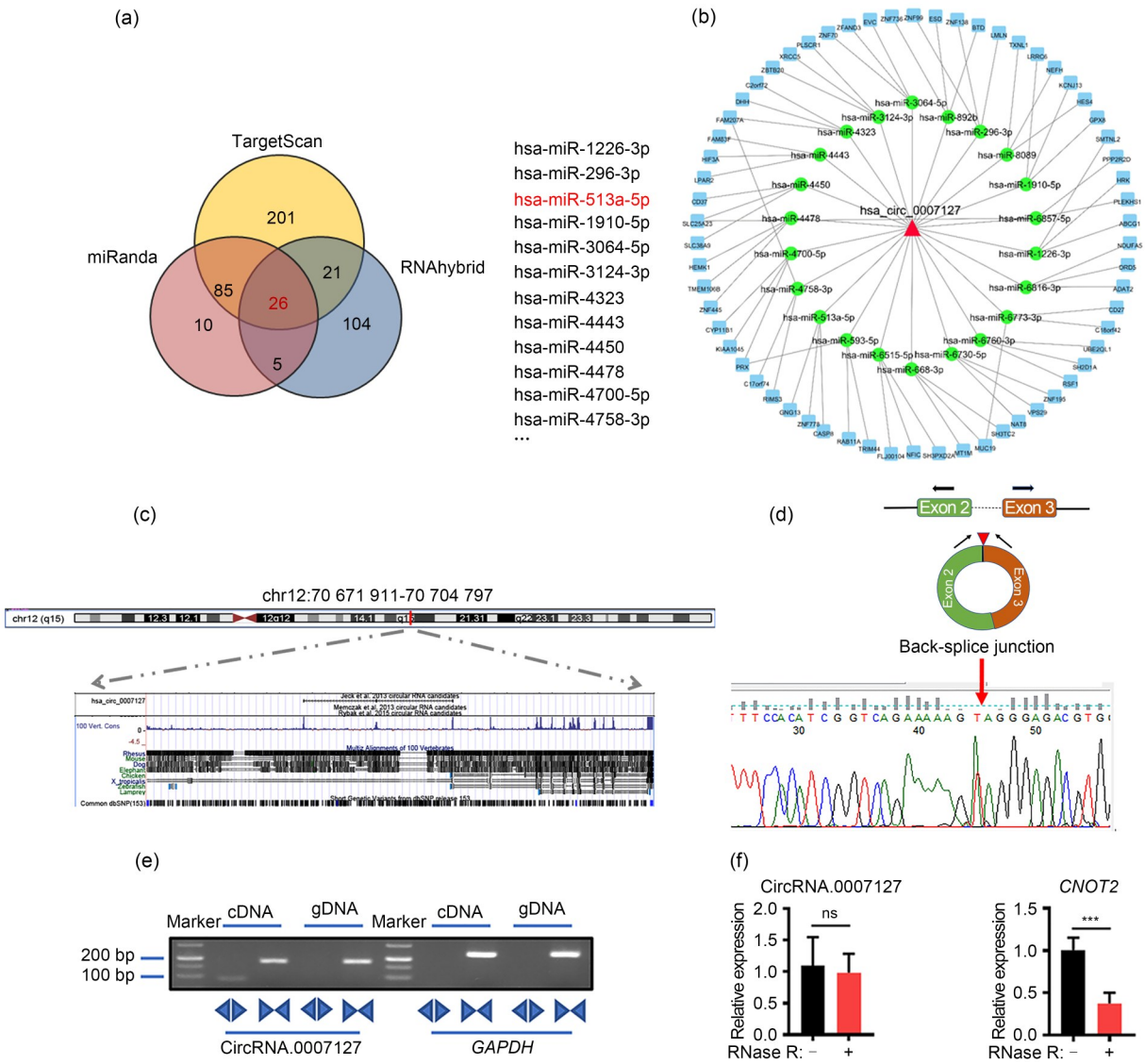

Fig. 1 Pathway prediction and identification of circRNA.0007127. (a) Bioinformatics analysis of the potential target miRNAs of circRNA.0007127 by miRanda, TargetScan, and RNAhybrid. (b) Network interaction between circRNA.0007127 and target miRNAs, as well as the downstream mRNAs of these miRNAs. (c) CircRNA.0007127 is formed from the human *CNOT2* gene through back-splicing. (d) Schematic diagram of the circularization of exon 2 and exon 3 of *CNOT2*-forming circRNA.0007127 (the arrowhead shows the junction), where the back-splice junction site (arrow) of circRNA.0007127 was detected by Sanger sequencing. (e) The divergent primers detected circRNA.0007127 in cDNA but not in gDNA. (f) The expression of circRNA.0007127 and *CNOT2* mRNAs treated or not treated with RNase R was determined by qPCR. The circRNA.0007127 was resistant to RNase R. Data were shown as mean±standard error of the mean (SEM), n=3. *** P< 0.001. CircRNA: circular RNA; miRNA: microRNA; mRNA: messenger RNA; *CNOT2*: carbon catabolite repression 4-negative on TATA-less (*CCR4-NOT*) complex subunit 2; cDNA: complementary DNA; gDNA: genomic DNA; RNase: ribonuclease; qPCR: quantitative real-time polymerase chain reaction; miR: microRNA; chr: chromosome; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; ns: not significant.

or genome rearrangement, the convergent primers were designed to amplify the *CNOT2* mRNA. The nucleic acid electrophoresis results showed that circRNA.0007127 was amplified from the cDNA only by the divergent primers (Fig. 1e). Furthermore, we confirmed the stable expression of circRNA.0007127 in K-562 cells using specific primers. CircRNA.0007127 was resistant to RNase R treatment due to its covalently closed loop structure, and *CNOT2* mRNA was significantly degraded by RNase R (Fig. 1f). These

findings indicated that circRNA.0007127 plays a special role in regulating the apoptosis pathway and can be amplified by specific divergent primers.

