Circ-SKA3 Enhances Doxorubicin Toxicity in AC16 Cells Through miR-1303/TLR4 Axis

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Summary

Doxorubicin (DOX) is a widely used anticancer drug, but its cardiotoxicity largely limits its clinical utilization. Circular RNA spindle and kinetochore-associated protein 3 (circ-SKA3) were found to be differentially expressed in heart failure patients. In this study, we investigated the role and mechanism of circ-SKA3 in DOX-induced cardiotoxicity.

The quantitative real-time polymerase chain reaction and western blot assays were applied to measure the expression of circ-SKA3, microRNA (miR)-1303, and toll-like receptor 4 (TLR4). The viability and apoptosis of AC16 cells were analyzed using cell counting kit-8, flow cytometry, and western blot assays. The interaction between miR-1303 and circ-SKA3 or TLR4 was verified using dual-luciferase reporter and RNA immunoprecipitation assays. Exosomes were collected from culture media by the use of commercial kits and then qualified by transmission electron microscopy.

The expression of circ-SKA3 and TLR4 was increased, whereas miR-1303 expression was decreased in DOX-treated AC16 cells. DOX treatment promoted cell apoptosis and inhibited cell viability in AC16 cells in vitro, which was partially reversed by circ-SKA3 knockdown, TLR4 silencing, or miR-1303 overexpression. Mechanistically, circ-SKA3 served as a sponge for miR-1303 to upregulate TLR4, which was confirmed to be a target of miR-1303. Additionally, circ-SKA3 contributed to DOX-induced cardiotoxicity through the miR-1303/TLR4 axis. Further studies suggested that circ-SKA3 was overexpressed in exosomes extracted from DOX-mediated AC16 cells, which could be internalized by surrounding untreated AC16 cells.

Circ-SKA3 enhanced DOX-induced toxicity in AC16 cells through the miR-1303/TLR4 axis. Extracellular circ-SKA3 was packaged into exosomes, and exosomal circ-SKA3 could function as a mediator in intercellular communication between AC16 cells.

Key words: Apoptosis, Variability, Cytotoxicity, Cardiomyocytes, Exosome, circRNA

Doxorubicin (DOX) is an anthracycline type of chemotherapy that is one of the most widely used anticancer drugs. It can be used to treat diverse types of cancers that affect the bladder, breast, ovaries, kidneys, lungs, thyroid, stomach, nerve tissues, bones, joints, and soft tissues; additionally, it has a marked therapeutic function on certain types of lymphomas and leukemia.1-3) Although extensive clinical utilization, the clinical benefit of DOX is largely limited by the toxic side effects on cardiotoxicity, which ultimately cause cardiomyopathy and congestive heart failure.4-6) Presently, it has been revealed that DOX can induce the death and apoptosis of cardiomyocytes, causing dangerous effects on the heart.6-7) Thus, further investigation on the mechanism underlying the death of cardiomyocytes may be an effective strategy for preventing DOX-induced cardiotoxicity.

CircRNAs are a new type of covalently closed, single-stranded transcripts that lack 3'- and 5'-ends and have a higher tolerance to degradation by RNase R exonuclease.8-9) CircRNAs are abundantly expressed in mammalian cells with high evolutionary conservation and stability across different species and have been revealed to participate in various physiological and pathological processes,9-11) rendering them ideal candidates for future clinical diagnosis, treatment, and prognosis. Presently, increasing studies have reported that circRNAs are related to the pathogenesis of cardiovascular diseases; differential expression of specific circRNAs was observed in the pathe-
logical condition and was involved in regulating cardiac development, heart function, cardiac hypertrophy, and heart failure.\textsuperscript{12-14} CircRNA spindle and kinetochore-associated protein 3 (SKA3) (circ-SKA3, ID: hsa_circ_0029696) is a circRNA derived from the SKA3 gene; it locates at chr13:21735928-21746820 with the genomic length of 10892 bp. A recent study showed that hsa_circ_0029696 was remarkably elevated in the blood of patients with heart failure.\textsuperscript{15} However, the researches on the function and mechanism of circ-SKA3 in DOX-stimulated cardiotoxicity are relatively rare.

