SOCS-6 promotes mitochondrial fission and cardiomyocyte apoptosis and is negatively regulated by QKI mediated miR-19b

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Research

Keywords: SOCS-6, Mitochondrial fission, Cardiomyocytes, miR-19b, QKI

Posted Date: August 2nd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-49963/v1>

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Abstract

Background

Ischemia/reperfusion (IR) injury following myocardial infarction can result in debilitating complications and morbidity. Mitochondrial dysfunction and abnormal mitochondrial fission have been implicated in the complications associated with IR injury as cardiomyocytes are abundant in mitochondria. SOCS-6 is known to participate in mitochondrial fragmentation but its exact involvement and the pathways associated are uncertain.

Results

In the present study, we examine the biological role and regulation of SOCS-6 in mitochondrial dynamics using hypoxia and reoxygenation (H/R) in cardiomyocytes and with a murine model of IR injury. We found that SOCS-6 inhibition by RNA interference attenuated H/R-induced mitochondrial fission and apoptosis in cardiomyocytes. A luciferase assay indicated that SOCS-6 is a direct target of miR-19b. The overexpression of miR-19b decreased mitochondrial fission and apoptosis in vitro . Moreover, the presence of miR-19b reduced the level of SOCS-6 and the injury caused by IR in vivo . There were less apoptotic cells in the myocardium of mice injected with miR-19b. In addition, we found that the RNA-binding protein, QKI, participates in the regulation of miR-19b expression.

Conclusions

Our results indicate that the inhibition of mitochondrial fission through downregulating SOCS-6 via the QKI/miR-19b/SOCS-6 pathway attenuated the damage sustained by IR. The QKI/miR-19b/SOCS-6 axis plays a vital role in regulation of mitochondrial fission and cardiomyocyte apoptosis and could form the basis of future research in the development of therapies for the management of cardiac diseases.

Background

Through advances in disease management, the mortality rate associated with myocardial infarction (MI) has declined worldwide [1,2]. However, the reperfusion strategies used to treat MI can result in ischemia/reperfusion (IR) injury, which can cause further complications such as chronic heart failure and myocardial dysfunction [3–6]. Several studies have implicated mitochondrial dysfunction and abnormal mitochondrial fission in the complications associated with MI and IR injury [7–9]. High-energy requiring cardiomyocytes are abundant in mitochondria, which occupy 30% of the total cell volume, and are therefore vulnerable to cellular changes that would impact mitochondrial function [10]. The inhibition of functional changes in the mitochondria may prevent some of the complications associated with IR injury.

Changes in the morphology of mitochondria occur frequently, mainly through either fusion or fission, and in response to the cellular environment [11]. Fusion enables adjacent mitochondria to combine and elongate whereas fission enables an equal division of mitochondria during cell division but also the

fragmentation and the removal of damaged mitochondria by mitophagy [12]. Disrupting the balance between fusion or fission by factors such as oxidative stress and ischemia is believed to increase mitochondrial permeability and promote apoptosis, thereby making the heart more vulnerable to IR injury [13]. Transplantation of autologously derived mitochondria into the heart of rabbits before the induction of IR was found to enhance the consumption of oxygen and cardiac function post-injury [14]. Moreover, dynamin-related protein 1 (DRP1), a mitochondrial division inhibitor, administered to rats before IR was able to prevent mitochondrial fragmentation and improve mitochondrial oxygen consumption [15]. Mutations in DRP1 prevent fission and leads to an excessive number of fused mitochondria [16]. Ser637 in DRP1 is a cyclic AMP-dependent kinase phosphorylation site that prevents that prevents DRP1 from being translocated from cytosol to mitochondria, however, calcineurin-dependent dephosphorylation of this site increases translocation [17]. Suppressor of cytokine signaling 6 (SOCS-6) is known to inhibit the phosphorylation of DRP1 thereby promoting its mitochondrial translocation [18]. Consequently, upregulated SOCS-6 induces mitochondrial fragmentation whereas its downregulation increases mitochondrial diffusion.

Recently, there has been interest in the epigenetic mechanisms which control the expression of genes in coronary heart disease and mitochondria [19,20]. Quaking (QKI) is a member of the transduction and activation of RNA (STAR) family of RNA-binding proteins (RBPs) involved in various forms of epigenetic processing, such as pre-mRNA splicing and controlling the stability and turnover of microRNA (miRNA) [21,22]. RBPs such as QKI recognize cis elements in 3' untranslated regions (UTR) and are responsible for the post-transcriptional regulation of multiple functions including maintaining the endothelial barrier, regulating smooth muscle, and responding to inflammatory stimuli [23,24]. Bioinformatic analysis predicted that the 3'-UTR of SOCS-6 mRNA contained a putative target site for miR-19b, which is highly conserved in mammals and among the miRNA specifically regulated by QKI [22,25]. The miR-19b expression levels are decreased when QKI is silenced. Moreover, there is known to be a relationship between miR-19b and patients who have ST-elevated MI and miR-19b is downregulated in the infarct area of a murine model of IR injury [26,27].

In the present study, we assess the involvement of mitochondrial dynamics in IR injury by using a hypoxia and reoxygenation (H/R) model in cardiomyocytes and a murine model of IR. In particular, we examine the roles of SOCS-6, QKI, and miR-19b expression on levels of mitochondrial fission and apoptosis *in vitro* and *in vivo*. Finally, we review the literature associated with the QKI/miR-19b/SOCS-6 axis and discuss whether it may have potential in the improved management of cardiac diseases.

