circRNA-79530 regulates Twist-mediated mitochondrial damage *via* sponging miR-214 affecting hypoxia/reoxygenation-induced injury in H9c2 cardiomyocytes

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ABSTRACT

Cardiomyocyte injury related to hypoxia/reoxygenation (H/R) is pivotal in myocardial infarction. The circular RNA circRNA-79530 (circ79530) may play a regulatory role in this process, though its exact function has yet to be elucidated. This research explores the role of circRNA-79530 in H9c2 cells under H/R, with a particular focus on its interactions with miR-214 and the transcription factor Twist. It also examines their subsequent effects on mitochondrial function and oxidative stress. H9c2 cardiomyocytes were subjected to H/R to model myocardial injury. We measured circRNA-79530, miR-214, and Twist levels *via* RT-qPCR, with Twist protein *via* Western blotting. ROS levels were quantified using DCFH-DA, and cell viability and injuries were assessed through CCK-8, LDH, SOD, and MDA assays, respectively. Mitochondrial performance was assessed through various methods, including the measurement of mitochondrial membrane potential using JC-1 staining, the quantification of ATP levels, and the examination of the protein levels of mitochondrial complexes as well as the expression of fusion proteins. Our findings indicated that downregulation of circRNA-79530 modulated miR-214 and Twist expression, influencing mitochondrial dynamics and ROS production. Knockdown of circRNA-79530 improved cell viability, reduced oxidative stress and enhanced mitochondrial function. Additionally, overexpression of miR-214 mitigated Twist expression, further supporting the effect of miR-214 in H/R conditions. circRNA-79530 could worse oxidative stress and mitochondrial dysfunction, and regulate twist-mediated mitochondrial damage *via* sponging miR-214 in H9c2 cells under H/R conditions.

Key words: cardiomyocyte injury; hypoxia/reoxygenation; circRNA-79530.

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Introduction

Cardiovascular disease (CVD) has emerged as the leading threat to human health, with acute myocardial infarction (AMI) being the primary cause of death in CVD patients.[1] Rapid restoration of coronary blood flow to relieve myocardial hypoxia remains the most effective treatment for AMI.[2,3] However, this reperfusion can exacerbate pathological damage to the myocardial tissue, a phenomenon known as myocardial ischemia-reperfusion injury (MIRI).[4] Studies have shown that MIRI leads to a series of adverse events with high incidence and mortality rates, particularly among younger populations.[5] Therefore, finding ways to mitigate or prevent MIRI has become a critical challenge in the treatment of myocardial infarction.

One of the key pathogenic mechanisms of ischemia/reperfusion (I/R) injury is the production of large amounts of reactive oxygen species (ROS) following reperfusion. The oxidative stress induced by ROS accumulation is the primary cause of myocardial damage after I/R injury.[5-8] ROS are generated through multiple pathways in myocardial tissue, including fatty acid oxidation, catecholamine oxidation, and mitochondrial oxidation.[9,10] Notably, mitochondrial damage induced by ischemia and hypoxia is recognized as a primary contributor to cardiomyocyte death.[11-13] Mitochondria, which occupy approximately 30% of cardiomyocyte volume, are essential for maintaining cellular function, but when ROS accumulate, they cause oxidative damage to respiratory chain complexes, leading to cell death.[14,15] Furthermore, ROS can activate pathways such as NF-kappa B, MAPK, and type I interferon during reperfusion, triggering inflammatory responses that exacerbate myocardial tissue damage.[16,17] Recent studies have shown that the Twist protein can inhibit apoptosis and alleviate hypoxia-induced damage in trophoblast cells by blocking the mitochondrial apoptotic signaling pathway involving Bax/Bcl-2/Caspase-9/Caspase-3.[18] Twist expression is known to increase in response to hypoxia in I/R models, but its role in ROS production and myocardial I/R injury remains unclear.[19] Understanding the relationship between Twist and reperfusion injury is crucial, as it could reveal novel therapeutic targets for reducing myocardial damage.

Circular RNAs (circRNAs) represent a distinct group of non-coding RNA molecules characterized by the absence of 5' caps and 3' poly(A) tails, and they form closed loop structures through covalent bonding.[20] These molecules serve as miRNA sponges, sequestering miRNAs and modulating the expression of genes they would otherwise target, a process referred to as the competitive endogenous RNA (ceRNA) mechanism. Emerging studies have underscored the importance of the pivotal role of circRNAs in the regulation of a variety of diseases, including those affecting the cardiovascular system. They primarily exert their effects by acting as miRNA sponges, which in turn modulate the activity of downstream genes. miRNAs themselves influence gene expression by interacting with the 3' untranslated region (UTR) of their target mRNAs.[21] For instance, knockdown of circ_SMG6 has been shown to protect against MIRI by inactivating the EGR1/TLR4/TRIF signaling pathway mediated by miR-138-5p.[22] Additionally, CircZNF609 exacerbates MIRI by modulating the miR-214-3p/PTGS2 axis.[23] These findings underscore the significant role of circRNAs as competing endogenous RNAs (ceRNAs) in MIRI.

Building on these findings, our research centered on the Twist gene in rats to identify circRNAs and miRNAs that regulate its expression. We discovered that circRNA-79530 overlaps with the 3' UTR of Twist mRNA, with both RNA sequences aligned in the same direction. Further investigation revealed multiple miRNA recognition sites on the circRNA-79530 sequence, including sites for miR-214 and miR-580. These miRNAs are known to inversely regulate Twist expression, suggesting a complex regulatory network involving circRNA-79530.[24-26] Our previous studies in a renal fibrosis cell model and a mouse model demonstrated that circRNA-79530 regulates Twist expression and influences the epithelial-mesenchymal transition process. Preliminary experiments revealed that both circRNA-79530 and Twist gene expression are higher in the hypoxia/reoxygenation (H/R) cell model compared to controls. Based on these findings, we hypothesize that downregulation of circRNA-79530 may improve ROS-mediated mitochondrial damage and thereby mitigate myocardial reperfusion injury by regulating miR-214 and targeting Twist expression.

Materials and Methods

H/R cell model establishment

A custom cell culture medium was prepared, consisting of 90% DMEM (Dulbecco's Modified Eagle's Medium), supplemented with 10% FBS (Fetal Bovine Serum, VISTECH, SE100-01, Hartford, CT, USA) and 1% penicillin-streptomycin-gentamicin (Gibco, 15140-122, Rockville, MD, USA). H9c2 rat cardiomyocytes were obtained from the China Center for Type Culture Collection (CCTCC: GDC0606) and were cultured in the previously described normal culture medium in a controlled environment incubator at 37°C under a 5% CO_2 atmosphere; to ensure optimal cell health, the medium was refreshed at intervals of 1-2 days, as recommended by established protocols.[27] After 24 h of standard culture, the culture medium was switched to DMEM (without FBS) and the H9c2 were subjected to hypoxic conditions (5% CO_2, 94% N_2, 1% O_2) for 12 h. Following this, the H9c2 were restored to normoxic conditions (5% CO_2 and 95% air) for an additional 2 h to establish the H/R cell model.[27,28]

Cell transfection and grouping

H9c2 cells ($4x10^5$ cells/well) were transfected with 50 nM of the respective agents as per the experimental grouping. Transfections were performed using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. After incubation at 37°C for 48 h, cells were harvested for subsequent experimental analysis.[28] The experiment groups were as follows in different stages: Control group: H9c2 cells were cultured in a standard medium throughout the experiment. H/R group: In the H/R group, H9c2 cells were subjected to the H/R protocol for establishing the H/R model, as detailed above. H/R+sh-NC group: concurrent with the H/R treatment, H9c2 cells were trans-

Table 1. Primer sequences for RT-qPCR (rat).

