

Trophoblast Stem-Cell-Derived Exosomes Improve Doxorubicin-Induced Dilated Cardiomyopathy by Modulating the let-7i/YAP Pathway

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Trophoblast stem cells (TSCs) have been confirmed to play a cardioprotective role in heart failure. However, whether TSC-derived exosomes (TSC-exos) can protect against cardiac injury remains unclear. In the present study, TSC-exos were isolated from the supernatant of TSCs using the ultracentrifugation method and characterized by transmission electron microscopy and western blotting. Utilizing the public Gene Expression Omnibus (GEO) database, we found that let-7i and Yes-associated protein 1 (YAP) could participate in the development of heart failure. *In vitro*, AC16 cardiomyocytes subjected to doxorubicin (DOX) were treated with TSC-exos or let-7i mimic. Flow cytometry showed that TSC-exos and let-7i both decreased cardiomyocyte apoptosis. *In vivo*, mice that were intraperitoneally injected into DOX received either PBS, TSC-exos, or AAV9-let7i^{up} for let-7i overexpression. Mice receiving TSC-exos and AAV9-let7i^{up} showed improved cardiac function and decreased inflammatory responses, accompanied by downregulated YAP signaling. Mechanistically, TSC-exos could transfer let-7i to cardiomyocytes and silence the YAP signaling pathway. In conclusion, TSC-exos could alleviate DOX-induced cardiac injury via the let-7i/YAP pathway, which sheds new light on the application of TSC-exos as a potential therapeutic tool for heart failure.

INTRODUCTION

Dilated cardiomyopathy (DCM), characterized by a dilated ventricular cavity and impaired myocardial contractility, is the most common cardiomyopathy worldwide.¹ Clinically, patients with dilated cardiomyopathy often develop progressive congestive heart failure with an increased risk of death.^{2,3} Multiple studies have revealed the molecular mechanisms of DCM, including ischemic or toxic damage and familial genetic defects.^{4,5} However, potential treatments are still lacking.

Exosomes are 50–150 nm extracellular vesicles with a double-layered phospholipid membrane structure secreted by different cells under different physiological conditions⁶ and effectively mediate information exchange between cells.⁷ Previous studies have reported that exosomes derived from mesenchymal stem cells (MSCs) can improve

cardiovascular diseases, such as DCM and myocardial infarction.^{8–10} Recently, trophoblast stem cells (TSCs), which are obtained from “abandoned” amniotic tissue after delivery, have attracted increased interest from researchers^{11,12} due to the advantages of convenient material collection, abundant sources without ethical controversy, low immunogenicity, and strong multidifferentiation potential.^{13–15} In addition, TSCs secrete a large number of exosomes and play a beneficial role in many biological processes.¹⁶

MicroRNAs (miRNAs), as a class of small noncoding RNAs, have emerged as post-transcriptional regulators by binding to the 3′-UTR of target genes.¹⁷ They may exert powerful effects on cellular processes such as growth, differentiation, metabolism, and apoptosis.^{18,19} Previous studies have also confirmed that these molecules are involved in cardiovascular disorders.^{18,20}

Yes-associated protein 1 (YAP1) is known as a core factor of the Hippo pathway²¹ that plays a vital role in controlling organ size.²² Numerous studies have revealed a pivotal role of the Hippo-YAP pathway in cardiac development, growth, homeostasis, disease, and regeneration.^{23,24} Ikeda et al.²⁵ reported that YAP mediated the exacerbation of pressure overload-induced heart failure. YAP knockdown decreased fibrogenic responses in cardiac fibroblasts.²⁶ However, the upstream regulators in cardiovascular diseases remain poorly understood.

It has been reported that the prognosis of women with perinatal cardiomyopathy is significantly better than that of other types of

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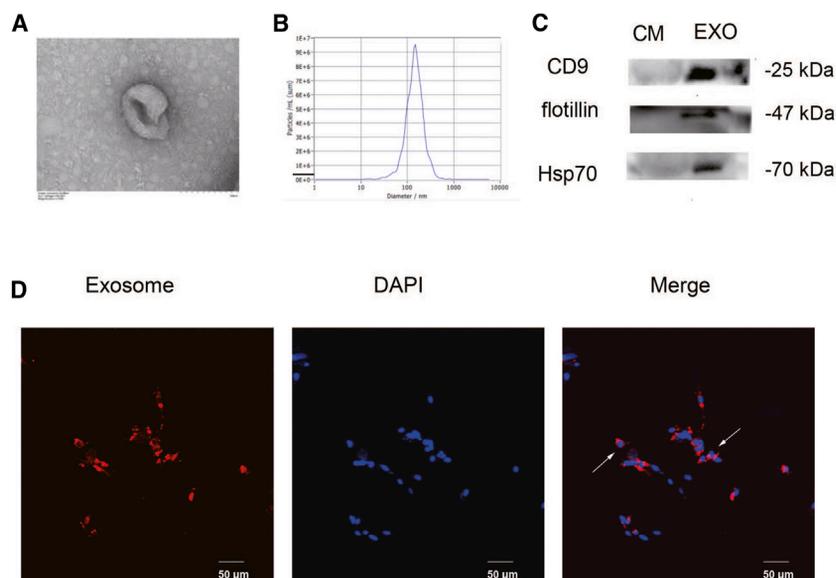