Results

SOCS-6 regulates H/R-induced mitochondrial fission and cardiomyocyte apoptosis in vitro

To determine the effects of SOCS-6 on levels of mitochondrial fission we first examined mitochondrial morphology with different expression levels of SOCS-6 in cardiomyocytes under H/R. Western blot analysis revealed that SOCS-6 levels increased in cardiomyocytes following H/R compared to normoxic

conditions, whereas the levels of DRP1 phosphorylation decreased (Fig. 1a). However, when SOCS-6 is downregulated, levels of DRP1 phosphorylation increased (Fig. 1b). Cardiomyocytes were assessed for injury using an assay to detect LDH leakage from cells. The release of LDH was significantly higher in cells exposed to H/R compared with control, however, cells transfected with SOCS-6 shRNA and subjected to H/R released significantly less LDH ($P<0.05$), indicating less damage (Fig. 1c). An assessment of apoptotic cells using TUNEL confirmed that H/R was severely detrimental to the number of viable cardiomyocytes ($P<0.01$). However, downregulating SOCS-6 resulted in significantly fewer TUNEL-positive cells ($P<0.05$) (Fig. 1d). An analysis of mitochondrial morphology using confocal images and MitoTracker Red indicated a reduced percentage of fragmented mitochondria in cardiomyocytes following H/R with SOCS-6 silenced (Fig. 1e). These results demonstrate that SOCS-6 may be involved in the regulation of mitochondrial fission and apoptosis in HL-1 cardiomyocytes.

SOCS-6 inhibition attenuates myocardial infarction and apoptosis in vivo

We next investigated whether the inhibition of SOCS-6 could attenuate MI and apoptosis *in vivo*. The hearts of adult male C57BL/6 mice were injected with lentivirus containing sh-SOCS-6 or a negative control and then exposed to MI followed by reperfusion. Western blot analysis confirmed that levels of SOCS-6 were reduced in mice with downregulated SOCS-6 following IR and at the same levels of control mice without IR (Fig. 2a). The infarct sizes in sections of myocardia following MI were significantly smaller in hearts with downregulated SOCS-6 (Fig. 2b, c). The myocardium of mice with SOCS-6 silenced appeared healthier with less apoptotic cells, as confirmed in a TUNEL assay (Fig. 2d). Moreover, TEM images of mitochondrial morphology in heart tissue sections with SOCS-6 silenced revealed lower fragmentation (Fig. 2e), confirming that the inhibition of SOCS-6 can attenuate MI and apoptosis in an animal model of IR injury.

miR-19b represses SOCS-6 expression and participates in the regulation of mitochondrial fission and apoptosis

We next considered whether miR-19b could control the regulation of mitochondrial fission and apoptosis by repressing SOCS-6 expression in a similar way to sh-SOCS-6. We mutated the predicted target site of miR-19b in the 3'-UTR of SOCS-6 mRNA and performed a luciferase reporter assay in HEK293T cells (Fig. 3a, b). We observed that relative luciferase activity for the wild-type 3'-UTR of SOCS-6 was significantly lower in the presence of miR-19b, whereas in the presence of anti-miR-19b, the luciferase activity was significantly higher. Additionally, it was evident that mutated 3'-UTR of SOCS-6 had no response to either miR-19b or anti-miR-19b. Western blotting using HL-1 cardiomyocytes transfected with miR-19b mimic or anti-miR-19b confirmed that levels of SOCS-6 were lower when miR-19b is overexpressed whereas they are higher when miR-19b is blocked (Fig. 3c). When HL-1 cardiomyocytes were subjected to H/R, the relative miR-19b levels detected by qRT-PCR were found to gradually decrease over 12 h to levels significantly lower than those under normal conditions (Fig. 3d). Moreover, in HL-1 cardiomyocytes transfected with miR-19b mimic and then exposed to H/R, the release of LDH, apoptosis, and level of fragmented mitochondria are all significantly lower compared with the negative

control ($P < 0.05$) (Fig. 3e–g), indicating that cells are subjected to less injury after H/R when miR–19b is overexpressed. Overall, these results suggest that miR–19b suppresses SOCS–6 and the overexpression of miR–19b generates a phenotype similar to that found with sh-SOCS–6.

Restoration of SOCS–6 reverses miR–19b-mediated inhibition of mitochondrial fission and cardiomyocyte apoptosis

We next examined whether the restoration of SOCS–6 could reverse miR–19b-mediated inhibition of mitochondrial fission and apoptosis in HL–1 cells. Following H/R, the overexpression of SOCS–6 was found to increase the release of LDH, apoptosis, and percentage of cardiomyocytes with fragmented mitochondria (Fig. 4a–d). However, the overexpression of miR–19b can significantly alleviate the increase in damage to cells caused by overexpressing SOCS–6. These results indicate that the restoration of SOCS–6 reverses the miR–19b-mediated inhibition of mitochondrial fission and cardiomyocyte apoptosis, which is further evidence of an association between SOCS–6 and miR–19b.

miR–19b suppresses myocardial infarction and apoptosis in vivo

After establishing that miR–19b suppresses damage to mitochondrial fragmentation and apoptosis *in vitro*, we determined whether it could suppress myocardial infarction and apoptosis in a mouse model of IR injury *in vivo*. miR–19b was injected into the hearts of adult male C57BL/6 mice which were then exposed to MI by ligating the coronary artery for 30 min. After 24 h perfusion, mice were euthanized and the infarct size was analyzed using planar morphometry of heart sections stained with TTC. Representative sections of myocardial infarction assayed by TTC staining are shown in Fig. 5a. Infarct size was significantly reduced in mice that had received miR–19b. Moreover, there were less apoptotic cells in the myocardium of mice receiving miR–19b and TEM images revealed that mitochondria were less fragmented (Fig. 5b–d). These results support *in vitro* findings, miR–19b also suppresses myocardial infarction and apoptosis *in vivo*.