Primer	Sequence(5'-3')	Product(bp)
circ79530-F	AGTTATCCAGCTCCAGAGTCT	165
circ79530-R	AGATCCGGTGTCTAAATGCAT	165
miR-214-F	GCCGAGAGTTGTCATGTGTCTAA	75
miR-214-R	GCTGTCAACGATACGCTACGTAAC	75
Twist -F	TACGAGGAGCTGCAGACACA	130
Twist -R	TCTTGCTCAGCTTGTCCGAG	130
U6-F	TCGCTTCGGCAGCACATATAC	99
U6-R	ATATGGAACGCTTCACGAATTTG	99
GAPDH-F	GCATCCTGGGCTACACTGAG	162
GAPDH-R	CCACCACCCTGTTGCTGTAG	162

fected with a lentivirus carrying non-specific shRNA (sh-NC). H/R+sh-79530 group: during H/R treatment, cells were transfected with lentivirus carrying shRNA targeting circRNA-79530. H/R+NC inhibitor group: H9c2 cells underwent H/R treatment while being treated with a non-specific shRNA inhibitor (NC inhibitor). H/R+miR-214 inhibitor group: H9c2 cells were treated with a miR-214 inhibitor during H/R treatment. H/R+sh-79530+miR-214 inhibitor group: H9c2 cells received both shRNA targeting circRNA-79530 and a miR-214 inhibitor during H/R treatment. H/R+mimics NC group: after H/R treatment, H9c2 cells were supplemented with non-specific miRNA mimic controls (mimics NC). H/R+miR-214 mimics group: H9c2 cells were treated with miR-214 mimics following H/R treatment. H/R+sh-79530+NC inhibitor group: after treatments with H/R and sh-79530, cells were additionally treated with a non-specific inhibitor to control non-specific effects. H/R+miR-214 mimics+oe-NC group: after H/R treatment, H9c2 cells were treated with miR-214 mimics to increase miR-214 levels and transfected with an empty vector as an overexpression control. H/R+miR-214 mimics+oe-Twist group: following H/R treatment, cells received both miR-214 mimics and a plasmid overexpressing Twist.

RT-qPCR

RNA was extracted from H9c2 cells utilizing the RNAiso Plus Reagent (catalog number 9109; TaKaRa, Tokyo, Japan). The RNA concentration was determined, and the purity was measured by using the A260/A280 ratio with a microspectrophotometer (Micro Drop; BIO-DL Corporation, Shanghai, China). To determine the expression levels of specific genes, including circRNA-79530, miR-214, and Twist mRNA, the RNA was reverse transcribed into cDNA using the Hifair® II 1st Strand cDNA Synthesis Kit (11119ES60; Yeason, Shanghai, China). DNA levels were quantified by conducting RT-qPCR on the StepOnePlus platform (ABI, ViiA 7, Foster City, CA, USA), using the synthesized cDNA as the template. The RT-qPCR protocol involved an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at the optimal temperature (Tm) for 30 s, extension at 60°C for 5 s, and a final step at 95°C for 30 s. The relative gene expression data were analyzed using the $2^{-\Delta\Delta Cq}$ method.[29]

Western blot analysis

H9c2 cells from various experimental groups were washed with PBS twice prior to being lysed using a cell lysis buffer supplemented with a comprehensive protease inhibitor mixture. Following a 30-min incubation period on ice, the samples were subjected to centrifugation at 10,000 rpm for 15 min to separate the supernatant. The supernatant's protein content was determined employing the BCA assay kit (BL521A; Biosharp, Hefei, China). For electrophoresis, approximately 20 μg of protein was applied to a 15% SDS-PAGE and then electrotransferred onto PVDF membrane (IPVH00010A; Merck Millipore, Billerica, MA, USA) at a constant current of 200 mA for 90 min. The membranes were then blocked with either 5% BSA or 5% non-fat dry milk for 2 h before being incubated with specific primary antibodies at 4°C overnight. The primary antibodies, all sourced from Proteintech Group and diluted according to the manufacturer's recommendations, targeted proteins such as Twist (1:2000), NDUFA9, SDHA, UQCRB, MTCO1, OPA1, Mfn1, Mfn2, Cytc, and GADPH (1:50000). The antigen-antibody interactions were visualized using peroxidase-labeled secondary antibodies (1:5000) and the immunoblots were developed with an ECL chemiluminescence detection reagent (BL520B; Biosharp, Hefei, China).[30] Band intensities were quantified using ImageJ software by measuring the integrated density of bands, normalized to loading controls with triplicate measurements for reproducibility.

ROS determination

To assess the alterations in ROS level change within H9c2 cells caused by H/R injury, the DCFH-DA fluorescent probe (MCE, 4091-99-0, Shanghai, China) was employed. The cells were prepared, and the DCFH-DA probe was loaded *in situ* by mixing it with serum-free medium (1:1000), achieving a final concentration of 10 μM. The cells were incubated with this DCFH-DA solution for 20 min at 37°C within a controlled incubator. After incubation, unbound DCFH-DA was removed by washing the cells thrice with serum-free medium. Finally, the cells were collected, and ROS levels were measured utilizing a fluorescence microplate reader (SpectraMax M5, Eugene, OR, USA).

Cell viability assay

The impact of H/R injury on H9c2 cell viability was evaluated using the CCK-8 assay kit (BS350C; Biosharp). Cells from various experimental groups were plated in 96-well plates with a concentration of 5×10^4 cells per well and incubated for different durations: 0 h, 24 h, 48 h, and 72 h. For each time interval, the CCK-8 solution was introduced into the wells in accordance with the supplier's guidelines to evaluate cell viability. After the incubation period, the absorbance at 450 nm for each well was recorded using a microplate spectrophotometer (model MK3; Thermo Fisher Scientific, Waltham, MA, USA). The calculation method of cell viability was referred to the manufacturer's instructions.

ELISA

The total protein concentration was determined using a BCA protein assay kit (BL521A; Biosharp,). The level of lactate dehydrogenase (LDH) in H9c2 cell supernatant (BL853B; Biosharp,), as well as levels of superoxide dismutase (SOD) (BC5165; Solarbio, Beijing, China) and malondialdehyde (MDA) (BC0020; Solarbio) in H9c2 cells, were measured using ELISA kits. The steps were all followed as per the guidelines provided by the manufacturer.

Flow cytometric evaluation of apoptotic cells

To evaluate apoptosis in H9c2 cells from different experimental groups, cells were first washed with PBS, digested using EDTA-free trypsin, and gently resuspended by pipetting. After centrifugation at 1500 rpm for 5 min, the supernatant was discarded. Cells were then resuspended in pre-cooled PBS, centrifuged again, and resuspended in diluted binding buffer to a final concentration of 1×10^6 cells/mL. Annexin V-FITC (BL110A; Biosharp) was added and the cells were incubated in the dark at room temperature for 10 min. Propidium iodide (PI) was subsequently added before flow cytometric analysis. Apoptotic cells were immediately analyzed using a BD FACSCanto™ II flow cytometer (BD Biosciences, San José, CA, USA). A total of 10,000 events per sample were recorded. Each experiment was performed in triplicate, and data were analyzed to quantify early and late apoptotic cell populations based on Annexin V-FITC (green fluorescence) and PI (red fluorescence) staining.[31]

Dual-luciferase assay

To explore the regulatory interaction between circRNA-79530/ Twist and miR-214, a dual-luciferase reporter assay can be utilized with minor modifications according to the literature.[32] The binding sites between circRNA-79530/ Twist and miR-214 were predicted *via* CircInteractome (https://circinteractome.nia.nih.gov/). This procedure involved constructing a vector containing the 3' UTR of circRNA-79530/ Twist linked to a luciferase reporter gene. The vector was transfected into 293T cells pre-seeded in a 96-well plate, reaching 70-80% confluency, along with miR-214 mimics or a non-targeting control using LipoTransTM Plus. After 48 h of

incubation at 37°C with 5% CO_2, the luciferase activity was measured. This assay quantitatively evaluated the interaction effects by measuring both firefly and *Renilla* luciferase activities, providing insights into the binding and regulatory impacts of miR-214 on circRNA-79530.

Evaluation of the cellular ATP content

To determine the ATP content in H9c2 cells, the assay was performed using a commercial ATP detection kit (BC0300; Solarbio). Initially, the cells underwent lysis using the provided buffer, tailored for effective cell disruption and release of ATP. Subsequently, the resulting lysates were mixed with an ATP detection reagent specific to this assay. The luminescence intensity, which correlates with ATP concentration, was measured using a microplate spectrophotometer (model MK3; Thermo Fisher Scientific). This method provides a quantitative evaluation of cellular energy levels, reflecting mitochondrial health and functionality.