3.2 Inhibition of apoptosis in K-562 cells by knockdown of circRNA.0007127

K-562 cells were selected to explore the biological functions of circRNA.0007127. Next, siRNAs specific to circRNA.0007127 were used to knock down its expression (Fig. 2a). As shown in Fig. 2b, the knockdown efficiency of three independent siRNAs was measured by qPCR; siRNA-1 showed high inhibitory efficiency, and it successfully knocked down the expression of circRNA.0007127 but did not affect *CNOT2* mRNA expression (Fig. 2c). Furthermore, we constructed an H_2O_2-induced apoptosis model involving K-562 cells. After treatment with H_2O_2, the mRNA expression levels of *CASP8*, Bcl-2-associated X (*Bax*), *FADD*, *CASP3*, Bcl-2-associated agonist of cell death (*Bad*), and *CASP6* increased in K-562 cells (Fig. 2d). All of these genes are involved in the regulation network of apoptosis, and their expression is upregulated after proapoptotic stimulation. When circRNA.0007127 was silenced by siRNA-1, the percentage of apoptotic K-562 cells decreased significantly compared with those in both apoptotic K-562 cells and siRNA control-transfected cells (Fig. 2e). Apoptotic cells were $7AAD^+$ (dead) or $7AAD^-$ (alive) and Annexin V^+ (phosphatidylserine$^+$). SiRNA-1 attenuated the decrease in the mitochondrial membrane potential, which indicates early cell apoptosis (Figs. 2f and 2g). These findings suggested that siRNA-1 specific to circRNA.0007127 decreases the apoptosis rate of K-562 cells, providing a new insight into the biological functions of circRNA.0007127.

3.3 Effects of circRNA.0007127 on apoptosis by affecting the expression of *CASP8*

In order to further explore the mechanism of apoptosis regulation by circRNA.0007127, the expression of apoptosis-related genes was verified by qPCR in apoptotic K-562 cells after siRNA-1 transfection. The knockdown of circRNA.0007127 suppressed the mRNA expression of *CASP8*, *CASP3*, *CASP6*, *FADD*, and *Bax* (Fig. 3a). However, knocking down circRNA.0007127 did not affect the mRNA level of *Bad*, suggesting that circRNA.0007127 might be mainly involved in regulating caspase family genes that encode the central regulatory molecules of apoptosis. Meanwhile, H_2O_2 treatment significantly increased the protein levels of CASP8, CASP3, CASP6, and p-FADD in K-562 cells, and these changes could be partly reversed by circRNA.0007127 knockdown (Figs. 3b and 3c). The protein bands showed that CASP8, CASP3, and p-FADD were the cleaved fragments of the protein, while CASP6 was the full-length protein. When siRNA-1 or siRNA NC was transfected into H_2O_2-treated K-562 cells, the immunofluorescence staining results indicated that the numbers of positive cells for CASP8 and CASP3 were significantly decreased in the siRNA-1 group compared to the NC group (Figs. 3d and 3e), suggesting that siRNA-1 inhibited the expression of the CASP8, which further suppressed the splicing and activation of the downstream effector CASP3, thereby decreasing H_2O_2-induced apoptosis. Next, K-562 cells were stimulated with H_2O_2 and then transfected with siRNA NC or siRNA-1. After 48 h of culture, cells were collected to detect the hydrolytic activity of CASP8. The absorbance value analysis indicated that siRNA-1 decreased the CASP8 hydrolytic activity induced by H_2O_2 in K-562 cells (Fig. 3f). In general, under the stimulation of pro-apoptotic signals in K-562 cells, the knockdown of circRNA.0007127 could protect cells from apoptosis by inhibiting the expression of CASP8 and its downstream effector caspases.

3.4 CircRNA.0007127-targeted hsa-miR-513a-5p

Next, the target genes of the predicted miRNAs were screened by employing online databases, and the results revealed that there were binding sites for CASP8 and circRNA.0007127 in the seed region of hsa-miR-513a-5p (Fig. 4a). Then, the dual luciferase reporting system was employed to verify whether circRNA.0007127 could adsorb miR-513a-5p. It was postulated that, if miR-513a-5p can bind to circRNA.0007127, the expression of the luciferase gene will be downregulated, and the expression level of luciferase will be inversely proportional to the bond strength. Therefore, we constructed a luciferase reporter plasmid containing the full length of WT circRNA.0007127. Subsequently, we mutated the predicted miR-513a-5p binding site that could not bind to circRNA.0007127, and constructed MUT circRNA.0007127 luciferase reporter plasmid vectors (Fig. 4b). These vectors were cotransfected with the

