

# Melatonin Protects Cardiomyocytes from Oxygen Glucose Deprivation And Reperfusion-Induced Injury by Inhibiting Rac1/JNK/Foxo3a/Bim Signaling Pathway

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## Original Article

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# Abstract

Melatonin has been shown to exert protective effect during myocardial ischemia/reperfusion (I/R). However, the underlying mechanism is not completely understood. Using the oxygen-glucose deprivation and reperfusion (OGD/R) model of H9c2 cells *in vitro*, we found that melatonin alleviated OGD/R-induced H9c2 cell injury via inhibiting Foxo3a/Bim signaling pathway. Inhibition of Rac1 activation contributed to the protective effect of melatonin against OGD/R injury in H9c2 cells. Additionally, melatonin inhibited OGD/R-activated Foxo3a/Bim signaling pathway through inactivation of Rac1. Furthermore, JNK inactivation was responsible for Rac1 inhibition-mediated inactivation of Foxo3a/Bim signaling pathway and decreased cell injury in melatonin-treated H9c2 cells. Taken together, these findings identified a Rac1/JNK/Foxo3a/Bim signaling pathway in melatonin-induced protective effect against OGD/R injury in H9c2 cells. This study provided a novel insight into the protective mechanism of melatonin against myocardial I/R injury.

## Introduction

Ischemia-reperfusion (I/R) injury is still one of the primary causes of death in ischemic heart disease [1, 2]. Cardiomyocyte apoptosis plays an essential role in acute myocardial I/R injury [3–5]. It is well known that cardiomyocytes are vulnerable to energy and nutrition depletion, and they will experience cell death immediately after I/R injury [5, 6]. Thus, alleviating I/R injury-induced cardiomyocyte apoptosis is of great importance for prevention and treatment of ischemic heart disease.

Melatonin is a naturally-occurring hormone with antioxidant property [7, 8]. Melatonin shows significant effects on numerous critical physiological and pathological processes due to its amphiphilic property [7, 8]. Previous studies demonstrated that melatonin has beneficial effects in myocardial I/R injury [5, 6, 9]. However, the underlying mechanisms about how melatonin plays beneficial role in myocardial I/R injury remains to be further elucidated.

Rac1, a member of the small GTPase proteins, is the predominant isoform of Rac expressed in cardiomyocytes and is pivotal in cardiomyocyte apoptosis [10, 11]. Previous studies demonstrated that Rac1 activation aggravates the damage of myocardial cells in the process of myocardial ischemia-reperfusion through reactive oxygen species (ROS) generation by activating the NADPH oxidase [12]. Rac1 activation was shown to be inhibited by melatonin in human umbilical vein endothelial cells (HUVECs) under hypoxic condition [13]. However, whether Rac1 signaling is involved in melatonin-ameliorated myocardial I/R injury remains unclear.

FOXO3a signaling pathway has been shown to play a critical role in regulating myocardial apoptosis *in vivo* and *in vitro* [14, 15]. It is revealed that FOXO3a serves as an important downstream of melatonin [16–18]. However, it is not known whether the protective effect of melatonin on cardiomyocytes is dependent on FOXO3a or not. Previous studies revealed that FOXO3a activity is regulated by Rac1 [19, 20], which is also reported to be as a critical downstream of melatonin [13]. Therefore, it is worthwhile to

determine the involvement of Rac1 and FOXO3a in the protective effect of melatonin against myocardial I/R injury.

In the present study, heart-derived H9c2 cells were cultured under oxygen-glucose deprivation and reperfusion (OGD/R) to mimic the myocardial I/R injury and to investigate whether Rac1 and FOXO3a are involved in the cardiac protective effect of melatonin and the potential mechanism.

## **Materials And Methods**

### **Cell culture and OGD/R model**

The H9c2 cardiomyocyte cell line was purchased from the Cell Line Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco/Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% (v/v) fetal bovine serum (FBS; Gibco/Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Carlsbad, CA, USA) at 37 °C in an atmosphere of 95% air and 5% CO<sub>2</sub>. For OGD/R model, H9c2 cells were exposed to hypoxic conditions (oxygen deprivation, a mixed atmosphere of 94% N<sub>2</sub>, 5% CO<sub>2</sub>, and 1% O<sub>2</sub>) for 4 h in glucose-free medium. After hypoxia, the cells were oxygenated under a normal oxygen concentration (reoxygenation) for 24 h in a normal medium.

### **Cell transfection**

Foxo3a siRNA and negative control siRNA were purchased from Genepharma Biotech., (Shanghai, China). Full-length Rac1-V12 were cloned into the pEGFP-N1 vector. The oligonucleotides and vectors were then transfected into H9c2 cells using the Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's manual. After 24 h, the expression of target genes was determined and cells were used in further experiments.

### **Subcellular fractionation**

H9c2 cells were treated as described in text and then subjected to subcellular fractionation using the cytoplasmic and nuclear protein extraction kit (Beyotime Biotechnology, Nanjing, China). The efficacy of fractionation was determined via Western blotting using anti-tubulin (cytosolic control) and anti-Histone3 (nuclear control), respectively.

