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ORIGINAL ARTICLE

LncRNA MALAT1 Promotes Oxygen-Glucose Deprivation and Reoxygenation Induced Cardiomyocytes Injury Through Sponging miR-20b to Enhance beclin1-Mediated Autophagy

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Abstract

Background/Aims LncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is reported to be highly expressed in myocardial I/R injury and closely related to autophagy. However, the exact biological role of MALAT1 and its underlying mechanism in myocardial I/R injury remain to be elucidated.

Methods We established incultured H9C2 cardiomyocytes an oxygen-glucose deprivation and reoxygenation (OGD/R) model for 6 h and then reoxygen-glucose for 4 h. We measured cell damage and autophagy levels after OGD/R by real-time quantitative PCR and Western blot. The relationships between miR-20b and MALAT1, beclin1 were confirmed by luciferase reporter assay.

Results We found that the expression of MALAT1 and beclin1, cell damage levels (lactate dehydrogenase (LDH) release, 222.4 ± 29.4 vs. 577.5 ± 27.4 U/L; creatine kinase MB isoenzyme (CK-MB), 1.0 ± 0.2 vs. 4.3 ± 0.4; cardiac troponin I (cTn-I), 1.0 ± 0.3 vs. 3.0 ± 0.3; $p < 0.05$), and autophagy levels were significantly increased after OGD/R model, while cell viability (100.0 vs. 54.2 ± 2.2%, $p < 0.05$) and the expression of miR-20b and P62 were reduced; the trend of all the above data was significantly reversed by MALAT1 siRNA. In addition, the luciferase reporter assay results confirmed that MALAT1 directly binds to miR-20b-5p and functions as a ceRNA for miR-20b-5p to regulate beclin1. As a result, MALAT1 overexpression antagonized while MALAT1 knockdown enhanced the inhibitory effects of miR-20b-5p on beclin1-related cardiomyocytes autophagy in OGD/R injury.

Conclusion LncRNA MALAT1 promotes OGD/R-induced cardiomyocytes injury through sponging miR-20b to enhance beclin1-mediated autophagy.

Keywords Ischemia reperfusion · MALAT1 · MiR-20b · Beclin1 · Autophagy

Introduction

Myocardial infarction is the leading cause of death worldwide, causing a huge burden on individuals and society [1]. Not only the disease of the patient itself can cause myocardial ischemia, but also many clinical operations and treatments (e.g., heart valve surgery) may cause myocardial ischemia [2]. The only effective measure for clinical treatment of myocardial infarction or ischemia is to restore reperfusion of blood flow. However, reperfused blood flow can cause the accumulation of harmful media such as reactive oxygen species, causing secondary damage to the cardiovascular system. Thus, understanding the mechanisms governing myocardial I/R injury is essential for the discovery of new strategies to control myocardial I/R injury.

Autophagy is an evolutionarily conserved intracellular degradation process that maintains cellular homeostasis

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by removing damaged proteins and organelle turnover [3]. Autophagy has been shown to exert a variety of physiological and pathophysiological effects in a wide variety of cells and tissues. Autophagy disorders are associated with many disorders, including dilated cardiomyopathy [4], ischemic heart disease [5], and heart failure [6]. Emerging evidence has suggested that inhibition of autophagy is an effective strategy to improve myocardial I/R injury [3] and beclin1 is the key egulatory molecule of autophagy in myocardial I/R injury in vitro and in vivo [7, 8]. However, the underlying mechanisms for regulating beclin1 have not yet been fully elucidated.

Non-coding RNAs (ncRNAs) are functional RNA molecules that are not translated into proteins but can regulate the expression and function of many protein-coding genes [9]. Studies have shown that myocardial I/R strongly affects the expression profiles of various ncRNA species such as microRNAs (miRNAs, 20–25 nt) and long noncoding RNAs (lncRNAs, > 200 nt) [10]. A lot of evidence has indicated miRNAs as crucial mediators of posttranscriptional gene silencing in pathogenic mechanisms of myocardial I/R injury, such as inflammation, apoptosis, and autophagy [11]. Furthermore, some subsets of miRNAs are also used to treat myocardial I/R injury [12]. Compared with that of miRNAs, the function of lncRNAs in myocardial I/R injury pathogenesis is much less well known. However, emerging evidence has suggested a functional involvement of lncRNAs in myocardial I/R injury by acting as competing endogenous RNAs (ceRNAs) that regulate specific RNA transcripts by competing for shared miRNAs [13]. This lncRNA–miRNA crosstalk might be a prominent mechanism controlling myocardial I/R injury and post-ischemia recovery.

LncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) controls key biological processes such as cellular proliferation and differentiation and the abnormal expression of MALAT1 has been found in the progression of a variety of organ I/R injury [14, 15]. Increasing evidence has suggested that MALAT1 is highly expressed in myocardial I/R injury and is a potential autophagy inducer in I/R injury [16]. Mukhopadhyay et al. confirmed that miR-20b has a significant counteracting effect on I/R-mediated vascular remodeling in vivo [11]. However, the regulation of MALAT1 on autophagy in myocardial I/R injury has not yet been elucidated. In addition, MALAT1 and beclin1 possess putative binding sites in miR-20b with bioinformatics. In this study, we investigated the regulatory function of MALAT1 and explored the role of MALAT1/miR-20b/beclin1 axis in oxygen-glucose-deprivation/reoxygenation (OGD/R)-induced autophagy in vitro, which may lead to novel therapeutic strategies for myocardial I/R injury.

Materials and Methods

Reagents and Antibodies

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, trypsin-EDTA, and the above related reagents for culturing cells were purchased from (GIBCO Laboratories, New York, USA). MALAT1 small interfering RNA (siRNA) (si-MALAT1), siRNA control (si-NC), pcDNA-MALAT1, pcDNA empty vector (pcDNA-NC), miR-20b mimics (miR-20b), miRNA control (mimic-NC), miR-20b inhibitor, and inhibitor control (inhibitor-NC) were all purchased from Suzhou GenePharma. Antibodies used in the experiment were as follows: anti-beta-actin (sc-47778; Santa Cruz, USA), anti-LC3 antibody (L7543, Sigma, USA), and anti-P62 antibody (ab56416, Abcam, USA), anti-beclin1 antibody (ab207612, Abcam, USA). Assay kits used in the experiment were as follows: cell counting kit-8 (CCK-8) (CK04; Shanghai Tongren, China), lactate dehydrogenase (LDH) assay kit (A020-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), rat creatine kinase-MB (CK-MB) ELISA kit (CSB-E14403r, Cusabio, China), rat cardiac troponin I (cTnI) elisa kit (CSB-E08594r, Cusabio, China).