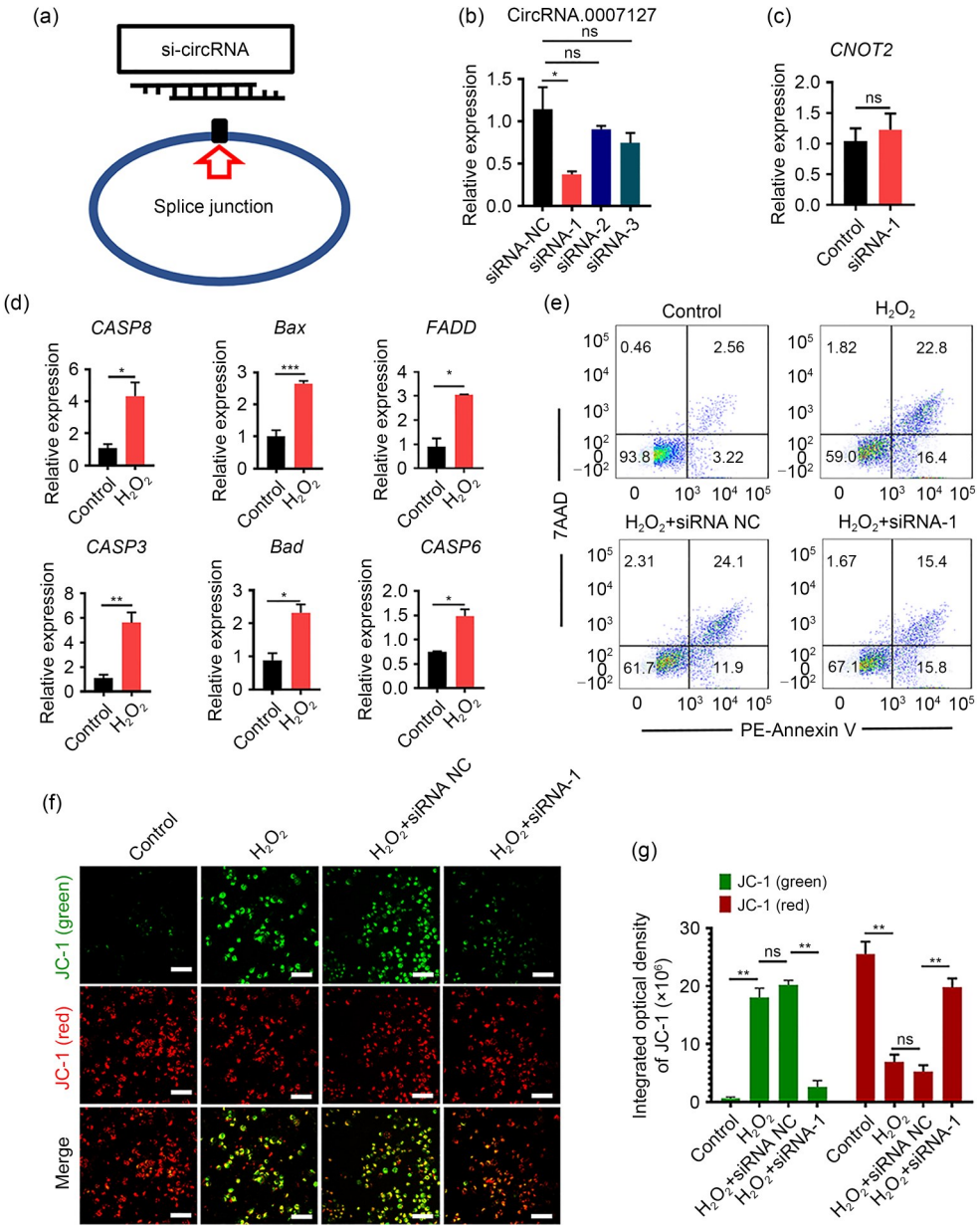

Fig. 2 Suppression of apoptosis in K-562 cells by knockdown of circRNA.0007127. (a) Schematic illustration of siRNA and target sites on circRNA. (b) Expression analyses of three different circRNA-targeting siRNAs in K-562 cells, showing that siRNA-1 had a high knockdown efficiency. (c) The expression of *CNOT2* after siRNA transfection was detected by RT-qPCR. (d) Quantification of *CASP8*, *Bax*, *FADD*, *CASP3*, *Bad*, and *CASP6* in K-562 cells treated with 1.76 mmol/L H_2O_2. (e) Flow cytometry assay showing the exposure of phosphatidylserine on the cell membrane. (f, g) H_2O_2-induced K-562 cells were transfected with siRNA-1, followed by JC-1 fluorescent mitochondrial imaging. The transformation of JC-1 from red fluorescence to green fluorescence was used as an indicator of early apoptosis (scale bar=40 μm). Data were shown as mean±standard error of the mean (SEM), n=3. * P<0.05, ** P<0.01, *** P<0.001. CircRNA: circular RNA; siRNA: small-interfering RNA; *CNOT2*: carbon catabolite repression 4-negative on TATA-less (*CCR4-NOT*) complex subunit 2; RT-qPCR: reverse transcription-quantitative real-time polymerase chain reaction; *CASP8*: caspase-8; *Bax*: Bcl-2-associated X; *FADD*: Fas-associated protein with death domain; *Bad*: Bcl-2-associated agonist of cell death; NC: negative control; ns: not significant; 7AAD: 7-amino-actinomycin.

miR-513a-5p mimic or inhibitor into HEK-293T cells. The luciferase reporter assay results suggested that miR-513a-5p significantly inhibited the luciferase activity of the luciferase reporter constructed in the circRNA.0007127 WT group, but not in the control groups (Fig. 4c). These results confirmed