Figure 1. Characterization of Trophoblast Exosomes

(A) Transmission electron microscopic analysis of exosomes. Scale bar, 200 nm. (B) The particle sizes of the extracted exosomes. (C) Western blot analysis of exosomal marker proteins, including CD9, flotillin, and Hsp70. CM, culture medium; EXO, exosomes. (D) Representative pictures of AC16 cell uptake of exosomes. White arrow indicated the cells with exosomes.

cardiomyopathy, with 50% of patients eventually recovering normal heart function,^{27,28} which could be attributed to some pregnancy-related factors. Therefore, we hypothesized that the TSC-derived exosomes can play a cardioprotective role in dilated cardiomyopathy.

RESULTS

Characterization of TSC-Derived Exosomes (TSC-exos)

TSC-derived exosomes were isolated from the conditioned medium of the HTR8-Svneo cell line and observed by transmission electron microscopy, which showed a double-layer membrane particle with a range of 50–150 nm (Figures 1A and 1B). Western blot analysis showed that TSC-exos highly expressed two exosomal markers: CD9 and flotillin (Figure 1C). In addition, coculture studies demonstrated that exosomes can be internalized by AC16 cells (Figure 1D).

Determination of miRNAs Involved in DCM from the GEO Dataset and *In Vitro*

To determine the differentially expressed miRNAs between healthy people and DCM patients, we analyzed the miRNA expression profiles from the Gene Expression Omnibus (GEO) dataset. As shown in Figure 2A, miR-27b and let-7i-5p were significantly decreased in the DCM group compared to the healthy control group. We then selected the top 30 miRNAs expressed in the TSC-exos from GSE93020 containing let-7i-5p (Figure 2B). We then examined the common miRNA let-7i-5p in DOX-induced DCM and DOX-exposed AC16 cells and found that let-7i-5p was significantly downregulated in both DOX-induced DCM and DOX-exposed AC16 cells (Figures 2C and 2D). Therefore, we speculated that TSC-exo-derived let-7i may play a role in DOX-induced DCM.

Effect of let-7i on the Expression of YAP1 in DCM

Further, we examined the GSE4172 dataset and discovered that the expression of Yap1 in the serum of patients with heart

failure was higher than that of healthy individuals (Figure 3A). In addition, our immunoblotting results confirmed a higher expression of YAP1 in the failing myocardial tissues (Figures 3B and 3C). An illustration of the seed sequence of let-7i binding to the wild-type and mutated form of the 3'-UTR of YAP1 mRNA is shown in Figure 3D based on bioinformatic prediction. The luciferase reporter results confirmed the direct binding of let-7i to YAP1 (Figure 3E), indicating that YAP1 was a direct target gene of let-7i-5p.

Effect of TSC-exo and let-7i on Apoptosis in DOX-Induced Cardiomyocytes *In Vitro* via YAP-Hippo Signaling

AC16 cells were treated with PBS and TSC-exos, as well as let-7i control and mimic. The flow cytometry and western blotting results showed that exosomes and let-7i mimic both inhibited cell apoptosis, characterized by a lower percentage of Annexin V+ cells (Figures 4A and 4B), lower expression of cleaved caspase 3 and higher expression of bcl2 (Figure 4C). However, early apoptosis (Annexin V⁺PI⁻ cells) and late apoptosis (Annexin V⁺PI⁺ cells) were not significantly different between the groups. Exosome and let-7i mimic-treated cardiomyocytes also had lower YAP1, CTGF, and TEAD1 expression than that of the other groups at both the protein level (Figure 4D) and mRNA level (Figures 4E–4G), suggesting decreased YAP1 activity.

Effect of Trophoblasts Exosomal let-7i on DOX-Injured Heart Function *In Vivo*

We treated Dox-challenged mice with PBS, TSC-exos, or AAV-let7i^{HP}. Echocardiography results (Figures 5A–5D) showed that TSC-exos and let-7i could restore impaired cardiac function and reverse cardiac remodeling caused by DOX. Moreover, they decreased the mRNA expression of heart failure markers, including ANP, β -MHC, and collagen I (Figure 5E). Pathological staining also confirmed that TSC-exos and let-7i partially maintained normal cardiac structure and alleviated cardiac fibrosis induced by DOX treatment (Figure 5G).