Cell Culture and Cell Oxygen-Glucose Deprivation and Reoxygenation (OGD/R) Model

H9C2 embryonic rat heart-derived (ventricular) cells (myoblasts) from ATCC, at the passages 5 to 10, were used in this study. H9C2 cells were cultured in an incubator with DMEM+10% FBS +100 units/mL penicillin and 100 mg/mL streptomycin under the condition of 37 °C 5% CO_2 and 95% O_2. Hypoxic environment was a moist and closed plastic vessel. For OGD treatment, the cells were placed in deoxygenated glucose-free medium and then placed onto the hypoxic vessel, filled with a mixture of 95% N_2 and 5% CO_2 for 5 min, and subjected to hypoxia for 6 h under 37 °C and then transferred to normal culture medium and maintained under normoxic conditions (5% CO_2) at 37 °C for 4 h, following. For detection of the methods of Chen et al. [17].

Cell Viability Measured by CCK-8 and LDH Assays

CCK-8 cell viability and LHD activity were measured according to the method of the kit instructions. Briefly, we added 10 μl CCK-8 reagent to a 96-well plate at 2 h of reoxygenation, and the absorbance at 450 nm was measured with a microplate reader at the end of reoxygenation. We hypothesized that the cell viability of the control group was 100%, and the cell viability of all treatment groups was shown as a percentage of the cell viability of the control group. When detected LDH activity, we collected the cell supernatant at the end of

reoxygenation, and the experimental procedure was followed according to the instructions, and finally the absorbance at 450 nm was measured with a microplate reader, following the methods of Deng et al. and Chen et al. [17, 18].

The Release of CK-MB and cTn-I

After the end of OGD/R, the cell supernatant was collected for detection of the levels of CK-MB and cTn-I release; all procedures were carried out according to the kit instructions and we followed the methods of Deng et al. and Chen et al. [17, 18]. The standard curve of the numerical test was established. After using the data from the control group to obtain the average, all the values including the control group were divided by the average to obtain the corresponding multiple, and then mean and SD were calculated.

Quantitative Real-time PCR

Total RNA was isolated from cultured H9C2 cells after H/R using TRIzol reagent (Invitrogen). One microgram of extracted RNA was then reverse transcribed into cDNA according to the manufacturer's instructions of a reverse transcriptase kit (Takara, Dalian, China). The RT-PCR was performed to quantify the expressions of MALAT1 and beclin1 with SYBR Green Real-time PCR Master Mix (Takara, Dalian, China) and miR-20bwith the miScript SYBR Green PCR Kit (Qiagen, German) on an ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The procedures of PCR were performed as follows: 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The relative quantitative of value was determined using the 2-ΔΔCt method and GAPDH expression was used as an internal control. Primer sequences used in PCR are as follows: MALAT1, 5'-GAATTGCGTCATTT AAAGCCTAGTT-3' (forward) and 5'-ACGUGACA CGUUCGGAGAATT-3' (reverse);GAPDH, 5'-TGTTGCCA TCAATGACCCCTT-3' (forward) and 5'-CTCCACGA CGTACTCAGCG-3' (reverse);U6, 5'-GCTTCGGC AGCACATATACT-3' (forward) and 5'-AACGCTTC ACGAATTTGCGT-3' (reverse);beclin1, 5'-CCTTGGAG GAGGAGAGGCTGATC-3' (forward) and 5'-AGCATGGA GCAACAACACTGTCTG-3' (reverse);miR-20b, 5'-TGCAGTAGTTTTGGCATGA-3' (forward) and 5'-TCAACAAGAGATTTGTTATCCAAGAG-3 (reverse), and we followed the methods of Wei et al. [19].

Western Blot Analysis

Total protein was extracted from cultured H9C2 using ice-cold RIPA lysis buffer together with a protein inhibitor mixture. For gel electrophoresis, total extracted proteins (30 μg) of each sample were separated by 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and then transferred to nitrocellulose membranes, followed by blocking with 5% nonfat milk for 2 h at room temperature. Subsequently, the membranes were incubated with primary antibodies against beclin1, LC3, P62, and β-actin as a control. After three washes, the membranes were further incubated with specific horseradish peroxidase conjugated secondary antibodies, following the methods of Deng et al. [20].

H9C2 Transfection

H9C2 were grown on 24-well plates (1×10^5 cells/well) and maintained for overnight before transfection. Cell transfection with si-MALAT1, miR-20b, miR-20b inhibitor, pcDNA-MALAT1, or corresponding controls, or cotransfection with miR-20b or mimic-NC and pcDNA-MALAT1, or vector into H9C2 cells was performed by Lipofectamine 2000, folllowing the methods of Chen et al. [17].

Luciferase Reporter Assay

The sequences of wild-type 3'-UTR of beclin1 gene (WT) and a mutant (MUT) containing the putative binding sites of miR-20b predicted by the TargetScan (http://www.targetscan.org) and the putative miR-20b target binding sequences in MALAT1 wild-type (WT) and a mutant (MUT) predicted by Starbase v.2.0 and miRcode were synthesized and cloned into the downstream of luciferase gene in the pmirGLO dual luciferase reporter vectors (GenePharma, Suzhou, China). The constructed luciferase vectors were named as beclin1-3'-UTR-WT, beclin1-3'-UTR-MUT, MALAT1-WT, and MALAT1-MUT, respectively. H9C2 cells were cotransfected either MALAT1-WT or MALAT1-MUT with miR-20b or mimic-NC, or either beclin1-3'-UTR-WT or beclin1-3'-UTR-MUT with miR-20b, mimic-NC, miR-20b + pcDNA-MALAT1, or miR-20b + pcDNA-NC using Lipofectamine 2000. Firefly and Renilla luciferase activities were measured consecutively by dual luciferase assay at 48 h after transfection. Renilla luciferase activity was used as the normalization, following the methods of Wei et al. [19].

Data Analysis and Statistics

All data are presented mean ± SD. All statistical analyses were conducted using SPSS 13.0 software (SPSS, Chicago, IL, USA). The normality distribution and homogeneity of variance of data were tested first. The difference between two groups was compared by unpaired *t* test and multiple groups were compared by one-way analysis of variance (ANOVA) when data conform to normality distribution and had homogeneity of variance, while the rank-sum test was used to test the data which did not conform to normal distribution or homogeneity of variance. Differences with $p < 0.05$ were considered statistically significant.

