p27<sup>kip1</sup> haploinsufficiency preserves myocardial function in the early stages of myocardial infarction via Atg5-mediated autophagy flux restoration

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Abstract. Myocardial infarction (MI) is a leading cause of mortality in adults worldwide. Over the last two decades, gene therapy has been a hot topic in cardiology, and there has been a focus on cell cycle inhibitors and their protective effects on the myocardium post-MI. In our previous study, the haploinsufficiency of p27<sup>kip1</sup> (p27) was demonstrated to improve cardiac function in mice post-MI by promoting angiogenesis and myocardium protection through the secretion of growth factors. Autophagy is an adaptive response of cells to environmental changes, such as nutrient deprivation, ischemia and hypoxia. The appropriate regulation of autophagy may improve myocardial function by preventing apoptosis of cardiomyocytes. In this study, we used immunoassays, transmission electron microscopy and cardiac ultrasound to confirm that p27 haploinsufficiency prevents myocardial apoptosis by restoring autophagy protein 5-mediated autophagy flux in the early stages of MI. The present study provides a novel method for studying MI or ischemic heart disease therapy.

Introduction

Myocardial infarction (MI) is a disease with the highest mortality rate, accounting for 1 in 5 deaths in the United States (1); in the past decade, 17.6 million individuals have suffered from MI. According to the American Heart Association, acute cardiac vascular occlusion and poor prognosis are the main causes of mortality (2). At present, instantly restoring blood flow to the ischemic area by percutaneous coronary intervention is the best approach to saving lives (3). Along with the development of gene technology and molecular biology, research into gene and genomic regulation is being conducted to reveal the cause of MI and to advance therapies (4). Cytokine and gene therapy have been widely utilized for therapeutic angiogenesis in myocardial ischemia (4). Various growth factors, such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), serve roles in balancing cell proliferation and survival under stress (5-7). Furthermore, cardiac remodeling and collateral angiogenesis improve early cardiac recovery (8). In our previous study, it was reported that p27<sup>kip1</sup> (p27) haploinsufficiency improved cardiac function in the early stages of MI by protecting the myocardium and increasing angiogenesis through IκB kinase activation (9). The protective effects of knocking down the cell cycle inhibitor p27 to stimulate the autocrine and paracrine effects of VEGF and HGF through the activation of inflammation and angiogenesis in the myocardium.

p27 is a potent cell cycle inhibitor that maintains cell proliferation by arresting the cell cycle in the G0/G1 phase; a previous study reported that the regulation of p27 not only affected cell cycle-related proliferation but was also involved in multifunctional molecular mechanisms in vivo and in vitro such as aging and inflammation regulating (10). The downstream activities of p27 include the regulation of inflammation and survival pathways (9,11,12). Through the regulation of p27 expression, development/tumorigenesis and metabolism are altered, which are implicated in energy modulation (13). Rossi et al (14) reported that gene knockdown may induce different biological effects than gene deletion; it was found that knockdown was more effective than knockout for mimicking pathological progression. In our previous study, p27 haploinsufficient mice (p27<sup>-/-</sup>) exhibited a different phenotype compared with wild-type (WT; p27<sup>+/+</sup>) or homozygous (p27<sup>++</sup>) mice (9).

Macroautophagy, more commonly referred to as autophagy, balances cell death and survival in response to stress and starvation (15). It is an important cellular homeostatic
process that cells use to degrade misfolded proteins and recycle damaged organelles, and is also related to a number of diseases, including cancer and neurodegeneration. The cup-shaped pre-autophagosomal double-membrane structure containing cytoplasmic material characterizes the formation of autophagosomes (16). As previously reported, the cardiomyocyte-specific abrogation of basal autophagy that results from a deficiency in autophagy protein 5 (Atg5) leads to spontaneous cardiac hypertrophy (17). Accordingly, autophagy acts as a protective mechanism that improves cardiomyocyte survival under stress (18). Serum withdrawal and hypoxia are common approaches used to mimic myocardial cell ischemia in vitro. Ding et al (19) reported that p27 levels were regulated by autophagy in serum-deprived mouse mesangial cells. It also has been reported that, in relation to autophagy and inflammation, cardiac remodeling and heart failure are promoted by the upregulation of p27 in the long term (20). To the best of our knowledge, no studies have investigated the relationship between p27 and autophagy in the early stages of MI.