Mitochondrial membrane potential (MMP) determination

To evaluate the MMP in H9c2 cells, the cells were stained with JC-1 dye following the protocol provided by the assay kit (M8650; Solarbio), with minor adjustments based on previously published methods.[33] This dye selectively accumulates in mitochondria and undergoes a color shift according to the membrane potential: high MMP is indicated by red fluorescence (excitation 525 nm, emission 590 nm), while lower MMP is marked by green fluorescence (excitation 485 nm, emission 529 nm). Following staining, the cells were examined under a Nikon Eclipse Ti 207 microscope at 100× magnification. The fluorescence intensity was assessed by capturing images of the cells, which were then analyzed using image analysis software to quantify the average fluorescence intensity within each microscope field. The ratio of red to green fluorescence intensity was calculated to evaluate the MMP.

Statistical analysis

Data were presented as the mean value ± SD of 3 replicates. Statistical analyses were conducted using one-way ANOVA through Statistic Package for Social Science (SPSS) 24 (IBM Corp., Armonk, NY, USA). The findings revealed that the differences observed were statistically significant with $^{**}p<0.01$.

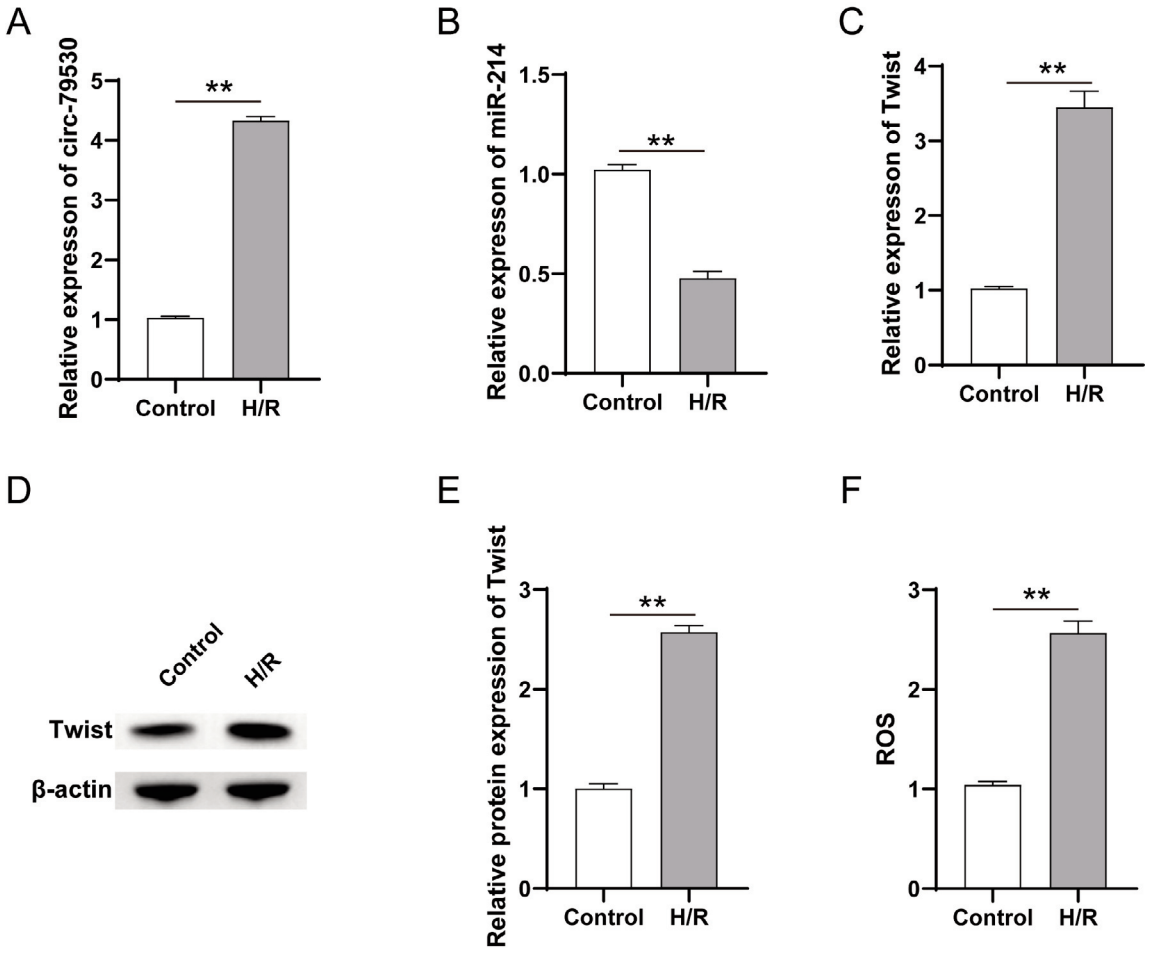

Figure 1. Relative gene expression changes of circ79530, miR-214, and Twist, and ROS levels in H9c2 cells stimulated by H/R. Relative gene expression levels of circRNA-79530 (**A**), miR-214 (**B**), Twist (**C**) in H9c2 cells treated with H/R compared to control. Relative gene expression levels of miR-214 in H9c2 cells under H/R conditions. **D,E**) The protein levels of circRNA-79530 in H9c2 cells following H/R treatment. **F**) The ROS levels in H9c2 cells subjected to H/R compared to control conditions. Control, untreated H9c2 cells; H/R, H9c2 cells subjected to H/R conditions.

Results

Effects of H/R on circRNA-79530, miR-214, Twist, and ROS levels in H9c2 cells

In order to evaluate the impact of the H/R model on H9c2 cells, the relative gene expression of circRNA-79530, miR-214, Twist and the levels of ROS were assessed. We observed significant upregulation of circRNA-79530, miR-214, and Twist gene expression in the H/R-treated group compared with the control group (Figure 1 A,C, $p<0.01$). Additionally, as illustrated in Figure 1 D,E, the protein level of Twist in H9c2 cells subjected to H/R was significantly elevated compared to those maintained under control conditions ($p<0.01$). We also confirmed that H/R stimulation significantly increased ROS levels in H9c2 cells (Figure 1F, $p<0.01$). These results suggested that the H/R condition induced an increase in the levels of circRNA-79530, miR-214, Twist, and ROS.

circRNA-79530 knockdown restored oxidative damage in H9c2 cells after H/R stimulation

To investigate the impact of circRNA-79530 on oxidative damage in H9c2 cells stimulated by H/R, we constructed lentiviruses carrying shRNAs targeting either a control sequence (sh-NC) or circRNA-79530 (sh-circRNA-79530). These were used to infect H9c2 cells under conditions simulating H/R. As depicted in Figure 2A, we designed three shRNAs to evaluate their transfection efficiency by assessing changes in the expression levels of circ79530. The shRNA showing the highest transfection efficiency, sh-circRNA-79530, was selected for further experimental analysis. As demonstrated in Figure 2B, the gene expression of circ79530 was significantly down-regulated following infection with sh-79530 in H9c2 cells compared with that infection with sh-NC ($p<0.01$). From Figure 2C, the effects of circ79530 knockdown on cell proliferation were observed over five days. The CCK-8 assay revealed that cells infected with sh-circRNA-79530 maintained closer to normal proliferation rates compared to those subjected to H/R treatment alone. As shown in Figure 2 D,E, we further explored the biochemical impacts of circ79530 knockdown. In Figure 2D, LDH activity, was significantly lower in the sh-circRNA-79530 group compared to both the H/R and H/R+sh-NC groups, suggesting circRNA-79530 has restored cell injury in H/R stimulation effectively. Additionally, a significant reduction in ROS level was observed in the sh-circRNA-79530-treated cells