Results

MALAT1, beclin1, and Autophagy Were Upregulated and miR-20b Was Downregulated in H9C2 OGD/R Models

As shown in Fig. 1, H9C2 cardiomyocytes subjected to OGD/R (6 h/4 h) exhibited cell damage (Fig. 1a–d). After OGD/R, cell viability was significantly decreased (Fig. 1a) (100.0 vs. 54.2 ± 2.2%, $p < 0.05$), while LDH release (Fig. 1b) (221.4 ± 29.4 U/L vs. 577.5 ± 27.4 U/L, $p < 0.05$), CK-MB level (Fig. 1c) (1.0 ± 0.2 vs. 4.3 ± 0.4, $p < 0.05$), and cTnI level (Fig. 1d) (1.0 ± 0.3 vs. 3.0 ± 0.3, $p < 0.05$) were increased. Furthermore, compared with normal control (NC) group, higher MALAT1 levels (Fig. 1e) (1.0 ± 0.2 vs. 4.0 ± 0.3, $p < 0.05$) and beclin1 levels (Fig. 1f) (1.0 ± 0.1 vs. 4.5 ± 0.2, $p < 0.05$) and lower miR-20b levels (Fig. 1g) (1.0 ± 0.1 vs. 0.5 ± 0.2, $p < 0.05$) were detected in OGD/R group. The western blot analysis revealed increased beclin1 expression (Fig. 1i) (1.0 ± 0.2 vs. 3.6 ± 0.3, $p < 0.05$) and ratio of LC3II/LC3I (Fig. 1j) (1.0 ± 0.1 vs. 2.1 ± 0.2, $p < 0.05$) along with decreased P62 expression (Fig. 1k) ($p < 0.05$).

Downregulation of MALAT1 Suppressed OGD/R-Induced Cell Injury and Autophagy

To explore the biological roles of MALAT1 in cardiomyocytes OGD/R injury, H9C2 cells were transfected with si-MALAT1 or si-control prior to OGD/R. As shown in Fig. 2, OGD/R-induced cell viability was significantly decreased (Fig. 2a) ($p < 0.05$) and LDH release (Fig. 2b) ($p < 0.05$), CK-MB levels (Fig. 2c) ($p < 0.05$), and cTnI levels (Fig. 2d) ($p < 0.05$) were notably increased in comparison with NC group while si-MALAT1 dramatically reversed this effect compared with si-control group. QRT-PCR results validated the inhibition of si-MALAT1 (Fig. 2e) ($p < 0.05$) and revealed increased expression of miR-20b (Fig. 2f) ($p < 0.05$) and decreased expression of beclin1 (Fig. 2g) ($p < 0.05$). Furthermore, si-MALAT1 significantly decreased beclin1 protein expression (Fig. 2i) ($p < 0.05$) and ratio of LC3II/LC3I (Fig. 2j) ($p < 0.05$) along with increased P62 expression (Fig. 2k) ($p < 0.05$).

MALAT1 Serves as a Molecular Sponge for miR-20b and Negatively Regulated Its Expression

Accumulating evidence has indicated that lncRNAs contain complementary binding sites to miRNAs and competitively inhibit miRNA expression and function. To examine the relationship between MALAT1 and miR-20b in OGD/R-induced injury, we constructed a luciferase reporter system carrying wild-type MALAT1 (MALAT1-WT) or mutant MALAT1 with mutations at the putative miR-20b-5p binding site (MALAT1-MUT) (Fig. 3a). We found that cotransfection with miR-20b-5p mimic decreased while cotransfection with miR-20b-5p inhibitor increased the luciferase activity in MALAT1-WT but not in MALAT1-MUT-transfected H9C2 cells (Fig. 3b, c) ($p < 0.05$), suggesting that miR-20b-5p binds to MALAT1 at the predicted binding site. Furthermore, MALAT1 overexpression in H9C2 cells led to reduced miR-20b-5p expression while expression of si-MALAT1 resulted in the opposite effects (Fig. 3d, e) ($p < 0.05$). Taken together, these data supported that MALAT1 may serve as a molecular sponge for miR-20b and negatively regulate its expression.

MALAT1 Abolished the Inhibitory Effect of miR-20b on OGD/R-Induced Cell Damage and Autophagy

To further explore the effect of MALAT1 on the function of miR-20b in OGD/R-induced cell damage and autophagy, H9C2 cells were cotransfected with miR-20b or mimic-NC and pcDNA-MALAT1 or pcDNA-NC prior to OGD/R. We found that cotransfection with miR-20b mimic significantly increased cell viability (Fig. 4a) (71.3 ± 2.4 vs. 54.2 ± 2.2%, $p < 0.05$) and decreased LDH release (Fig. 4b) (397.0 ± 25.4 U/L vs. 585.8 ± 27.4 U/L, $p < 0.05$), CK-MB level (Fig. 4c) (2.6 ± 0.2 vs. 4.5 ± 0.3, $p < 0.05$) and cTnI level (Fig. 4d) (1.8 ± 0.2 vs. 3.1 ± 0.3, $p < 0.05$) in comparison with mimic-NC group under OGD/R condition while cotransfection with miR-20b mimic and pcDNA-MALAT1 dramatically reversed this effect compared with cotransfection with miR-20b-5p mimic and pcDNA-NC. QRT-PCR results confirmed the effect of cotransfection with miR-20b mimic or/and pcDNA-MALAT1 (Fig. 4e, f) ($p < 0.05$). Furthermore, cotransfection with miR-20b mimic reduced while cotransfection with miR-20b mimic and pcDNA-MALAT1 dramatically increased mRNA expression (Fig. 4g) ($p < 0.05$) and protein expression (Fig. 4i) ($p < 0.05$) of beclin1. In addition, western blot analysis showed that the ratio of LC3II/LC3I (Fig. 4j) ($p < 0.05$) was significantly reduced and P62 expression (Fig. 4k) ($p < 0.05$) was significantly increased after overexpressing miR-20b during OGD/R condition, while the above change trend was reversed by cotransfection with miR-20b mimic and pcDNA-MALAT1.

MALAT1 Positively Regulated the Derepression of beclin1 by Sponging miR-20b

Although there is no related reported between miR-20b-5p and beclin1, MALAT1 and beclin1 possess putative binding sites in miR-20b with bioinformatics. In order to explore the relationship between MALAT1, miR-20b, and beclin1, we constructed luciferase reporter systems carrying the wild-type 3'-UTR of beclin1 (beclin1-3'-UTR-WT) or a mutant 3'-UTR of beclin1 with mutations at the predicted miR-20b-5p binding site (beclin1-3'-UTR-MUT) (Fig. 5a). The results showed that