### **Western blot analysis**

Total proteins from each group was extracted from cells using RIPA lysis buffer (Sigma, MO, USA). The concentration of each sample was quantified by BCA assay (Beyotime, China). A total of 50 µg of protein was separated by 10% SDS-PAGE, and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA) by wet transfer. The membranes were blocked with 5% milk for 1 h at room temperature, and then washed in TBST, subsequently incubated with primary antibodies specific to Bcl-2, Bax, Bad, caspase-3, Foxo3a, Bim, Rac1, Histone3, α-tubulin and GAPDH (all 1:1000; Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. The blots were treated with secondary antibodies conjugated to

HRP-linked secondary antibodies (1:2000, anti-rabbit; 1:5000, anti-mouse) for 1 h at room temperature. The blots were visualized with an enhanced chemoluminescence kit (Pierce; Thermo Fisher Scientific, Inc.). The band density was analyzed by Image J software.

## **Cell viability assay**

Cell viability was measured by a cell counting kit-8 (CCK-8) assay. Cells were seeded in 96-well cell culture plates at a density of  $2 \times 10^4$  cells/well. After treating as indicated in the text, the supernatant fluid was removed and a medium containing 10% CCK-8 reagent was added for 3 h incubation at 37 °C. The absorbance at 450 nm was measured using the Emax-precision microtiter plate reader (Molecular Devices, USA).

## **Cell apoptosis assay**

Annexin V-FITC/PI apoptosis detection kit (BD Bioscience, USA) was used to detect apoptosis by flow cytometry. Briefly, after treatments, H9c2 cells were harvested, washed twice with PBS, and resuspended in 500  $\mu$ l of binding buffer. 5  $\mu$ l of Annexin V-FITC and 5  $\mu$ l of propidium iodide were added and the mixture was incubated for 15 min in the dark. The apoptotic rates were determined using FACS Calibar (BD, CA, USA) and the data were analyzed by Modfit V3.0.

## **Pull-Down Assay**

Rac1 activity was measured using pull-down assay as described previously. Briefly, after treatments, equal volumes of total cellular protein were incubated with GST-PBD beads captured on MagneGST glutathione particles (Promega, Madison, WI). After incubation at 4 °C with constant rotation for 60 min, the particles were washed with washing buffer for three times and then resuspended in SDS sample buffer. The particles were then subjected to immunoblotting analysis by using anti-Rac1 antibody (Cell Signaling Technology).

## **Lactate dehydrogenase (LDH) activity detection**

Cells were incubated for the indicated treatments and the supernatants were collected for the measurement of LDH activity using a commercial available LDH kit (Nanjing Jian-cheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's manual.

## **Statistical analysis**

The results were presented as the mean  $\pm$  standard deviation (SD). Statistical analysis was performed using the SPSS 16.0 statistics software (SPSS, Chicago, IL). Significant differences were determined using One-way ANOVA followed by the Tukey's Honestly Significant Difference test.  $P < 0.05$  was considered statistically significant.

## **Results**

### **Melatonin attenuated OGD/R-induced H9c2 cell injury**

The protective effect of melatonin on OGD/R-induced H9c2 cell injury was firstly investigated. The results of CCK-8 and LDH assays showed that OGD/R exposure resulted in significant decrease in cell viability and increase in LDH release, which was attenuated by pretreatment with different concentrations of melatonin (Fig. 1A and 1B). The results indicated that the use of 0.1 mM and 0.5 mM melatonin were less effective, and no comparable attenuation effects on cell viability and LDH release were observed at the concentrations of 1 mM and 5 mM. Thus, 1 mM melatonin was chosen in the subsequent experiments. As shown in Fig. 1C, the cell apoptosis rates under OGD/R were significantly higher than those under OGD/R with melatonin treatment. Western blot analysis revealed that melatonin pretreatment significantly down-regulated pro-apoptotic proteins (Bad and Bax) and up-regulated the anti-apoptotic Bcl-2 in OGD/R-treated H9c2 cells (Fig. 1D). These data suggest that melatonin attenuated OGD/R-induced H9c2 cell injury.

### **Melatonin protected H9c2 cells against OGD/R injury via inhibiting Foxo3a/Bim signaling pathway**

As Foxo3a has been demonstrated to play a critical role in I/R-induced cardiomyocyte apoptosis [14, 15, 21], we then determined whether melatonin exerts any influence on Foxo3a in H9c2 cells upon OGD/R exposure. As shown in Fig. 2A, OGD/R exposure induced up-regulation of Foxo3a, whereas melatonin treatment reversed OGD/R-induced up-regulation of Foxo3a and Bim, the well-known downstream of Foxo3a. OGD/R exposure promoted the nuclear translocation of Foxo3a, which could be inhibited by melatonin treatment (Fig. 2B). Moreover, under OGD/R condition, Foxo3a knockdown by siRNA significantly up-regulated cell viability (Fig. 2C), down-regulated LDH release (Fig. 2D) and Bim expression (Fig. 2E). Together, these data indicate that melatonin protected H9c2 cells against OGD/R injury via inhibiting Foxo3a/Bim signaling pathway.