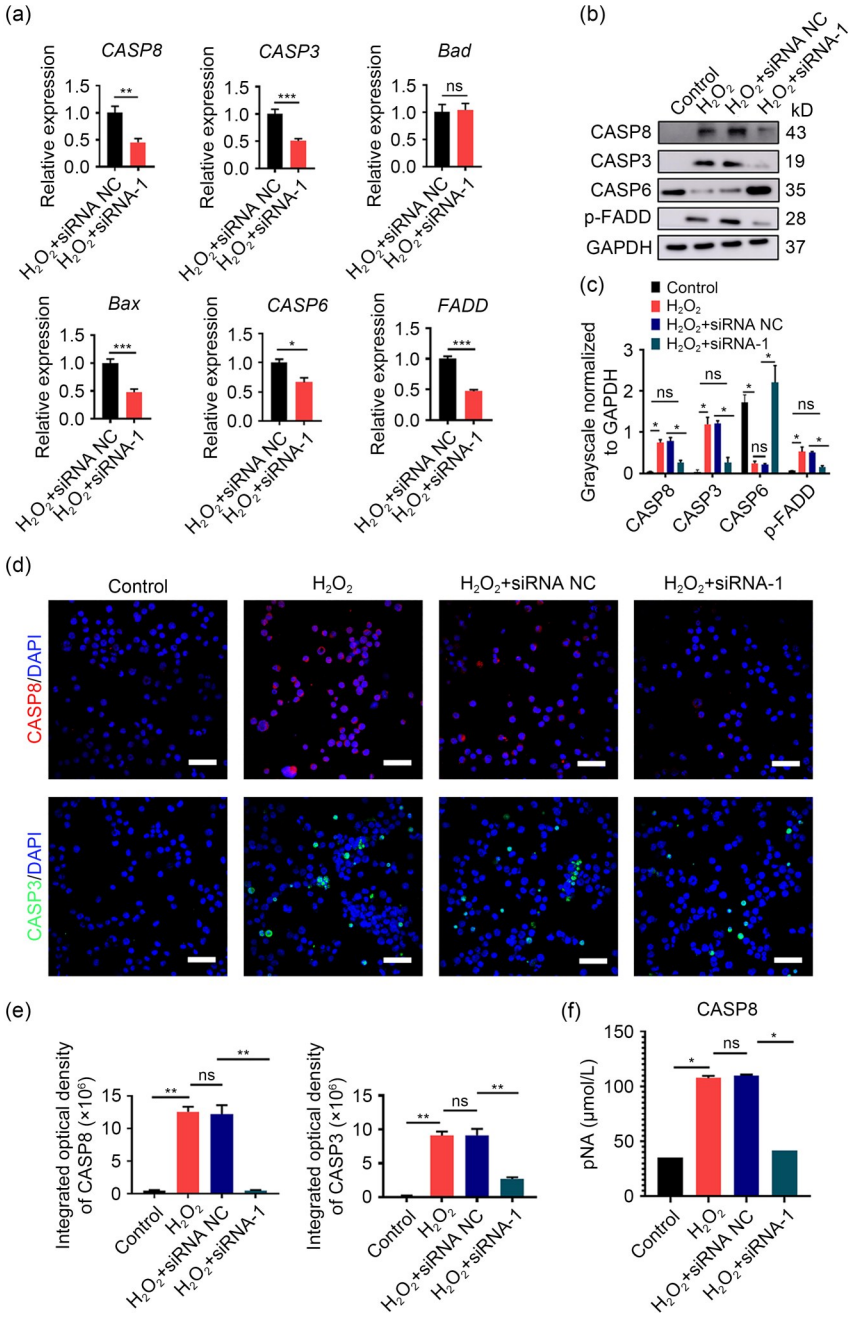

Fig. 3 Effects of circRNA.0007127 on apoptosis by suppressing the expression of CASP8. (a) The mRNA expression levels of *CASP8*, *CASP3*, *Bad*, *Bax*, *CASP6*, and *FADD* after knockdown by circRNA.0007127 were determined by qPCR. (b) Representative western blot images showing the protein expression of CASP8, CASP3, p-FADD, and CASP6 in K-562 cells treated with H_2O_2 and siRNA NC or siRNA-1 for 48 h. GAPDH was used as a loading control. (c) The histogram analysis of the western blot in (b) was performed. (d) Immunofluorescence photomicrographs of CASP8 and CASP3 in circRNA.0007127 siRNA-1 (or siRNA NC)-transfected K-562 cells followed by treatment with H_2O_2. Red, green, and blue represent CASP8, CASP3, and nuclei, respectively (scale bar=40 μm). (e) The integrated optical density of CASP8 or CASP3 in (d) was quantified. (f) The hydrolytic activity of CASP8 in K-562 cells with different treatments was measured by absorption value analysis. Data were shown as mean±standard error of the mean (SEM), n=3. * P<0.05, ** P<0.01, *** P<0.001. CircRNA: circular RNA; qPCR: quantitative real-time polymerase chain reaction; *CASP8*: caspase-8; *Bax*: Bcl-2-associated X; *FADD*: Fas-associated protein with death domain; *Bad*: Bcl-2-associated agonist of cell death; siRNA: small-interfering RNA; NC: negative control; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; p-FADD: phospho-FADD; ns: not significant; DAPI: 4,6-diamidinos-2-phenylindole; pNA: *p*-nitroaniline.