QKI participates in the regulation of miR–19b expression

Finally, we assessed whether QKI may participate in the regulation of miR–19b. HL–1 cardiomyocytes were infected with lentiviral constructs of QKI shRNA or a negative control and QKI was confirmed to be suppressed by western blotting (Fig. 6a). Relative miR–19b levels were then measured by qRT-PCR (Fig. 6b), which indicated that suppressing levels of QKI reduced the expression of miR–19b. Moreover, following H/R, silencing QKI, significantly increases LDH release, the number of apoptotic cells, and mitochondrial fission but overexpressing miR–19b reverses this effect (Fig. 6c–e). We also assessed the influence of QKI on SOCS–6 levels in HL–1 cells infected with lentiviral constructs of QKI shRNA (Fig. 6f). Higher levels of SOCS–6 were observed when QKI was silenced but the overexpression of miR–19b reversed this trend (Fig. 6g). Overall, these results suggest a QKI/miR–19b/SOCS–6 pathway, whereby miR–19b can suppress SOCS–6 but is in turn regulated by QKI.

Discussion

The disruption of mitochondrial dynamics during myocardial IR injury presents problems in clinical practice and may contribute to the severity of the infarct size [28,29]. Inhibiting mitochondrial fission is thought to protect the heart against IR injury [13,15], and the delivery of mitochondria to cardiovascular tissue by vascular perfusion has been successfully applied in animal models to significantly reduce the infarct size [30,31]. SOCS-6 forms a complex with DRP1, a GTPase of the dynamin superfamily that is essential for mitochondrial fission, and PGAM5, a mitochondrial phosphatase, to facilitate DRP1 mitochondrial translocation [18]. The upregulation of SOCS-6 is known to have a detrimental effect on mitochondrial function by inhibiting the phosphorylation of DRP1 [32,33].

In the present study, we assessed the impact of SOCS-6, QKI, and miR-19b expression on mitochondrial morphology in IR injury using cardiomyocytes and a murine model. Consequently, we found that SOCS-6 upregulation led to decreased DRP1 phosphorylation and a greater percentage of fragmented mitochondria and that the silencing of SOCS-6 was able to reduce the increases in the levels of mitochondrial fragmentation. Downregulated SOCS-6 expression also led to a decreased number of apoptotic cells and lowered cell injury. Moreover, in a mouse model of IR injury, the infarct size was greater in mice hearts expressing SOCS-6 than in those with SOCS-6 silenced. Nishimura et al. [34] reported that DRP1 interacts with actin-binding protein filamin A through guanine nucleotide exchange to mediate myocardial senescence via mitochondrial fission in mice after MI. They found that hypoxic stress promoted the interaction of filamin A with the GTPase domain of DRP1 which consequently led to increased actin binding by DRP1, and they suggest that developing an inhibitor of DRP1 may be useful in the management of IR injury. Lin et al. [35] first studied SOCS-6 because its inactivation occurred frequently in gastric cancer. In later studies that found that it promotes intrinsic apoptosis by targeting mitochondria and inducing fragmentation through its interaction with DRP1 [18]. Our results support these findings, H/R was severely detrimental to the number of viable cardiomyocytes. However, downregulating SOCS-6 in cardiomyocytes resulted in significantly fewer apoptotic cells and a reduced the percentage of fragmented mitochondria.

In this study, we found that miR-19b targeted and downregulated SOCS-6 in cardiomyocytes, which led to less injury and fragmented mitochondria. We chose to study miR-19b because it was the only member of the miR-17-92 cluster to be downregulated in the infarct area following IR injury [27]. miR-19 has also been found to promote the progression of osteosarcoma by targeting and downregulating SOCS-6 in association with JAK2/STAT3 signaling [36]. The JAK2/STAT3 signaling pathway is involved in the progression of several cancers and is associated with other SOCS proteins through feedback regulation in various cell processes [37-40]. Although the research on JAK2/STAT3 signaling and mitochondrial fragmentation is limited, one study has proposed that JAK2/STAT3 signaling can prevent myocardial IR injury by reducing IR-induced mitochondrial oxidative damage [41]. Given this association with IR injury and mitochondrial oxidative damage, the regulation of Soc6 by miR-19 in association with JAK2/STAT3 signaling would be worthy of further investigation. In microglia, miR-19 is also known to inhibit oxidative stress and cell apoptosis by its association with PTEN/PI3K/Akt pathway-related proteins [42]. The numerous target genes associated with miR-19 may be due to non-canonical binding related to the RBP HuR. In a study screening for miRNA involved in breast cancer therapy resistance, UTR sequences

responded to miR-19b despite lacking a canonical binding site, however, the sequences contained a consensus site for HuR [43]. It may be worthwhile investigating the number of target genes associated with miR-19 during IR to determine if any associate through non-canonical binding.

In our study, suppressing the levels of QKI reduced the expression of miR-19b, which then significantly increases LDH release, the number of apoptotic cells, and mitochondrial fission. Downregulating QKI also increased the expression of SOCS-6. Overall, we found that miR-19b can suppress SOCS-6 but is in turn regulated by QKI.

QKI deficiency was found to increase susceptibility to IR injury in a mouse model of diabetes [44]. The QKI gene is known to exist in three alternative isoforms, QKI-5, QKI-6, and QKI-7, which can dimerize with each other [22]. The isoforms all share the same RNA-binding domain but have different amino acid sequences at the C-terminus and can translocate between the nucleus and cytoplasm. In the diabetic model mentioned previously, the deficiency of QKI5 overactivated FoxO1, which increases sensitivity to stress and contributed to IR injury whereas QKI5 overexpression destabilized FoxO1 mRNA. In another study, miR-208a/b was found to exacerbate H/R injury by downregulating QKI5 and QKI6 in cardiomyocytes [45]. Therefore, QKI downregulation seems to be a recurring factor in susceptibility to IR injury. The ratio and involvement of different isoforms may be important and is worthy of further investigation as different isoforms could be involved in the regulation of miR-19b.

Conclusions

In agreement with other studies [13,15], the present study found that the inhibition of mitochondrial fission attenuated the damage sustained after IR. This inhibition of mitochondrial fission through downregulating Soc6 via a QKI/miR-19b/SOCS-6 axis is an intricate process that may even involve non-canonical interactions and could form the basis of future research.