### **Inhibition of Rac1 activation contributed to the protective effect of melatonin against OGD/R injury in H9c2 cells**

Rac1 plays a crucial role in the modulation of OGD/R-induced cell injury [22, 23]. The regulatory effect of melatonin on Rac1 was then investigated. As shown in Fig. 3A, OGD/R induced an increase in Rac1-GTP, while the level of total Rac1 protein in H9c2 cells remained unmodified. The increased Rac1 activation by OGD/R exposure was suppressed by melatonin. To determine the involvement of Rac1 activation in OGD/R-induced cell injury, H9c2 cells were pretreated with Rac1-GTP inhibitor, NSC23766. The results showed that when Rac1 activity was inhibited by NSC23766 (Fig. 3B), cell viability was increased (Fig. 3C) and LDH release was decreased (Fig. 3D) in OGD/R-treated H9c2 cells. These data indicated that Rac1 activation is required for OGD/R-induced H9c2 cell injury. To further determine whether the inactivation of Rac1 was contributed to melatonin mediated protection against OGD/R, H9c2 cells were transfected with Rac1-V12, an active mutant of Rac1. The results showed that Rac1-V12 overexpression (Fig. 3E) significantly reversed the effect of melatonin on cell viability (Fig. 3F) and LDH release (Fig. 3G) in OGD/R-treated H9c2 cells. Collectively, these results indicate that inhibition of Rac1 activation contributed to the protective effect of melatonin against OGD/R-induced injury in H9c2 cells.

### **Melatonin inhibited OGD/R-activated Foxo3a/Bim signaling pathway through inactivation of Rac1**

The above results indicated that Rac1 and Foxo3a signaling mainly contribute to the protective effect of melatonin on OGD/R-induced H9c2 cell injury. Previous studies have shown that activation of Foxo3a/Bim signaling pathway is primarily regulated by Rac1 [19, 20]. Thus, we investigated whether Rac1 is involved in melatonin-induced activation of Foxo3a/Bim signaling pathway in our system. The results showed that inactivation of Rac1 by NSC23766 significantly inhibited expression of Foxo3a and Bim in OGD/R-treated H9c2 cells (Fig. 4A). Furthermore, the inhibitory effects of melatonin on OGD/R-induced up-regulation of Foxo3a and Bim were reversed by overexpression of Rac1-V12 (Fig. 4B). Together, these results indicated that melatonin inhibited OGD/R-activated Foxo3a/Bim signaling pathway through inactivation of Rac1.

### **JNK inactivation was responsible for Rac1 inhibition-mediated inactivation of Foxo3a/Bim signaling pathway and decreased cell injury in melatonin-treated H9c2 cells**

To explore the mechanism underlying Rac1-mediated inactivation of Foxo3a/Bim signaling pathway in our system, we first examined the activation of JNK pathway in the melatonin-treated H9c2 cells. The western blotting results demonstrated that the level of phosphorylated JNK was increased by OGD/R exposure (Fig. 5A). However, the promotion to the phosphorylated JNK was inhibited by melatonin treatment (Fig. 5A). The inhibitory effect of melatonin on OGD/R-induced phosphorylation of JNK was reversed by overexpression of Rac1-V12 (Fig. 5B). Moreover, Rac1-V12 overexpression-induced up-regulation of Foxo3a and Bim in melatonin-treated H9c2 cells were reversed by pretreatment with SP600125, a JNK inhibitor (Fig. 5C). These results indicated that JNK acts as a downstream effector of Rac1 in mediating inactivation of Foxo3a/Bim signaling pathway in melatonin-treated H9c2 cells.

To investigate the role of JNK de-phosphorylation in the protective effect of melatonin, we then measured the cell viability and LDH release of melatonin-treated H9c2 cells, in the presence of anisomycin, a potent JNK agonist. The results showed that anisomycin treatment markedly deteriorated melatonin-mediated viability increase and LDH release decrease of OGD/R-treated H9c2 cells (Fig. 5D and 5E). Together, these experiments demonstrated that JNK inactivation was responsible for Rac1 inhibition-mediated inactivation of Foxo3a/Bim signaling pathway and decreased cell injury in melatonin-treated H9c2 cells.

## **Discussion**

The present study firstly demonstrated that melatonin-induced Rac1 inactivation contributes to the protective effect of melatonin against OGD/R injury in cardiomyocytes. Previous studies have demonstrated that increased levels of active myocardial Rac1 renders the heart susceptible to I/R injury [11, 24, 25]. As other Rho GTPases, Rac1 cycle between an active and inactive state thanks to specific regulators that catalyze exchange of GDP into GTP (Rac1-GEF) or hydrolysis of GTP into GDP (Rac1-GAP) [26, 27]. It has been demonstrated that the activity of Rac1 was regulated by specific GEF or GAP in different cell settings [26, 27]. Thus, it will be worthwhile to investigate which cardiomyocyte-specific GAP mediates melatonin-induced Rac1 inactivation in our system.

The FOXO3a/Bim signaling pathway plays a crucial role in the modulation of I/R-induced cell apoptosis [21, 28, 29]. Previous studies demonstrated that melatonin inhibits I/R-induced cell apoptosis in many types of cells, including cardiomyocyte[5, 6, 9]. Importantly, FOXO3a has been shown to serve as an important downstream target of melatonin and mediate melatonin-induced transcriptional regulation of proapoptotic Bim [30, 31]. Thus, we investigate whether FOXO3a/Bim signaling is involved in the protective effect of melatonin against OGD/R injury in cardiomyocytes. Our data showed that melatonin treatment significantly reduced OGD/R-induced activation of FOXO3a/Bim signaling pathway and FOXO3a knockdown significantly inhibited OGD/R-induced injury in H9c2 cells. These findings indicate that melatonin protected H9c2 cells against OGD/R injury via inhibiting Foxo3a/Bim signaling pathway.

