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ORIGINAL PAPER

Microarray analysis and functional characterization revealed NEDD4-mediated cardiomyocyte autophagy induced by angiotensin II

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Abstract

Autophagy is a highly regulated intracellular process to maintain cellular homeostasis by degrading damaged proteins and organelles. Dysregulation of autophagic activity in cardiomyocytes is implicated in various heart diseases. However, the underlying mechanisms of cardiomyocyte autophagy are not yet known. In this study, the enhanced cardiomyocyte autophagy was induced by angiotensin II (0.1 μmol/L), demonstrated by the increase of double-membraned autophagosomes, BECN1 expression, and the conversion of LC3-I to LC3-II. Microarray assay showed that a total of 197 genes were differentially expressed in angiotensin II–treated cardiomyocytes, including 22 upregulated and 175 downregulated. Gene ontology functional enrichment analysis showed that nearly 50% of differentially expressed genes were related to metabolism and energy maintenance in biological process. Pathway analysis showed that most frequently represented pathways were involved in metabolism and the citric acid cycle and respiratory electron transport. Based on KEGG database, 10 differentially expressed genes were found to be involved in autophagic signaling pathways. The hub genes with high degree were predicted to regulate cardiomyocyte autophagy activity by PPI network analysis. NEDD4, the top focus hub gene, showed a clear time-dependent increased expression pattern in cardiomyocytes during angiotensin II treatment. Moreover, inhibition of NEDD4 could significantly reduce cardiomyocyte autophagy induced by angiotensin II. In summary, the cardiomyocyte autophagy–related genes were screened by microarray assay combining with bioinformatics analysis. The role of NEDD4 on cardiomyocyte autophagy might provide valuable clues to finding therapeutic targets for heart diseases.

Keywords Cardiomyocytes · Autophagy · Angiotensin II · Bioinformatics analysis · NEDD4

Introduction

Autophagy is a lysosome-mediated cellular degradation process for recycling and removal of misfolded proteins, insoluble aggregates, damaged organelles, and external pathogens (Mizushima 2018; Wang et al. 2016b). Autophagy occurs in all eukaryotes, and normally activated in response to diverse types of cellular stress, including nutrient deprivation and hormone stimulation (Li et al. 2016; Zhang et al. 2015). Dysregulation of cardiomyocyte autophagic activity is implicated in various heart diseases. Constitutive autophagy is essential for the maintenance of cardiomyocyte homeostasis, whereas excess autophagic activity can aggravate cardiac hypertrophy, and may contribute to the pathogenesis of heart failure (Mialet-Perez and Vindis 2017; Rifki and Hill 2012). However, the underlying mechanisms of cardiomyocyte autophagy are not yet known.

Ying Gu, Fan Yang and Yongchao Yu contributed equally to this work.

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Angiotensin II can induce cardiac hypertrophy by mediating through angiotensin II type 1 (AT1) receptors (Demos-Davies et al. 2014). Sustained activation of the angiotensin II-AT1 receptor axis contributes to cardiac remodeling, hypertrophy, and dysfunction. The activation of p38 mitogen–activated protein kinase (p38/MAPK), c-Jun N-terminal kinase (JNK), and nuclear factor κB (NF-κB) signaling had been identified as important mechanisms in cardiomyocyte hypertrophy and heart failure induced by angiotensin II (Das et al. 2004; Sabri et al. 2003; Schluter and Wenzel 2008). Recently, it had been reported that the elevation of autophagic activity in cardiomyocytes could also be induced by angiotensin II (Kishore et al. 2015). Based on the double-edged sword of autophagy modulation in cardiac hypertrophy, the mechanism exploration of cardiomyocyte autophagy induced by angiotensin II would help us deeply understand the development of cardiac hypertrophy.

Advances in molecular genetics (microarray and high-throughput sequencing technologies) have enabled the screening of differentially expressed genes during various physiological and pathological processes. It has been widely used for investigating the underlying regulatory network involved in diverse diseases to improve the clinical diagnosis, and discover new drug targets (D'Andrea 2010; Meng et al. 2016). In the present study, we screened the differentially expressed genes involved in cardiomyocyte autophagy through microarray and bioinformatics analysis, and hoped to provide valuable clues to finding therapeutic targets for heart diseases.

Methods

Cardiomyocyte isolation and treatment

Primary neonatal rat cardiomyocytes were isolated from 1- to 2-day-old Sprague-Dawley rats as previously reported (Gu et al. 2017). Briefly, isolated ventricles were digested with collagenase (1 mg/mL) at 4 °C for 14 h. After differential preplating for 2 h, cardiomyocytes were plated and cultured in DMEM supplement with 10% fetal bovine serum and BrdU (0.1 mmol/L). Having been cultured in DMEM for 24 h, the cardiomyocytes were stimulated by angiotensin II (0.1 μmol/L) for 12 h.