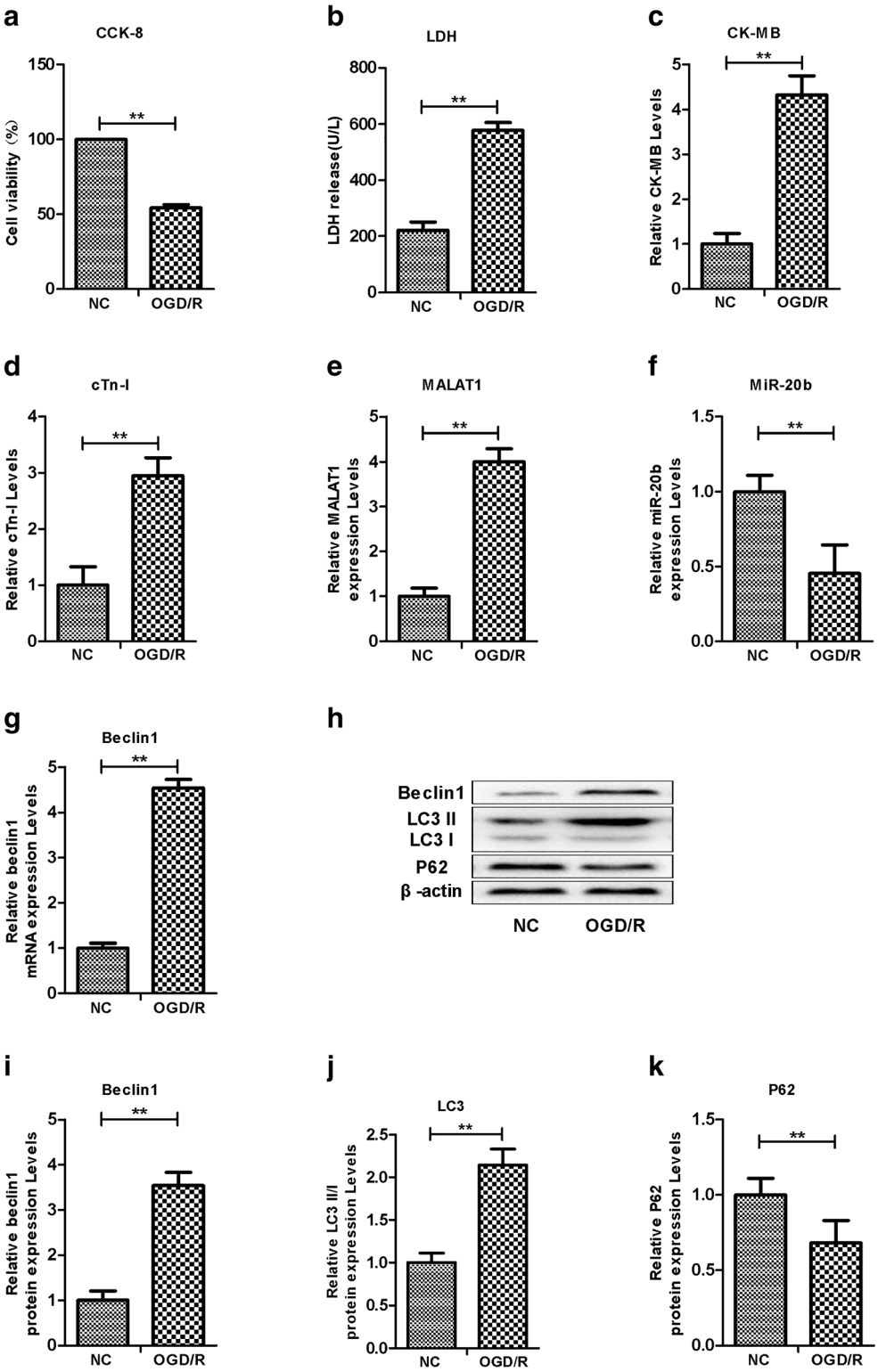

Fig. 1 MALAT1, beclin1, and autophagy were upregulated and miR-20b was downregulated in H9C2 OGD/R models. **a** Cell viability as detected by CCK-8. **b** Lactate dehydrogenase (LDH) activity assay. **c**, **d** The Elisa method detects the CK-MB and cTnI released by H9C2 cells. **e**–**g** The expressions of MALAT1, miR-20b, and beclin1 were examined by qRT-PCR in H9C2 cells after OGD/R. **f**–**i** The expressions of beclin1, LC3, and P62 were examined by western blot in H9C2 after OGD/R. Mean ± SD are from 5 different experiments. *refers to $p < 0.05$, ** refers to $p < 0.01$

cotransfection with miR-20b-5p mimic decreased the luciferase activity in beclin1 3'-UTR-WT but not in beclin1 3'-UTR-MUT-transfected H9C2 cells (Fig. 5b), indicating that miR-20b-5p directly targets beclin1 by binding to beclin1 3'-UTR at the predicted binding site. In contrast, MALAT1 overexpression increased while MALAT1 knockdown decreased the beclin1 3'-UTR-dependent luciferase reporter expression (Fig. 5c, d). In addition, MALAT1 overexpression reversed the inhibitory effects of miR-20b mimics on beclin1 3'-UTR-dependent luciferase reporter expression (Fig. 5e).