The above findings in the present study suggested that Rac1 and Foxo3a/Bim pathway were involved in the protective effect of melatonin against OGD/R injury in cardiomyocytes. We then investigated the relationship between Rac1 and Foxo3a/Bim pathway in our system. The result showed that the inhibitory effects of melatonin on OGD/R-induced activation of FOXO3a/Bim signaling pathway were reversed by Rac1 activation, suggesting that Rac1 served as an upstream modulator of melatonin-induced inactivation of FOXO3a/BIM signaling pathway in our system. Our findings are consistent with previous reports which also revealed that Rac1 acted as an upstream regulator of FOXO3a/Bim signaling pathway in different cell types or cell settings[19, 20].

Activation of JNK signaling pathway has been demonstrated to be required for I/R-induced cardiomyocyte apoptosis [32–36]. A recent study by Lu et al reported that inactivation of JNK signaling pathway is an important mechanism for melatonin-induced apoptosis inhibition in cardiomyocyte during myocardial infarction[37]. Activation of JNK signaling pathway has also demonstrated to serve as a critical downstream of Rac1 during cardiomyocyte apoptosis[38]. Furthermore, JNK has been reported to be an upstream signaling in regulation of Foxo3a activity[39, 40]. We then investigated whether JNK is involved in Rac1-mediated inactivation of Foxo3a/Bim signaling pathway in our system. Our data showed that melatonin-induced JNK inactivation was reversed by overexpression of activated Rac1 in OGD/R-treated H9c2 cells. Inhibition of JNK activity by its inhibitor markedly reversed Rac1 activation-induced activation of FOXO3a/Bim signaling pathway in melatonin-treated H9c2 cells under OGD/R condition. Moreover, upregulation of JNK activity by its agonist obviously destroyed melatonin-ameliorated OGD/R injury in H9c2 cells. These findings suggested that JNK acts as a downstream of Rac1 in mediating inactivation of Foxo3a/Bim signaling pathway in melatonin-treated H9c2 cells.

## Conclusion

In conclusion, our findings revealed that melatonin exerted protective effects against OGD/R-induced H9c2 cell injury by inhibiting Rac1/JNK/Foxo3a/Bim signaling pathway. Our findings provided a novel insight into the protective mechanism of melatonin against myocardial I/R injury and warrant further study of targeting melatonin-Rac1 signaling as a potential clinical treatment for myocardial I/R injury. A limitation of current study is that all experimental results were obtained from the level of cells *in vitro*. Therefore, it will be important to verify our experimental results using animal experiments.

## Declarations

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## Author contributions

WY wrote the manuscript. JY and ZX performed the experiments. NB and WM analyzed the data. PC supervised the study.

## Data availability

The datasets used during the present study are available from the corresponding author upon reasonable request.

## Conflicts of interest

The authors declare that they have no competing interests.