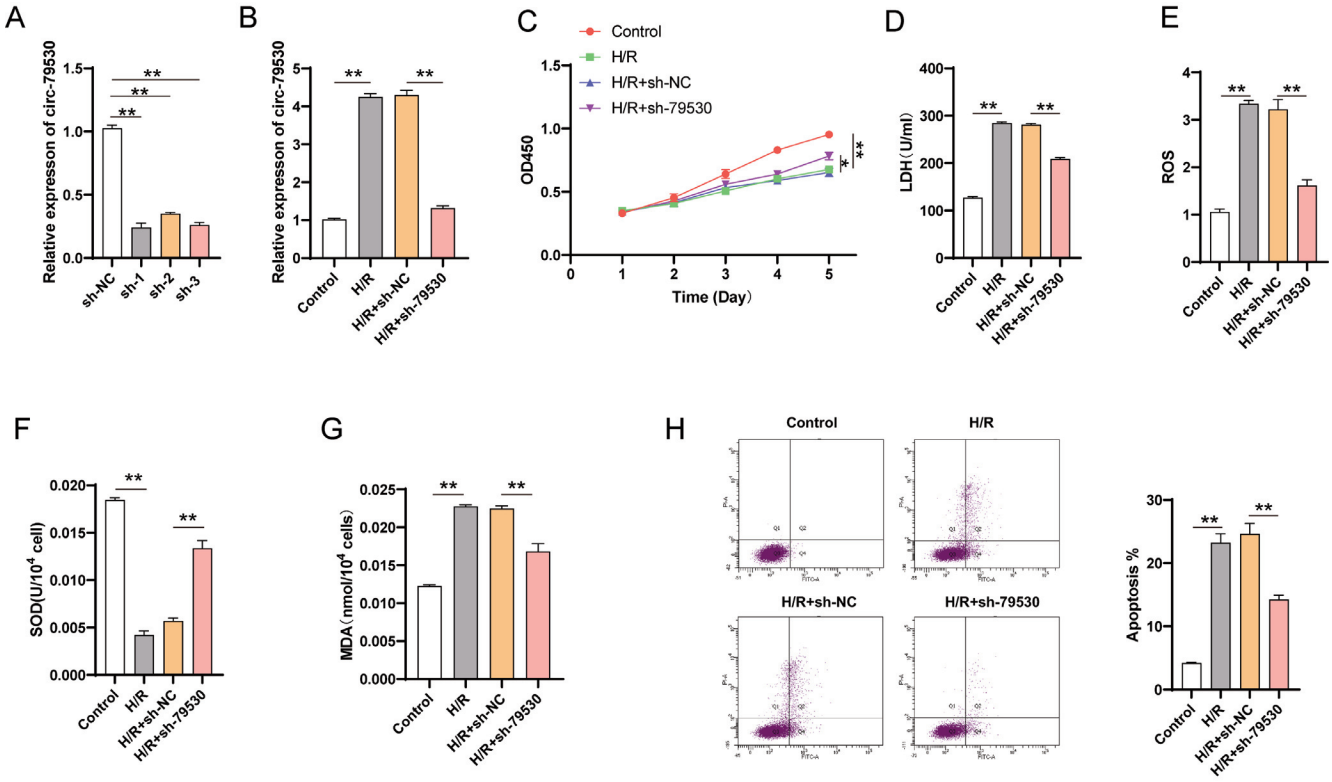

Figure 2. The influence of circRNA-79530 knockdown on oxidative damage in H9c2 cells stimulated by H/R. **A**) Evaluation of transfection efficiency of shRNAs targeting circRNA-79530. **B**) Gene expression level change of circRNA-79530 in H9c2 cells following infection with sh-circRNA-79530 compared to sh-NC. **C**) Cell proliferation rates over five days as assessed by the CCK-8 assay under different conditions: Control group, H/R group, H/R + sh-NC group, and H/R + sh-79530 group. **D**) LDH activity in H9c2 cells under different conditions as described before. **E**) Reactive ROS levels in H9c2 cells under different conditions as described before. **F**) SOD levels in H9c2 cells under different conditions as described before. **G**) MDA levels in H9c2 cells under different conditions as described before. **H**) Flow cytometry analysis of apoptosis levels in H9c2 cells under different conditions as described before.

(p<0.01), underscoring the protective role of circ79530 knockdown against oxidative stress induced by H/R. In Figure 2F, it is illustrated that SOD activity was significantly enhanced in H2ce cells treated with sh-circRNA-79530 compared to the H/R and H/R + sh-NC groups, indicating enhanced antioxidative defense mechanisms upon circ79530 knockdown. MDA activity followed a similar trend, with a marked increase in the H/R + sh-circRNA-79530 group compared to those only subjected to H/R or H/R + sh-NC. This elevation in catalase activity further corroborates the protective effect of circ79530 knockdown against oxidative stress induced by H/R treatment (p<0.01). As shown in Figure 2G, the MDA levels were significantly reduced in the H/R + sh-circRNA-79530 group compared to both the H/R and H/R + sh-NC groups. This reduction indicated a decrease in lipid peroxidation and suggests a protective effect of circ79530 knockdown against oxidative stress induced by H/R treatment. In Figure 2H, the effects of circRNA-79530 knockdown on apoptosis were analyzed. Flow cytometry results showed the apoptosis levels in H9c2 cells under different conditions. The percentage of apoptotic cells, as indicated by the population in the upper-right quadrant, was significantly