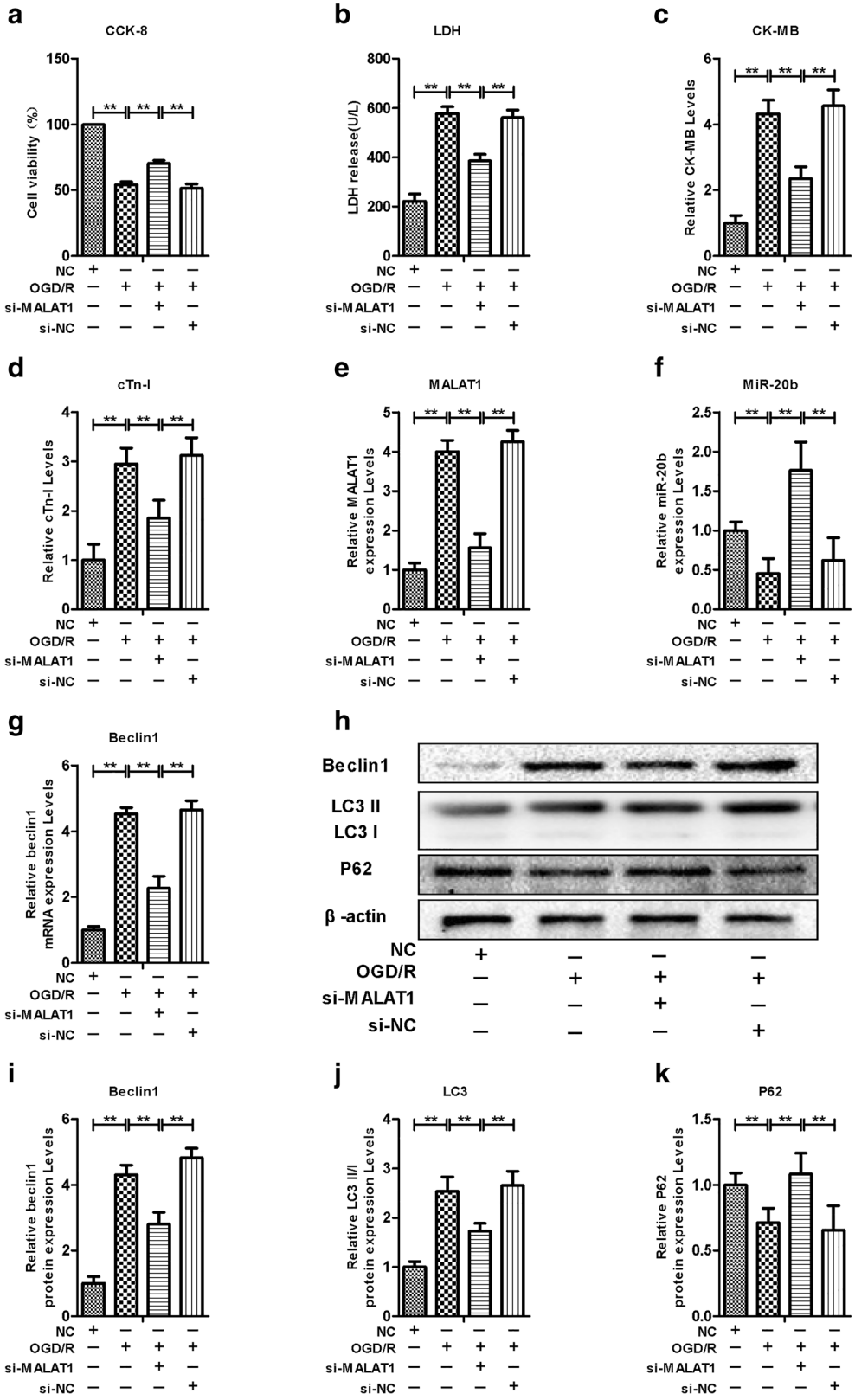

Fig. 2 Downregulation of MALAT1 suppressed OGD/R-induced cell injury and autophagy. **a** Cell viability as detected by CCK-8. **b** Lactate dehydrogenase (LDH) activity assay. **c**, **d** The Elisa method detects the CK-MB and cTnI released by H9C2 cells. **e**–**g** The expressions of MALAT1, miR-20b, and beclin1 were examined by qRT-PCR in H9C2 cells after OGD/R. **f**–**i** The expressions of beclin1, LC3, and P62 were examined by western blot in H9C2 after OGD/R. Mean ± SD are from 5 different experiments. *refers to $p < 0.05$, ** refers to $p < 0.01$

These data, taken together with the fact that we have detected a direct binding between MALAT1 and miR-20b-5p, indicated that MALAT1 regulates beclin1 by acting as a ceRNA for miR-20b-5p.

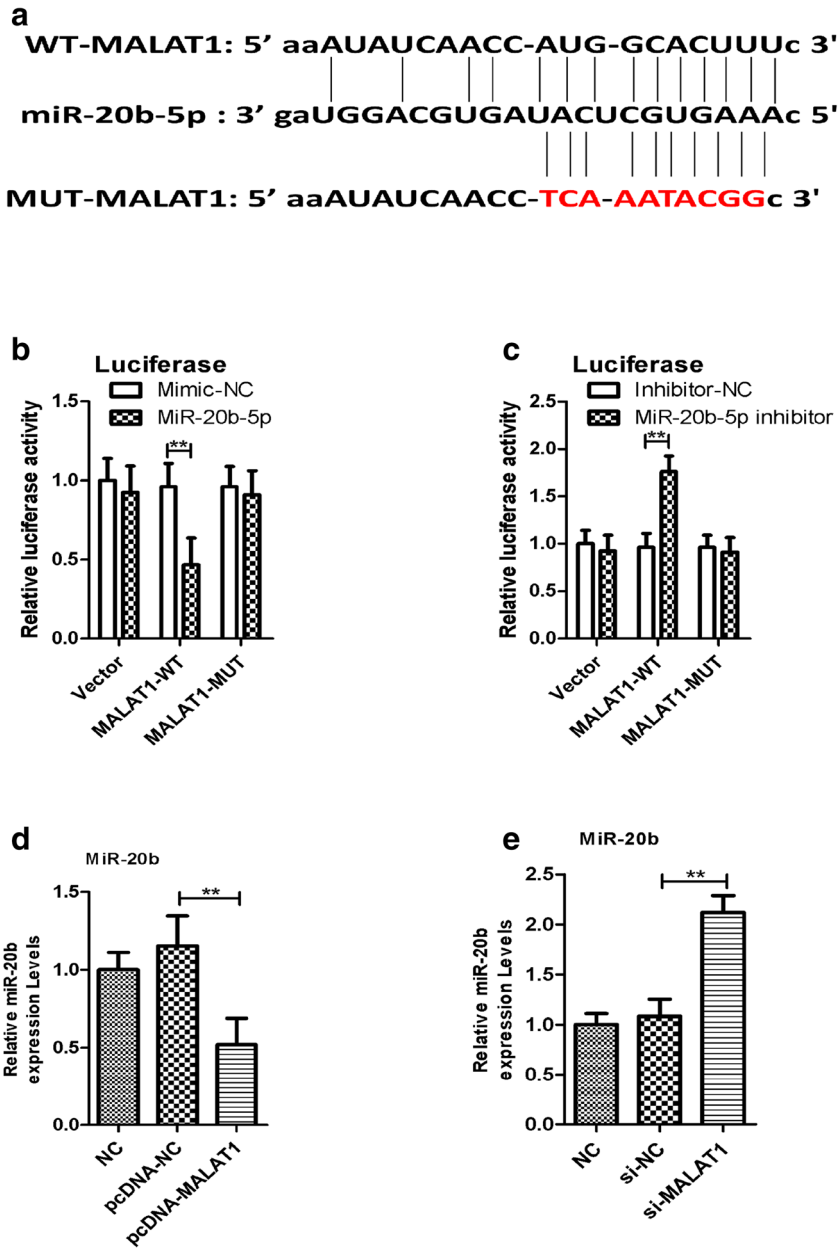

Fig. 3 MALAT1 serves as a molecular sponge for miR-20b and negatively regulated its expression. **a** The predictive binding sites between MALAT1 and miR-20b. **b**, **c** The luciferase reporter vectors (MALAT1-WT and MALAT1-MUT) were cotransfected with miR-20b mimics, miR-20b inhibitor, or matched controls into H9C2 and luciferase activity was detected by luciferase reporter assay. **d**, **e** The relative expression of miR-20b in H9C2 transfected with pcDNA-MALAT1, si-MALAT1 or corresponding controls was examined by qRT-PCR. Mean ± SD are from 5 different experiments. *refers to $p < 0.05$, ** refers to $p < 0.01$