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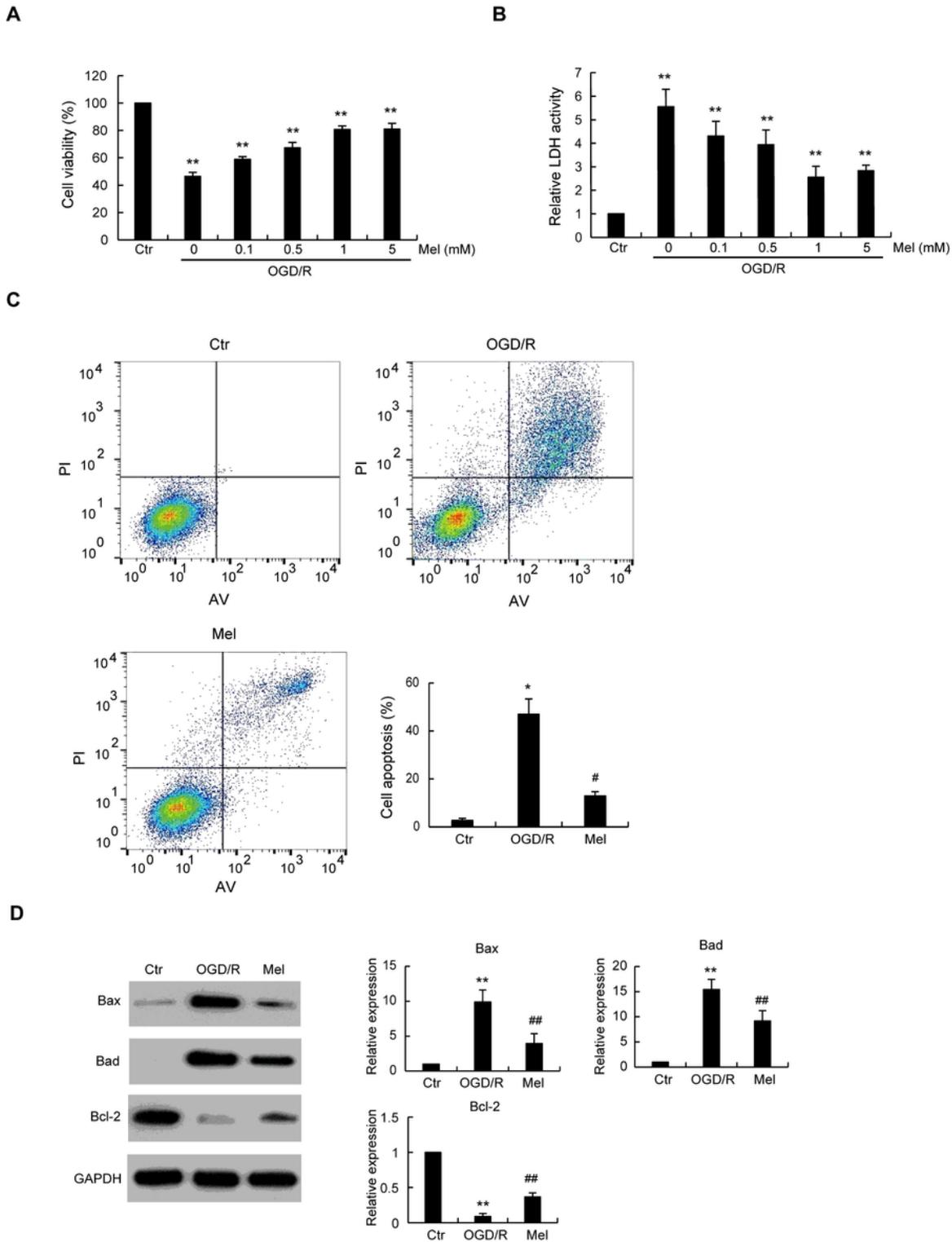
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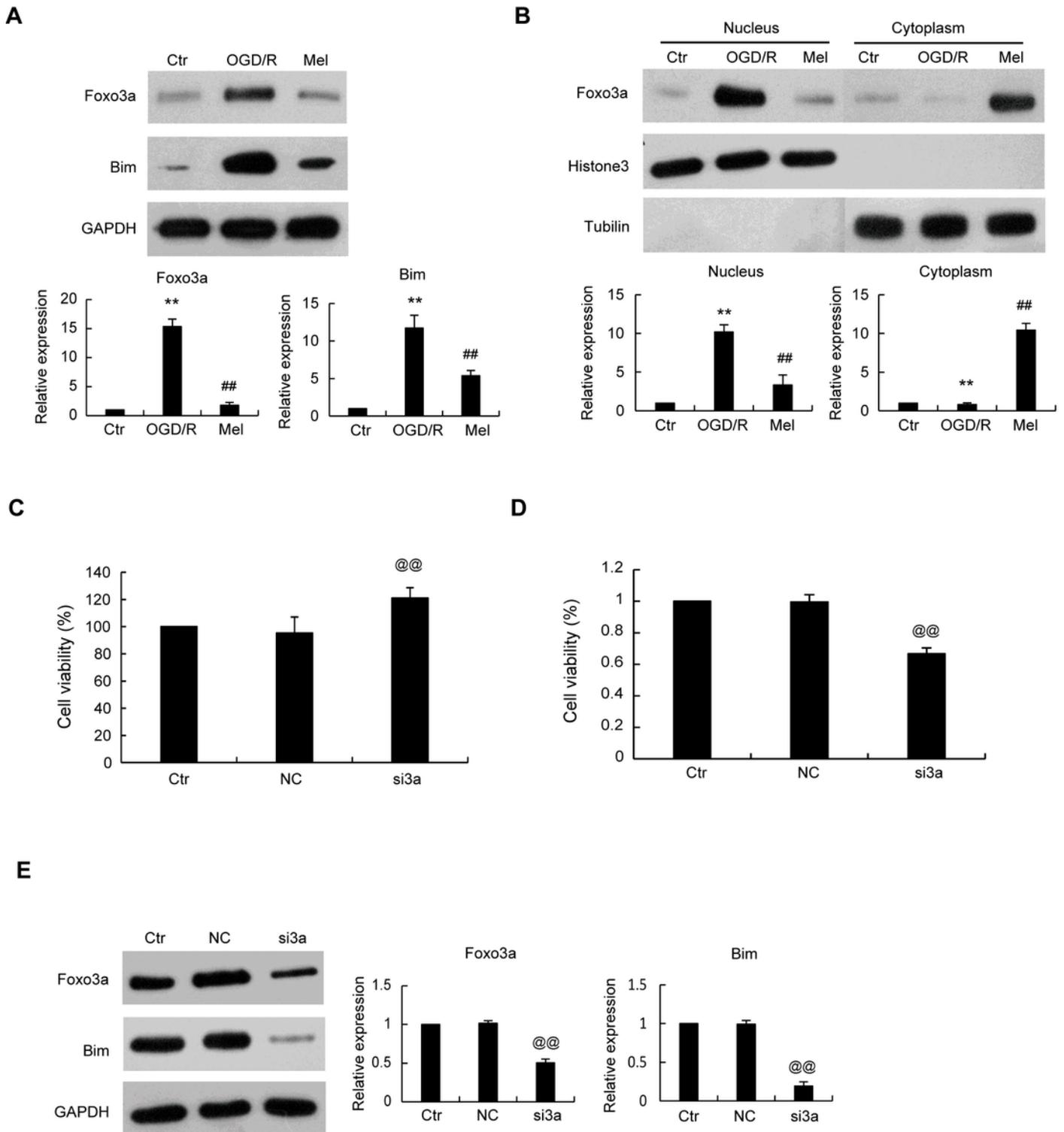
## Figures