Cardiomyocyte immunofluorescence

After permeabilizing with Triton X-100, the paraformaldehyde-fixed cardiomyocytes on coverslips were blocked with 5% goat serum for 1 h at room temperature. Then, the cells were incubated with α-actinin antibody (1:1000 dilution) overnight at 4 °C, followed by incubation with Alexa Fluor 594–conjugated secondary antibody at room temperature. Subsequently, the coverslips were mounted onto glass slides using Vectashield mounting media with DAPI for nuclei staining, and visualized on a fluorescence microscopy.

Transmission electron microscopy

The cardiomyocyte ultrastructure was visualized by transmission electron microscopy. Briefly, cardiomyocytes with or without angiotensin II treatment were fixed with 2.5% glutaraldehyde overnight at 4 °C, and post-fixed with 1% osmium tetroxide for 30 min. Followed by dehydration and embedding, the ultrathin sections were observed using an H-7650 (HITACHI) transmission electron microscopy.

Small interfering RNA transfection

The siRNAs targeting NEDD4 (si-NEDD4) and negative control (si-NC) were designed by RiboBio Co., Ltd. (China). The synthesized siRNAs (50 nmol/L) were transfected to cardiomyocytes using riboFECT™ CP Transfection (Ribobio) according to the manufacturer's protocol. The cardiomyocytes were then incubated for 24 h before angiotensin II stimulation.

Quantitative real-time PCR

Total RNA was extracted from cardiomyocytes by RNA Extraction Kit (TaKaRa Bio) according to the protocol of the manufacturer. After quantified, equal amount of RNA samples (500 ng) was generated cDNA by using PrimeScript RT reagent Kit (TaKaRa Bio) with oligo-dT and random primers. Quantitative real-time PCR (qRT-PCR) was performed on a LightCycler 480 II PCR system (Roche) by using SYBR Green. GAPDH was used as internal control. The specificity of amplification was determined by melting temperature curve analysis. The PCR products were kept to 60 °C and were then heated to 95 °C at a rate of 0.5 °C/s. Fluorescence signals were continuously monitored during the whole process of melting temperature curve analysis.

Western blot

Western blotting was performed as described previously (Wang et al. 2016a). Equal amount of protein was separated by SDS-PAGE and transferred to the PVDF membrane. After blocking, the membrane was incubated with diluted primary antibodies (BECN1, 1:2000 dilution. LC3β, 1:1000 dilution. NEDD4, 1:5000 dilution. GAPDH, 1:2000 dilution) overnight followed by incubation with HRP-conjugated secondary antibody. The immunoreactive bands were visualized by ECL plus Kit according to the manufacturer's instructions. GAPDH was used as loading control.

Microarray analysis

Total RNA integrity was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies). The sample labeling, microarray hybridization, and washing were performed based on the manufacturer's standard protocols. Briefly, total RNAs were transcribed to double strand cDNA, then synthesized into cRNA and labeled with Cyanine-3-CTP. After hybridization, the microarrays were scanned by the Agilent Scanner G2505C (Agilent Technologies). The data was analyzed by Genespring 13.1. The threshold values of ≥ 2-fold change and false discovery rate (FDR) < 0.01 were employed as cutoff criterion.

Functional enrichment analysis

Gene ontology (GO) functional analysis and pathway enrichment analysis were performed by using Funrich analysis software (Pathan et al. 2015). *P* value < 0.05 was used as a reference value.

PPI network construction

Protein-protein interaction (PPI) network was constructed based on Search Tool for the Retrieval of Interacting Genes (STRING) database and Biological General Repository for Interaction Datasets (BioGRID). All the differentially expressed genes were imported into Cytoscape plugin to create network visualizations. The module analysis was performed by the plugin Molecular Complex Detection.

Statistical analysis

All statistical analyses were performed by SPSS 21.0. The quantitative data were expressed as mean ± standard deviation, and analyzed by Student's *t* test. *P* values less than 0.05 were considered statistically significantly different.

Results

Cardiomyocyte autophagy induced by angiotensin II

The purity of isolated cardiomyocytes was above 90% identified by α-actinin immunofluorescence assay (Fig. 1a). After stimulation of angiotensin II, significantly increased double-membraned autophagosomes were formed in cardiomyocytes by transmission electron microscopy (Fig. 1b). Meanwhile,