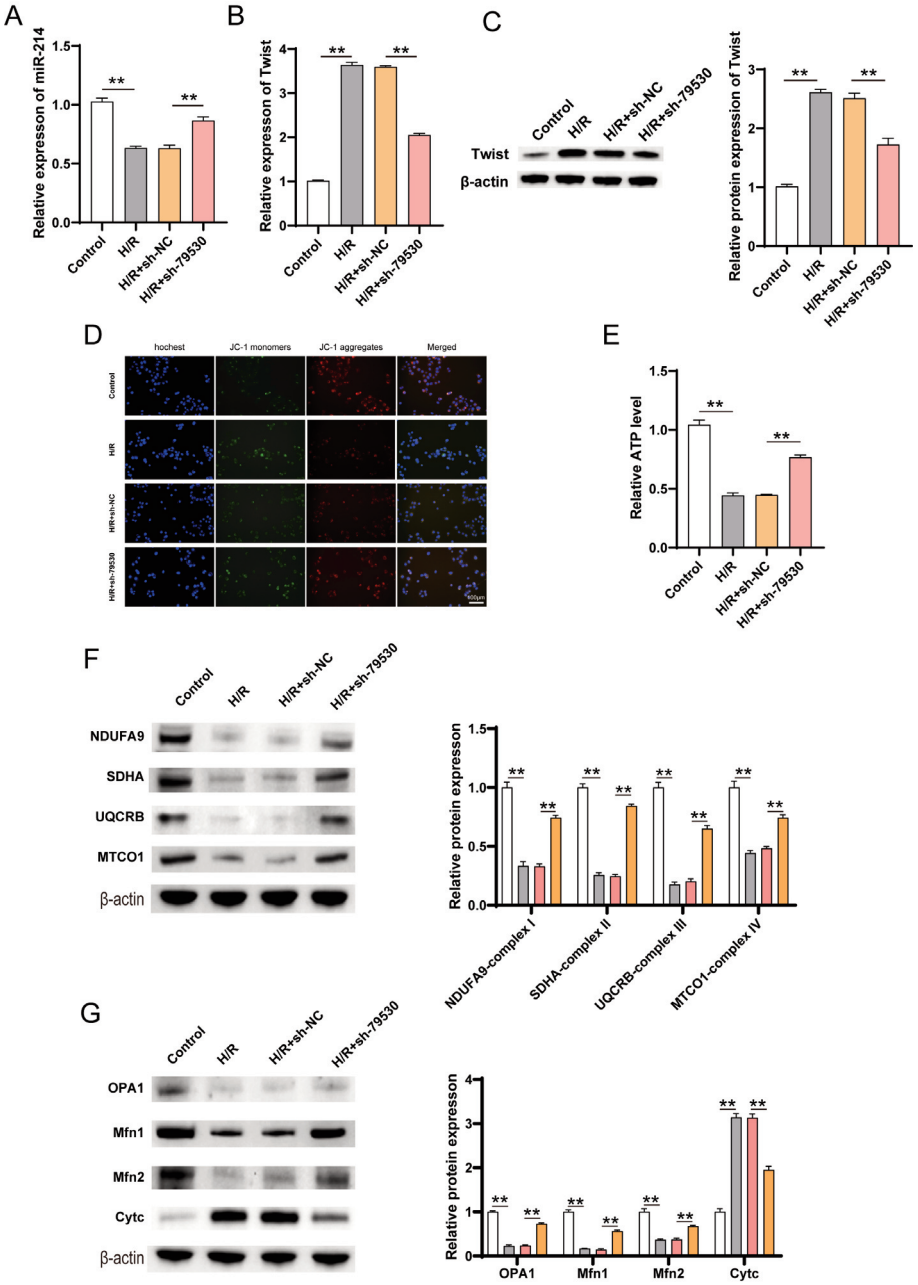

Figure 3. Impact of circRNA-79530 knockdown on miR-214, Twist expression, and mitochondrial function in H9c2 cells stimulated by H/R. **A**) miR-214 gene expression levels in H9c2 cells under different treatment conditions: Control, H/R, H/R + sh-NC, and H/R + sh-79530. **B**) Twist gene expression levels in H9c2 cells under different treatment conditions as described before. **C**) Twist protein levels in H9c2 cells under different treatment conditions as described before. **D**) JC-1 staining showing mitochondrial membrane potential; scale bar: 100 μm. **E**) Relative ATP levels in H9c2 cells under different treatment conditions as described before. **F**) Protein levels of mitochondrial respiration complex proteins in H9c2 cells under different treatment conditions as described before. **G**) Protein levels of mitochondrial dynamics markers and cytochrome c in H9c2 cells under different treatment conditions as described before.

higher in the H/R and H/R + sh-NC groups compared to the control. However, knockdown of circRNA-79530 (H/R + sh-79530) markedly reduced the percentage of apoptotic cells compared to the H/R and H/R + sh-NC groups (Figure 2H, $p<0.01$), indicating that circRNA-79530 knockdown can mitigate H/R-induced apoptosis. Collectively, these results underscore the potential of circRNA-79530 as a therapeutic target for ameliorating oxidative damage and enhancing cell survival under H/R conditions. Further studies will be aimed at elucidating the molecular pathways involved in this protection, with a particular focus on the modulation of apoptosis and oxidative stresses.

circRNA-79530 knockdown modulated miR-214, Twist, and mitochondrial function in H9c2 cells under H/R conditions

To investigate the effects of circRNA-79530 on miR-214, Twist, and mitochondrial function in H9c2 cells, various assays were conducted using lentiviruses carrying shRNAs targeting either a control sequence (sh-NC) or circRNA-79530 (sh-circRNA-79530). As shown in Figure 3A, the gene expression of miR-214 was significantly reduced in the H/R group compared to the control ($p<0.01$). However, in the H/R + sh-circRNA-79530 group, miR-214 expression was restored significantly ($p<0.01$), indicating that knockdown of circRNA-79530 upregulated miR-214 expression. In Figure 3B, the gene expression of Twist, a known target of miR-214, was examined. The H/R group exhibited increased Twist gene expression compared to that of the control group ($p<0.01$), while the knockdown of circRNA-79530 significantly reduced Twist expression, consistent with the regulatory relationship between miR-214 and Twist. Figure 3C showed an increase in Twist protein levels in H2ce cells following H/R ($p<0.01$). However, the H/R + sh-circRNA-79530 group exhibited a significant decrease in Twist protein levels compared to the H/R and H/R + sh-NC groups ($p<0.01$). It has been illustrated the impact of circRNA-79530 knockdown on mitochondrial function using JC-1 staining in Figure 3D. Mitochondrial membrane potential was impaired in the H/R group, indicated by the increased JC-1 monomers (green fluorescence) and decreased JC-1 aggregates (red fluorescence). Knockdown of circRNA-79530 improved mitochondrial membrane potential, as evidenced by the restoration of red fluorescence. Additionally, the relative ATP levels were measured (Figure 3E), showing that the knockdown of circRNA-79530 partially restored ATP production compared to the H/R group. In Figure 3F, the expression of key proteins involved in

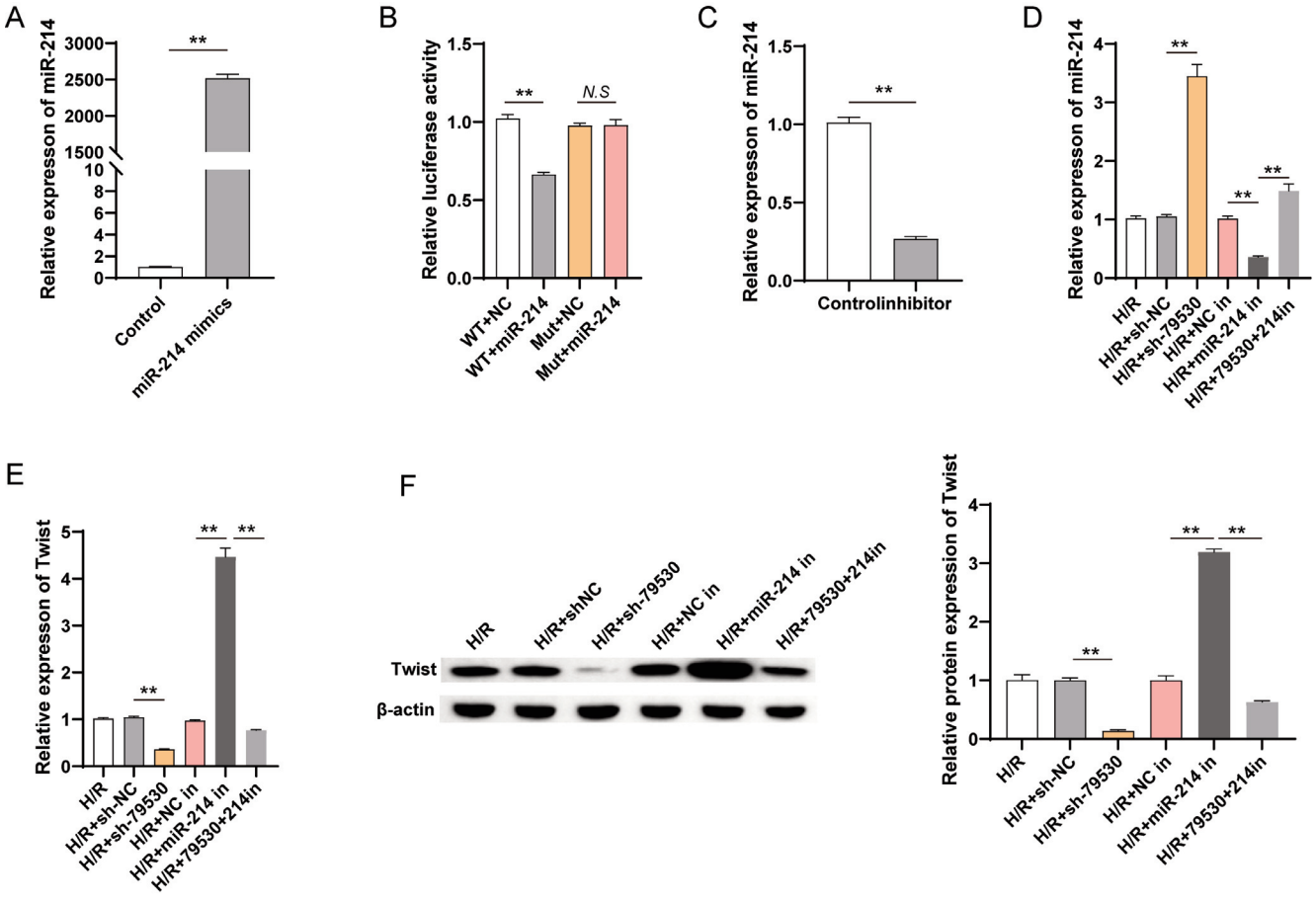

Figure 4. Functional analysis of circRNA-79530, miR-214, and Twist Expression in H9c2 cells stimulated by H/R. **A**) miR-214 expression levels following transfection with miR-214 mimics. **B**) Dual-luciferase reporter assay assessment. (**C**) miR-214 gene expression levels after application of a miR-214 inhibitor. **D**) Relative gene expression levels of miR-214 across different experimental groups: H/R group, H/R+ sh-NC, H/R+sh-79530, H/R+ NC inhibitor, H/R ? miR-214 inhibitor, H/R? 79530+ miR-214 inhibitor. **E**) Twist mRNA levels across different experimental groups as described before. **F**) The protein level of Twist across different experimental groups as described before.