**Figure 1**

Effect of melatonin on OGD/R-induced H9c2 cell injury. (A-B) H9c2 cells were incubated under OGD/R with or without different concentrations of melatonin pretreatment, cell viability (A) and cytotoxicity (B) were determined by CCK-8 assay and LDH release assay, respectively. (C-D) H9c2 cells were cultured for 24h under three conditions: normal (Ctr), OGD/R, OGD/R with 1mM melatonin (Mel), apoptosis of H9c2 cells was measured by Annexin V/PI staining (C) and the levels of apoptosis-related proteins were

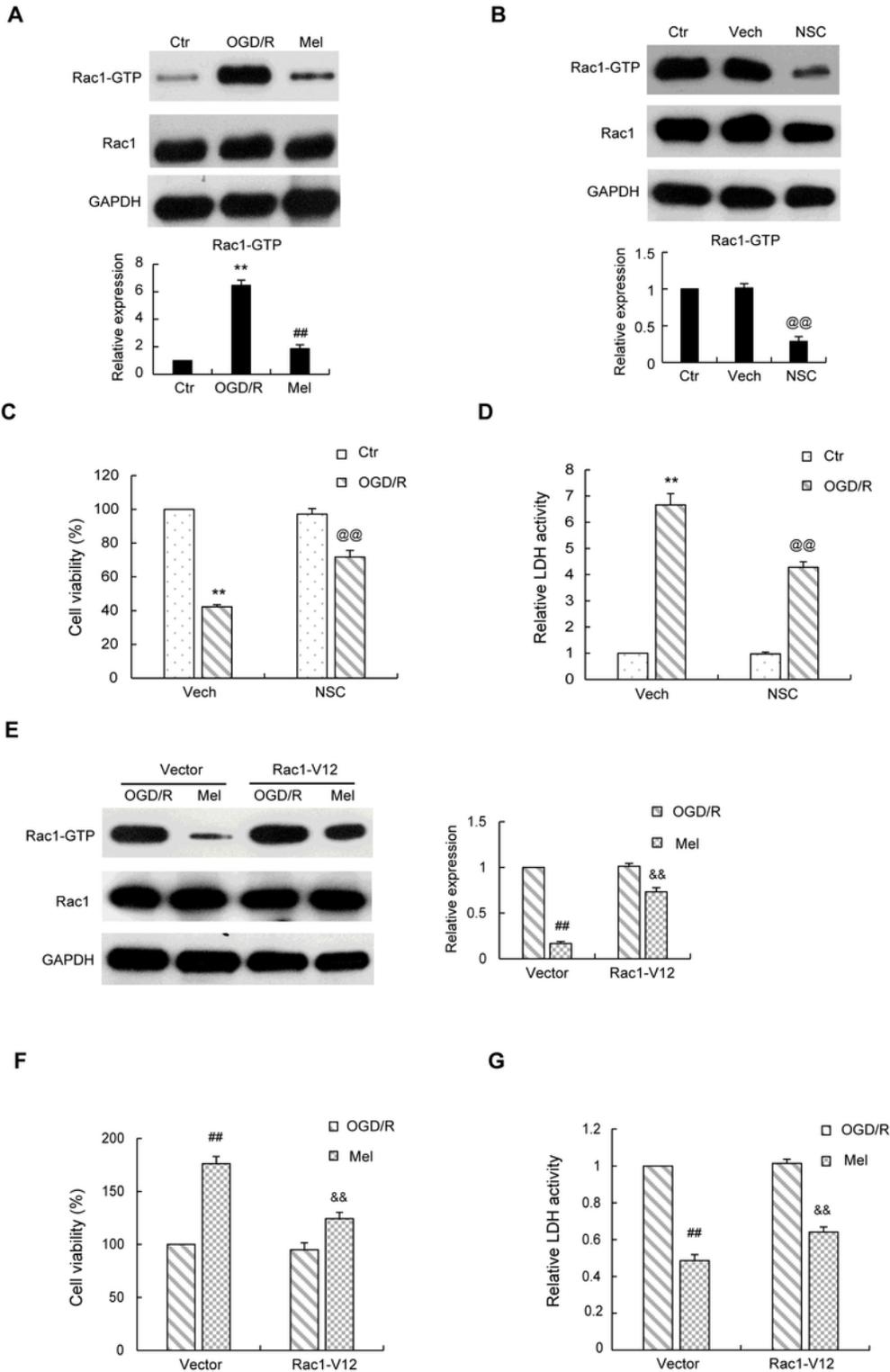
measured by western blot (D). \*P<0.05, \*\*P<0.01 compared with ctr; #P<0.05, ##P<0.01 compared with OGD/R.