Materials And Methods

Cell cultures and H/R treatment

Cardiomyocytes from a mouse HL-1 cardiac cell line (American Type Culture Collection, Manassas, VA, USA) were cultured in Claycomb media with 10% fetal bovine serum, 0.1 mmol/L noradrenaline, 2 mmol/L L-glutamine, and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO₂. To induce H/R, cells were exposed to 1% O₂, 94% N₂, and 5% CO₂ for 2 h (hypoxia) followed by 95% air and 5% CO₂ for 12 h (reoxygenation).

Western blot analysis

To prepare cells for western blotting they were first lysed in RIPA buffer (Biotime, Jiangsu, China). Proteins in the lysate (30 µg) were separated using SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked in 5% skimmed milk and 0.1% Tween-20 for 1 h and then incubated with

primary antibodies at 4°C overnight. The primary antibodies and dilutions used in this study were Anti-SOCS-6 (1:1000, Abcam, Cambridge, UK), Anti-DRP1 (1:1000, Abcam), Anti-DRP1 (phospho S637) (1:500, Abcam), anti-QKI (1:1000, Cell Signaling Technology, Boston, MA, USA), and anti- β -actin (1: 5000; Sigma-Aldrich, St. Louis, MO, USA). Membranes were either probed with horseradish peroxidase-conjugated with goat anti-rabbit IgG or goat anti-mouse IgG (Santa Cruz Biotechnology, Dallas, TX, USA). Final blots were visualized by an Amersham ECL Western Blotting Detection Reagent (GE Healthcare, Amersham, UK). The intensity of protein bands was quantified by ImageJ software.

Quantitative reverse transcription-PCR

Trizol was used to extract total RNA was extracted from cells (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription (RT) was performed on 2 μ g of RNA to produce cDNA using HiScript Reverse Transcriptase (RNase H; *Vazyme, Nanjing, China*). Quantitative PCR was performed using SYBR Green Master Mix (*Vazyme*) on a QuantStudio 6 Flex Real-Time PCR System (*Applied Biosystems, Foster City, CA, USA*). The level of expression was normalized to U6 expression. The relative expression of genes was presented as fold change and was calculated using the $2^{-\Delta\Delta CT}$ method.

Lactate dehydrogenase (LDH) assay, TUNEL, and analysis of mitochondrial fission

LDH concentrations were measured in the supernatant of cell cultures after H/R to assess leakage. Cell culture supernatants were harvested and LDH content was measured using an LDH kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

The level of apoptosis was determined in cells following H/R by using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). Following H/R, cells were cultured on coverslips, fixed in 4% paraformaldehyde for 5 min and then the TUNEL procedure was performed using a TUNEL kit (Roche, Hamburg, Germany). Nuclei were counterstained with DAPI. The percentage of TUNEL-positive cells was determined from a mean of at least 10 fields under a confocal laser scanning microscope (Nikon C2, Nikon Corporation, Tokyo, Japan).

To determine mitochondrial morphology, cardiomyocytes plated onto collagen treated coverslips were stained with MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) for 20 min. The percentage of cells with fragmented mitochondria from at least 300 cells per group was determined under a confocal laser scanning microscope (Nikon C2) and presented as the mean $\pm$ SEM.

Luciferase reporter assay

To validate the interaction between SOCS-6 and miR-19b we first transfected HEK293 cells with either a wild-type or mutant construct of mouse SOCS-6 with miR-19b mimic or anti-miR-19b. The 3'-UTR of SOCS-6 was amplified by PCR using the forward primer 5'-CATGTTGGGGTAAGGAAGTCTCA-3' and the reverse primer 5'-GCGCGACATACTGTATCTAGAAG-3'. Mutations were created using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and both wild-type and mutant were

subcloned separately into pGL3 vectors. Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to measure the luciferase activity after 48 h (following manufacturer's instructions).

Lentiviral constructions, plasmid, and oligonucleotide transfection

Lentiviral constructs were designed to knockdown SOCS-6 and QKI. SOCS-6 short hairpin RNAs (shRNA) (5'-GCAGAATAACCCAATCCAAAG-3') and QKI shRNA target sequence (5'-GCTGATGGAGCTTGCAATTCT-3') were cloned into GV248 vectors (Genechem, Shanghai, China). A non-silencing scrambled shRNA was used as a negative control. Using HEK293T cells, lentiviral particles were produced. Further, lentiviral stocks were made after concentrating the samples using ultracentrifugation.

For Sosc6 overexpression, mouse SOCS-6 CDS was amplified with the primers 5'-GCGCGATGAAGAAAATCAGTCTG-3' (Forward) and 5'-TCAGTAGTGCTTCTCCTGCAAA-3' (Reverse), subcloned into pcDNA3.1 and transfected into cells using Lipofectamine 2000. pcDNA3.1 empty vector (EV) were used as a control. For miR-19b expression experiments, miR-19b and anti-miR-19b oligonucleotides, and their corresponding controls (miR-NC and anti-miR-NC) were obtained from GenePharma (Shanghai, China). Lipofectamine 2000 was used for their successive transfection into the cells.

In vivo model of I/R

The use of animals in all experiments followed the established guidelines published by the National Institutes of Health (1996) following scientific, humane, and ethical principles on the use and care of laboratory animals for biomedical research. Animal experiments were approved by the Ethical Committee of Minhang Hospital, Fudan University. To induce myocardial IR injury, C57BL/6 male mice from the Shanghai SLAC Laboratory Animal Center (Shanghai, China) were anesthetized by 5% isoflurane. Anesthetization was maintained at 1.5–2% in a 100% oxygen flow and body temperature of 37°C was maintained throughout the surgery. The heart was first exposed, and then using the 8–0 silk ligature, the left anterior descending coronary artery was ligated for 30 min occlusion. The ligature was then released and reperfusion occurred for 3 h, the analysis for cell apoptosis assay and mitochondrial morphology/function assessment were performed at this time point. Alternatively, at 24 h, infarct size determination was performed. Following reperfusion for the times indicated, the mice were euthanized (CO₂ inhalation) and hearts were recovered for further analysis.