Effect of Exosomes on YAP1/Hippo Signaling Pathway by Delivery of let-7i

The results (Figures 6A and 6B) showed that the exosome and let-7i-5p AAV groups had higher let-7i-5p expression and lower YAP1 expression than DCM-NC group. Both groups also had lower

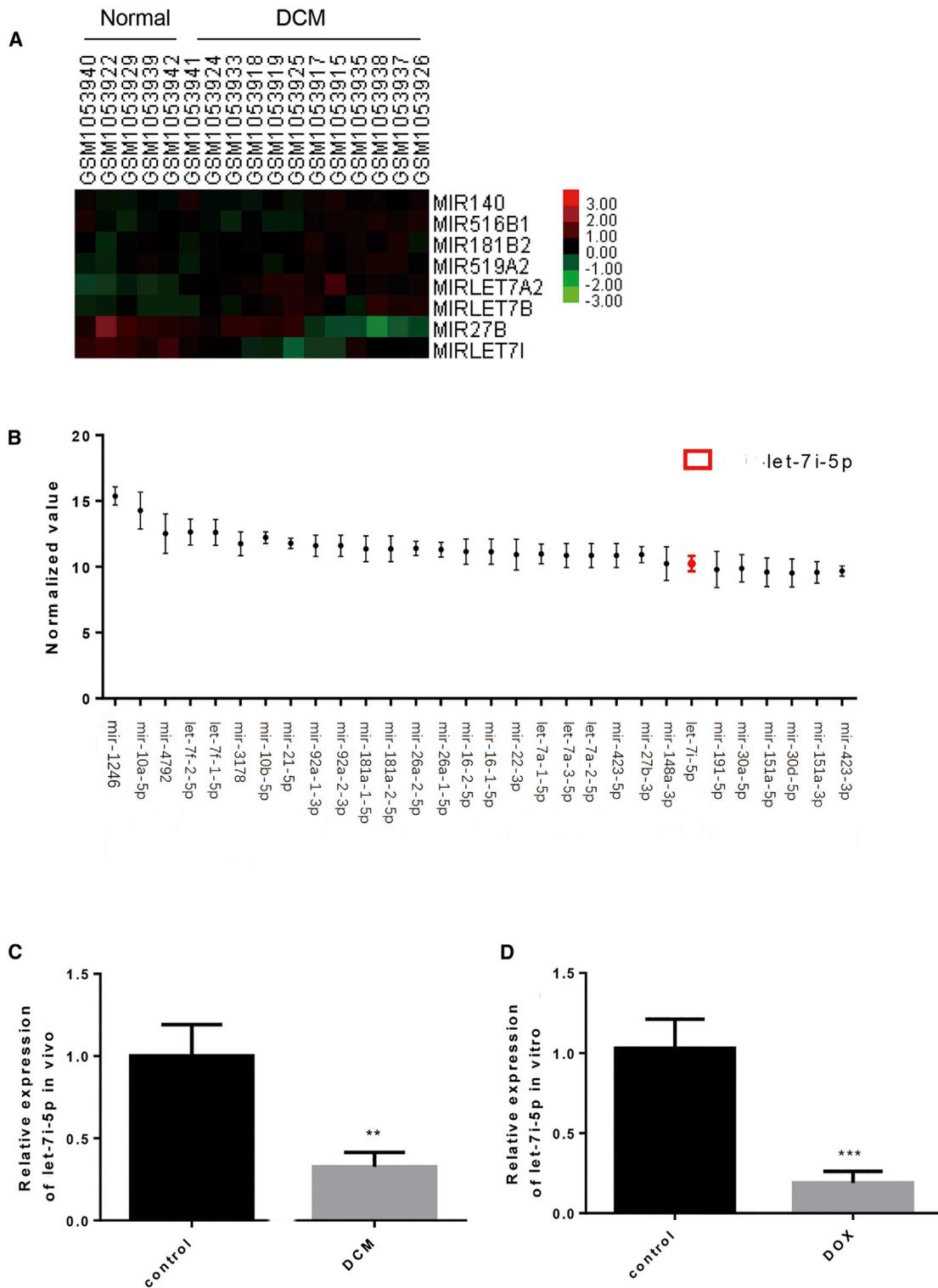


Figure 2. Differentially Expressed microRNAs in DCM and Enriched in Trophoblast Exosomes

(A) Differentially expressed miRNAs in DCM patients. (B) Highly expressed miRNAs in trophoblast exosomes. (C) The expression level of let-7i-5p in the control and dilated cardiomyopathy groups. (D) The expression level of let-7i-5p in the control and DOX-exposed AC16 cells by qPCR assays. All data are presented as the mean \pm SEM, $n = 6$ /group. ** $p < 0.01$; *** $p < 0.001$, compared to the control group.