Exosomes are nano-sized vesicles described as 30-150 nm in diameter, which are actively secreted from different cell types.\textsuperscript{16} They contain various bioactive molecules, such as proteins or nucleic acids (e.g., circRNAs), and can communicate a wide range of content to target cells in local environments or distant metastatic sites through docking and fusing to the cytomembrane.\textsuperscript{17-19} Inter-cellular communication mediated by exosomes has an important role in modulating the physiological status and pathological processes.\textsuperscript{20} Presently, circulating exosomes and exosomal cargos are increasingly being discovered to involve in cardiovascular pathophysiology.\textsuperscript{21,22} However, investigations on exosomal circRNA in DOX-stimulated cardiotoxicity are lacking.

This study aimed to explore the biological functions and the specific regulatory mechanism of circ-SKA3 in DOX-stimulated death of AC16 cells; additionally, we investigated whether circ-SKA3 exerted its effects by exosomes.

**Methods**

**Cell culture and treatment:** Human cardiomyocyte-like AC16 cells were purchased from Shanghai Institute of Cell Biology (Shanghai, China) and then grown in a humidified incubator of 5% CO\textsubscript{2} at 37°C with the Dulbecco’s Modified Eagle’s Medium (Invitrogen, Carlsbad, CA, USA) that contains 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen).

For in vitro assay, AC16 cells were exposed to different concentrations of doxorubicin (DOX, 2.5, 5, and 10 μM) (Sigma, St. Louis, MO, USA) for 24 hours. Cells treated with the same volume of vehicle (saline) were used as control. The 5 μM was selected as the final concentration for subsequent analysis.

**Cell transfection:** Oligonucleotide miR-1303 mimics (miR-1303), inhibitors (anti-miR-1303), and their respective negative control oligonucleotides (miR-NC or anti-miR-NC) were purchased from GenePharma (Shanghai, China). The pCD5-cir-circ-SKA3 overexpression plasmid (circ-SKA3) and mock vector with no circ-SKA3 sequence (pCD5-cirR), the specific siRNA targeting circ-SKA3 (circ-SKA3), siRNA against TLR4 (si-TLR4), and their respective negative controls (si-NC or si-con) were synthesized by Geneseed (Guangzhou, China). The pCD5-ciR-circ-SKA3 overexpression plasmid (circ-SKA3) and mock vector with no circ-SKA3 sequence (pCD5-ciR) and 1% penicillin-streptomycin (Invitrogen).

**Cell viability assay:** After assigned transected and/or treatment, the viability of AC16 cells was evaluated by adding a 10 μL cell counting kit-8 (CCK-8) solution (Beiyotime, Shanghai, China) to the culture medium and incubated for an additional 2 hours at 37°C. The absorbance of each sample was examined at 450 nm using a microplate reader.

**Cell apoptosis assay:** AC16 cells were plated into six-well plates and subjected to assigned transfection and/or treatment; then, AC16 cells were trypsinated, washed, and stained with Annexin V-fluorescein isothiocyanate (10 μL) and propidium iodide (10 μL) (BD Biosciences, Franklin Lakes, NJ, USA) for 20 minutes under darkness. Lastly, cell apoptosis was assessed using a FACS can flow cytometer (BD Biosciences).

**Western blot:** Total protein was extracted by using RNA immunoprecipitation (RIP) assay lysis buffer (Beiyotime), and then, protein concentrations were detected using the bicinchoninic acid assay. Approximately 50 μg protein was fractionated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, shifted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), and then blocked with 5% skim milk for 1.5 hours. Afterward, the membranes were probed with specific primary antibodies at 4°C overnight, followed by incubation with appropriate secondary antibodies at 37°C for 2 hours. The primary antibodies included anti-B-cell lymphoma (anti-Bcl-2) (1:1000, ab32124), anti-Bcl-2 associated X (anti-Bax) (1:1000, ab32203), anti-Cleaved caspase-3 (1:1000, ab2302) anti-CD63 (1:1000, ab13405), anti-CD9 (1:2000, ab92726), anti-TLR4 (1:500, ab13556), and anti-GAPDH (ab18602, 1:5000), obtained from Abcam (Cambridge, MA, USA). Immunoreactive bands were detected using an enhanced chemiluminescence detection kit (Millipore).