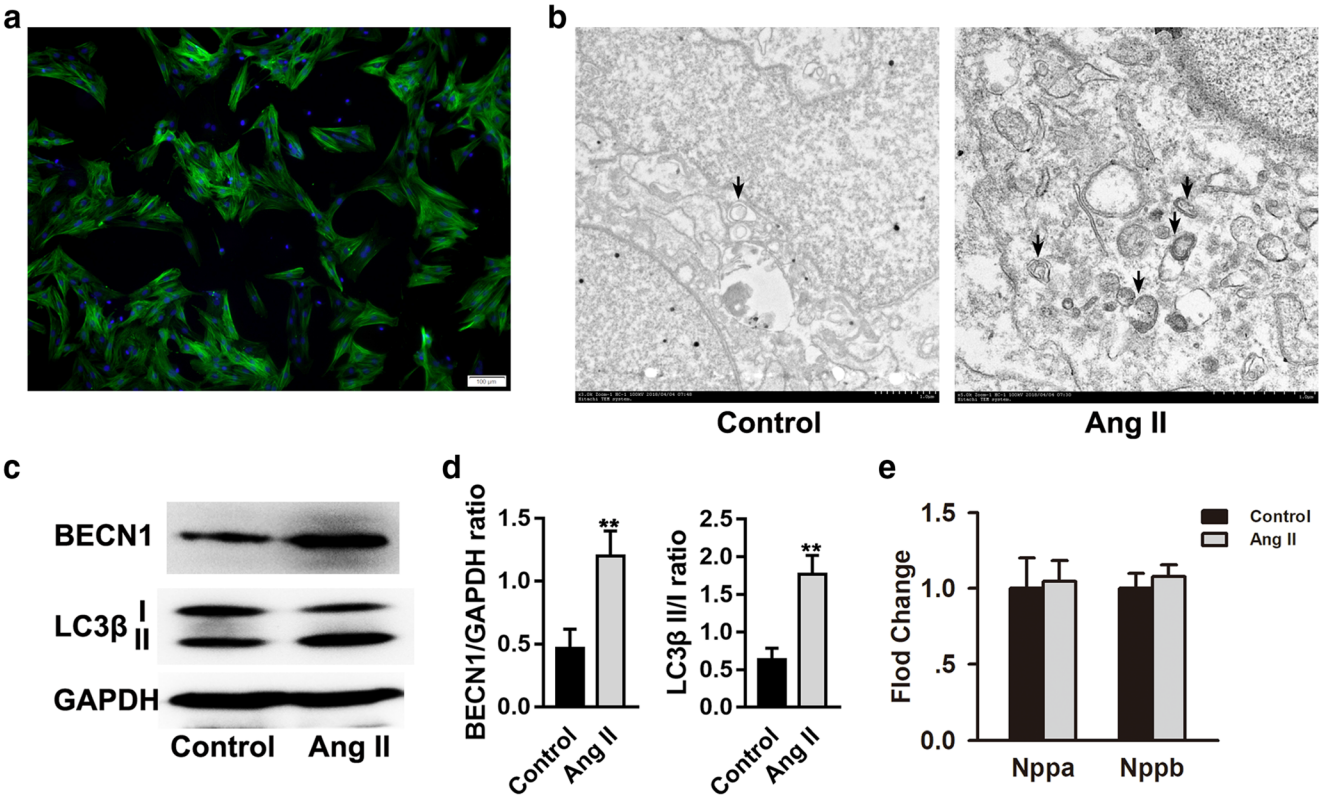

Fig. 1 The increased cardiomyocytes autophagy was induced by angiotensin II. **a** Representative image of α-actinin immunofluorescence staining in cardiomyocytes. **b** Increased double-membraned autophagosomes were found in cardiomyocytes by transmission electron microscopy. The black arrows indicated autophagic vacuoles or early double membrane structure. **c** and **d** BECN1 and LC3β expression in cardiomyocytes were analyzed by Western blot. The protein levels were determined according to the densitometry evaluated by Image J software. $^{**}P < 0.01$ vs. control group. **e** The relative expression of Nppa and Nppb in cardiomyocytes after angiotensin II stimulation. GAPDH was used as internal control

Western blot assay confirmed that the expression of BECN 1 and the conversion of soluble LC3-I to lipid bound LC3-II (LC3 II/I) were significantly increased in angiotensin II–treated cardiomyocytes (Fig. 1c, d). However, qRT-PCR assay found the expression of hypertrophic markers (Nppa and Nppb) did not notably change after angiotensin II treatment (Fig. 1e).

Differentially expressed genes in angiotensin II–treated cardiomyocytes

A microarray analysis was applied to detect differentially expressed genes in cardiomyocytes stimulated by angiotensin II for 12 h. A total of 197 differentially expressed genes were identified (Fold change ≥ 2, and FDR < 0.01), including 22 upregulated and 175 downregulated (Supplemental Table S1). The top 10 upregulated and downregulated genes were shown in hierarchical clustering heatmap (Fig. 2a). Among them, the most upregulated and downregulated differentially expressed genes were DNAJC7 and UBQLN2, respectively. Then, 10 mRNAs were randomly selected and analyzed by qRT-PCR in cardiomyocytes with or without angiotensin II stimulation, including 5 upregulated genes (FTH1, ROM1, ABCA7, RNASE2, and RBP36) and 5 downregulated genes (JUNB, DLAT, ACTA1, HSDL2, and NDRG4). As shown in Fig. 2b, all the genes showed a consistent expression pattern with the results of microarray assay, indicating high reliability of analysis.

GO and pathway functional enrichment analysis

GO functional analysis found that the majority of differentially expressed genes were proved to be located in the cytoplasm, and extracellular and exosome in cellular components analysis (Fig. 3a). The most enriched gene ontology was related to catalytic activity and structural molecule activity in the molecular function analysis (Fig. 3b), and was correlated with energy pathways, metabolism, and cell growth and/or maintenance in the biological process analysis (Fig. 3c). Biological pathway analysis showed that the most frequently represented pathways were involved in metabolism, the citric acid cycle, and respiratory electron transport, and metabolism of lipids and lipoproteins (Fig. 3d).