MALAT1 Antagonizes the Inhibitory Effects of miR-20b Mimics on beclin1 to Regulate OGD/R-Induced H9C2 Cell Autophagy

To test the function of MALAT1 in cardiomyocytes I/R injury, we prepared H9C2 cells stably transfected with pcDNA-MALAT1, pcDNA-NC, si-MALAT1, or si-NC. In alignment with the effects of MALAT1 on beclin1 3'-UTR-dependent luciferase reporter expression, MALAT1 overexpression increased while MALAT1 knockdown decreased beclin1 expression in H9C2 cells as indicated by qRT-PCR and western blot analysis (Fig. 6a–c). Moreover, MALAT1 overexpression antagonized while MALAT1 knockdown enhanced the inhibitory effects of miR-20b-5p mimics on mRNA expression and protein expression of beclin1 (Fig. 6d–f). Consequently, MALAT1 overexpression restored OGD/R-induced H9C2 cell autophagy inhibited by miR-20b-5p mimics while MALAT1 knockdown had the opposite effects as revealed by western blot analysis of autophagy-related proteins (Fig. 6).

Discussion

Myocardial I/R injury caused by various pathways such as disease or clinical treatment has caused widespread concern. Emerging evidence has suggested a functional engagement of lncRNAs in myocardial I/R injury by acting as competing endogenous RNAs (ceRNAs) that regulate specific RNA

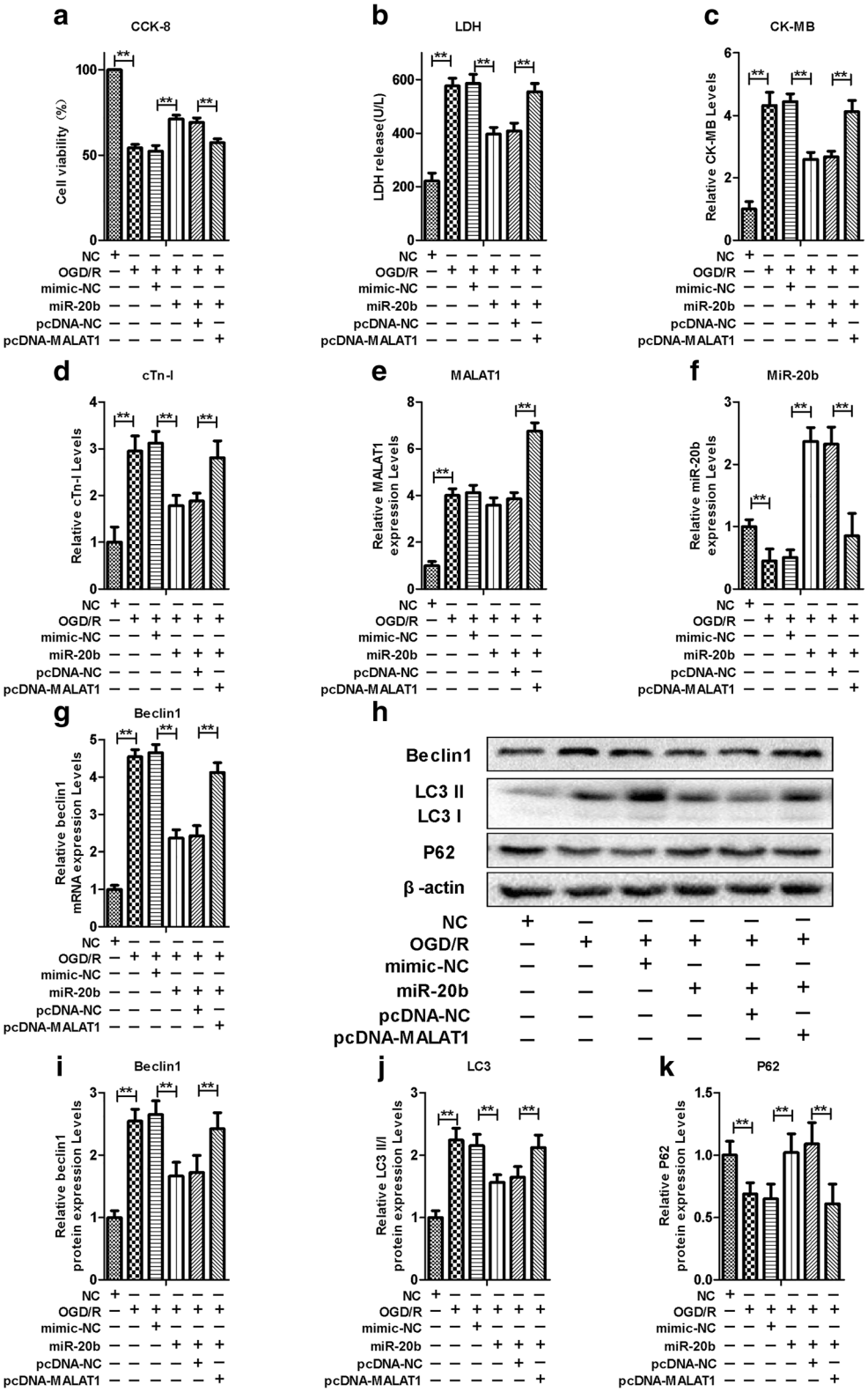

Fig. 4 MALAT1 abolished the inhibitory effect of miR-20b on OGD/R-induced cell damage and autophagy. H9C2 cells were cotransfected with miR-20b or mimic-NC and pcDNA-MALAT1 or pcDNA-NC to OGD/R. **a** Cell viability as detected by CCK-8. **b** Lactate dehydrogenase (LDH) activity assay. **c**, **d** The Elisa method detects the CK-MB and cTnI released by H9C2 cells. **e**–**g** The expressions of MALAT1, miR-20b, and beclin1 were examined by qRT-PCR in H9C2 cells after OGD/R. **f**–**i** The expressions of beclin1, LC3, and P62 were examined by western blot in H9C2 after OGD/R. Mean ± SD are from 5 different experiments. *refers to $p < 0.05$, ** refers to $p < 0.01$

transcripts through competing for shared miRNAs. This lncRNA-miRNA crosstalk might be a prominent mechanism controlling myocardial I/R injury and post-ischemia recovery. In this study, we found that MALAT1 was significantly upregulated in cardiomyocytes subjected to OGD/R, consistent with a previous study [14]. Furthermore, our study first revealed that downregulation of MALAT1 attenuated OGD/R-induced beclin1-dependent autophagy by regulating miR-