**Quantitative real-time polymerase chain reaction (qRT-PCR):** Total RNA was isolated using TRizol reagent (Invitrogen). The RNA from nuclear and cytoplasm fractions of cells was extracted according to the manufacturer’s recommended protocol of RNA Subcellular Isolation Kit (Yasen, Shanghai, China). For circRNA confirmation, 3 μg of total RNAs were treated without or with 3 U/μg of RNase R at 37°C for 15 minutes. The synthesis of complementary DNA was performed using the Transcriptor First Strand cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA), and then, the SYBR Green PCR Master Mix (Applied Biosystems) was mixed with primers for qRT-PCR detection. The relative expression levels of the molecules were calculated using the 2-ΔΔCt method and normalized to U6 or GAPDH. Primer sequences used in qRT-PCR were listed:

| CircRNA | Forward | Reverse |
|---------|---------|---------|
| circ-SKA3: F 5′-ACAGAATTCAGGCTCAATGAT-3′, R 5′-AGAGTCTGACACTTCTGAATGGA-3′; | SKA3: F 5′-TACAGGACAAAGACCATGATAC-3′, R 5′-GGATACGATGTACCGCTCAAGT-3′; | miR-1303: F 5′-ACGGGGTCTTGCTCTAAAAA-3′, R 5′-CATGGCGTGTCCGTGGAGT-3′; |
|  | F 5′-GCAGCTTGACTAGACTCTCCA-3′ | miR-1303: F 5′-ACGGGGTCTTGCTCTAAAAA-3′, R 5′-CATGGCGTGTCCGTGGAGT-3′; |
|  | R 5′-AGAGTCTGAACTTCTGAATGAAGG-3′ |  |
|  | R 5′-CAGTGCGTGTCGTGGAGT-3′; |  |
|  |  | R 5′-GCAGCTTGACTAGACTCTCCA-3′; |
|  |  | GAPDH: F 5′-CTCACCAGATGACCAATGTGTT-3′, R 5′-CGCGTGCTCAATAATGTGTT-3′; |
|  |  | U6: F 5′-AAAGCAGAATTCAGGCTCAATGAT-3′, R 5′-ACAGAATTCAGGCTCAATGAT-3′; |
5'-GTACAACACATTGTTTCCTCGGA-3'.

**Dual-luciferase reporter assay:** The binding sites between miR-1303 and circ-SKA3 or TLR4 3'UTR were predicted by circinteractome or Targetscan online databases. The sequences of a wild type (WT) and a mutant variant (MUT) were synthesized and cloned into the pmirGLO luciferase vectors (GeneCreat, Wuhan, China) to generate pmirGLO-WT/MUT-circ-SKA3 (WT-circ-SKA3 or MUT-circ-SKA3) and pmirGLO-WT/MUT-TLR 43'UTR (WT-TLR43'UTR or MUT-TLR4 3'UTR). Then, AC16 cells were seeded into 48-well plates and cotransfected with these constructed luciferase reporter vectors with miR-1303 mimic or miR-NC. Cells were harvested 48 hours after transfection, firefly activity was analyzed with a dual-luciferase reporter system (GeneCreat).

**RIP assay:** AC16 cells were lysed and the lysate was incubated with the protein A/G magnetic beads (Merck, Darmstadt, Germany) coupled with anti-Ago2 or anti-IgG antibody overnight at 4°C. Thereafter, the sample was interacted with protease K buffer to remove the protein, and immunoprecipitated RNAs were eluted, purified, and subjected to qRT-PCR analysis.

**Exosome isolation:** The culture medium of AC16 cells was centrifuged at 3000 g for 15 minutes, and then a 0.22 μm PVDF filter (Millipore) was used to filter cells and cellular debris. Afterward, the filtered culture medium was treated with the Exoquick exosome precipitation solution (System Biosciences, CA, USA) (1:5) and the mixture was centrifuged for at least 12 hours. After further refrigeration at 1,500 g for 30 minutes, the supernatant was discarded and exosomes were collected. According to the ratio of the volume of the initial culture medium and the suspension (10:1), exosomes were resuspended in phosphate-buffered saline (PBS), and the morphology of exosomes was determined using a transmission electron microscopy (TEM) (×200) (JEOL, Akishima, Japan).