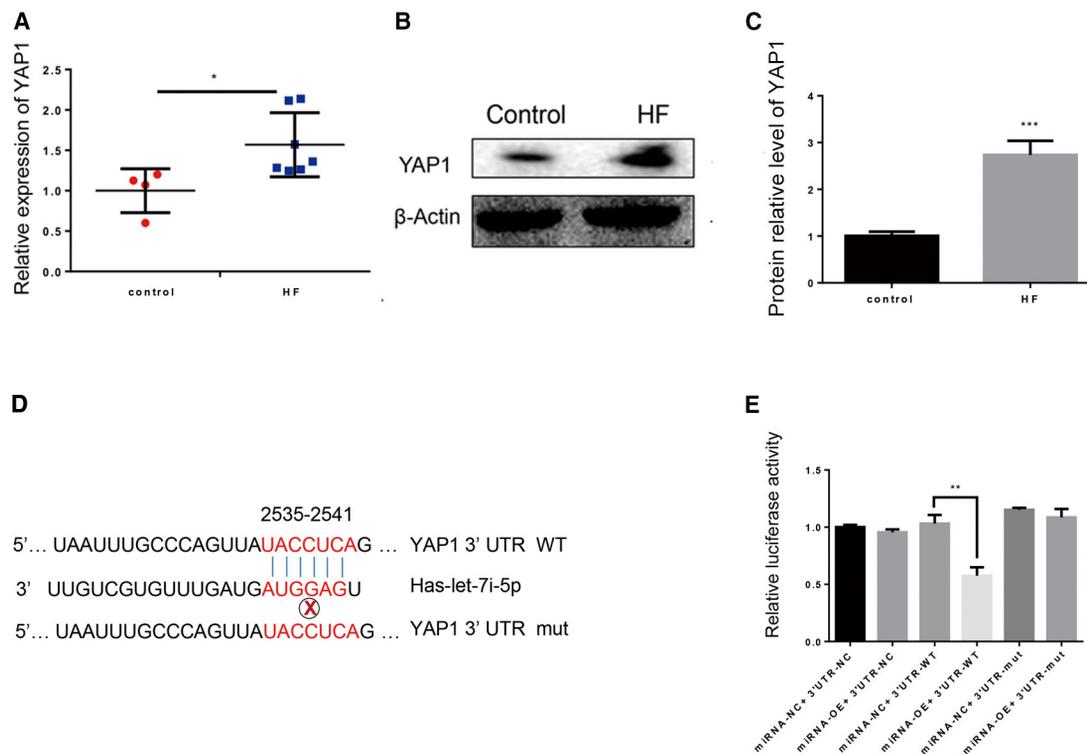


Figure 3. Effect of let-7i on the Expression of YAP1 in DCM

(A) The relative expression level of YAP1 from the serum of healthy individuals and HF patients from the GEO dataset. (B) Western blot analysis of YAP1 in the myocardial tissue of DOX-induced HF mice. (C) Relative protein levels were analyzed by densitometric analysis. (D) An illustration of the seed sequence of let-7i binding to the wild-type and mutated 3'-UTR of YAP1 mRNA based on bioinformatic prediction. (E) Dual luciferase reporter results of HEK293T cells cotransfected with luciferase reporter vectors (YAP1 3'-UTR-WT or YAP1 3'-UTR-mut) and miRNA (let-7i-5p-NC or let-7i-5p-OE). NC, negative control mimics; OE, overexpression. All data are presented as the mean \pm SEM, $n = 6$ /group. ** $p < 0.01$; *** $p < 0.001$, compared to the control group.

interleukin-1 (IL-1) and IL-6 expression than DCM-NC group (Figures 6C and 6D), suggesting an anti-inflammatory role of exosomes and let-7i. Furthermore, immunoblotting showed lower YAP1, cleaved-caspase3, CTGF, and ANP expression in the exosome and the let-7i-5p AAV group (Figures 6E and 6F), suggesting that TSC-exos may transfer let-7i to inhibit the YAP1 signaling pathway and exert a protective effect.

DISCUSSION

Recent studies have reported that exosomes derived from various stem cells enhanced myocardial viability and prevented adverse remodeling in pathological settings.²⁹ For example, exosomes secreted by cardiac progenitor cells were reported to stimulate the migration of endothelial cells³⁰ and protect against ischemia/reperfusion injury.³¹ Embryonic stem-cell-derived exosomes promote endogenous repair and enhance cardiac function following myocardial infarction.³² Stem-cell-derived exosomes are distinguished by different enriched proteins, mRNAs or miRNAs. TSCs are obtained from placentas and embryonic stem cells and are characterized by their special cargos. Fetal maternal stem cell transfer appears to be a critical mechanism in the maternal response to cardiac injury, which provides a foundation for TSCs as a novel cell type for potential use in

cardiovascular therapy.^{27,33} In our study, it is also observed, in the histological and functional studies, that TSC exosomes improve both cardiac function and fibrosis.

A previous study found that let-7i participated in angiogenesis in dilated cardiomyopathy.³⁴ Wang et al.³⁵ demonstrated that let-7i acts as a novel negative regulator of angiotensin II-induced cardiac inflammation and fibrosis by suppressing the expression of IL-6 in the heart and may represent a new potential therapeutic target for hypertensive cardiac fibrosis. Moreover, let-7i was negatively correlated with Toll-like receptor 4 levels in patients with coronary artery disease, contributing to the beneficial effects of atorvastatin.³⁶ Utilizing the online GEO database and qPCR for TSC-derived exosomes, we verified that let-7i was enriched in TSC-exos. Consistent with previous studies, our results demonstrated that increased expression of let-7i mediated the protective role of TSC-exos on doxorubicin cardiotoxicity.

miRNAs play a role by targeting functionally related gene networks.^{18,19} By bioinformatic prediction and luciferase validation, we identified YAP1 as the target of let-7i. Previous publications demonstrated that YAP activation contributed to cardiac fibrosis in heart