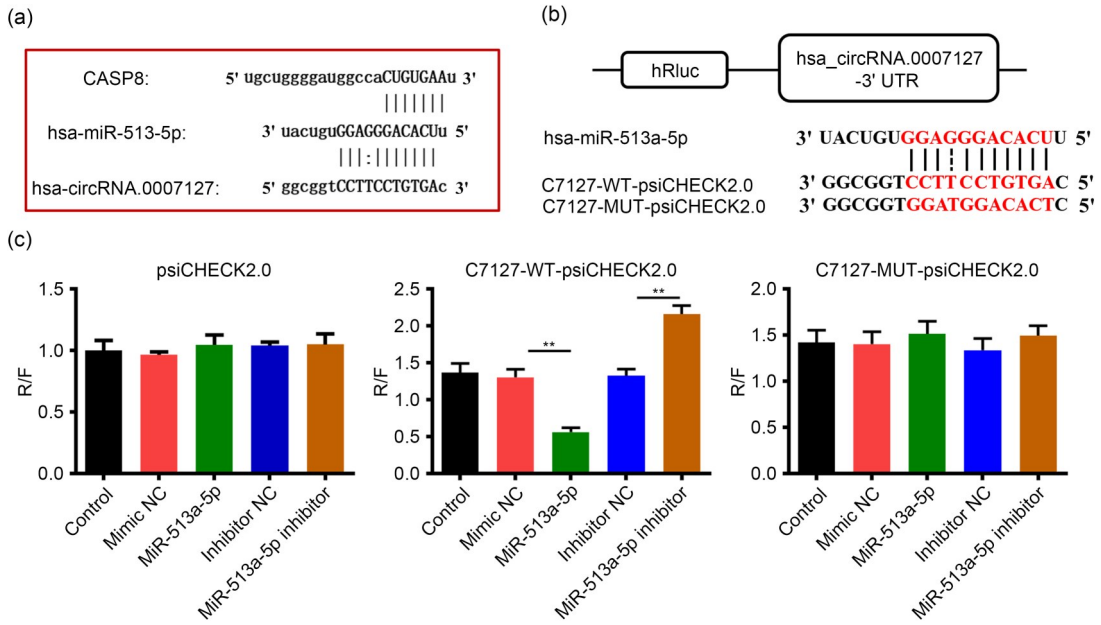

Fig. 4 CircRNA.0007127-targeted hsa-miR-513a-5p. (a) The seed region of miR-513a-5p, the binding sites of circRNA.0007127 with miR-513a-5p, and those of CASP8 with miR-513a-5p. (b) Schematic diagram of the hsa-miR-513a-5p binding sequences of C7127-WT-psiCHECK2.0 and C7127-MUT-psiCHECK2.0. Here, C7127-WT-psiCHECK2.0 represents the WT vector, and C7127-MUT-psiCHECK2.0 represents the MUT vector. (c) MiR-513a-5p mimic or NC as well as miR-513a-5p inhibitor or NC was cotransfected into HEK-293T cells with luciferase reporter containing C7127-WT-psiCHECK2.0 or C7127-MUT-psiCHECK2.0. The luciferase activity of each group was analyzed. Here, psiCHECK2.0 represents the blank vector. Data were shown as mean±standard error of the mean (SEM), n=3. ** P<0.01. CircRNA: circular RNA; miRNA: microRNA; Mimic NC: miRNA mimic negative control; Inhibitor NC: miRNA inhibitor negative control; *CASP8*: caspase-8; WT: wild type; MUT: mutant; NC: negative control; UTR: untranslated region; R/F: relative activity of Renilla luciferase/firefly luciferase.

that circRNA.0007127 could efficiently bind to miR-513a-5p.

3.5 Effects of miR-513a-5p on apoptosis by binding to the 3' UTR region of *CASP8*

In order to further confirm that the cellular phenotype was caused by the binding of *CASP8* with miR-513a-5p, luciferase vectors were constructed (Fig. S1). The bioinformatics analysis showed that the *CASP8* 3' untranslated region (UTR) contains a sequence that is complementary to the miR-513a-5p seed sequence. Luciferase activity assay was conducted to investigate the targeting relationship between miR-513a-5p and *CASP8*. Compared with the mutant constructs, the miR-513a-5p mimic significantly downregulated the luciferase activity of the *CASP8*-WT constructs (Fig. 5a). MiR-513a-5p mimic or inhibitor was transfected into K-562 cells to explore its biological function. First, the effects of miR-513a-5p on *CASP8* expression in apoptotic K-562 cells transfected with mimic or inhibitor were analyzed by qPCR (Fig. 5b). Overexpressed miR-513a-5p downregulated the expression of *CASP8*, while the inhibitor promoted the expression of *CASP8*. The CASP8 activity was also measured after treatment with the miR-513a-5p mimic. As shown in Fig. 5c, in H_2O_2-stimulated K-562 cells, when the expression of miR-513a-5p was upregulated, the hydrolysis activity of CASP8 was reduced. The flow cytometry results showed substantially increased expression of miR-513a-5p and fewer apoptotic cells, while apoptosis was elevated by the miR-513a-5p inhibitor (Fig. 5d). Moreover, early apoptosis was observed using the JC-1 probe (Fig. S2). The proportion of early apoptotic cells in the mimic group decreased, and the proportion of apoptotic cells in the inhibitor group increased compared with those in the control group. Subsequently, the protein levels of CASP8 and CASP3 were determined by western blotting. The results revealed that CASP8 and CASP3 were concurrently decreased by the mimic and increased by the inhibitor (Fig. 5e). Immunofluorescent staining assays showed that the expression level of miR-513a-5p was negatively associated with the protein levels of CASP8 and CASP3 (Figs. 5f and 5g). Thus, these results