mitochondrial respiration complexes was assessed. The H/R group exhibited a reduction in the expression of NDUFA9 (Complex I), SDHA (Complex II), UQCRB (Complex III), and MTCO1 (Complex IV). However, the knockdown of circRNA-79530 resulted in the restoration of these protein expression levels, suggesting an improvement in mitochondrial function ($p<0.01$). Lastly, Figure 3G showed the effects of circRNA-79530 knockdown on mitochondrial dynamics. The expression levels of OPA1 (a marker for mitochondrial fusion), Mfn1, Mfn2 (markers for mitochondrial fusion), and Cytc (cytochrome c, a marker for apoptosis) were analyzed. The H/R group showed disrupted mitochondrial dynamics, with altered expression of these proteins. Knockdown of circRNA-79530 restored the protein expression of OPA1, Mfn1, and Mfn2, while reducing the release of cytochrome c, indicating a protective role of circRNA-79530 knockdown in maintaining mitochondrial integrity and reducing apoptosis.

circRNA-79530 knockdown modulated Twist expression through miR-214 in H/R-treated H9c2 cells

To further explore the regulatory relationship between circRNA-79530, miR-214, and Twist expression in H/R-treated H9c2 cells, we conducted a series of experiments, the results of which were illustrated in the accompanying results. In Figure 4A, transfection with miR-214 mimics significantly elevated miR-214 expression ($p<0.01$), validating the successful overexpression of miR-214. To determine whether miR-214 directly targets the 3' UTR of Twist, a dual-luciferase reporter assay was performed (Figure 4B). The results indicated that miR-214 significantly suppressed luciferase activity in cells transfected with wild-type Twist (WT + miR-214), whereas this suppressive effect was abrogated in cells transfected with a mutated Twist 3' UTR (Mut + miR-214), confirming that miR-214 targets Twist *via* direct binding. The specific binding site of miR-214 within the 3' UTR of Twist is illustrated by the complementary sequences shown below: Wild-type Twist 3' UTR sequence: 5' ...ATAAATCTATATGACAAAGATTT...3'; rno-miR-214-5p sequence: 5' UCUGUGUACUGUUGAGA 3'. Furthermore, as shown in Figure 4C, the application of a miR-214 inhibitor effectively reduced miR-214 expression compared to the control group ($p<0.01$). The relative gene expression levels of miR-214 across various experimental groups were depicted in Figure 4D. miR-214 expression was upregulated in the H/R + sh-79530 group compared to H/R and H/R + sh-NC groups ($p<0.01$), indicating that the knockdown of circRNA-79530 pro-

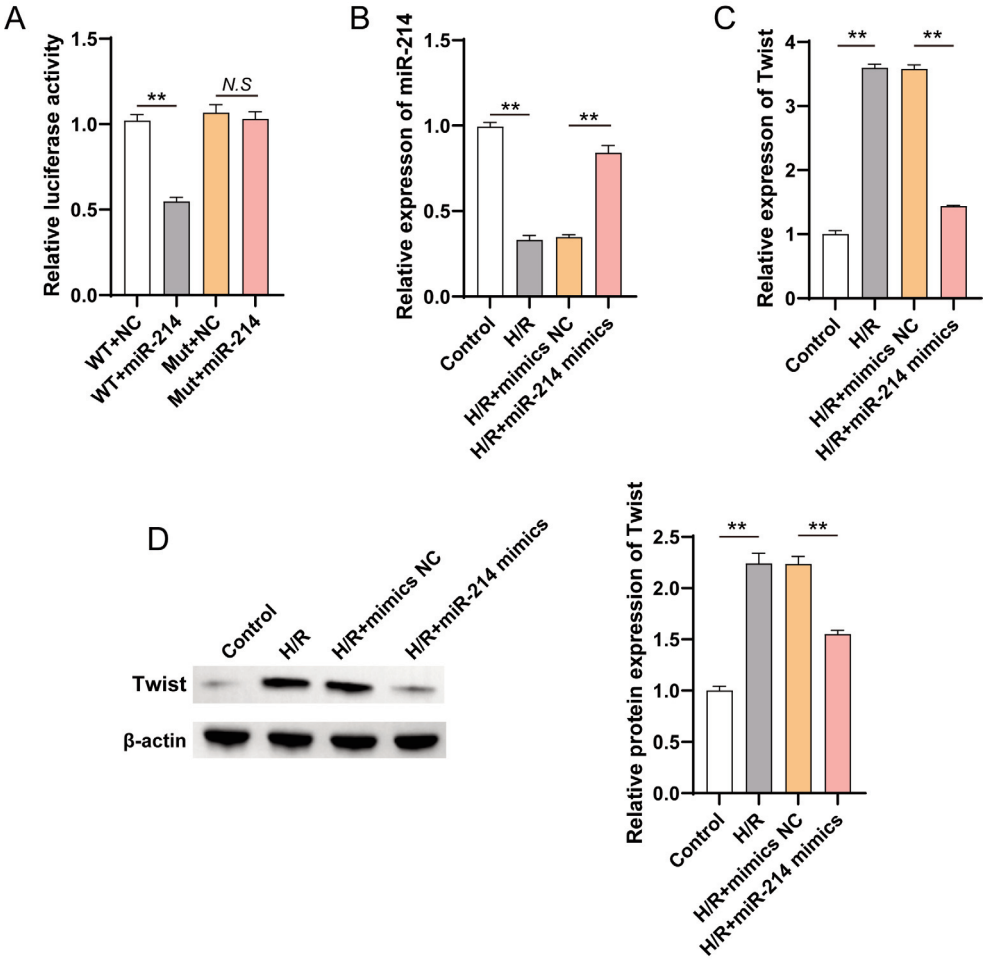

Figure 5. Role of miR-214 in regulating Twist expression in in H9c2 cells stimulated by H/R. **A**) Dual-luciferase reporter assay demonstrating miR-214 targeting of wild-type Twist. **B**) miR-214 gene expression levels in H9c2 cells under different experimental conditions: Control, H/R, H/R +mimics NC, H/R+miR-214 mimics group. **C**) Twist gene expression levels in H/R-treated H9c2 cells under different experimental conditions as described before. **D**) Twist protein levels in H/R-treated H9c2 cells under different experimental conditions as described before.