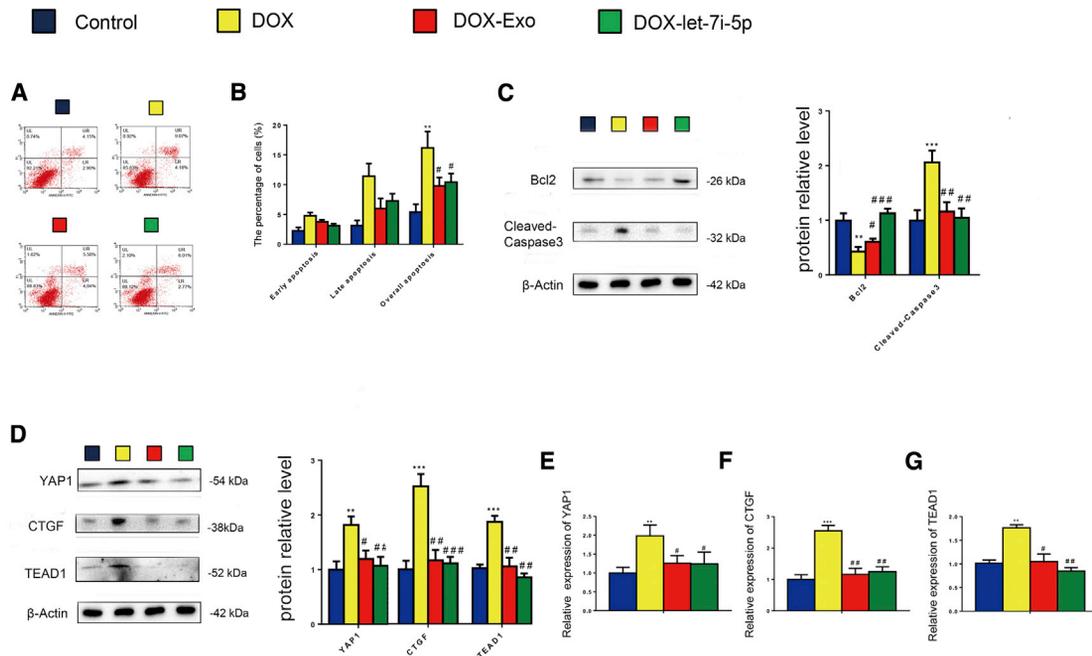


Figure 4. Effect of TSC-exo and let-7i on Apoptosis in DOX-Induced Cardiomyocytes *In Vitro* via YAP-Hippo Signaling

The groups were categorized into the control, DOX, DOX-Exo, and DOX-let-7i-5p groups based on the treatment of DOX or let-7i. (A) Apoptosis results detected by flow cytometry. (B) The percentage of early apoptotic cells (Annexin V⁺ PI⁻), late apoptotic cells (Annexin V⁺ PI⁺), and overall apoptotic cells (Annexin V⁺). (C and D) Western blot of Bcl2 and cleaved caspase 3 (C) and (D) western blot of YAP1, CTGF, and TEAD1 (D) in the control group and the groups treated with DOX, DOX-Exos, and DOX-let-7i-5p. And relative protein levels were analyzed by densitometric analysis. (E–G) The mRNA expression levels of (E) YAP1, (F) CTGF, and (G) TEAD1 in the group treated with control, DOX, DOX-Exos, and DOX-let-7i-5p were measured by real-time PCR. All data are presented as the mean \pm SEM, n = 6/group. **p < 0.01, ***p < 0.001 compared to the control group; #p < 0.05, ##p < 0.01, ###p < 0.001 compared to the DOX-treated group.

failure.^{25,26} By AAV-mediated let-7i overexpression, we demonstrated that let-7i could inactivate YAP signaling and protect against doxorubicin-induced dilated cardiomyopathy. In addition, our luciferase reporter assay confirmed that YAP1 was a downstream target of let-7i.

In summary, our studies revealed for the first time that TSC-derived exosomes could protect against doxorubicin-induced dilated cardiomyopathy. Mechanistically, exosomal let-7i inhibited myocardial apoptosis and mitigated cardiac fibrosis by downregulating YAP signaling. In addition, our AAV-mediated let-7i injection provides a potential strategy to manipulate miRNAs for the treatment of heart diseases.

MATERIALS AND METHODS

GEO Database

The GEO is a free database containing a microarray of RNA-seq profiles, from which expression datasets were downloaded for the following analysis. GEO: GSE42955 is a microarray expression profile comprised of 5 control human hearts and 12 dilated cardiomyopathy samples based on the GPL6244 platform. GEO: GSE4172 is a microarray profile consisting of 12 endomyocardial biopsies from 4 healthy individuals and 8 dilated cardiomyopathy patients. GEO: GSE93020 is a dataset from miRNA sequencing of exosomes from extravillous trophoblast cells based on the GPL18573 platform.