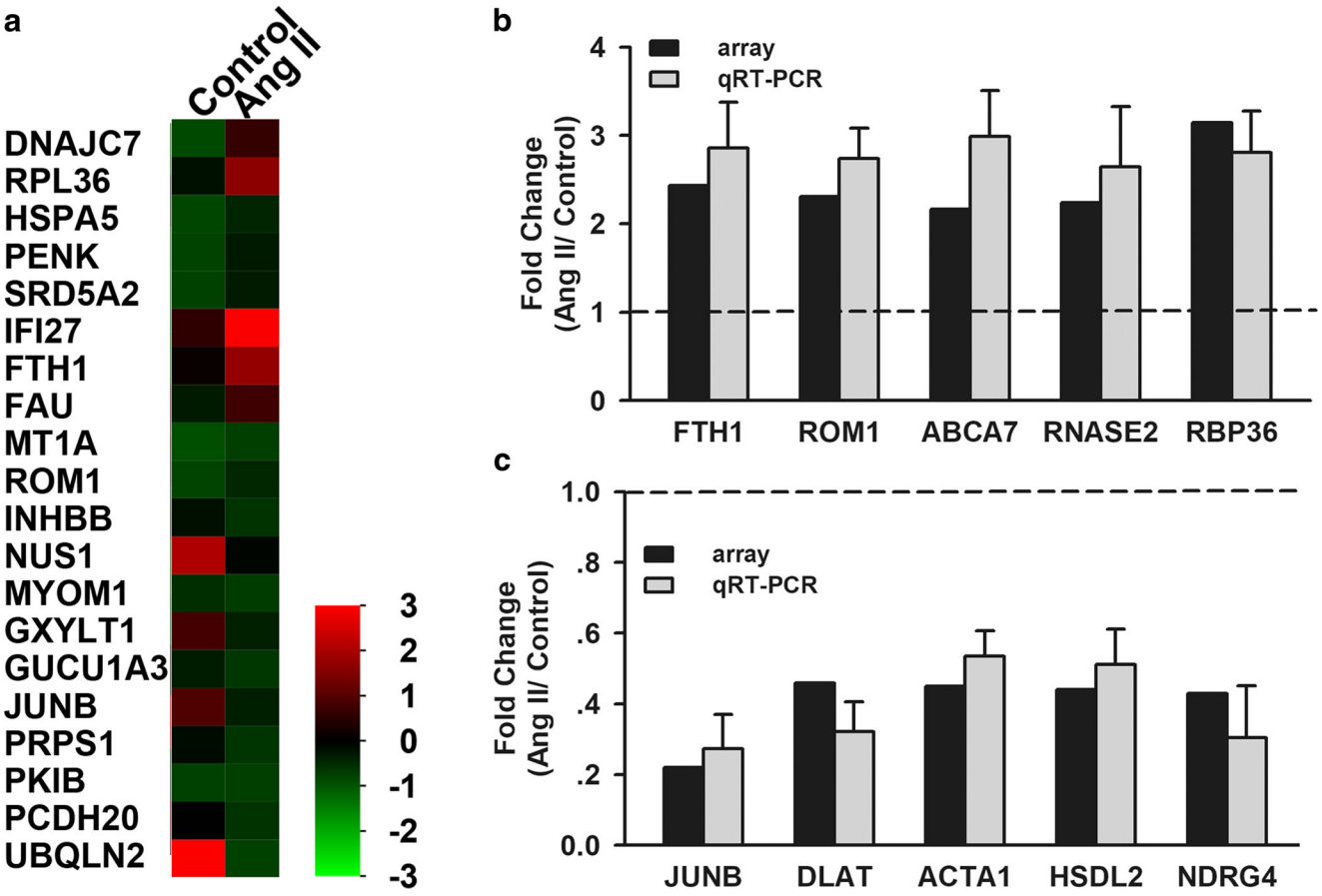

Fig. 2 Differentially expressed genes in angiotensin II–treated cardiomyocytes. **a** The top 10 upregulated and downregulated differentially expressed genes in cardiomyocytes from control and angiotensin II groups. The heat map was generated based on log-transformed normalized intensity, with red as the highest value and green as the lowest value. **b** Validation of differentially expressed genes by qRT-PCR