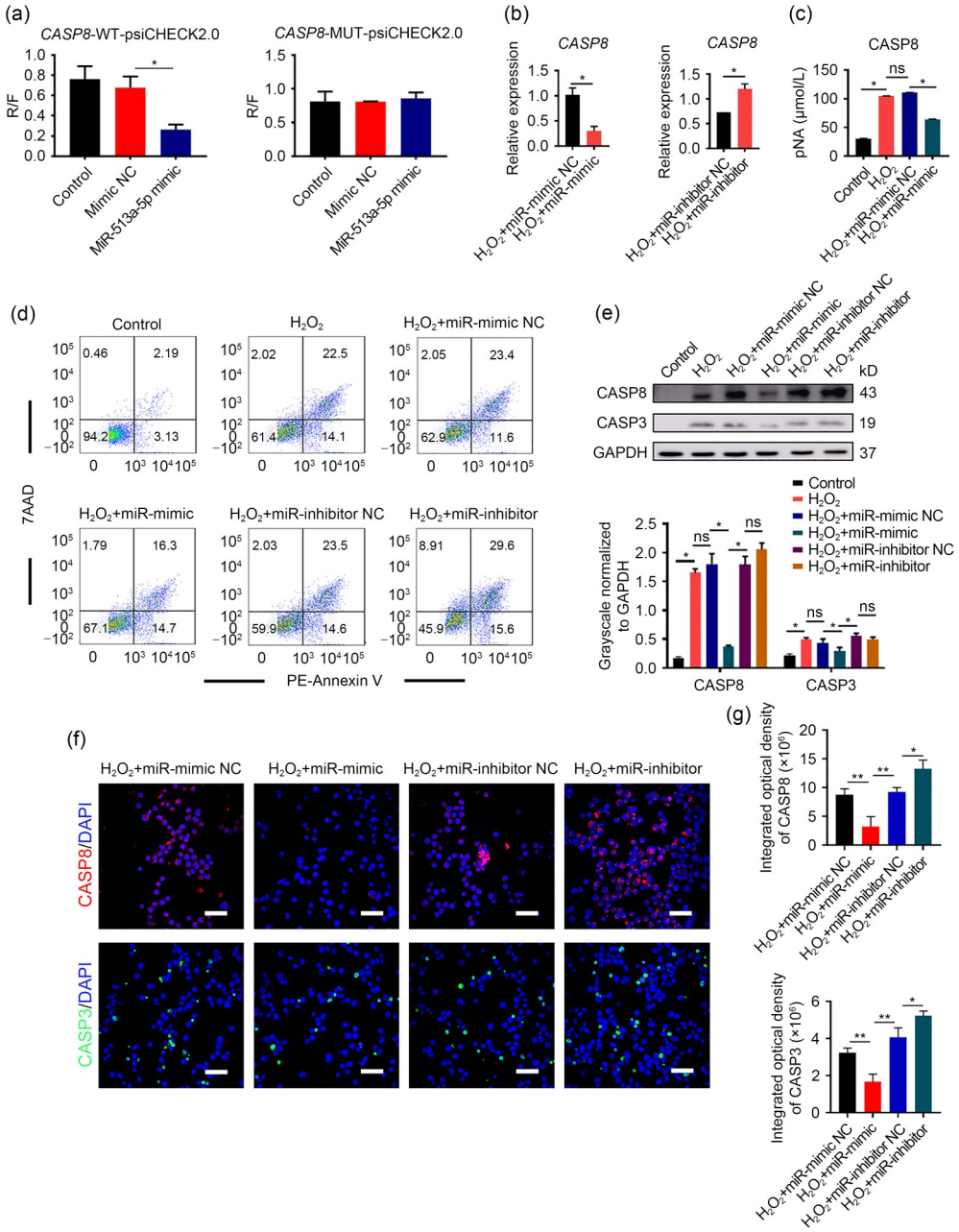

Fig. 5 Effects of miR-513a-5p on apoptosis by binding to the 3' UTR region of *CASP8*. (a) Luciferase reporter assay was performed after HEK-293T cells were cotransfected with CASP8-WT-psiCHECK2.0 or CASP8-MUT-psiCHECK2.0 and miR-513a-5p mimic or mimic NC. (b) The effects of miR-513a-5p on *CASP8* expression in H_2O_2-induced K-562 cells transfected with mimic or inhibitor were analyzed by qPCR. (c) The absorbance values of K-562 cells in different treatment groups were detected by enzyme plate analyzer, indicating the hydrolytic activity of CASP8. (d) Flow cytometry experiments indicated that the miR-513a-5p mimic reduced the apoptosis of K-562 cells, while its inhibitor further promoted apoptosis. (e) The protein levels of CASP8 and CASP3 were determined by western blotting in apoptotic K-562 cells with knockdown or overexpression of miR-513a-5p (top panel). Representative corresponding densitometry analyses of protein expression levels were performed by western blotting (bottom panel). (f, g) CLSM images showing the expression of CASP8 and CASP3. Red, green, and blue represent CASP8, CASP3, and nuclei, respectively (scale bar=40 μm). Data were shown as mean±standard error of the mean (SEM), n=3. * P<0.05, ** P<0.01. R/F: relative activity of Renilla luciferase/firefly luciferase; CASP8: caspase-8; miR: microRNA; WT: wild type; MUT: mutant; qPCR: quantitative real-time polymerase chain reaction; CLSM: confocal laser scanning microscopy; ns: not significant; 7AAD: 7-amino-actinomycin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DAPI: 4,6-diamidinos-2-phenylindole; Mimic NC: miRNA mimic negative control; Inhibitor NC: miRNA inhibitor negative control; UTR: untranslated region; NC: negative control.

demonstrated that miR-513a-5p could significantly inhibit H_2O_2-induced apoptosis by targeting CASP8.

3.6 Effects of circRNA.0007127 on apoptosis in H_2O_2-treated K-562 cells via the miR-513a-5p/*CASP8* axis

We explored whether circRNA.0007127 promoted apoptosis through the miR-513a-5p/*CASP8* axis. siRNA-1, an interfering RNA specifically targeting circRNA.0007127, and a miR-513a-5p inhibitor were co-transfected into K-562 cells after H_2O_2-induced apoptosis. Flow cytometry revealed that circRNA.0007127 knockdown reduced the apoptosis rate of H_2O_2-treated K-562 cells, while the miR-513a-5p inhibitor reversed this effect (Fig. 6a). The above results implied that siRNA-1 could inhibit the gene expression of *CASP8* and *CASP3* in apoptotic K-562 cells. However, the miR-513a-5p inhibitor antagonized the effect of siRNA-1 (Fig. 6b). Similarly, JC-1 fluorescence probe detection revealed that the miR-513a-5p inhibitor antagonized the effect of siRNA-1 on mitochondrial membrane potential loss, thus promoting apoptosis (Fig. S3). Then, a rescue experiment was performed to detect the changes in the activity of CASP8 after siRNA-1 and the inhibitor were co-transfected into apoptosis-stimulating K-562 cells (Fig. 6c). The hydrolytic activity of CASP8 was blocked by siRNA-1 but promoted by the miR-513a-5p inhibitor, indicating that the latter increased the activation of the CASP8 zymogen. After the transfection of H_2O_2-induced K-562 cells with siRNA-1 or siRNA-1 together with the miR-513a-5p inhibitor, the expression of the CASP8 protein in the siRNA-1 group was decreased, while that in the rescue group was increased (Fig. 6d). Furthermore, the immunofluorescence results showed that the miR-513a-5p inhibitor restored the expression of CASP8 and CASP3, which was suppressed by the knockdown of circRNA.0007127 in apoptotic K-562 cells (Figs. 6e and 6f). Taken together, these findings revealed that circRNA.0007127 acts as a miR-513a-5p sponge to regulate apoptosis in cells by affecting *CASP8* expression (Fig. 6g).