Exosome Isolation

HTR8-Svneo cells (Novobio, Shanghai, PR China), a type of TSC, were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution under 5% CO₂ at 37°C. When the cells reached 70% to 80% confluency, they were cultured with fresh DMEM containing 5% exosome-depleted FBS (System Biosciences, USA) for 48 h. Exosomes were isolated using differential centrifugation based on previously described methods with slight modifications^{37,38} Briefly, HTR8-Svneo cells were removed by centrifugation at 300 \times g for 10 min; the supernatant was then cleared of apoptotic bodies by centrifugation at 2,000 \times g for 20 min; microvesicles were preferentially pelleted at 10,000 \times g for 30 min; exosomes were then purified from the supernatant by ultracentrifugation at 100,000 \times g for 60 min. After isolation, exosomes were diluted in 100 μ L of filtered PBS and stored at -80° C.

Exosome Identification

Exosomes were fixed in 2.5% glutaraldehyde at 4°C, dehydrated with gradient alcohol and embedded in epoxy resin. Sections were stained with uranyl acetate and citrate acid lead. The photos were taken under a transmission electron microscope (JEM-1010, JEOL, Tokyo, Japan). Exosomes were dissolved in radioimmunoprecipitation assay (RIPA) buffer and quantified using a Bicinchoninic acid (BCA) protein analysis kit (Thermo Fisher Scientific, USA). The exosome markers (CD9,

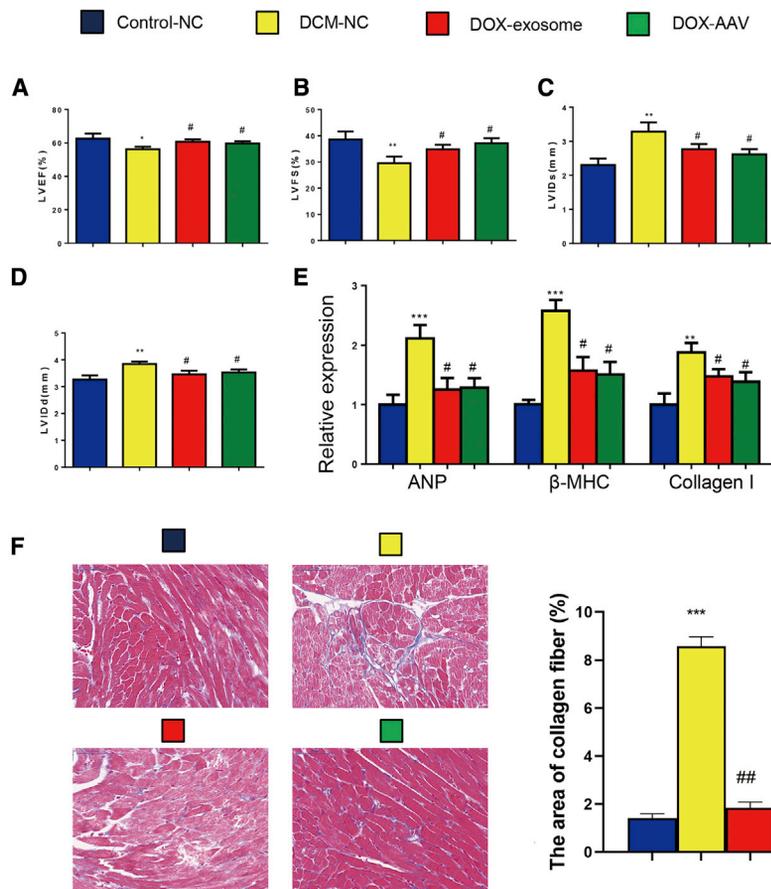


Figure 5. Effect of Trophoblasts Exosomal let-7i on DOX-Injured Heart Function *In Vivo*

8-week-old male mice were divided into four groups: control, DOX, DOX-Exo, and DOX-AAV^{up-let-7i-5p}. (A) Left ventricular ejection fraction (LVEF, %). (B) Left ventricular fraction shortening (LVFS, %). (C) Left ventricular internal diameter in diastole (LVIDs, %). (D) Left ventricular end-diastolic diameter (LVEDd, %). (E) The mRNA expression levels of ANP, β -MHC, and type I collagen in myocardial tissue were measured by real-time PCR. (F) Representative micrographs of Masson staining of heart tissue sections. Scale bar, 1,000 μ m. All data are presented as the mean \pm SEM, n = 6 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control group; #p < 0.05, compared to the DOX treatment group.

into the left ventricle myocardium at multiple points. AAV-let-7i_{vp} group, AAV9 vector carrying let-7i_{vp} (3×10^{10} per mouse) was injected through the tail vein. Recombinant AAV9 vectors carrying let-7i or GFP were manufactured by GeneChem (Shanghai, PR China).

Echocardiography

Cardiac function was assessed by transthoracic echocardiography (Visual Sonics, USA) 2 weeks later. Mice were anesthetized with isoflurane (1.5% in air) and monitored for respiratory frequency and temperature. End diastole was measured at the time of the apparent maximal

left ventricle (LV) diastolic dimension, and end systole was measured at the time of the most anterior systolic excursion of the posterior wall. LV internal dimensions at end-diastole (LVIDd) and at end-systole (LVIDs) were measured digitally on the M-mode tracings from 3 cardiac cycles. LV ejection fraction (LVEF) and LV fractional shortening (LVFS) were calculated accordingly.