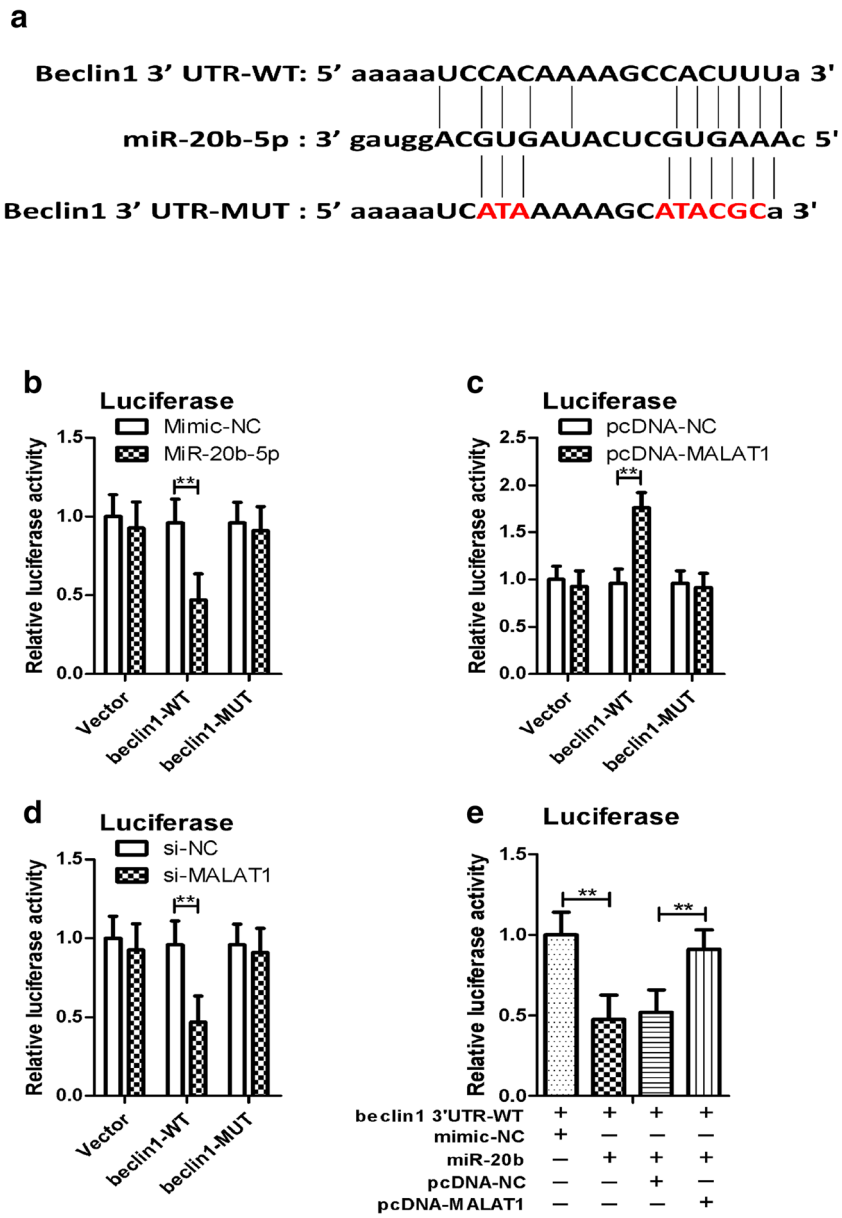

Fig. 5 MALAT1 positively regulated the derepression of beclin1 by sponging miR-20b. **a** The predictive binding sites between MALAT1 and miR-20b. **b**–**e** The luciferase reporter vectors (beclin1-WT and beclin1-MUT) were cotransfected with miR-20b mimics, pcDNA-MALAT1, si-MALAT1, or matched controls into H9C2 and luciferase activity was detected by luciferase reporter assay. Mean ± SD are from 5 different experiments. *refers to $p < 0.05$, ** refers to $p < 0.01$

20b in cardiomyocytes. Besides, our study demonstrated a novel lncRNA-miRNA-mRNA regulatory network that is MALAT1-miR-20b-beclin1 in myocardial I/R-induced myocardial autophagy, contributing to a better understanding the pathogenesis and progression of I/R injury.

MiRNAs are generally considered to be "initiators" and unilateral modulators of mRNA expression. However, advances in research over the past decade have shown that pseudogenes, lncRNAs, and circular RNAs (circRNAs) can act as ceRNAs by binding to consensus MREs (miRNA response element), thereby reducing the level of miRNA available for target mRNA. We found that advances in epigenetic genomics have focused on ncRNAs involved in I/R injury. Zhu et al. reported that MALAT1 may act as a competing endogenous RNA for miR-200c to upregulate the expression of HMGB1 and downregulate cardiac transient outward potassium current [21]. Wang et al. found that lncRNA-AK088388 can competitively bind to miR-30a, promoting the expression of beclin-1 and LC3-II, autophagy, and eventually myocardial I/R injury [22]. Tang et al. revealed the long non-coding RNA HOTAIR can function as competing endogenous RNA for miR-20b-5p and attenuates its inhibitory effect on ATG7 and that HOTAIR regulates autophagy via the miR-20b-5p/ATG7 axis in hepatic I/R injury, which may serve as a basis to develop novel therapeutic strategies to treat hepatic I/R injury [13]. Although miRNAs and lncRNA has been shown to play an important role in regulating autophagy, we have not found the role of miR-20b in regulating myocardial I/R-induced autophagy. In this study, we have identified the mechanism of miR-20b regulates beclin1-related autophagy