**Statistical analysis:** Each experiment was performed in triplicate, and all data were manifested as mean ± standard deviation (SD). The differences between groups were analyzed using the one-way analysis of variance or the Student t test as appropriate on Graphpad Prism7 software. *P* < 0.05 indicated statistically significant.

**Results**

**DOX suppresses AC16 cells viability and promotes cell apoptosis in vitro:** AC16 cells were treated with varied doses of DOX (2.5, 5, and 10 μM) for 24 hours, and then, cell viability and apoptosis were investigated. CCK-8 assay showed that DOX treatment decreased AC16 cell viability in a concentration-dependent manner (Figure 1A). Conversely, with the increasing doses of DOX, the apoptosis of AC16 cells was increased by DOX (Figure 1B, C). Furthermore, western blot analysis suggested that the expression of proapoptotic Bax protein was increased, whereas the expression of antiapoptotic protein Bcl-2 was decreased in AC16 cells with increasing doses of DOX (Figure 1D). Caspase-3 is responsible for the majority of proteolysis during apoptosis, and the detection of Cleaved caspase-3 is therefore considered a reliable marker for cells that are dying or have died by apoptosis. We also found that with the increased concentrations of DOX, Cleaved caspase-3 protein level was elevated (Figure 1D). These data indicated that DOX could induce the death of AC16 cells.

**Circ-SKA3 is highly expressed in DOX-treated AC16 cells:** To explore the function of circ-SKA3 on DOX-induced cardiotoxicity in vitro, the effect of DOX on circ-SKA3 expression was first detected. As shown in Figure 2A, DOX remarkably elevated the expression level of circ-SKA3 in AC16 cells in a concentration-dependent manner, suggesting that circ-SKA3 might be involved in DOX-induced cardiotoxicity. Then, qRT-PCR analysis confirmed that circ-SKA3 was predominantly distributed in the cytoplasmic fraction of AC16 cells (Figure 2B). Additionally, it was discovered that circ-SKA3 could resist RNase R digestion, whereas the linear form of SKA3 decreased sharply under the RNase R treatment (Figure 2C), indicating the circular characteristic of circ-SKA3.

**Knockdown of circ-SKA3 ameliorates DOX-induced cytotoxicity in AC16 cells:** Next, the role of circ-SKA3 in DOX-treated AC16 cells was analyzed. AC16 cells were transfected with siRNA targeting circ-SKA3 (si-circ-SKA3) and then treated with 5 μM DOX for 24 hours. qRT-PCR analysis showed that the transfection of si-circ-SKA3 significantly reduced the DOX-induced elevation of circ-SKA3 expression in AC16 cells (Figure 3A). Afterward, functional experiments were performed; we demonstrated that circ-SKA3 knockdown notably attenuated DOX-evoked cell viability arrest (Figure 3B) and apoptosis promotion (Figure 3C, D) in AC16 cells. Taken together, the knockdown of circ-SKA3 protected AC16 cells from DOX-induced cytotoxicity.

**MiR-1303 is a target of circ-SKA3:** To decipher the mechanism related to the role of circ-SKA3, we predicted the latent miRNAs that could bind to circ-SKA3 through circinteractome online databases. Results showed that numerous miRNAs were found to have base pairs complementary to circ-SKA3, and miR-1303 was selected for subsequent interaction analysis because of the high predicted score and the decreased level in DOX-exposed cardiomyocytes.23 The potential binding sites between circ-SKA3 and miR-1303 were shown in Figure 4A. Then, the dual-luciferase reporter assay was conducted, and we found that miR-1303 overexpression significantly reduced the luciferase activity of the WT-circ-SKA3 reporter relative to the control group, whereas there was no change in the luciferase activity of the MUT-circ-SKA3 reporter after the upregulation of miR-1303 levels (Figure 4B). Moreover, RIP assay showed that circ-SKA3 and miR-1303 were markedly pulled down by anti-Ago2 in AC16 cells compared with IgG antibody (Figure 4C). These results confirmed that circ-SKA3 directly targeted miR-1303.