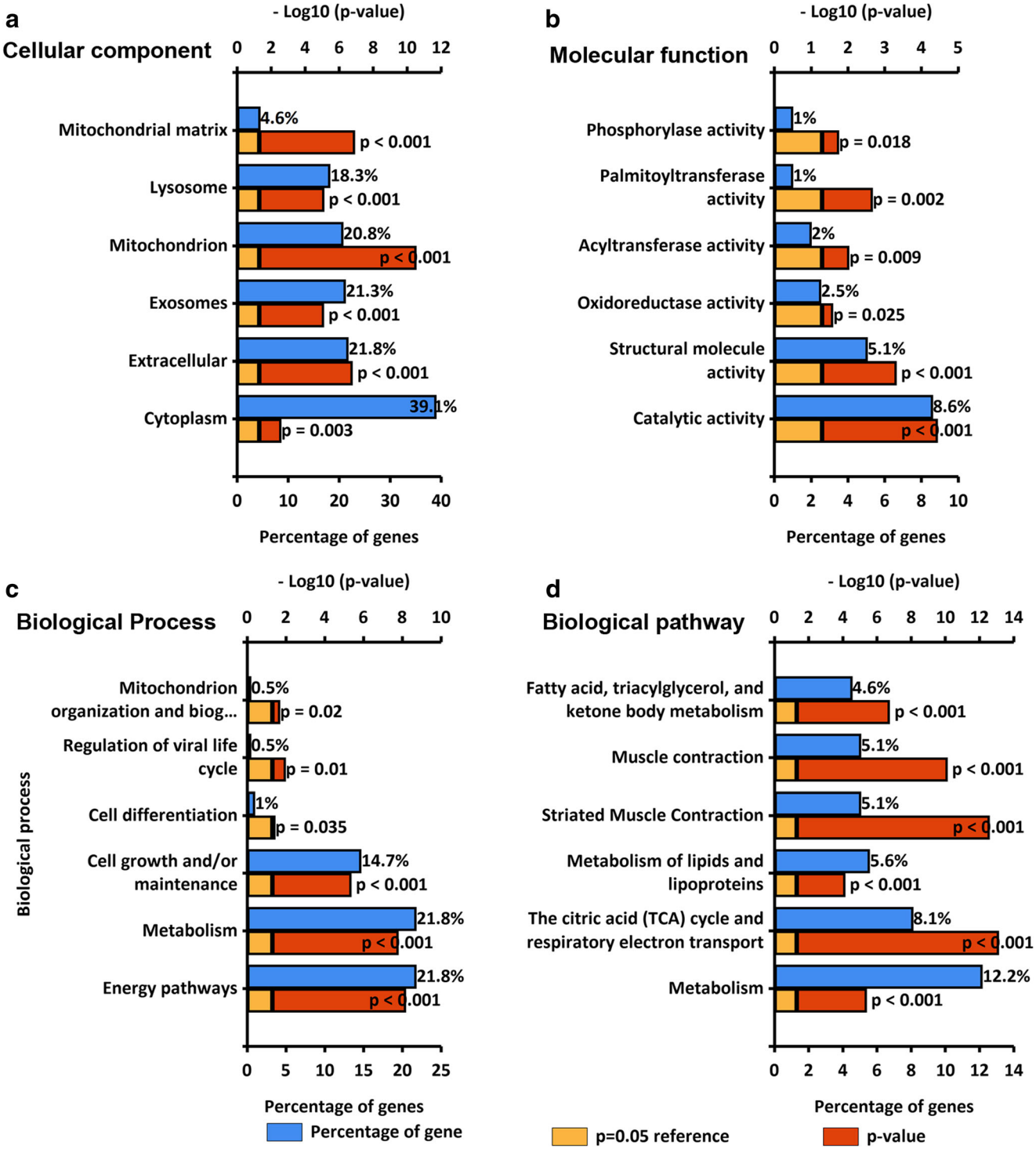

Fig. 3 GO and pathway functional enrichment analysis. GO functional analysis and pathway enrichment analysis were performed by using Funrich analysis software. **a**, **b** and **c** The top 6 GO enrichment analysis of dysregulated genes in cellular components, molecular function, and biological process. **d** The top 6 enriched pathways of differentially expressed genes

The potential genes involved in autophagic signaling pathways

There are 4 signaling pathways reported to be involved in regulation of autophagy, including MAPK signaling pathway, AMPK signaling pathway, insulin signaling pathway, and mTOR signaling pathway. All the differently expressed genes were analyzed based on database of Kyoto Encyclopedia of Genes and Genomes (KEGG). A total of 10 genes were involved in autophagic signaling pathways, including 2