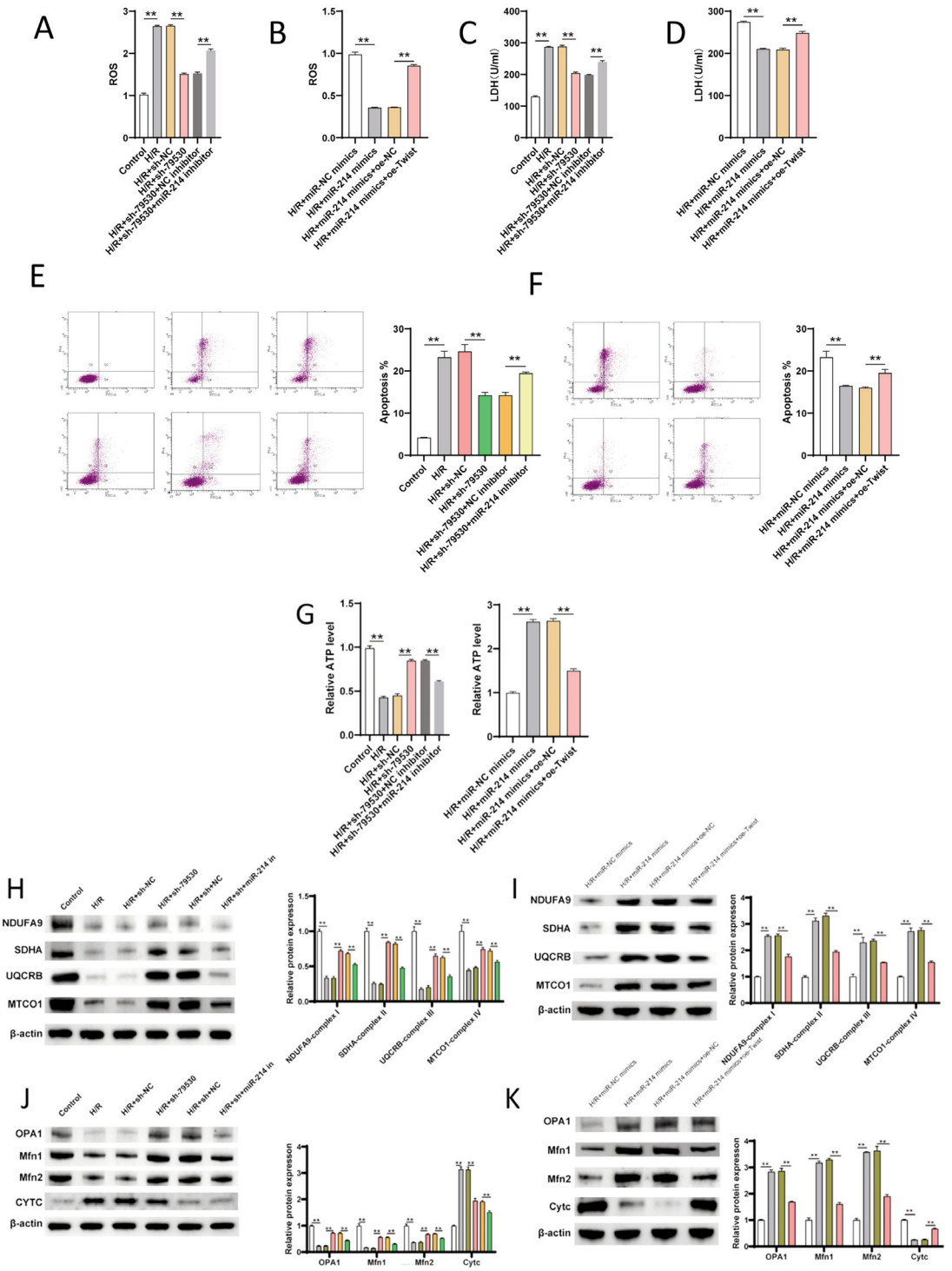

Figure 6. Effect of circRNA-79530 knockdown on ROS production, LDH release, apoptosis, ATP levels, and mitochondrial protein expression in H9c2 cells stimulated by H/R. **A**) ROS levels in H9c2 cells in different experimental groups: Control, H/R, H/R + sh-NC, H/R+sh-79530, H/R+sh-79530+NC inhibitor, H/R+sh-79530+miR-214 inhibitor group. **B**) ROS levels in H9c2 cells in different experimental groups: H/R +miR-NC mimics group, H/R+miR-214 mimics group, H/R+miR-214 mimics +oe-NC group, H/R+miR-214 mimics +oe-Twist group. **C**) LDH release in different experimental groups as described in panel A. **D**) LDH release in different experimental groups as described in panel B. **E**) Flow cytometry analysis of apoptosis and corresponding quantification of apoptotic cells in different experimental groups as described as described in panel A. **F**) Flow cytometry analysis of apoptosis and corresponding quantification of apoptotic cells in different experimental groups as described as described in panel B. **G**) Relative ATP levels indicate cellular energy status under different treatment conditions as described before (left, panel A; right, panel B). **H,J**) Western blot analysis of mitochondrial function-related proteins (NDUFA9, SDHA, UQCRB, MTCO1) and mitochondrial dynamics-related proteins (OPA1, Mfn1, Mfn2, Cytc) in different experimental groups as described panel A. (**I,K**) Western blot analysis of mitochondrial function-related proteins (NDUFA9, SDHA, UQCRB, MTCO1) and mitochondrial dynamics-related proteins (OPA1, Mfn1, Mfn2, Cytc) in different experimental groups as described panel B.

moted miR-214 expression. The addition of miR-214 inhibitor in the H/R + sh-79530 group led to a significant reduction in miR-214 levels compared to the H/R + sh-79530 group without the inhibitor, demonstrating that the effect of circRNA-79530 knockdown on miR-214 expression could be reversed by inhibiting miR-214 activity ($p<0.01$). To further investigate the downstream effects, we assessed Twist gene and protein expressions, as shown in Figure 4E, the gene expression levels of Twist were analyzed in various groups. Notably, Twist mRNA levels were significantly reduced in the H/R + sh-79530 group compared to both the H/R and H/R + sh-NC groups, indicating an effective downregulation through circRNA-79530 knockdown ($p<0.01$). Upon the addition of a miR-214 inhibitor to the H/R + sh-79530 group, Twist mRNA levels were partially restored ($p>0.01$), suggesting that the suppression of Twist is mediated through miR-214 activity. Finally, as depicted in Figure 4F, the Twist protein level followed a similar trend, supporting the hypothesis that miR-214 mediated the effect of circRNA-79530 on Twist expression. Together, these findings elucidate the regulatory axis of circRNA-79530/miR-214/ Twist in the context of H/R-induced injury.

miR-214 regulated Twist expression in H/R-induced H9c2 cell injury

To further investigate whether miR-214 could target Twist in H/R-treated H9c2 cells, we conducted a series of experiments to examine the relationship between miR-214 and Twist expression. As shown in Figure 5A, a dual-luciferase reporter assay confirmed that miR-214 directly targets Twist by significantly reducing luciferase activity in cells transfected with wild-type Twist (WT + miR-214), while no significant reduction was observed in cells transfected with a mutated Twist 3' UTR (Mut + miR-214). The specific binding site of miR-214 within the 3' UTR of Twist is illustrated by the complementary sequences shown below: Wild-type Twist 3' UTR sequence: 5' ...ATAAATCTATATGACAAA-GATTT...3'; rno-miR-214-5p sequence: 5' UCUGUGUACUGU-UGAGA 3'. To assess the effect of H/R treatment on miR-214 expression, we compared miR-214 levels across different experimental groups: Control group, H/R group, H/R+mimics NC, H/R+miR-214 mimics group (Figure 5B). The results demonstrated that H/R treatment led to a substantial downregulation of miR-214 compared to the control group ($p<0.01$). However, the introduction of miR-214 mimics (H/R + miR-214 mimics group) effectively restored miR-214 expression ($p<0.01$). Subsequently, we examined the impact of miR-214 on Twist gene expression levels (Figure 5C), where H/R treatment significantly upregulated Twist expression ($p<0.01$). Notably, overexpression of miR-214 attenuated this upregulation, indicating that miR-214 can negatively regulate Twist expression at the mRNA level. Finally, as shown in Figure 5D, Twist protein levels were elevated following H/R treatment but were significantly reduced upon miR-214 overexpression. These results collectively suggested that miR-214 targets and downregulates Twist, thereby playing a critical role in modulating the cellular response to H/R-induced injury in H9c2 cells.

circRNA-79530 downregulation ameliorated cellular and mitochondrial damage through the miR-214/ Twist axis