**Figure 2**

Melatonin reduced OGD/R-induced H9c2 cell injury via inhibiting Foxo3a/Bim signaling pathway. (A-B) H9c2 cells were cultured for 24h under three conditions: normal (Ctr), OGD/R, OGD/R with 1mM melatonin (Mel), then the levels of Foxo3a and Bim were measured by western blot (A), and the extracts

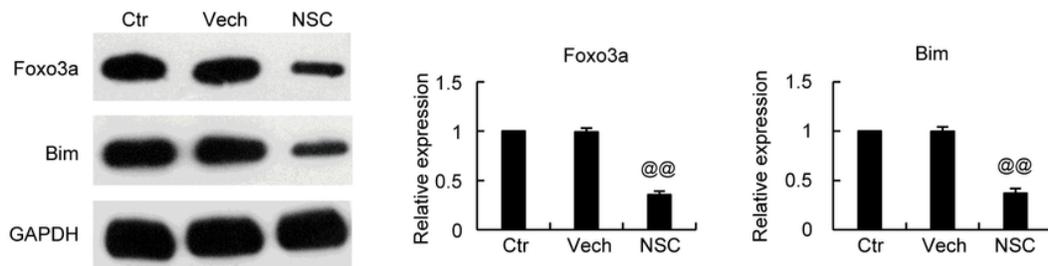
of plasma and nuclear section of H9c2 cells were subjected to immunoblotting analysis to detect the location and expression of Foxo3a separately. Tubulin and Histone3 were used for detecting cytoplasm and nucleus part. (C-E) H9c2 cells were transfected with negative control siRNA (NC) or Foxo3a siRNA (si3a), then cells were exposed to OGD/R, cell viability (C) was determined by CCK-8 assay, cell cytotoxicity (D) was determined by LDH release assay, and the levels of Foxo3a and Bim were measured by western blot (D). \* $P < 0.05$ , \*\* $P < 0.01$  compared with ctr; # $P < 0.05$ , ## $P < 0.01$  compared with OGD/R; @ $P < 0.05$ , @@ $P < 0.01$  compared with NC.



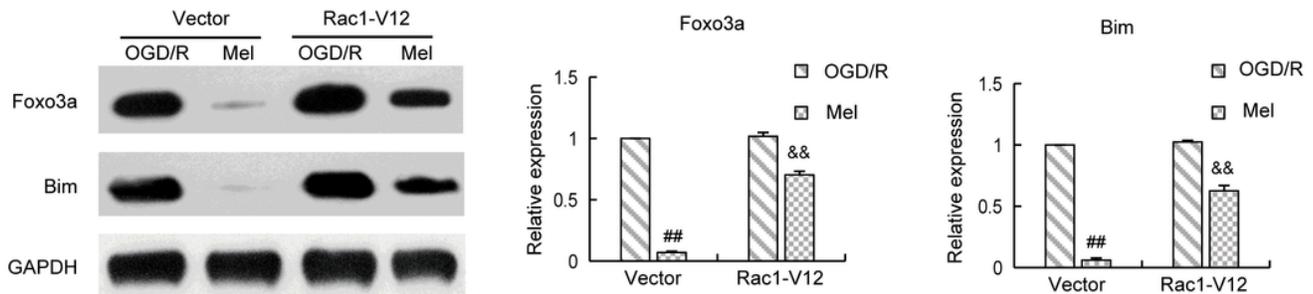
### Figure 3

Melatonin reduced OGD/R-induced H9c2 cell injury via inactivating Rac1. (A) H9c2 cells were cultured for 24h under three conditions: normal (Ctr), OGD/R, OGD/R with 1mM melatonin (Mel), then the levels of Rac1-GTP and total Rac1 were measured by western blot. (B-D) H9c2 cells were treated with Vehicle (Vech) or NSC23766 (NSC), then the cells were exposed to OGD/R or cultured in normal medium. The levels of Rac1-GTP and Rac1 were measured by western blot (B), cell viability (C) was determined by CCK-8 assay, cell cytotoxicity (D) was determined by LDH release assay. (E-G) H9c2 cells were transfected with empty plasmid (Vector) or Rac1-V12, then the cells were treated with 1mM melatonin (Mel) followed by OGD/R exposure. The levels of Rac1-GTP and Rac1 were measured by western blot (E), cell viability (F) was determined by CCK-8 assay, cell cytotoxicity (G) was determined by LDH release assay. \* $P < 0.05$ , \*\* $P < 0.01$  compared with ctr; # $P < 0.05$ , ## $P < 0.01$  compared with OGD/R; @ $P < 0.05$ , @@ $P < 0.01$  compared with Vech; & $P < 0.05$ , && $P < 0.01$  compared with Vect;