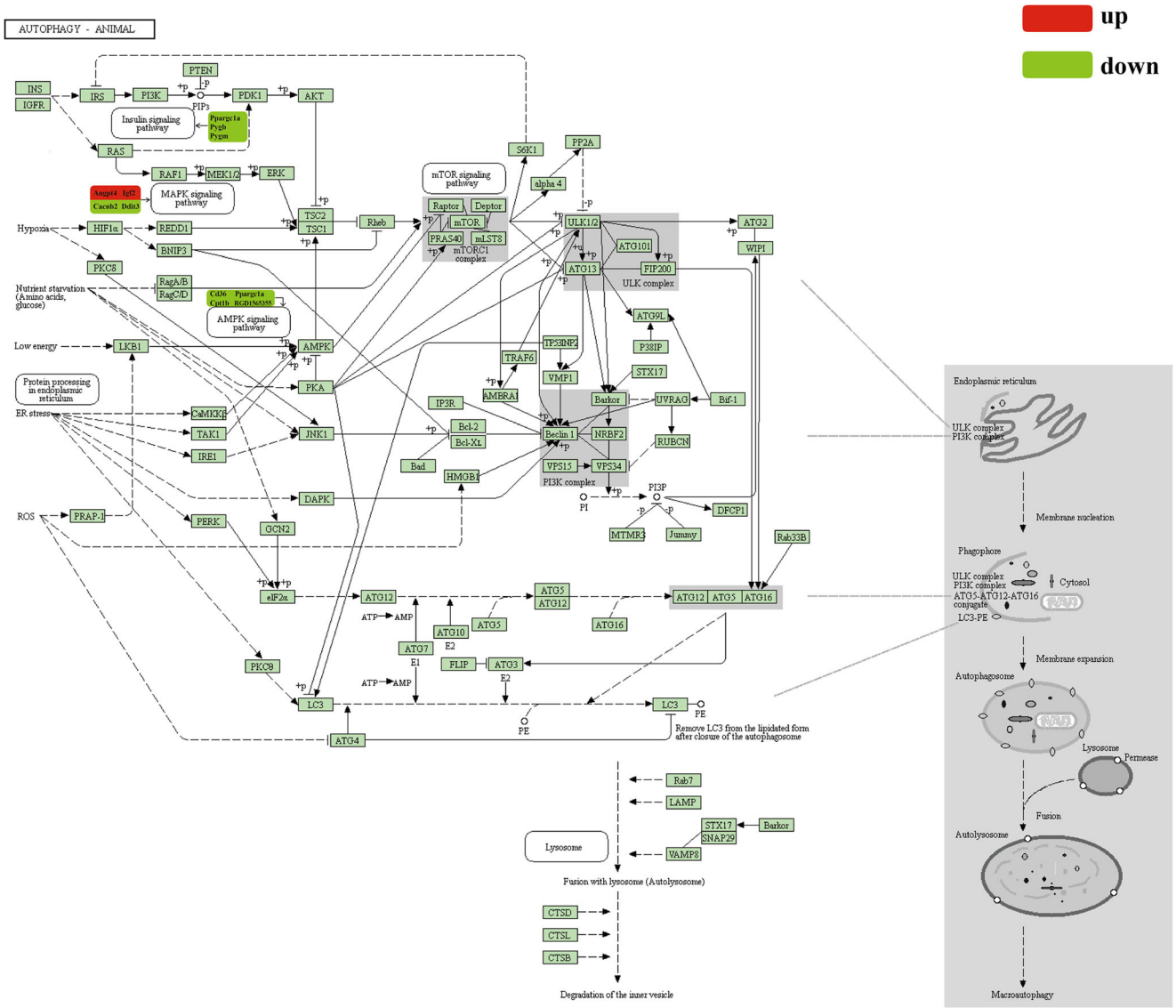

Fig. 4 The potential genes involved in autophagic signaling pathways. Based on KEGG database, 10 genes were involved in autophagic signaling pathways. Red and green indicated upregulated and downregulated expressed genes, respectively

upregulated genes (ANGPT4 and IGF2) and 8 downregulated genes (PPARGC1A, PYGB, PYGM, CACNB2, DDIT3, CD36, CPT1B, and RGD1565355) (Fig. 4). Among them, CD36, CPT1B, PPARGC1A, and RGD1565355 were involved in AMPK signaling pathway. PPARGC1A, PYGB, and PYGM participated in insulin signaling pathway. ANGPT4, CACNB2, DDIT3, and IGF2 were involved in MAPK signaling pathway. PPARGC1A, also named PGC-1α, was the only gene participated in 2 signaling pathways (AMPK and MAPK signaling pathway).

PPI network analysis

The 197 differentially expressed genes were analyzed based on the database of STRING and BioGRID. A total of 141 genes were filtered into the PPI network complex, constituted by 308 nodes and 655 interactions (Fig. 5). According to the result from topological analysis, 10 hub genes were identified with the criteria of degree > 20 (Supplemental Table S2). The top 5 node degree genes were NEDD4, ATP5A1, ACO2, ATP5B, and ACTA1. Among them, NEDD4 (degree = 100, and topological coefficient = 0.012) could interact with 109 node genes, suggesting important biological functions in cardiomyocyte autophagy.

Inhibition of NEDD4-attenuated cardiomyocytes autophagy induced by angiotensin II

To identify whether NEDD4 participated in cardiomyocyte autophagy, the expression of NEDD4 was detected in cardiomyocytes with angiotensin II stimulation. QRT-PCR assay found that treatment of angiotensin II could induce a