4 Discussion

Non-coding RNAs (ncRNAs) play important regulatory roles in the functional activities of normal or abnormal cells, and can be used as molecular therapeutic targets of diseases (Slack and Chinnaiyan, 2019). New evidence suggests that miRNAs, long ncRNAs (lncRNAs), and circRNAs are involved in the occurrence and development of chronic myeloid leukemia, treatment resistance, and treatment prognosis (Litwińska and Machaliński, 2017; Benetatos et al., 2020; de Acha et al., 2020; Wen et al., 2020; Yu and Li, 2020). In our previous circRNA sequencing study of red blood cells (accession to cite for these sequence read archive (SRA) data: PRJNA698384), we found a new highly expressed circRNA numbered 0007127, which arises from the host gene *CNOT2*; however, the functional role of circRNA.0007127 has not been reported. Bioinformatics analysis revealed that circRNA.0007127 might act as a molecular regulator of apoptosis, and the apoptosis K-562 cell model through H_2O_2-induced oxidative stress was established to assess the biological significance of circRNA.0007127 in the process of cell apoptosis caused by oxidative damage.

We used interfering RNA to silence the expression of circRNA.0007127 in the K-562 cell apoptosis model, and the expression of the host gene *CNOT2* was unaffected. In the K-562 cell model, the knockdown of circRNA.0007127 reduced phosphatidylserine exposure on the cell membrane and increased the mitochondrial membrane potential while decreasing the expression level of oxidative stress-related caspase genes. Moreover, circRNA.0007127, as a function of competitive endogenous RNA (ceRNA), competitively binds to miR-513a-5p and then eliminates the endogenous inhibitory effect of miR-513a-5p targeting the *CASP8* gene. The reduction in CASP8 inhibits the expression of CASP3 and the activation of mitochondria. This finding revealed that circRNA.0007127 regulates the apoptosis of K-562 cells via a ceRNA mechanism.

The expression of apoptosis-related genes is downregulated in most hematological malignancies. Therapeutic agents targeting the apoptotic mechanisms of hematologic tumor cells can specifically inhibit antiapoptotic molecules that are overexpressed in tumor cells, such as Bcl-2-associated proteins and inhibitors of apoptosis proteins (Droin et al., 2013). Studies have shown that a variety of drugs or genes regulate the apoptosis of chronic myeloid leukemia cells through the caspase apoptosis signaling pathway, inhibiting the proliferation of tumor cells and inducing their death (Jung et al., 2019; Song et al., 2020). The