In vivo delivery of lentivirus and miRNA

Mice were anesthetized as described in section 2.7. A micro-catheter was introduced through an incision made in the middle of the neck, into the right common carotid artery. Lentivirus expressing sh-SOCS-6 (1×10^7 PFU) was injected (100 μ L) via the micro-catheter. The micro-catheter was then removed and the incision closed. To inject miR-19b mimic into the hearts, the hearts were exposed in mice anesthetized as described in section 2.7. A microsyringe with a 30 G needle was inserted 1 mm deep into the left ventricular wall and 5 μ g of miR-19b mimic was delivered. The chest was closed and the mice were

allowed to recover. Myocardial IR injury was induced 72 hours after the injection of lentivirus or miR-19b mimic into hearts.

Measurement of myocardial infarct size and apoptosis

To measure the impact of IR on infarct size, hearts were first divided into five biventricular sections of equal thickness and then stained with 1.5% triphenyltetrazolium chloride (TTC, Sigma-Aldrich) solution for 15 min at 37°C. The unstained regions represented infarcted tissue and were quantified under a digital camera with NIH image software. To determine myocardial apoptosis, paraffin-embedded tissue was cut into 4–5 µm sections and incubated in 50 µL of TUNEL solution. The percentage of TUNEL-positive nuclei were calculated and defined as the apoptotic index.

Transmission electron microscopy

Tissue sections (1–2 mm) were cut perpendicular to the long axis of the left ventricular wall of the hearts isolated from mice. They were incubated overnight in 4% glutaraldehyde before being fixed in 1% osmium tetroxide for 1 h. Dehydration of the sections were carried out using a graded ethanol immersion series and embedded in resin. An ultramicrotome was then used to cut the resin embedded tissue into 80 nm-thick sections. The sections were placed under a JEM-1400 electron microscope (JEOL, Tokyo, Japan) and the images were captured with a CCD camera (Olympus, Tokyo, Japan). The TEM allowed mitochondria to be observed at ×10000 magnification.

Statistical analysis

Student's *t* test was used to compare samples between two groups and to compare samples among multiple groups one-way analysis of variance (ANOVA) was used. All the data are presented as the mean ± SEM of three independent experiments and a value of $P < 0.05$ was considered significant.

Declarations

Ethics approval and consent to participate

Animal experiments were approved by the Ethical Committee of Minhang Hospital, Fudan University.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the Shanghai Natural Science Foundation (19ZR1446000), the fusion grant of Minhang Hospital-College of Pharmacy, Fudan University (RO-MY201713), and the project from Minhang Science and Technology Commission (2017MHZ72).

Authors' contributions

PZ, PG, YLS, and WH designed the experiments. PZ and PG performed the experiments, acquired and analyzed the data, wrote the manuscript. XMY, YL, and YWH performed the experiments, acquired and analyzed the data. YLS and WH conceived and designed study, analyzed the data. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References

- [1]G. W. Reed, J. E. Rossi, C. P. Cannon, Acute myocardial infarction, *Lancet*. 389 (2017) 197–210. doi:10.1016/S0140–6736(16)30677–8.
- [2]J. González-Montero, R. Brito, A. I. Gajardo, R. Rodrigo, Myocardial reperfusion injury and oxidative stress: Therapeutic opportunities, *World J. Cardiol*. 10 (2018) 74–86. doi:10.4330/wjc.v10.i9.74.
- [3]M. Neri, I. Riezzo, N. Pascale, C. Pomara, E. Turillazzi, Ischemia/reperfusion injury following acute myocardial infarction: A critical issue for clinicians and forensic pathologists, *Mediators Inflamm*. 2017 (2017) 1–14. doi:10.1155/2017/7018393.
- [4]N.-B. Liu, M. Wu, C. Chen, M. Fujino, J.-S. Huang, P. Zhu, X.-K. Li, Novel molecular targets participating in myocardial ischemia-reperfusion injury and cardioprotection, *Cardiol. Res. Pract*. 2019 (2019) 1–16. doi:10.1155/2019/6935147.
- [5]A. Binek, R. Fernández-Jiménez, I. Jorge, E. Camafeita, J. A. López, N. Bagwan, C. Galán-Arriola, A. Pun, J. Agüero, V. Fuster, B. Ibanez, J. Vázquez, Proteomic footprint of myocardial ischemia/reperfusion injury: Longitudinal study of the at-risk and remote regions in the pig model, *Sci. Rep*. 7 (2017) 12343. doi:10.1038/s41598–017–11985–5.
- [6]V. K. Shah, K. K. Shalia, Reperfusing the myocardium - a damocles Sword., *Indian Heart J*. 70 (2018) 433–438. doi:10.1016/j.ihj.2017.11.009.