Cell Culture

The human cardiomyocyte cell line AC16 was obtained from the American Type Culture Collection (USA) and cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin solution under 5% CO₂ at 37°C. After reaching 60%–70% confluence, AC16 cells were treated with doxorubicin (1 μ L; 1 μ M) to model cardiac injury *in vitro*. In addition, let-7i mimic or negative control mimic was also transfected into AC16 cells. Briefly, at 60%–70% confluence, the cells were transfected with let-7i mimic (50 nM) using Lipo2000 (RiboBio, PR China) following the manufacturer's instructions. The let-7i mimic and negative control were designed by RiboBio (Guangzhou, PR China). The let-7i mimic was a mature miRNA.

Quantitative RT-PCR

Total RNA was extracted using TRIzol (TaKaRa, Japan), and 1 μ g mRNA or miRNA was transcribed into cDNA using a commercial

Alix, and flotillin) were detected by immunoblotting. The diameter of exosomes (EVs) was measured using DLS, in which the Zetasizer Nano-ZS90 instrument (Malvern, UK) was used to activate the light at a wavelength of $\lambda = 532$ nm. For tracking *in vitro*, exosomes were labeled with the PKH67 Red Fluorescent Cell Linker Kit (Sigma, USA).

Animal Treatment

A total of 24 C57BL/6 male mice (8–10 weeks) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, PR China) and were fed a normal diet under specific pathogen-free (SPF) conditions. All animal procedures were in accordance with the Care and Use of Laboratory Animals published by the National Institutes of Health and approved by the Institutional Ethics Committee of Nanjing Drum Tower Hospital.

All mice were randomly divided into 4 groups, namely, the control group, DOX group, exosome group, and AAV9-let7i^{up} group.³⁹ The latter 3 groups were established as a dilated cardiomyopathy model by administration of doxorubicin hydrochloride (Pfizer Pharmaceutical, USA) via intraperitoneal injection (5 mg/kg) every other day for a cumulative dose of 35 mg/kg.^{40,41} In the exosome group, exosomes (100 μ L per mouse; 1 μ g/ μ L) were injected

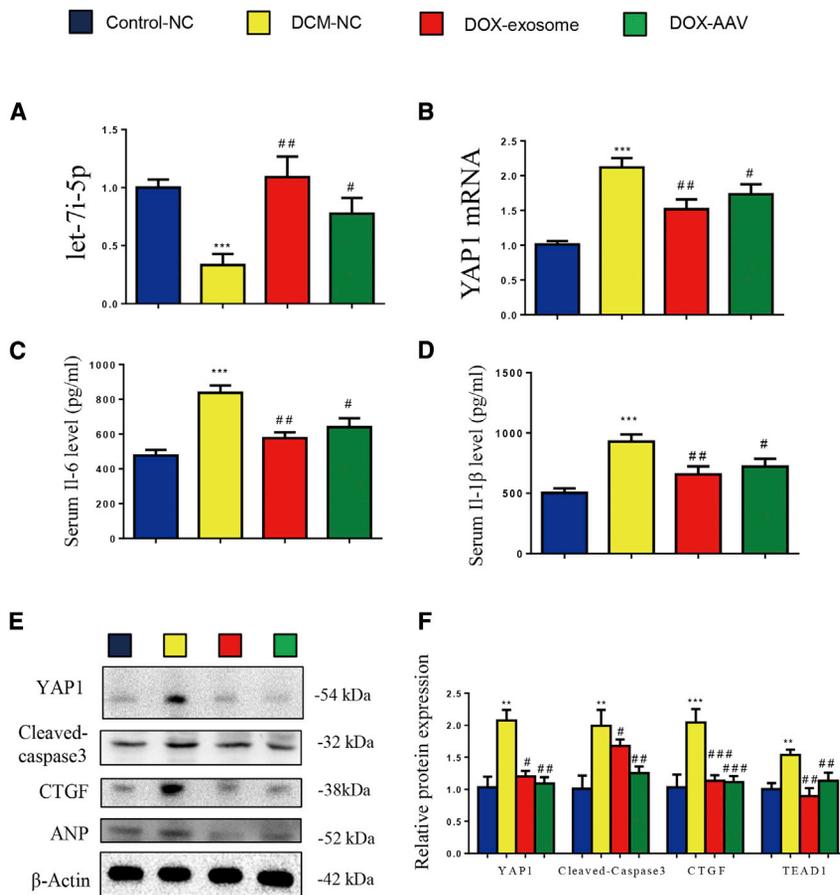


Figure 6. Effect of Exosomes on YAP1/Hippo Signaling Pathway by Delivery of let-7i

8-week-old male mice were divided into four groups: control, DOX, DOX-Exo, and DOX-AAV^{let-7i-5p}. (A) The mRNA expression level of let-7i-5p in myocardial tissue. (B) The mRNA expression level of YAP1 in myocardial tissue. (C) The serum level of IL-6. (D) The serum level of IL-1β. (E) Western blot analysis of YAP1, cleaved caspase 3, CTGF, and ANP. (F) Relative protein levels were analyzed by densitometric analysis. All data are presented as the mean ± SEM, n = 6 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control group; #p < 0.05, compared to the DOX treatment group.

polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Then, the membranes were incubated with primary rabbit anti-mouse antibodies (CD9, flotillin, Hsp70; YAP1, bcl-2, cleaved-caspase 3, CTGF, TEAD1, ANP, and β-actin; Abcam; USA) at a dilution of 1:1,000. After overnight incubation at 4°C, the membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG; 1:10,000) for 1 h at room temperature. The immunobands were visualized using an enhanced chemiluminescence (ECL) detection kit (Beyotime, PR China).