The present study aimed to determine the functional role of p27 haploinsufficiency in the induction of autophagy by hypoxia and serum withdrawal in vitro and in vivo for cardiomyocyte survival, and protection from apoptosis. The results demonstrated that p27+/- resulted in the increased induction of Atg5-related autophagosomes and suppressed serum withdrawal-induced caspase-3 activation and apoptosis in MI hearts. It was demonstrated that p27 knockout governed the regulation of autophagy by increasing the expression of Atg5 in the early stages of MI in mice and in MI mimick cells. In the present study, 3-methyladenine (3-MA) and rapamycin were used to investigate the rescue of autophagy and cell survival following MI.

Materials and methods

Animals and establishment of an MI model. The establishment of the MI animal model was described in our previous study (9). p27+/- and strain-specific C57BL/6 WT p27+/- mice were bred from a breeding pair of heterozygous p27 mice (kindly donated by Professor Dengshun Miao, McGill University; Fig. S1A) and housed in the Animal Research Center of Nanjing Medical University. The mice were bred in a specific pathogen-free rodent feeding room and kept at 15% humidity and 25°C conditions and a 12 h-light/dark cycle. Tail fragment genomic DNA was used to genotype the mice; a total of 40 adult male 3-month-old C57BL/6 (25±5 g) WT (n=20) and p27+/- (n=20) mice were split into MI and Sham control groups (10 mice/group). Left anterior descending (LAD) artery ligation was performed to induce MI, as previously describe (9). Briefly, the LAD coronary artery was ligated permanently using an 8-0 polypropylene suture; the Sham animals underwent the same procedure, but without ligation of the LAD coronary artery. During the experimental trial, all mice were allowed ad libitum access to food and water. The protocols used in the present study were approved by the Ethics Review of Lab Animal Use Application of Nanjing Medical University, and the procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (9).

Echocardiogram. Cardiac function was determined using a high-frequency ultrasound system Vevo2100 (FUJIFILM VisualSonics, Inc.) equipped with a 30-MHz transducer. The ejection fraction (EF) and fractional shortening (FS) were calculated according to the following formulas: EF=[(LVEDV-LVESV)/LVEDV] x100, where LVEDV is left ventricular end-diastolic volume and ESV is LV end-systolic volume; and FS=[(LVEDd-LVESd)/LVEDd] x100, where EDD is end-diastolic dimension and ESd is end-systolic dimension. Each parameter was the average of three values. Mice were anesthetized with 4-5% chloral hydrate at 300-4500 mg/kg; no signs of peritonitis, pain or discomfort were observed. The heart rates of the mice were maintained at 250±35 beats/min under anesthesia.

Immunohistochemistry (IHC) and Masson's trichrome staining. Mouse hearts were collected and left ventricular tissues fixed in 4% buffered paraformaldehyde at room temperature for 24 h and embedded in paraffin at 60°C for 1 h. The paraffin embedded tissues were cut into 5 µm-thick sections, which were subsequently deparaffinized and rehydrated with a graded xylene and ethanol series. Antigen retrieval was performed by high temperature and pressure: 1 mM EDTA (Beijing Solarbio Science & Technology Co., Ltd.) was heated to 100°C, the sections were soaked in EDTA and heated for 5 min at a pressure of 110 kPa. Sections were incubated in 3% H2O2 at room temperature for 10 min. Subsequently, the sections were blocked in 10% BSA (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 30 min. IHC was performed using the SuperPicture™ 3rd Gen IHC Detection kit (cat. no. 878973; Invitrogen; Thermo Fisher Scientific, Inc.), following manufacturer's protocol. Briefly, the sections were incubated with a primary antibody against microtubule-associated proteins 1A/1B light chain (LC3) (1:500; cat. no. ab128025; Abcam) overnight at 4°C. The sections were subsequently incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody [1:500: cat. no. 7074/6; Cell Signaling Technology, Inc. (CST)] for 2 h at 25°C. A two-step technique was used for visualization using components from the SuperPicture™ 3rd Gen IHC Detection kit, and hematoxylin was used as a counterstain. The heart tissue sections from the left ventricle of mice 28 days post-MI and fixed, embedded, cut into 5-µm-thick sections were also stained using Masson's trichrome to confirm successful model establishment by infarction and fibrotic areas (Fig. S1B). A total of six fields were analyzed per sample and images were captured using an Olympus CX41 light microscope (CX41; Olympus Corporation, Tokyo, Japan) and Image-Pro Plus 6.0 (Media Cybernetics, Inc.) was used to detect LC3 expression.