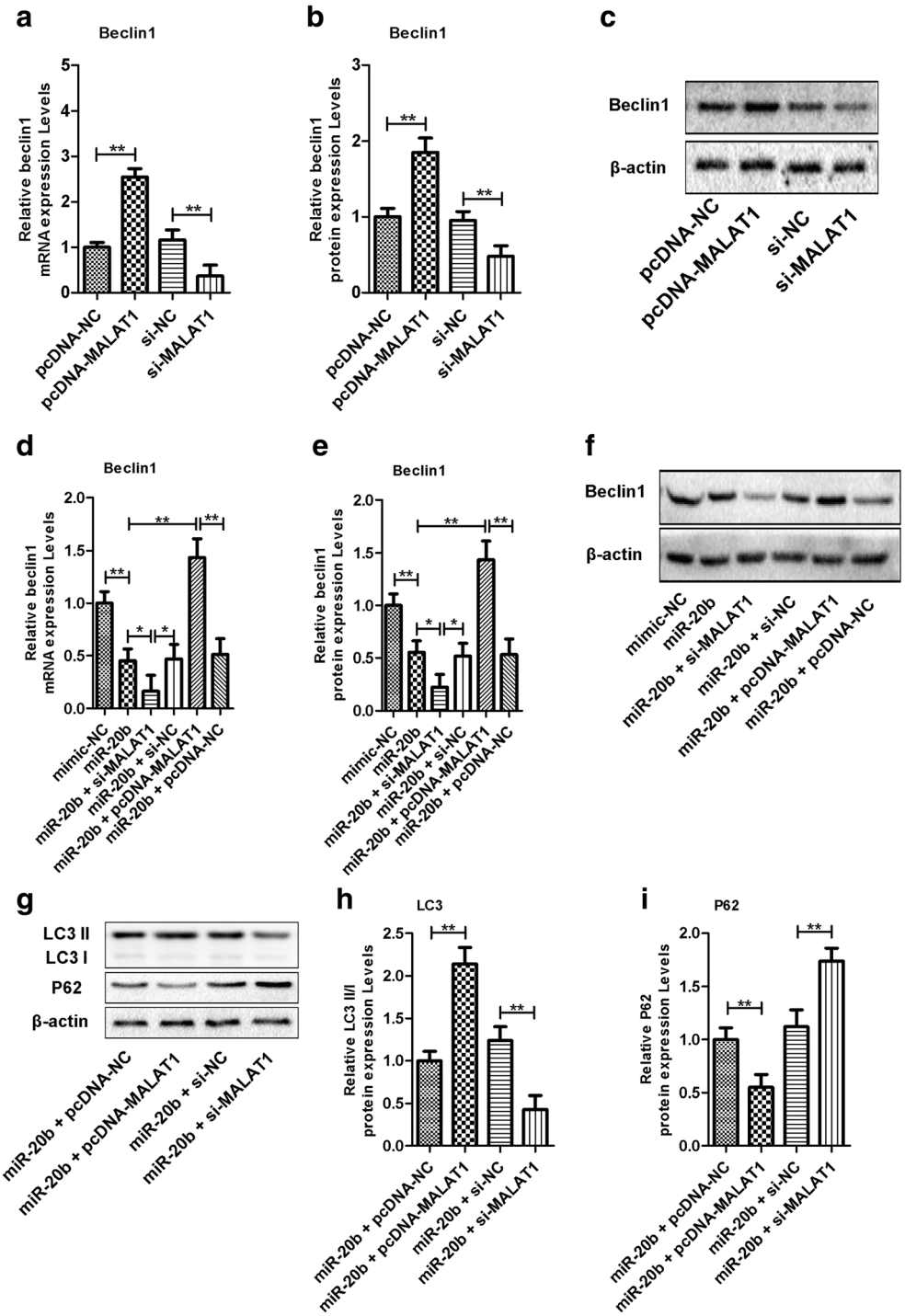

Fig. 6 MALAT1 antagonizes the inhibitory effects of miR-20b-5p mimics on beclin1 to regulate OGD/R-induced H9C2 cell autophagy. **a** The relative beclin1 mRNA levels in H9C2 cells stably transfected with pcDNA-MALAT1, pcDNA-NC, si-MALAT1, or si-NC detected by qRT-PCR. **b**, **c** The relative beclin1 protein levels in H9C2 cells stably transfected with pcDNA-MALAT1, pcDNA-NC, si-MALAT1, or si-NC detected by western blot. **d** The relative beclin1 mRNA levels in H9C2 cells cotransfected with pcDNA-MALAT1, pcDNA-NC, si-MALAT1, or si-NC and miR-20b detected by qRT-PCR. **e**, **f** The relative beclin1 protein levels in H9C2 cells cotransfected with pcDNA-MALAT1, pcDNA-NC, si-MALAT1, or si-NC and miR-20b detected by western blot. **g**–**i** The relative LC3 and P62 protein levels in H9C2 cells cotransfected with pcDNA-MALAT1, pcDNA-NC, si-MALAT1, or si-NC and miR-20b detected by western blot. Mean ± SD are from 5 different experiments. *refers to $p < 0.05$, ** refers to $p < 0.01$

in myocardial I/R injury for the first time, and confirmed the specific mechanism by which MALAT1 regulates miR-20b expression in myocardial I/R injury for the first time.

Recent studies have demonstrated a novel regulatory mechanism that lncRNAs serve as a miRNA sponge, thus modulating the derepression of miRNA target at a posttranscriptional level. For example, Zhu et al. found that MALAT1 may act as a competing endogenous RNA for miR-200c to upregulate the expression of HMGB1 and downregulate cardiac transient outward potassium current [21]. Li et al. reported that a new MALAT1-miR-26b-ULK2 regulatory axis in which MALAT1 served as a competing endogenous RNA by sponging miR-26b and upregulating ULK2 expression, thereby promoting brain microvascular endothelial cells autophagy and survival under OGD/R condition [23]. Ren et al. uncovered inhibitory functions of miR-145 on angiogenesis through directly targeting on VEGF-A and ANGPT2 for the first time and proved the protective role of lncRNA-MALAT1 for brain

microvascular endothelial cells under OGD/R condition through the direct regulation of miR-145 [24]. Although MALAT1 has been shown to be an autophagy inducer and plays an important role in I/R injury, its specific mechanism for regulating autophagy and I/R injury has not yet been elucidated. In our study, we demonstrated that MALAT1 could directly interact with miR-20b and negatively regulated its expression. Moreover, function analyses revealed that overexpression of MALAT1 partly overturned the inhibitory effect of miR-20b overexpression on autophagy and cardiomyocytes I/R injury in vitro, suggesting that MALAT1 served as a sponge of miR-20b, inhibiting both miR-20b expression and function. Additionally, the autophagy biomarker beclin1 was demonstrated to be a target of miR-20b, which was consistent with previous studies. More importantly, luciferase reporter system further revealed that MALAT1 overexpression partly abolished miR-20b-induced repressing activity on the beclin1 3'-UTR, as well as protein expression of beclin1. Collectively, these data suggested that MALAT1 regulated the derepression of beclin1 by sponging miR-20b. Overall, these results uncovered that downregulation of MALAT1 exerted its biological roles through inhibiting beclin1-dependent autophagy by regulating miR-20b expression in myocardial I/R injury.

Our research has some limitations. For example, the role of the MALAT1/miR-20b/beclin1 axis in improving myocardial I/R injury needs to be verified in vivo. Although our study may help to better understand the pathogenesis of myocardial I/R injury, how to better use it in the treatment of myocardial I/R patients needs further investigations in clinical settings.

Authors' Contributions Shuang Wang, Tao Yao, and Zhihua Ruan conceived and designed the project. Shuang Wang and Tao Yao performed the experiments with the help of Fan Deng, Wenqian Yu, Yiting Song, and Jingyi Chen. Fan Deng and Shuang Wang analyzed the data. Shuang Wang wrote the manuscript. All authors discussed the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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