Subsequently, the effect of DOX on miR-1303 expression was investigated; the results exhibited that miR-1303 expression was decreased by DOX in a concentration-dependent manner (Figure 4D). Then, the transfection efficiency of circ-SKA3 was detected (Figure 4E), and we found that the knockdown of circ-SKA3 elevated the expression of miR-1303 in DOX-mediated AC16 cells, whereas circ-SKA3 overexpression reversed the ef-
Figure 1. DOX suppresses AC16 cell viability and promotes cell apoptosis in vitro. AC16 cells were treated with a varied dose of DOX (2.5, 5, and 10 μM) for 24 hours. A: The viability analysis of AC16 cells using CCK-8 assay. B, C: Flow cytometry for AC16 cell apoptosis. D: Detection of Bcl-2, Cleaved caspase-3, and Bax protein expression using western blot assay. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 2. Circ-SKA3 is highly expressed in DOX-induced AC16 cells. A: qRT-PCR analysis of circ-SKA3 expression in AC16 cells treated with a varied dose of DOX (2.5, 5, and 10 μM) for 24 hours. B: qRT-PCR analysis of the levels of circ-SKA3, GAPDH, and U6 in purified nuclear and cytoplasmic fractions of AC16 cells. C: qRT-PCR analysis of circ-SKA3 and linear SKA3 mRNA expression in AC16 cells treated with RNase R or not. *P < 0.05, ***P < 0.001, ****P < 0.0001.
Figure 3. Knockdown of circ-SKA3 ameliorates DOX-induced cytotoxicity in AC16 cells. AC16 cells were transfected with si-circ-SKA3 or si-NC and then treated with 5 μM DOX for 24 hours. A: Measurement of circ-SKA3 expression using qRT-PCR in AC16 cells. B: CCK-8 assay of the viability of AC16 cells. C: Flow cytometry for AC16 cell apoptosis. D: Western blot analysis for the expression of Bcl-2, Cleaved caspase-3, and Bax protein expression in AC16 cells. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Effect (Figure 4F). Altogether, circ-SKA3 directly targeted miR-1303 and negatively regulated its expression.

Knockdown of circ-SKA3 ameliorates DOX-induced cytotoxicity in AC16 cells via miR-1303: We then investigated whether miR-1303 is involved in the function of circ-SKA3 on DOX-treated AC16 cells. AC16 cells were transfected with si-circ-SKA3 together with or without anti-miR-1303 and then subjected to the treatment of 5 μM DOX for 24 hours. The relative protein expression of Bcl-2, Cleaved caspase-3, and Bax was measured using Western blotting. The results showed that knockdown of circ-SKA3 significantly increased the relative protein expression of Bcl-2 (P < 0.001) and decreased the relative protein expression of Cleaved caspase-3 (P < 0.01) and Bax (P < 0.05) compared with the DOX group, suggesting that circ-SKA3 ameliorates DOX-induced cytotoxicity in AC16 cells via miR-1303.
miR-1303 is a target of circ-SKA3. A: The binding sites between circ-SKA3 and miR-1303. B: Dual-luciferase reporter assay for the luciferase activity in AC16 cells cotransfected with WT-circ-SKA3, MUT-circ-SKA3 reporter, and miR-1303 mimic or miR-NC. C: qRT-PCR analysis of circ-SKA3 and miR-1303 expression after RIP assay. D: Detection of miR-1303 expression in AC16 cells treated with a varied dose of DOX (2.5, 5, and 10 μM) for 24 hours using qRT-PCR. E: AC16 cells transfected with circ-SKA3 or pCDS-cIR were treated with 5 μM DOX, and then, the expression of circ-SKA3 was detected using qRT-PCR. F: AC16 cells transfected with circ-SKA3, pCDS-cIR, si-NC, or si-circ-SKA3 were treated with 5 μM DOX, and then, the expression of miR-1303 was examined using qRT-PCR. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

μM DOX for 24 hours. qRT-PCR analysis showed that miR-1303 inhibition reduced the elevation of miR-1303 expression induced by circ-SKA3 knockdown in DOX-treated AC16 cells (Figure 5A). Afterward, it was proved that knockdown of circ-SKA3 promoted cell viability (Figure 5B) and inhibited cell apoptosis (Figure 5C-E) in DOX-treated AC16 cells, which were abolished by a miR-1303 inhibitor (Figure 5B-E). These data suggested that circ-SKA3 exerted its role via miR-1303 in DOX-treated AC16 cells.