- [7]A. J. Lautz, B. Zingarelli, Age-dependent myocardial dysfunction in critically ill patients: Role of mitochondrial dysfunction, *Int. J. Mol. Sci.* 20 (2019) 3523. doi:10.3390/ijms20143523.
- [8]H. Zhou, P. Zhu, J. Wang, H. Zhu, J. Ren, Y. Chen, Pathogenesis of cardiac ischemia reperfusion injury is associated with CK2 α -disturbed mitochondrial homeostasis via suppression of FUNDC1-related mitophagy., *Cell Death Differ.* 25 (2018) 1080–1093. doi:10.1038/s41418-018-0086-7.
- [9]Q. Chen, J. Thompson, Y. Hu, J. Dean, E. J. Lesnefsky, Inhibition of the ubiquitous calpains protects complex I activity and enables improved mitophagy in hearts following ischemia-reperfusion, *Am. J. Physiol. Physiol.* (2019) ajpccell.00190.2019. doi:10.1152/ajpccell.00190.2019.
- [10]S.-B. Ong, A. B. Gustafsson, New roles for mitochondria in cell death in the reperfused myocardium., *Cardiovasc. Res.* 94 (2012) 190–6. doi:10.1093/cvr/cvr312.
- [11]S.-B. Ong, S. B. Kalkhoran, H. A. Cabrera-Fuentes, D. J. Hausenloy, Mitochondrial fusion and fission proteins as novel therapeutic targets for treating cardiovascular disease., *Eur. J. Pharmacol.* 763 (2015) 104–14. doi:10.1016/j.ejphar.2015.04.056.
- [12]I. Scott, R. J. Youle, Mitochondrial fission and fusion., *Essays Biochem.* 47 (2010) 85–98. doi:10.1042/bse0470085.
- [13]S.-B. Ong, S. Subrayan, S. Y. Lim, D. M. Yellon, S. M. Davidson, D. J. Hausenloy, Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury, *Circulation.* 121 (2010) 2012–2022. doi:10.1161/CIRCULATIONAHA.109.906610.
- [14]A. Masuzawa, K. M. Black, C. A. Pacak, M. Ericsson, R. J. Barnett, C. Drumm, P. Seth, D. B. Bloch, S. Levitsky, D. B. Cowan, J. D. McCully, Transplantation of autologously derived mitochondria protects the heart from ischemia-reperfusion injury, *Am. J. Physiol. Circ. Physiol.* 304 (2013) H966–H982. doi:10.1152/ajpheart.00883.2012.
- [15]M.-H. Disatnik, J. C. B. Ferreira, J. C. Campos, K. S. Gomes, P.M. M. Dourado, X. Qi, D. Mochly-Rosen, Acute inhibition of excessive mitochondrial fission after myocardial infarction prevents long-term cardiac dysfunction., *J. Am. Heart Assoc.* 2 (2013) e000461. doi:10.1161/JAHA.113.000461.
- [16]E. Smirnova, L. Griparic, D.-L. Shurland, A.M. van der Bliek, Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells, *Mol. Biol. Cell.* 12 (2001) 2245–2256. doi:10.1091/mbc.12.8.2245.
- [17]C.-R. Chang, C. Blackstone, Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology., *J. Biol. Chem.* 282 (2007) 21583–7. doi:10.1074/jbc.C700083200.
- [18]H.-Y. Lin, R.-H. Lai, S.-T. Lin, R.-C. Lin, M.-J. Wang, C.-C. Lin, H.-C. Lee, F.-F. Wang, J.-Y. Chen, Suppressor of cytokine signaling 6 (SOCS6) promotes mitochondrial fission via regulating DRP1 translocation., *Cell*

Death Differ. 20 (2013) 139–53. doi:10.1038/cdd.2012.106.

[19]S. Y. Kim, C. R. Morales, T. G. Gillette, J. A. Hill, Epigenetic regulation in heart failure., *Curr. Opin. Cardiol.* 31 (2016) 255–65. doi:10.1097/HCO.0000000000000276.

[20]S. Andalib, A. A. Divani, T. M. Michel, P. F. Høilund-Carlsen, M. S. Vafaei, A. Gjedde, Pandora's Box: mitochondrial defects in ischaemic heart disease and stroke, *Expert Rev. Mol. Med.* 19 (2017) e5. doi:10.1017/erm.2017.5.

[21]J. Mukohyama, Y. Shimono, H. Minami, Y. Kakeji, A. Suzuki, Roles of microRNAs and RNA-binding proteins in the regulation of colorectal cancer stem cells, *Cancers (Basel)*. 9 (2017) 143. doi:10.3390/cancers9100143.

[22]A.-J. Chen, J.-H. Paik, H. Zhang, S. A. Shukla, R. Mortensen, J. Hu, H. Ying, B. Hu, J. Hurt, N. Farny, C. Dong, Y. Xiao, Y. A. Wang, P. A. Silver, L. Chin, S. Vasudevan, R. A. Depinho, STAR RNA-binding protein Quaking suppresses cancer via stabilization of specific miRNA., *Genes Dev.* 26 (2012) 1459–72. doi:10.1101/gad.189001.112.

[23]T. A. Ebersole, Q. Chen, M. J. Justice, K. Artzt, The quaking gene product necessary in embryogenesis and myelination combines features of RNA binding and signal transduction proteins., *Nat. Genet.* 12 (1996) 260–5. doi:10.1038/ng0396–260.

[24]A. B. Herman, M. V Autieri, Inflammation-regulated mRNA stability and the progression of vascular inflammatory diseases., *Clin. Sci. (Lond)*. 131 (2017) 2687–2699. doi:10.1042/CS20171373.

[25]Y. Wang, G. Vogel, Z. Yu, S. Richard, The QKI–5 and QKI–6 RNA binding proteins regulate the expression of microRNA 7 in glial cells, *Mol. Cell. Biol.* 33 (2013) 1233. doi:10.1128/MCB.01604–12.

[26]S. Katta, S. Karnewar, D. Panuganti, M. K. Jerald, B. K. S. Sastry, S. Kotamraju, Mitochondria-targeted esculetin inhibits PAI–1 levels by modulating STAT3 activation and miR–19b via SIRT3: Role in acute coronary artery syndrome, *J. Cell. Physiol.* 233 (2018) 214–225. doi:10.1002/jcp.25865.

[27]J. Xu, Y. Tang, Y. Bei, S. Ding, L. Che, J. Yao, H. Wang, D. Lv, J. Xiao, miR–19b attenuates H₂O₂-induced apoptosis in rat H9C2 cardiomyocytes via targeting PTEN., *Oncotarget*. 7 (2016) 10870–8. doi:10.18632/oncotarget.7678.

[28]D. Garcia-Dorado, A. Rodríguez-Sinovas, M. Ruiz-Meana, J. Inserte, Protection against myocardial ischemia-reperfusion injury in clinical practice, *Rev. Española Cardiol. (English Ed)*. 67 (2014) 394–404. doi:10.1016/j.rec.2014.01.010.

[29]T. Wai, J. García-Prieto, M. J. Baker, C. Merkwirth, P. Benit, P. Rustin, F. J. Rupérez, C. Barbas, B. Ibañez, T. Langer, Imbalanced OPA1 processing and mitochondrial fragmentation cause heart failure in mice., *Science*. 350 (2015) aad0116. doi:10.1126/science.aad0116.