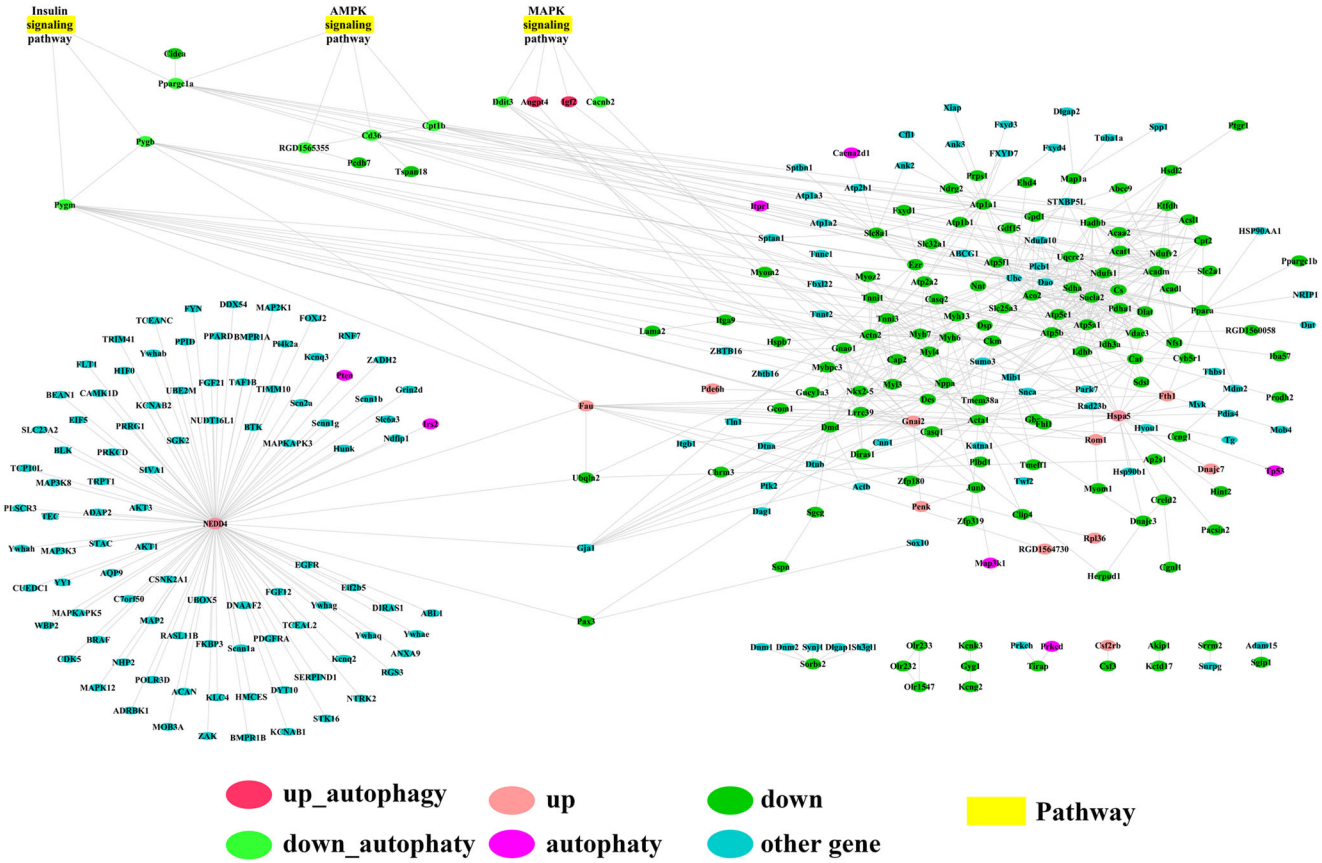

Fig. 5 PPI network complex of differentially expressed genes. Red nodes represent the upregulated genes and green nodes represent the downregulated genes, and the blue points represent genes which had no differentially expressed genes but is closely related to the differentially expressed genes

significant increase of NEDD4 expression with a time-dependent manner (Fig. 6a). The unanimous result was also confirmed by Western blot assay (Fig. 6b, c). Subsequently, a synthetic small interfering RNA (si-NEDD4) was applied to investigate the role of NEDD4 on cardiomyocyte autophagy. NEDD4 expression was significantly reduced in cardiomyocytes after transfection, which was accompanied with the low autophagy level as indicated by the decreased expression of BECN1 and LC3 II/I (Fig. 6d, e). According to PPI network analysis result, we further detected the expression of NEDD4-related genes (UBQLN2, PTEN, IRS2, and PAX3). Silencing of NEDD4 in cardiomyocytes could significantly promote the PAX3 expression, but reduce the expression of UBQLN2, PTEN, and IRS2 (Fig. 6f).

Discussion

Autophagy, an evolutionary conserved catabolic process mediated by lysosome, has been identified to be crucial for cellular homeostasis and quality control (Hosseinpour-Moghaddam et al. 2018; Kim and Lee 2014). Increasing evidence shows cardiomyocyte autophagy plays critical roles in progress of cardiovascular diseases, including myocardial infarction, cardiac hypertrophy, and heart failure (Liu et al. 2018; Sasaki et al. 2017; Sciarretta et al. 2018). The mechanism exploration of cardiomyocyte autophagy would provide potential drug targets for heart disease therapy.

Autophagy is characterized by the mobilization of intracellular nutrients to meet energy requirements in the event of nutrient deficiency. Dysregulation of autophagy could contribute to the development of metabolic disorders, including insulin resistance, diabetes mellitus, obesity, atherosclerosis, and osteoporosis (Jansen et al. 2012; Kovsan et al. 2011; Martinet and De Meyer 2009; Masini et al. 2009). Thus, autophagy is essentially a metabolic process that can control energy balance. In the present study, nearly 50% of differentially expressed genes were predicted to be involved in the regulation of metabolism and energy maintenance through bioinformatics analysis.