Cell culture and treatment. The rat H9c2 cell line (cat. no. GNR5; Cell Bank of the Chinese Academy of Science) was seeded at 1.25x10⁴ cells/well in DMEM (Gibco; Thermo Fisher Scientific, Inc.) in 6-well culture plates and were allowed to grow for 2 days in incubated box at 37°C, 5% CO₂, pH 7.2-7.4. FBS (10%; Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 µg/ml streptomycin were added to the culture medium before cell culture. When the cells reached 80-85% confluency the media was replaced with DMEM containing 1% FBS, to mimic ischemia, and the cells were placed into an anoxic box (95% nitrogen, 5% CO₂ at 37°C)
to induce hypoxia for 3 or 12 h. Confirmation of hypoxia was determined by western blotting for increased protein expression levels of hypoxia-inducible factor-1α (HIF-1α; Fig. S1C).

**Western blot analysis.** Tissues and cells were lysed using RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin]. Protein concentrations were determined by BCA; 20 μg of protein lysates were separated by 10% SDS-PAGE and transferred to PVDF membranes. Membranes were washed with PBS, blocked with 5% non-fat dry milk for 1 h at room temperature, and subsequently incubated over night at 4°C with primary antibodies (all 1:1,000) against p27 (cat. no. 610241; BD Biosciences), Cleaved-caspase-3 (cat. no. 9664; CST), Bcl-2 (cat. no. 2870; CST), Beclin 1 (cat. no. 3495; CST), Atg5 (cat. no. 12994; CST), LC3 (cat. no. ab128025; Abcam), HIF-1α (cat. no. 36169; CST) and GAPDH (cat. no. 2118; CST). Subsequently, the membranes were incubated with HRP-conjugated goat anti-mouse (cat. no. sc-2002; Santa Cruz Biotechnology, Inc.) or goat anti-rabbit HRP (cat.no. sc-2005; Santa Cruz Biotechnology, Inc.) secondary antibodies overnight at 4°C (both 1:2,000). Protein bands were visualized using the Gel-Pro analyzer 4.0 and were considered to indicate a statistically significant difference.

**Statistical analysis.** Data are presented as the mean ± SEM from three independent experiments. All statistical analysis was conducted using SPSS 19.0 statistical software (IBM Corp.). Statistical significance between groups was determined using one-way ANOVA and Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

p27 haploinsufficiency improves cardiac function after MI. p27<sup>−/−</sup> has been shown to have either a proangiogenic or an antiapoptotic effect depending on cell cycle re-entry (9). LAD artery ligation was used to mimic MI in WT and p27<sup>−/−</sup> mice. In our previous study, it was reported that p27<sup>−/−</sup> and WT mice exhibited high mortality following this procedure, but that p27<sup>−/−</sup> mice had a moderate mortality (9). Significant systolic dysfunction was observed in WT MI compared with WT Sham (P<0.05), p27<sup>−/−</sup> MI compared with p27<sup>−/−</sup> Sham (P<0.05) at 28 days following MI and in WT MI compared with p27<sup>−/−</sup> MI as assessed using the percentage of LV FS and EF in the MI and control groups (Fig. 1A). However, LV dysfunction was lower in p27<sup>−/−</sup> mice compared with the WT mice after infarction (P<0.05; Fig. 1A). These data suggested that the haploinsufficiency of p27 may preserve cardiac function following MI. p27<sup>−/−</sup> mice exhibited reduced cardiac injury after MI by promoting the expression of the anti-apoptotic protein Bcl-2 at 3 days post-MI compared with WT MI at day 3 (P<0.01) and by reducing the protein expression level of cleaved caspase-3 at 3 and 28 days post-MI compared with the WT MI counterpart (P<0.05 and P<0.01, respectively) (Fig. 1B and C).