TLR4 is a target of miR-1303: The downstream genes of miR-1303 were further searched. Through Targetscan bioinformatic analysis software, miR-1303 was suggested to bind to TLR4 via complementary base pairing (Figure 6A). Results from the dual-luciferase reporter assay showed that the luciferase activity was markedly decreased in AC16 cells cotransfected with miR-1303 mimics and WT-TLR4 3’UTR vector, but not with the mutant-type vector (Figure 6B). RIP assay further suggested that TLR4 and miR-1303 were significantly abundant in Ago2-containing microribonucleoproteins relative to control IgG (Figure 6C). All these data confirmed that miR-1303 directly targeted TLR4.

Knockdown of TLR4 attenuates DOX-induced cytotoxicity in AC16 cells: To elucidate the role of TLR4 on DOX-induced cardiotoxicity, the siRNA targeting TLR4 was designed and transfected into AC16 cells; then, transfected cells were treated with 5 μM DOX for 24 hours. Western blot analysis showed that transfection of si-TLR4 reversed DOX-triggered elevation of TLR4 level (Figure 7A). Then, the results of functional experiments exhibited that the silencing of TLR4 reversed DOX-evoked cell viability inhibition (Figure 7B) and apoptosis enhancement (Figure 7C, D) in AC16 cells. Hence, we demonstrated that TLR4 knockdown protected AC16 cells against DOX-induced cytotoxicity.

MiR-1303/TLR4 axis regulates DOX-induced cytotoxicity in AC16 cells: Given the relationship between miR-1303 and TLR4, whether miR-1303/TLR4 axis was responsible for DOX-induced cardiotoxicity was then explored. AC16 cells were transfected with miR-1303 combined with or without TLR4, followed by treatment with 5 μM DOX for 24 hours. Western blot analysis suggested that TLR4 transfection rescued miR-1303-induced decrease of TLR4 expression in DOX-treated AC16 cells (Figure 8A). Next, the CCK-8 assay showed miR-1303 reexpression in DOX-treated AC16 cells promoted cell viability, which was reversed via TLR4 upregulation (Figure 8B). Meanwhile, TLR4 upregulation also abolished miR-1303 reexpression-induced inhibition of cell apoptosis in DOX-mediated AC16 cells (Figure 8C-E). Collectively, miR-1303 played a protective role in DOX-induced cytotoxicity in AC16 cells via regulating TLR4. Importantly, we also observed that silencing of circ-SKA3 reduced DOX-evoked increase of TLR4 expression in AC16 cells;
Figure 5. Knockdown of circ-SKA3 ameliorates DOX-induced cytotoxicity in AC16 cells via miR-1303. AC16 cells were cotransfected with si-circ-SKA3 and anti-miR-1303 and then subjected to the treatment of 5 μM DOX for 24 hours. A: Detection of the expression of miR-1303 using qRT-PCR in AC16 cells. B: The viability analysis of AC16 cells using CCK-8 assay. C, D: Flow cytometry for AC16 cell apoptosis. E: Detection of Bcl-2, Cleaved caspase-3, and Bax protein expression using western blot assay. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Additionally, miR-1303 inhibition rescued circ-SKA3 silencing-induced decrease of TLR-4 expression in DOX-treated AC16 cells (Figure 8F), suggesting that circ-SKA3 could regulate TLR4 expression via sponging miR-1303.