- [30]J. D. McCully, D. B. Cowan, C. A. Pacak, I. K. Toumpoulis, H. Dayalan, S. Levitsky, Injection of isolated mitochondria during early reperfusion for cardioprotection, *Am. J. Physiol. Circ. Physiol.* 296 (2009) H94–H105. doi:10.1152/ajpheart.00567.2008.
- [31]D. B. Cowan, R. Yao, V. Akurathi, E. R. Snay, J. K. Thedsanamoorthy, D. Zurakowski, M. Ericsson, I. Friehs, Y. Wu, S. Levitsky, P. J. del Nido, A. B. Packard, J. D. McCully, Intracoronary delivery of mitochondria to the ischemic heart for cardioprotection, *PLoS One*. 11 (2016) e0160889. doi:10.1371/journal.pone.0160889.
- [32]E. Smirnova, D.-L. Shurland, S. N. Ryazantsev, A.M. van der Blik, A human dynamin-related protein controls the distribution of mitochondria, *J. Cell Biol.* 143 (1998) 351–358. doi:10.1083/jcb.143.2.351.
- [33]S. C. Kamerkar, F. Kraus, A. J. Sharpe, T. J. Pucadyil, M. T. Ryan, Dynamin-related protein 1 has membrane constricting and severing abilities sufficient for mitochondrial and peroxisomal fission., *Nat. Commun.* 9 (2018) 5239. doi:10.1038/s41467-018-07543-w.
- [34]A. Nishimura, T. Shimauchi, T. Tanaka, K. Shimoda, T. Toyama, N. Kitajima, T. Ishikawa, N. Shindo, T. Numaga-Tomita, S. Yasuda, Y. Sato, K. Kuwahara, Y. Kumagai, T. Akaike, T. Ide, A. Ojida, Y. Mori, M. Nishida, Hypoxia-induced interaction of filamin with Drp1 causes mitochondrial hyperfission-associated myocardial senescence., *Sci. Signal.* 11 (2018) eaat5185. doi:10.1126/scisignal.aat5185.
- [35]R.-H. Lai, Y.-W. Hsiao, M.-J. Wang, H.-Y. Lin, C.-W. Wu, C.-W. Chi, A. F.-Y. Li, Y.-S. Jou, J.-Y. Chen, SOCS6, down-regulated in gastric cancer, inhibits cell proliferation and colony formation., *Cancer Lett.* 288 (2010) 75–85. doi:10.1016/j.canlet.2009.06.025.
- [36]Z. Sun, Q. Liu, H. Hong, H. Zhang, T. Zhang, miR-19 promotes osteosarcoma progression by targeting SOCS6, *Biochem. Biophys. Res. Commun.* 495 (2018) 1363–1369. doi:10.1016/j.bbrc.2017.10.002.
- [37]Y. Fu, Y. Xu, S. Chen, Y. Ouyang, G. Sun, MiR-151a-3p promotes postmenopausal osteoporosis by targeting SOCS5 and activating JAK2/STAT3 signaling, *Rejuvenation Res.* (2019) rej.2019.2239. doi:10.1089/rej.2019.2239.
- [38]Y.-T. Zeng, X.-F. Liu, W.-T. Yang, P.-S. Zheng, REX1 promotes EMT-induced cell metastasis by activating the JAK2/STAT3-signaling pathway by targeting SOCS1 in cervical cancer, *Oncogene*. (2019). doi:10.1038/s41388-019-0906-3.
- [39]S. Ebong, C.-R. Yu, D. A. Carper, A. B. Chepelinsky, C. E. Egwuagu, Activation of STAT signaling pathways and induction of suppressors of cytokine signaling (SOCS) proteins in mammalian lens by growth factors, *Investig. Ophthalmology Vis. Sci.* 45 (2004) 872. doi:10.1167/iovs.03-0311.
- [40]H. Xiong, W. Du, Y.-J. Zhang, J. Hong, W.-Y. Su, J.-T. Tang, Y.-C. Wang, R. Lu, J.-Y. Fang, Trichostatin A, a histone deacetylase inhibitor, suppresses JAK2/STAT3 signaling via inducing the promoter-associated

histone acetylation of SOCS1 and SOCS3 in human colorectal cancer cells, *Mol. Carcinog.* 51 (2012) 174–184. doi:10.1002/mc.20777.

[41]Y. Yang, W. Duan, Z. Jin, W. Yi, J. Yan, S. Zhang, N. Wang, Z. Liang, Y. Li, W. Chen, D. Yi, S. Yu, JAK2/STAT3 activation by melatonin attenuates the mitochondrial oxidative damage induced by myocardial ischemia/reperfusion injury, *J. Pineal Res.* 55 (2013) 275–286. doi:10.1111/jpi.12070.

[42]W. Liu, L. Han, R. Xiang, Protection of miR-19b in hypoxia/reoxygenation-induced injury by targeting *PTEN*, *J. Cell. Physiol.* 234 (2019) 16226–16237. doi:10.1002/jcp.28286.

[43]J. L. Thorne, S. Battaglia, D. E. Baxter, J. L. Hayes, S. A. Hutchinson, S. Jana, R. A. Millican-Slater, L. Smith, M. C. Teske, L. M. Wastall, T. A. Hughes, MiR–19b non-canonical binding is directed by HuR and confers chemosensitivity through regulation of P-glycoprotein in breast cancer, *Biochim. Biophys. Acta - Gene Regul. Mech.* 1861 (2018) 996–1006. doi:10.1016/j.bbagr.2018.08.005.

[44]W. Guo, T. Jiang, C. Lian, H. Wang, Q. Zheng, H. Ma, QKI deficiency promotes FoxO1 mediated nitrosative stress and endoplasmic reticulum stress contributing to increased vulnerability to ischemic injury in diabetic heart, *J. Mol. Cell. Cardiol.* 75 (2014) 131–140. doi:10.1016/j.yjmcc.2014.07.010.