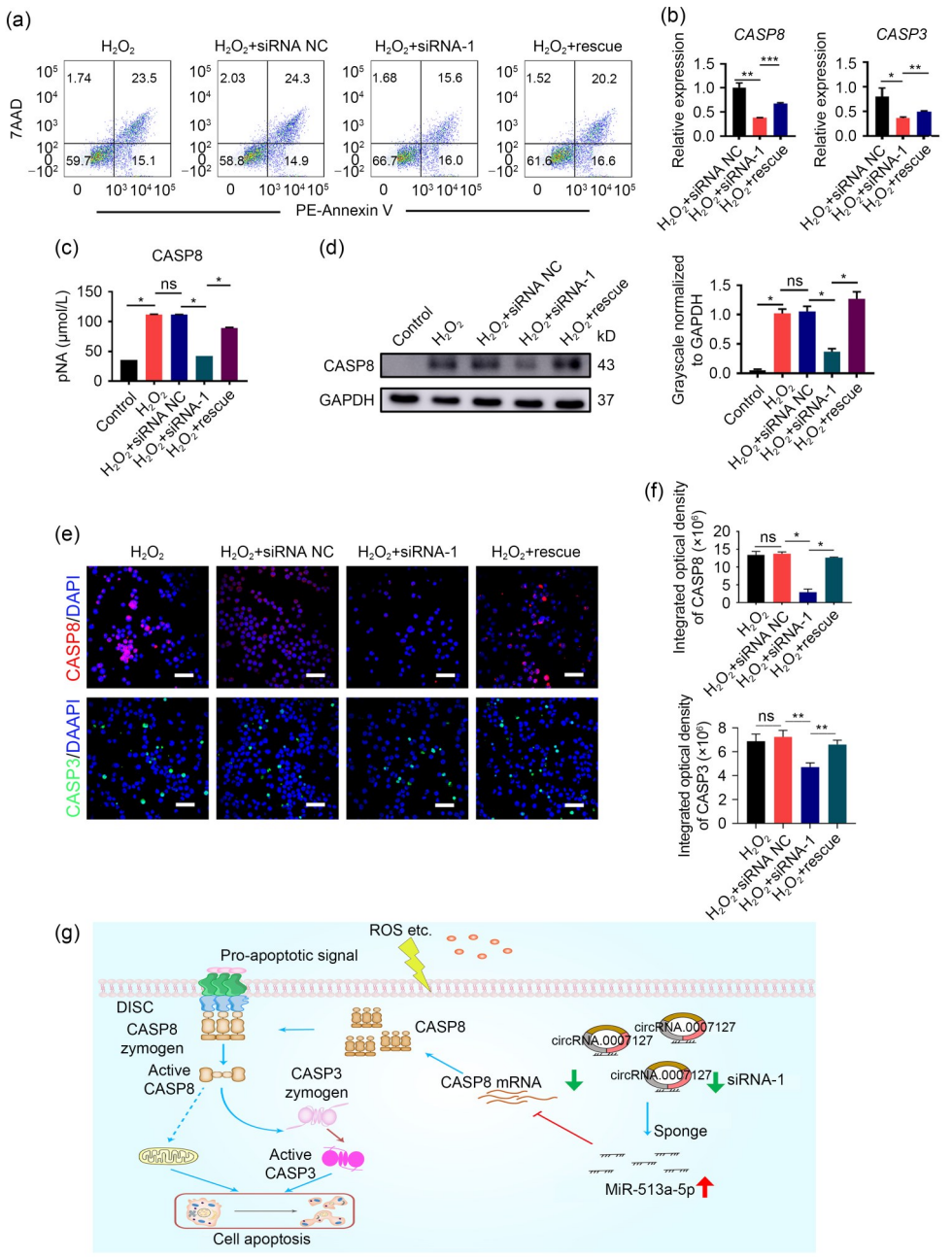

Fig. 6 Effects of circRNA.0007127 on apoptosis in H_2O_2-treated K-562 cells via the miR-513a-5p/*CASP8* axis. (a) The cell apoptosis in each treatment group was measured by flow cytometry. (b) RT-qPCR showed that the miR-513a-5p inhibitor antagonized the effects of siRNA-1 on *CASP8* and *CASP3* in apoptotic K-562 cells. (c) siRNA NC, siRNA-1, or siRNA-1 together with miR-513a-5p inhibitor was transfected into apoptotic K-562 cells. Then, the absorbance of each treatment group was detected. (d) Western blotting was performed to measure the protein expression of CASP8 in H_2O_2-induced K-562 cells transfected with siRNA NC, siRNA-1, or siRNA-1 with the miR-513a-5p inhibitor (left panel). Representative corresponding densitometry analyses of protein expressions were performed by western blotting (right panel). (e) CLSM images of K-562 cells labeled with CASP8 or CASP3 in each group. The proportion of CASP3-positive cells increased after transfection with the miR-513a-5p inhibitor. Red, green, and blue represent CASP8, CASP3, and nuclei, respectively (scale bar=40 μm). (f) The integrated optical density of CASP8 or CASP3 in (e) was quantified. (g) The hypothesis diagram illustrates the function and mechanism of circRNA.0007127 in the miR-513a-5p/*CASP8* axis apoptosis pathway. Data were shown as mean±standard error of the mean (SEM), *n*=3. * *P*<0.05, ** *P*<0.01, * *P*<0.001. CircRNA: circular RNA; siRNA: small-interfering RNA; miR: microRNA; CASP8: caspase-8; RT-qPCR: reverse transcription-quantitative real-time polymerase chain reaction; NC: negative control; CLSM: confocal laser scanning microscopy; 7AAD: 7-amino-actinomycin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; ns: not significant; mRNA: messenger RNA; DISC: death-inducing signaling complex; pNA: *p*-nitroaniline.**

SD-HA fusion protein was found to activate apoptotic signals by binding to the CASP8 domain and induced K-562 cell apoptosis (Huang et al., 2018). Moreover, the inosine phosphate dehydrogenase inhibitor FF-10501 activates CASP8 or induces endoplasmic reticulum stress in K-562 cells by producing ROS to trigger K-562 cell apoptosis and necrosis (Matsumoto et al., 2019).

CASP8 is the key initiating protein of the exogenous activation pathway of apoptosis. Activated CASP8 causes a cascade of caspase proteolysis and further activates proteins such as CASP6 and CASP3 (Keller et al., 2018). The activation of CASP8 further initiates intracellular pathways, induces mitochondria to release cytochrome *c*, and effectively amplifies the apoptotic signal (Tummers and Green, 2017). We found that decreased circRNA.0007127 expression could inhibit the activation of CASP8. In addition, luciferase activity assays revealed that miR-513a-5p has a regulatory pattern opposite to that of the upstream molecule circRNA.0007127 and the downstream molecule *CASP8*, suggesting that circRNA.0007127 may act as a ceRNA to regulate *CASP8* in apoptotic K-562 cells.

It has been reported that miR-513a-5p stimulates apoptosis by controlling the DNA damage response induced by apurinic/apyrimidinic (AP) endonuclease 1 (APE1) irradiation in patients with osteosarcoma (Dai et al., 2018). LINC01436 can significantly reduce the expression of miR-513a-5p through the action of ceRNA, thus promoting the proliferation, metastasis, and radiation tolerance of gastric cancer cells (Lu et al., 2020). In addition, miR-513a-5p has carcinogenic potential and can be used as a long-term biomarker of breast cancer risk (Muti et al., 2018). In our experiment, miR-513a-5p significantly reduced the luciferase activity of *CASP8* luciferase reporter gene and inhibited the H_2O_2-induced apoptosis of K-562 cells. Although this is contrary to the effect of miR-513a-5p on apoptosis stimulation reported above, it fully conforms to the regulatory characteristics of miRNA, and thus the same miRNA can regulate multiple different genes.

Overall, circRNA.0007127 silencing was shown to reduce phosphatidylserine exposure on the cell membrane and reduce the mitochondrial membrane potential, inhibiting the apoptosis of K-562 cells. Mechanistic studies revealed that circRNA.0007127 enhances the expression of *CASP8* through competitive binding of miR-513a-5p. However, the application value of circRNA.0007127 in clinical diagnostics and treatment still needs further investigation.

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Author contributions

Xiajing LI designed and conducted experiments, obtained and analyzed data, and was a major contributor in writing the manuscript. Yiyu ZHANG provided sequencing data and experimental design. Ning WANG conducted experiments and analyzed the data. Zhaohu YUAN provided information support, and analyzed and interpreted the data. Xiaojie CHEN collected and processed experimental samples. Qicong CHEN conducted experiments. Hui DENG and Xinxin TONG collected experimental samples and performed statistical analysis. Honglin CHEN participated in paper revision and performed statistical analysis. Yuyou DUAN provided experimental design, performed statistical analysis, analyzed and interpreted the data, and participated in paper revision. Yaming WEI provided experimental design and financial support, conceptualized the outline and topic of the article, and participated in paper revision. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Xiajing LI, Yiyu ZHANG, Ning WANG, Zhaohu YUAN, Xiaojie CHEN, Qicong CHEN, Hui DENG, Xinxin TONG, Honglin CHEN, Yuyou DUAN, and Yaming WEI declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Tables S1 and S2; Figs. S1–S3