Extracellular circ-SKA3 is packaged into exosomes and can be internalized by recipient AC16 cells: Exosomes are well-known critical mediators of cell-to-cell communication, and exosome-mediated transfer of circRNAs can mediate signals between cells.24-26 Thus, we explored the existing pattern of extracellular circ-SKA3. The morphology of exosomes was confirmed via TEM (Supplemental Figure A), and the identification of surface hallmarks (CD63 and CD9) using western blot further validated that the isolated particles were exosomes (Supplemental Figure B). Afterward, we found that circ-SKA3 expression was remarkably higher in isolated exosomes than in the supernatant of AC16 cells (Supplemental Figure C). Exosomal circ-SKA3 level was elevated in DOX-stimulated AC16 cells in a concentration-dependent manner (Supplemental Figure D). Additionally, DOX-treated AC16 cells were cocultured with or without GW4869, an inhibitor of exosome generation, it was discovered that exosomal circ-SKA3 expression was reduced in GW4869 treated groups (Supplemental Figure E). All these results suggested that extracellular circ-SKA3 was enriched and packaged in exosomes. Moreover, untreated AC16 cells were incubated with PBS or exosomes, derived from DOX-treated AC16 cells or 293T cells, we found that compared with the incubation of PBS or 293T-EXO, the circ-SKA3 level was significantly upregulated after AC16-EXO incubation (Supplemental Figure F). These data suggested that extracellular circ-SKA3 was packaged into
Figure 6. TLR4 is a target of miR-1303. A: The binding sites between TLR4 and miR-1303. B: Dual-luciferase reporter assay for the luciferase activity in AC16 cells cotransfected with WT-TLR4 3’UTR, MUT-TLR4 3’UTR reporter, and miR-1303 mimic or miR-NC. C: qRT-PCR analysis of TLR4 and miR-1303 expression after RIP assay. D: Detection of TLR4 expression in AC16 cells treated with a varied dose of DOX (2.5, 5, and 10 μM) for 24 hours with Western blot. E, F: AC16 cells were transfected with miR-1303, miR-NC, anti-miR-1303, and anti-miR-NC and then treated with 5 μM DOX for 24 hours. E: qRT-PCR analysis of miR-1303 expression in AC16 cells. F: Western blot analysis of TLR4 protein in AC16 cells. **P < 0.01, ***P < 0.001, ****P < 0.0001.

Discussion

DOX is one of the most widely available antibiotic antineoplastic agents with high efficacy, which has made great contributions to the therapy of many types of malignant cancers; nevertheless, the cumulative dose-related cardiac toxicity seriously restricts its clinical benefit and usage.27) Apoptosis comprises a distinct form of cell death, which has emerged as a key determinant of target organ damage in cardiovascular diseases; blocking apoptosis is cardioprotective and can prevent the development of heart failure.28) Recently, cardiomyocyte apoptosis have been identified in DOX-induced cardiotoxicity; the reduction of apoptosis and death of cardiomyocytes during the use of DOX might largely rescue DOX-evoked cardiotoxicity.6,29) In the present study, in vitro experiments showed that DOX treatment impaired cell viability and induced cell apoptosis in AC16 cells. Presently, emerging studies have reported that circRNAs may function as suitable potential therapeutic targets or diagnostic biomarkers for DOX-induced cardiotoxicity.30,31) In this study, we found circ-SKA3 was highly expressed in DOX-mediated AC16 cells. CircRNAs processing and function are dependent on RNA-binding proteins (RBPs).32) Qki5 is an RBP and abundant in the heart, it has been reported to regulate the formation of numerous circRNAs during the epithelial to mesenchymal transition.31) Additionally, DOX downregulated Qki5, thereby suppressing circRNAs derived from Ttn, Fhod3, and Strn3 in mouse cardiomyocytes, leading to an increase of DOX-induced apoptosis rate and an extent of cell atrophy.31,33) Thus, we speculate that DOX may induce the upregulation of circ-SKA3 via regulating RBPs, which still needs further investigation. Afterward, further functional experiments suggested that the knockdown of circ-SKA3 in AC16 cells reversed DOX-triggered cell viability arrest and apoptosis promotion. Hence, we demonstrated that circ-SKA3 promoted DOX-mediated cardiotoxicity. Moreover, our study unveiled that circ-SKA3 was mainly located in exosomes, which could be taken up by target cells, leading to a transfer of exosomal circ-SKA3 to target cells, which might affect RNA expression and function in target cells. Exosomes are small spherical packages continually secreted by cells; they are found to be stably present in body fluids and have the ability to stimulate immune responses, which endowing them are promising biomarkers for the detection of disease and the development of novel approaches to vaccination.34,35) Therefore, exosomal circ-SKA3 might be an ideal biomarker for preventing DOX-triggered cardiotoxicity.