[45]F. Wang, Y. Yuan, P. Yang, X. Li, Extracellular vesicles-mediated transfer of miR–208a/b exaggerate hypoxia/reoxygenation injury in cardiomyocytes by reducing QKI expression, *Mol. Cell. Biochem.* 431 (2017) 187–195. doi:10.1007/s11010-017-2990-4.

Figures

Figure 1

SOCS-6 regulates mitochondrial fission and apoptosis in HL-1 cardiomyocytes. A. HL-1 Cardiomyocytes were treated with hypoxia and reoxygenation (H/R) at the times indicated. SOCS-6 and phosphorylated DRP1 levels were analyzed by western blot assays. B. HL-1 Cardiomyocytes were infected with lentiviral constructs of SOCS-6 shRNA or a negative control (sh-NC) and then exposed to H/R. SOCS-6 and phosphorylated DRP1 levels were analyzed by western blot assays. C. the release of lactate

dehydrogenase (LDH) in HL-1 Cardiomyocytes was assayed. D. Representative images of apoptosis analyzed by TUNEL staining, TUNEL-positive cells were counted and calculated. Scale bar, 50 μ m. E. Representative confocal images of mitochondrial morphology stained with MitoTracker Red and the percentage of cardiomyocytes with fragmented mitochondria are shown. Nuclei were visualized by DAPI. Scale bar, 20 μ m. *P<0.05

Figure 2

SOCS-6 inhibition attenuates myocardial infarction and apoptosis in mice. A. Hearts of adult male C57BL/6 mice were injected with lentivirus. After 72 h they were exposed to 30 min of myocardial ischemia (MI) followed by indicated time of reperfusion. A. SOCS-6 levels were detected by western blotting. B. Representative sections of MI assayed by TTC staining after 24 h of reperfusion. The infarct size is presented as the percentage area at risk. C. Representative images of heart sections stained with hematoxylin and eosin. Scale bar, 50 μ m. D. Apoptotic cells were detected by TUNEL assay after 3 h of reperfusion. Scale bar, 50 μ m. E. Images of mitochondrial morphology obtained by transmission electron microscopy after 3 h of reperfusion. Scale bar, 1 μ m.

Figure 3

miR-19b represses SOCS-6 expression and participates in the regulation of mitochondrial fission and apoptosis. A. The putative target site of miR-19b located in in the 3'-UTR of SOCS-6 mRNA as predicted by TargetScan software. B. Luciferase activity was determined 48 h after HEK293T cells were co-transfected with wild-type or mutated 3'-UTR of mouse SOCS-6 with miR-19b mimic or anti-miR-19b. C. Western blot analysis of SOCS-6 levels in HL-1 cells transfected with miR-19b mimic, anti-miR-19b, or a

negative control for 24 h. D. HL-1 cardiomyocytes were treated with H/R at the times indicated. Relative miR-19b levels were detected by qRT-PCR. E–G. HL-1 Cardiomyocytes were transfected with miR-19b mimic or a negative control (miR-NC) and then exposed to H/R. E. Lactate dehydrogenase (LDH) release in HL-1 cardiomyocytes. F. Apoptosis analyzed by percentage of TUNEL-positive cells. G. Mitochondria were stained by MitoTracker Red and the percentages of fragmented mitochondria are shown. Nuclei were visualized with DAPI. *P<0.05.

Figure 4

Restoration of SOCS-6 reverses miR-19b-mediated inhibition of mitochondrial fission and cardiomyocyte apoptosis. HL-1 cells were co-transfected with miR-19b mimic and SOCS-6 overexpression vectors for 24 h, or miR-NC and control empty vector (EV). A. Western blot analysis of SOCS-6 levels. B. Release of lactate dehydrogenase (LDH) from HL-1 cardiomyocytes after hypoxia and reoxygenation (H/R). C.

Apoptosis calculated by the percentage of TUNEL-positive cells. D. Percentage of cardiomyocytes with fragmented mitochondria. Mitochondria were stained with MitoTracker Red. Nuclei were visualized by DAPI. *P<0.05.

Figure 5

miR-19b suppresses myocardial infarction and apoptosis in vivo. The hearts of adult male C57BL/6 mice were injected with miR-19b. After 72 h they were exposed to 30 min of myocardial ischemia, followed by

indicated time of reperfusion. A. Representative sections of myocardial infarction assayed by TTC staining after 24 h of reperfusion. Infarct size is presented as a percentage of the area at risk. B. Representative images of heart sections stained with hematoxylin and eosin. Scale bar, 50 μ m. C. Apoptotic cells were detected by TUNEL assay after 3 h of reperfusion. Scale bar, 50 μ m. D. Representative transmission electron microscopy images of mitochondrial morphology after 3 h of reperfusion. Scale bar, 1 μ m.

Figure 6

QKI participates in the regulation of miR-19b expression. A. HL-1 cardiomyocytes were infected with lentiviral constructs of QKI shRNA or a negative control (sh-NC). SOCS-6 levels were analyzed by western blotting. B. Relative miR-19b levels were detected by qRT-PCR. C. Release of lactate dehydrogenase (LDH) from HL-1 cardiomyocytes exposed to hypoxia and reoxygenation (H/R). D. Representative images of apoptosis analyzed by TUNEL staining. Percentages of TUNEL-positive cells are shown. E. Representative confocal images of mitochondrial morphology in cells stained with MitoTracker Red. Percentage of fragmented mitochondria in cardiomyocytes is shown. Nuclei were visualized by DAPI. F. Western blotting analysis of SOCS-6 levels in HL-1 cells infected with lentiviral constructs of QKI shRNA or its negative control (sh-NC). G. Lentiviral constructs of QKI shRNA infected HL-1 cells were transfected with miR-19b mimic for 24 h, and then SOCS-6 levels were detected by western blotting. *P<0.05