To further investigate whether the downregulation of circRNA-79530 mediates its effects on cellular and mitochondrial damage through the miR-214/ Twist axis under H/R conditions, we conducted a series of experiments. As shown in Figure 6 A,B, ROS levels were significantly elevated in cells subjected to H/R compared to the control group ($p<0.01$). However, the knockdown of circRNA-79530 led to a marked decrease in ROS levels. This protective effect was reversed by miR-214 inhibition, suggesting that circRNA-79530 may reduce oxidative stress through miR-214-mediated targeting of Twist ($p<0.01$). In the H/R + miR-214 mimics group, ROS levels were also reduced compared to the H/R group, supporting the role of miR-214 in oxidative stress regulation. This reduction was reversed in the H/R + miR-214 mimics + oe-Twist group ($p<0.01$), indicating that Twist overexpression can counteract the protective effects of miR-214 overexpression. H9c2 cells exposed to H/R conditions exhibited increased LDH release (Figure 6 C,D), indicating significant cell damage. The knockdown of circRNA-79530 reduced LDH release, which was again reversed by miR-214 inhibition ($p<0.01$). LDH release was significantly reduced compared to the H/R group ($p<0.01$), highlighting the protective role of miR-214. This protective effect was diminished in the H/R + miR-214 mimics + oe-Twist group ($p<0.01$), where LDH levels were restored, further implicating Twist as a key mediator of cellular damage under these conditions. It depicted flow cytometry analysis of apoptosis, with corresponding quantification in the bar graphs (Figure 6 E,F). H/R conditions treatment notably elevated the proportion of cells undergoing apoptosis ($p<0.01$). This increase in apoptosis was attenuated by circRNA-79530 knockdown, while inhibition of miR-214 diminished the anti-apoptotic effect, implicating the miR-214/ Twist pathway in the regulation of cell survival ($p<0.01$). Similarly, in the H/R + miR-214 mimics group, apoptosis was reduced compared to the H/R group, consistent with miR-214's role in promoting cell survival. However, in the H/R + miR-214 mimics + oe-Twist group, apoptosis levels were partially restored, indicating that Twist overexpression can counteract miR-214's protective effects on apoptosis. ATP levels, as depicted in Figure 6G, were substantially reduced under H/R conditions, reflecting compromised cellular energy status. circRNA-79530 knockdown restored ATP levels, and this effect was attenuated by miR-214 inhibition ($p<0.01$). In the H/R + miR-214 mimics group, ATP levels were also restored compared to the H/R group ($p<0.01$), further supporting miR-214's role in energy homeostasis. This restoration was reversed in the H/R + miR-214 mimics + oe-Twist group ($p<0.01$), suggesting that Twist overexpression can interfere with the beneficial effects of miR-214 on cellular energy status. Finally, Figure 6H-K demonstrated that mitochondrial function-related proteins (NDUFA9, SDHA, UQCRB, MTCO1) and mitochondrial dynamics-related proteins (OPA1, Mfn1, Mfn2, Cytc) were dysregulated under H/R conditions ($p<0.01$). Knockdown of circRNA-79530 restored the expression of these proteins ($p<0.01$), while the protective effect was diminished by miR-214 inhibition. In the H/R + miR-214 mimics group, the expression of these mitochondrial proteins was also improved compared to the H/R group, underscoring the role of miR-214 in maintaining mitochondrial integrity. However, in the H/R + miR-214 mimics + oe-Twist group, these protein levels were partially reduced, further confirming Twist's involvement in mitochondrial dysfunction under H/R stress. These results collectively suggested that circRNA-79530 downregulation ameliorates cellular and mitochondrial damage in an H/R model by modulating the miR-214/ Twist axis, providing insights into potential therapeutic strategies for conditions characterized by oxidative stress and mitochondrial dysfunction.

Discussion

In the present study, we explored the effect of circRNA-79530 in modulating cellular and mitochondrial damage in cardiomyocytes under H/R conditions. Our findings demonstrate that downregulation of circRNA-79530 exerts a significant protective effect against H/R-induced oxidative stress, cellular injury, and mitochondrial dysfunction, primarily through the miR-214/Twist axis.

By investigating circRNA-79530, we aim to uncover novel interventions for I/R injury, which is crucial in the pathophysiology of ischemic heart disease.

H/R is a well-established model for simulating I/R injury, a critical event in the pathophysiology of ischemic heart disease.[34,35] Notably, ROS accumulation during reoxygenation is critical in driving cellular and mitochondrial damage, leading to mitochondrial function impairment and apoptosis.[36,37] In our study, circRNA-79530 knockdown markedly attenuated these effects, reducing ROS production, LDH release, and apoptotic cell count. This positions circRNA-79530 as a potential protective factor in I/R scenarios. LDH elevation, a hallmark of cellular injury in the H/R model, signifies compromised cell membrane integrity.[38,39] Concurrently, apoptosis acts as a crucial ischemic injury response, with ROS as a primary trigger for the mitochondrial-dependent cell death pathways.[40] Our results show that the modulation of circRNA-79530 emerges as a promising therapeutic avenue, potentially reducing the detrimental impacts of I/R by attenuating oxidative stress and enhancing cell survival. Our results suggest that circRNA-79530 modulation could be a viable therapeutic strategy, potentially diminishing the adverse effects of I/R by alleviating oxidative stress and enhancing cell survival.

The mechanism by which circRNA-79530 exerts these effects appears to involve the regulation of miR-214 and its downstream target, Twist.[25,41] Previous studies have established that miR-214 plays a pivotal role in cardiac protection by targeting pro-apoptotic and stress-responsive genes.[42] Consistent with this, our study shows that inhibiting miR-214 partially reverses the protective effects of circRNA-79530 knockdown, elevating ROS levels, LDH release, and apoptosis rates. This highlights miR-214 as a pivotal mediator in the circRNA-79530 regulatory pathway, influencing the expression of Twist, a transcription factor implicated in cell survival and mitochondrial function.[42-44] Interestingly, we also observed that circRNA-79530 knockdown led to a significant restoration of mitochondrial function, as evidenced by increased ATP production and the re-expression of key mitochondrial proteins such as NDUFA9, SDHA, UQCRB, and MTCO1 that are critically involved in the electron transport chain and are crucial for ATP production and overall cellular energy metabolism.[45-47] NDUFA9 is part of Complex I and initiates electron transfer, while SDHA, a component of Complex II and the TCA cycle, further facilitates this electron flow.[46,48] UQCRB, located in Complex III, continues the transfer process to cytochrome c, and MTCO1, a key component of Complex IV, completes the electron transfer, reducing oxygen to water and helping maintain the proton gradient necessary for ATP synthesis.[49,50] Together, these proteins ensure efficient mitochondrial function and energy production, playing vital roles in maintaining cellular health and function. The mitochondrial membrane potential, assessed by JC-1 staining, was also preserved in the circRNA-79530 knockdown group, further underscoring the role of circRNA-79530 in maintaining mitochondrial integrity under stress conditions. This aligns with the emerging understanding of mitochondria as central regulators of cell death and survival, particularly under ischemic conditions.

The role of Twist in this context is particularly noteworthy. Twist is known to be involved in epithelial-mesenchymal transition and has been associated with increased cell motility and survival in various cancer models.[51] During the hypoxic process, the expression of the Twist gene is activated and in a rat I/R model, the hypoxic conditions can lead to an increased expression of the Twist gene.[19] However, its role in cardiomyocytes under stress conditions has been less well characterized. Our findings suggested that the downregulation of circRNA-79530 leads to decreased Twist expression, which correlated with reduced cellular damage and improved mitochondrial function. This raises the possibility that Twist may act as a detrimental factor in H/R-induced cardiomyocyte injury, potentially through the modulation of mitochondrial dynamics and function.

In conclusion, our study provides new insights into the molecular mechanisms by which circRNA-79530 modulates cellular and mitochondrial responses to H/R stress. By targeting the miR-214/Twist axis, circRNA-79530 downregulation confers protection against oxidative stress and mitochondrial dysfunction, highlighting its potential as a therapeutic target for ischemic heart disease. Future studies should aim to further elucidate the specific pathways involved and explore the therapeutic potential of modulating circRNA-79530 and its downstream effectors *in vivo*.

In this study, we demonstrated that the downregulation of circRNA-79530 plays a crucial protective role in mitigating cellular and mitochondrial damage in cardiomyocytes subjected to H/R stress. Our findings reveal that circRNA-79530 exerts its effects through the miR-214/Twist axis. Specifically, circRNA-79530 knockdown led to a significant reduction in ROS levels, LDH release, and apoptosis, all of which are hallmarks of cellular injury under H/R conditions. Furthermore, the restoration of mitochondrial function, as evidenced by improved ATP levels and the expression of key mitochondrial proteins, underscores the protective impact of circRNA-79530 downregulation against mitochondrial dysfunction. The inhibition of miR-214 partially reversed these protective effects, indicating that miR-214 is a critical mediator in the circRNA-79530/Twist regulatory pathway. Additionally, the downregulation of circRNA-79530 led to decreased Twist expression, which is known to contribute to cellular damage and mitochondrial dysfunction. These findings suggest that targeting the circRNA-79530/miR-214/Twist axis could be a promising therapeutic strategy for reducing oxidative stress and preserving mitochondrial integrity in conditions such as ischemic heart disease.

In conclusion, our study highlights the potential of circRNA-79530 as a novel target for therapeutic intervention in mitigating H/R-induced cardiomyocyte injury, paving the way for further investigations into the molecular mechanisms underlying ischemic heart disease and other related conditions.

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