Luciferase Reporter Measurement

The wild-type luciferase reporter was constructed with the 3'-UTR of Yap1 containing let-7i binding sites, while the mutant type had no let-7i binding sites (GeneCopoeia, Rockville, MD, USA). In this vector, the 3'-UTR sequence was inserted downstream of a secreted Gaussia luciferase (GLuc) reporter gene. A chimeric mRNA was transcribed consisting of GLuc and a 3'-UTR target sequence.⁴² The wild-type reporter or mutant reporter and let-7i mimic or the negative controls were transfected into HEK293 cells using Lipo 2000 (RiboBio, PR China). After 24 h, the cell medium was collected for luciferase activity measurement. Firefly luciferase activity was calculated normalized to Renilla luciferase activity. Luciferase activities were detected using a Multimode Detector (Promega).

Cell Apoptosis

The cardiomyocytes obtained from the hearts of the different groups of treated mice were collected at a concentration of 1×10^6 cells/mL. Then, the cells were resuspended in 200 μL of binding buffer, and 5 μL of Annexin-fluorescein isothiocyanate (FITC) and propidium iodide (PI; Keygen, China) were added. After incubation for 30 min in the dark, the cells were analyzed by flow cytometry (BD, USA). The data were analyzed by FlowJo (TreeStar, USA).

Histology Assay

Murine hearts were harvested after 4 weeks. Then, the heart tissues were fixed in 4% paraformaldehyde. After fixation and paraffin

kit (TaKaRa, Japan) according to the manufacturer's instructions. Quantitative RT-PCR was carried out on a real-time system (Applied Biosystems, USA) using SYBR Green PCR Master Mix (TaKaRa, Japan) for mRNA or miRNA Universal SYBR qPCR Master Mix for miRNA (Vazyme, PR China). The primers were as follows: ANP F: 3'-GCTTCCAGGCCATATTGGAG-5'; R: 3'-GGGGGCATGACCTC ATCTT-5'; βMHC F: 3'-ACTGTCAACACTAAGAGGGTCA-5'; R: 3'-TTGGATGATTTGATCTTCCAGGG-5'. Collagen I F: 3'-GCTC CTCTTAGGGGCCACT-5'; R: 3'-CCACGTCTCACCATTGGGG-5'; YAP F: 3'-ACCCTCGTTTTGCCATGAAC-5'; R: 3'-TGTGCTGGGA TTGATATCCGTA-5'; GAPDH F: 3'-AGGTCGGTGTGAACGG ATTTG-5'; R: 3'-TGTAGACCATGTAGTTGAGGTCA-5'; let-7i F: 3'-GGGGTGAGGTAGTAGTTTGT-5'; R: 3'-TGCGTGTCTGGG GTC-5'; U6 F: 3'-CTCGCTTCGGCAGCACA; R: 3'-AACGCTTCAC GAATTTGCGT-5'. U6 and GAPDH were used as internal controls for miRNAs or mRNAs, respectively. The relative expression level for each gene was calculated using the 2^{-ΔΔCt} method.

Western Blotting

Heart tissues, exosomes, and cells were lysed using RIPA buffer (Beyotime, Jiangsu). Protein concentration was measured by the BCA method (Thermo Fisher Scientific, USA). Equal amounts of protein were loaded on SDS-PAGE gels, separated, and then transferred onto

embedding, the cardiac tissues were cut into 5 μm -thick slices. The sections were stained with Masson (Servicebio, PR China) to analyze global heart morphology and cardiac fibrosis. The slices were observed on a high-resolution microscope and a fluorescence microscope (Leica, Japan).

Enzyme-Linked Immunosorbent Assay (ELISA)

Murine blood was collected from the orbital sinus and centrifuged at 3,000 rpm for 5 min at 4°C to acquire serum. The serum IL-1 β and IL-6 levels were measured using commercial ELISA kits according to the manufacturer's instructions (R&D, UK).

Statistical Analysis

Data are presented as the mean \pm standard deviation. All statistical analyses were performed with SPSS (23.0). Differences were analyzed with one-way analysis of variance (ANOVA) for multiple groups and Student's t test for only two groups. $p < 0.05$ was considered statistically significant.

AUTHOR CONTRIBUTIONS

R.G., B.X., and D.S. designed this study; J.N. and Y.L. performed experiments; and K.W., M. W., and L.K. participated in writing the article.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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