**A**

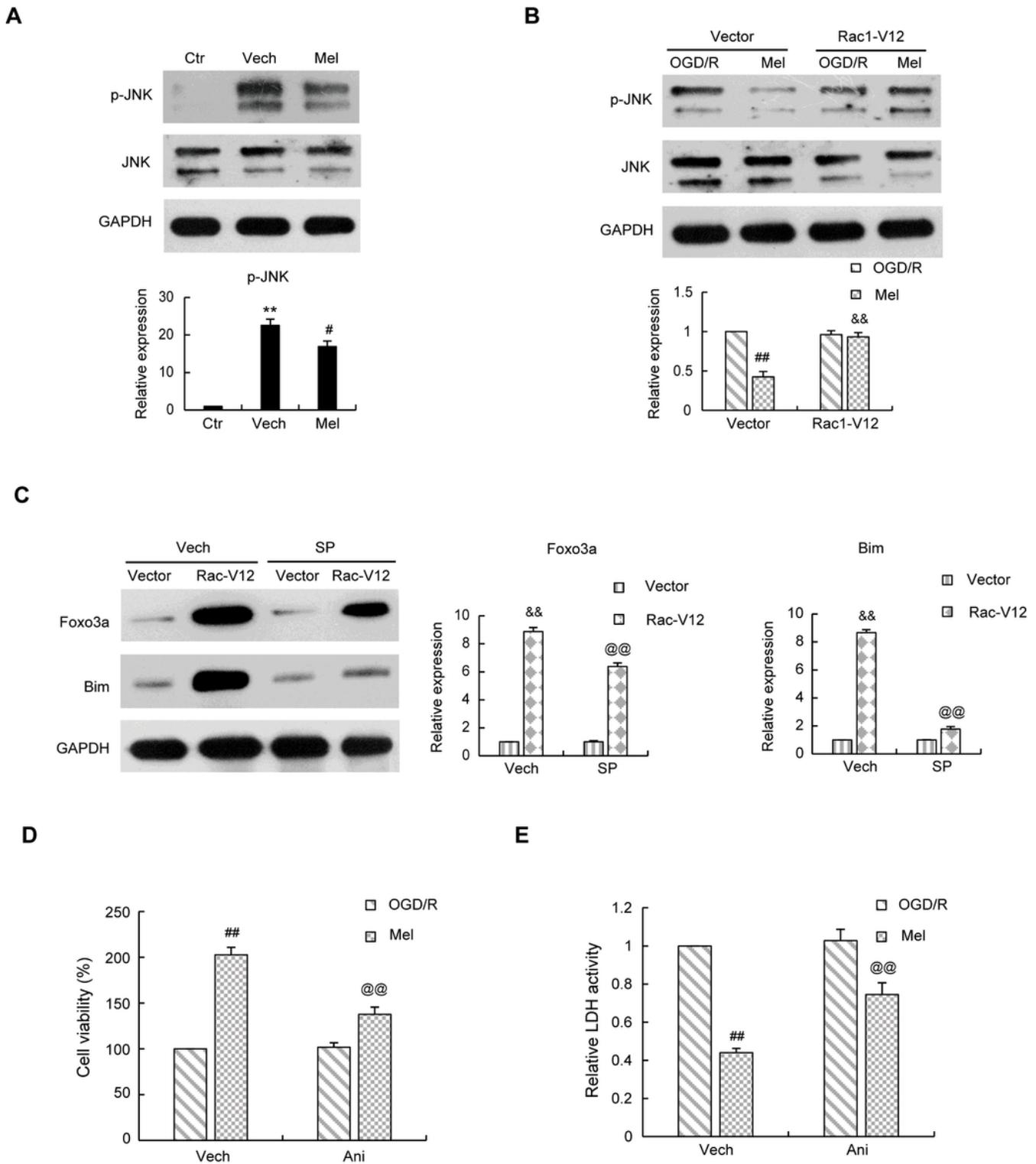


**B**



### Figure 4

Melatonin inhibited OGD/R-activated Foxo3a/Bim signaling pathway through via inactivating Rac1. (A) H9c2 cells were treated with Vehicle (Vech) or NSC23766 (NSC), then the cells were exposed to OGD/R or cultured in normal medium. The levels of Foxo3a and Bim were measured by western blot. (B) H9c2 cells were transfected with empty plasmid (Vector) or Rac1-V12, then the cells were treated with 1mM melatonin (Mel) followed by OGD/R exposure. The levels of Foxo3a and Bim were measured by western blot. @ $P < 0.05$ , @@ $P < 0.01$  compared with Vech; & $P < 0.05$ , && $P < 0.01$  compared with Vect.



**Figure 5**

JNK acted as a downstream effector of Rac1 in mediating melatonin-induced inactivation of Foxo3a/Bim signaling pathway and cell protective effect in OGD/R-treated H9c2 cells. (A) H9c2 cells were cultured for 24h under three conditions: normal (Ctr), OGD/R, OGD/R with 1mM melatonin (Mel), then the levels of p-JNK and total JNK were measured by western blot. (B) H9c2 cells were transfected with empty plasmid (Vector) or Rac1-V12, then the cells were treated with 1mM melatonin (Mel) followed by OGD/R exposure.

The levels of p-JNK and total JNK were measured by western blot. (C) H9c2 cells were transfected with empty plasmid (Vector) or Rac1-V12, the cells were treated with Vehicle (Vech) or SP600125 (SP) and 1mM melatonin (Mel) followed by OGD/R exposure. The levels of Foxo3a and Bim were measured by western blot. (D-E) (B) H9c2 cells were pretreated with Vehicle (Vech) or anisomycin (Ani), then the cells were treated with 1mM melatonin (Mel) followed by OGD/R exposure. Cell viability (D) was determined by CCK-8 assay, cell cytotoxicity (E) was determined by LDH release assay. \*P<0.05, \*\*P<0.01 compared with ctr; #P<0.05, ##P<0.01 compared with OGD/R; @P<0.05, @@P<0.01 compared with Vech; &P<0.05, &&P<0.01 compared with Vect.