p27 haploinsufficiency attenuates ischemic injury through a pro-autophagy-induced antiapoptotic pathway in vivo. As shown in Fig. 2A, MI induced the expression of the autophagic vesicle-associated form of LC3-II within 28 days after LAD...
artery ligation. It was observed that in both the Sham and MI groups at 28 days the autophagy marker LC3 was expressed at significantly higher levels in the p27^+/^- groups compared with expression levels in the WT group (P<0.01; Fig. 2A and B).
In addition, Beclin 1 and downstream Atg5 expression levels were affected. Beclin 1 was expressed at significantly higher levels in the p27<sup>−/−</sup> and WT MI groups compared with expression levels in the respective p27<sup>−/−</sup> Sham and WT MI groups at 3 days (P<0.01), and at 28 days Beclin 1 expression was significantly higher in the p27<sup>−/−</sup> MI group compared with the WT MI 28 days group (P<0.01) (Fig. 2A and B). Atg5 was also expressed at significantly higher levels in the p27<sup>−/−</sup> groups compared with expression levels in the respective WT groups (P<0.05 or P<0.01; Fig. 2A and B). These data suggested that p27 haploinsufficiency contributes to the observed increased levels of autophagy in the Sham and MI groups. Furthermore, IHC was used to detect the levels of LC3 in WT and p27<sup>−/−</sup> mice at 28 days post-surgery; fewer LC3-positive areas were observed in the WT mice compared with the p27<sup>−/−</sup> mice (Fig. 2C). Co-IP experiments were performed on WT mouse heart tissue lysates. Binding was detected between Atg5 and p27 in heart tissue (Fig. 2D). In addition, TEM also detected the formation of autophagosomes and autolysosomes in WT tissue 28 days after MI (Fig. 2E). These results suggested...
that p27 haploinsufficiency attenuated ischemic injury by mitigating apoptosis via Atg5-related autophagy.

*p27* <sup>KD</sup> restores autophagy flux in the early stages of hypoxia/ischemia in vitro. The lentiviral-mediated stable p27<sup>KD</sup> H9c2 cell line (~50% efficiency; Fig. S1D) was used to mimic the effect of hypoxia/ischemia in cardiomyocytes. Western blotting was used to determine the expression of the autophagy-related proteins Beclin 1, Atg5 and LC3 in H9c2 cells. The results demonstrated that both Beclin 1 and Atg5 expression were higher in the p27<sup>KD</sup> H9c2 cells compared with WT cells after 3 h of hypoxia/ischemia, but attenuated after 12 h (Fig. 3A and B). The ratio of LC3-II/I, a hallmark of autophagosomes, coincided with the expression of Beclin 1 and Atg5 (Fig. 3B). Western blotting revealed that after 3 h of hypoxia/ischemia, Beclin 1 and Atg5 protein expression levels increased to a peak expression level compared with WT NC (*P*<0.01), and the levels in p27<sup>KD</sup> with 3 h of hypoxia were
significantly higher compared with WT with 3 h of hypoxia (P<0.01) (Fig. 3a and B). H9c2 cells were infected with the mRFP-GFP-lc3 adenovirus and then exposed to 3 h of hypoxia; the results demonstrated that, after 3 h, there was a significantly increased ratio of GFP and mRFP puncta per cell in p27<sup>Kd</sup> cells compared with WT (P<0.01; Fig. 3c), whereas the effects were reversed by a tg5Kd co-knockdown (P<0.01; Fig. 3c). Flow cytometry results revealed that total early+late apoptotic rates in atg5<sup>Kd</sup> cells were significantly increased after 12 h of hypoxia and serum deprivation compared with WT cells (P<0.05; Fig. 3D). p27<sup>Kd</sup> cells had lower apoptosis compared with WT (P<0.01), and co-knockdown of Atg5 reversed this effect of p27 haploinsufficiency (P<0.05). These data indicated that the mechanisms by which p27 insufficiency protects against hypoxia/ischemia may be through restoring the autophagy flux.

*p27-mediated autophagy flux is affected by 3-MA/rapamycin.* To further investigate whether p27<sup>Kd</sup> improved autophagy flux, cells were treated with the autophagy promoter rapamycin and the autophagy inhibitor 3-MA. Rapamycin treatment resulted in an increased level of GFP and mRFP puncta/cell compared with untreated cells, whereas 3-MA treatment significantly decreased the number of puncta/cell (Fig. 4). In addition, p27<sup>Kd</sup> increased expression of the autophagic vesicle-associated form of LC3II following treatment with rapamycin or 3-MA with 3 h in hypoxic conditions. *P<0.05. 3-MA, 3-methyladenine; GFP, green fluorescent protein; KD, knockdown; LC3, microtubule-associated proteins 1A/1B light chain; mRFP, monomeric red fluorescent protein; p27, p27<sup>kip1</sup>.