It has been revealed that circRNAs can serve as endogenous sponges of miRNAs to prevent miRNA-mediated degradation of target mRNAs.36,37) Thus, the mechanism related to the role of circ-SKA3 was deciphered. This study uncovered that circ-SKA3 directly bound to miR-1303, and miR-1303 directly targeted TLR4; importantly, circ-SKA3 could indirectly regulate TLR4 via sponging miR-1303. Hence, a circ-SKA3/miR-1303/...
The TLR4 axis in AC16 cells was identified. Then, whether circ-SKA3 exerted its role in DOX-induced cardiotoxicity via miR-1303/TLR4 axis was explored. MiR-1303 was previously found to be decreased in DOX-exposed cardiomyocytes. In this study, we also discovered that DOX reduced miR-1303 expression in AC16 cells; further functional experiments suggested that miR-1303 reexpression in AC16 cells abolished DOX-induced death of cells.
Figure 8. MiR-1303/TLR4 axis regulates DOX-induced cytotoxicity in AC16 cells. AC16 cells were cotransfected with miR-1303 and TLR4, followed by treatment with 5 μM DOX for 24 hours. A: Western blot analysis of TLR4 expression in AC16 cells. B: CCK-8 assay of the viability of AC16 cells. C, D: The apoptosis analysis of AC16 cells using flow cytometry. E: Western blot analysis for the expression of Bcl-2, Cleaved caspase-3, and Bax protein expression in AC16 cells. AC16 cells were cotransfected with si-NC, si-circ-SKA3, si-circ-SKA3 + anti-miR-NC, or si-circ-SKA3 + anti-miR-1303 and then treated with 5 μM DOX for 24 hours. F: The TLR4 expression in AC16 cells was detected using Western blot. **P < 0.01, ***P < 0.001, ****P < 0.0001.

Moreover, the inhibition of miR-1303 also reversed the cardioprotective action of circ-SKA3 knockdown on DOX-induced cardiotoxicity. Hence, we verified that circ-SKA3/miR-1303 axis was engaged in DOX-induced cardiotoxicity.

TLR4, a transmembrane protein, belongs to the pattern recognition receptor family, the activation of which can result in the production of inflammatory cytokines and intracellular signaling pathway NF-κB, and plays a fundamental role in pathogen recognition and activation of innate immunity. Previous studies showed that TLR4 abolishment could ameliorate DOX-evoked cardiotoxicity. In this study, TLR4 was found to be increased in DOX-treated AC16 cells, the silencing of TLR4 also abolished DOX-evoked cardiotoxicity and death in AC16 cells, which were consistent with previous findings. Additionally, we confirmed that TLR4 overexpression abrogated the inhibitory function of miR-1303 in DOX-evoked cardiotoxicity. Thus, the miR-1303/TLR4 axis was responsible for DOX-triggered cardiotoxicity. Nonetheless, although some interesting results were found in this work, there remain some limitations. The data presented are based on a limited number of AC16 cells in vitro. The expression profile of circ-SKA3 in human primary cardiomyocytes and whether circ-SKA3 silencing can restore the viability of DOX-induced cardiomyocytes still needed further study. Additionally, the in vivo assay using animal models with high or low circ-SKA3 expression is essential to verify the function of circ-SKA3 on heart damage induced by DOX.
In conclusion, this work demonstrated that the knockdown of circ-SKA3 could abolish DOX-induced cardiotoxicity in AC16 cells via the miR-1303/TLR4 axis. Moreover, it was confirmed that extracellular circ-SKA3 was packaged into exosomes and exosomal circ-SKA3 could function as a mediator in intercellular communication between AC16 cells. All these findings suggested the potential involvement of exosomal circ-SKA3 in DOX-triggered cardiotoxicity, providing a promising strategy to protect cardiomyocytes from DOX-evoked cardiotoxicity.

Disclosure

Conflicts of interest: The authors declare that they have no financial conflicts of interest.

Authors’ contributions: Bin Li and Xinyong Cai conceived and designed the experiments; Yunxia Wang and Hongmin Zhu performed the experiments and funding acquisition; Ping Zhang, Panpan Jiang, and Xu Yang contributed reagents/materials/analysis tools; and Lang Hong and Liang Shao wrote the paper. All authors read and approved the final manuscript.

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

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**Supplemental Files**

Supplemental Figure
Please see supplemental files; https://doi.org/10.1536/ihj.20-809