**Discussion**

Myocardial infarction is induced by cardiac coronary vascular and branch occlusion, resulting in nutrient and oxygen insufficiency (21,22). As a result, the ischemic myocardium dies
and is replaced by fibrotic scar tissue (23). Fibroblast cells are implicated in the dysfunction of contractility, leading to heart failure (24). Furthermore, in cardiomyocytes, our previous study reported that 3 and 28 days of hypoxia in vivo, and 3 and 12 h of hypoxia in vitro induces ischemia/hypoxia that can be used to mimic the early stages of natural heart attack, according to immunodetection and ultrasonic cardiograms (9). Recently, an association between autophagy and cardiac disorders such as myocardial infarction has been demonstrated (25). p27 is a potent cell cycle inhibitor implicated in survival, proliferation, antiapoptotic functions and migration (10). Cardiomyocytes undergo ischemia in the early stages of MI, and p27KD may activate immunoreactivity and autophagy. It has been hypothesized that by promoting the cell cycle, p27 haploinsufficiency could improve cell survival and basic autophagy naturally (26). Previously, it was reported that the knockdown of p27 preserved cardiac function in the early stage of MI by regulating VEGF and HGF, and their receptors, promoting cardiomyocyte survival and leading to proangiogenic effects (9). Similarly, results from the present study demonstrated that p27KD mice exhibited improved cardiac function and higher cardiomyocyte viability following acute MI compared with WT mice. The reduced survival found in p27KD mice in the earliest stages after MI, but not in WT and p27+/- mice, has also been described (9). The present study revealed that p27+/- mice had augmented autophagy in the early stages after MI.

Autophagy can be stimulated by various forms of cellular stress, including serum withdrawal, nutrient deprivation, damaged organelles, hypoxia, DNA damage and protein misfolding, which resist apoptosis and necrosis (27,28). Proper autophagy levels are required to maintain cell homeostasis and function. In the heart, uncontrolled autophagy has been demonstrated to contribute to pathological cardiac remodeling and subsequent heart failure (18). Although p27 has been reported to be involved in regulating the process of autophagy (20), few studies have investigated the relationship between p27 and autophagy in the heart. In the present study, it was found that p27KD cardiomyocytes increased LC3 protein expression in vivo and in vitro. Furthermore, restored autophagy flux in p27KD ischemic H9C2 cells were observed by GFP/mRFP LC3 fluorescence and IHC. Therefore, the results indicated that p27 may inhibit the autophagy process of cardiomyocytes in the early stages after MI, which may lead to subsequent cardiac dysfunction. In addition, to evaluate the potential contribution of changes in the autophagic flux in p27KD cardiomyocytes, the tandem fluorescence reporter mRFP-GFP-LC3 was used to measure flux. The data from the present study revealed a significantly increased number of GFP and mRFP puncta/cell ratios in p27KD cells, indicating that flux is blocked in the early stages of MI.

Atg5 is an important upstream mediator of autophagy through its binding to LC3, which blocks the formation of autophagosomes (26). It has also been found to serve an important role in the maintenance of cardiac function (29). In the present study, co-IP using Atg5 was used to analyze the relationship between Atg5 and LC3. The results demonstrated that p27 can bind to the Atg5 protein in cardiomyocytes. p27KD increased Atg5 expression and promoted autophagy in the early stages of MI. Furthermore, it was found that Atg5KD reversed the effects of increased autophagy and prevented apoptosis in cultured cardiomyocytes that underwent hypoxia/ischemia. Pathways such as AMP-activated protein kinase/mTOR/ErB/AKT regulate autophagy and apoptosis in cardiomyocytes (16); however, a limited number of studies have attempted to reveal a relationship with p27. The results from the present study provide a new insight of autophagy and apoptosis in cardiomyocytes under hypoxic/ischemic conditions and indicate mechanisms of immunologic injury protection and autophagy flux restoration. The present study indicated that p27 may serve as an inhibitor of Atg5-mediated autophagy activation.

In conclusion, the results from the present study revealed that the p27/Atg5/LC3 pathway modulates the regulation of autophagy in cardiomyocytes under hypoxic/ischemic conditions, providing an insight into the regulation of cardiomyocyte autophagy through p27. The present study paves the way for further studies investigating therapeutic targets of p27 in the early stages of MI.

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

Authors’ contributions
NZ and JW wrote the manuscript and edited the figures. NZ, WC, QH, JW, DL and YG carried out the molecular laboratory work and participated in the data analysis. DL and QH revised the manuscript and acquired funding. DL, JW and YG supervised all experimental process. QH prepared the supplementary data.

Ethics approval and consent to participate
The protocols of the present study were approved by the Ethics Review of Lab Animal Use Application of Nanjing Medical University (Nanjing, China), and the procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