Multiple signaling pathways are known to be involved in autophagy, such as the mTOR, insulin, and AMPK pathways. Autophagy-related genes in these pathways are essential to drive the process of autophagy (Frankel and Lund 2012; Munson and Ganley 2015; Wang et al. 2017). PGC-1α is a transcriptional co-activator known as the master regulator of mitochondrial biogenesis. It had been reported that PGC-1α/KLF4/ERR transcriptional complex played important roles on

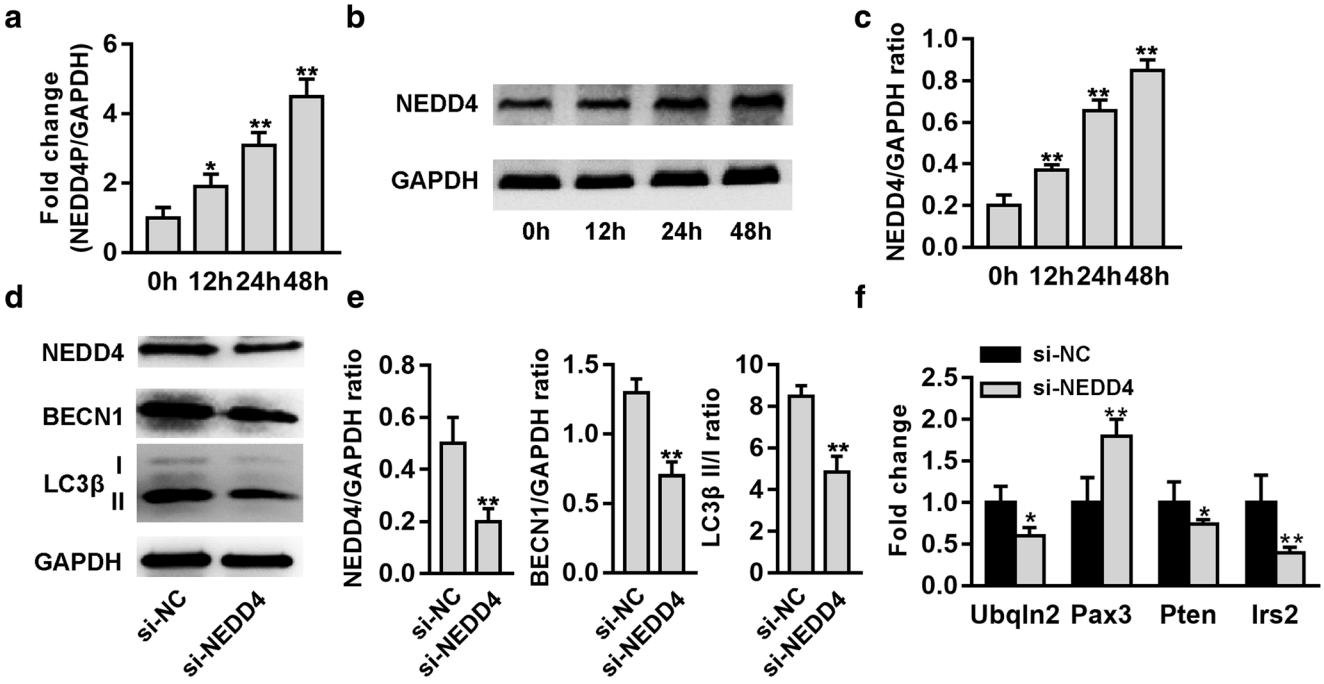

Fig. 6 The role of NEDD4 in cardiomyocytes autophagy induced by angiotensin II. **a** The relative expression of NEDD4 mRNA in cardiomyocytes during angiotensin II stimulation. $*P < 0.05$ and $**P < 0.01$ vs. 0-h group. **b** and **c** The protein expression of NEDD4 in cardiomyocytes was analyzed by Western blot. The protein levels were determined according to the densitometry evaluated by Image J software. GAPDH was used as a loading control. $**P < 0.01$ vs. 0-h group. **d** and **e** The protein expression of autophagy-related protein in cardiomyocytes following angiotensin II with or without si-NEDD4 transfection. NC, negative control. $**P < 0.01$ vs. NC group. **f** The relative expression of NEDD4-related genes in cardiomyocytes stimulated by angiotensin with or without NEDD4 inhibition. $*P < 0.05$ and $**P < 0.01$ vs. NC group

regulation of cardiomyocyte autophagy (Liao et al. 2015). In our study, a total of 10 differentially expressed genes were found in the 3 autophagic signaling pathways. In spite of unclear function, pharmacological activation/inactivation of autophagic signaling pathways by targeting these genes might be of therapeutic benefit.

PPI networks are critical for understanding underlying mechanisms as these vital activities are ascribed to interactions between proteins to form a signal transduction network system when confronted with external and internal environmental stimuli. As several vital activities are associated with the combination and dissociation of proteins, hub genes are important in this process. In the present study, NEDD4 was found to be the top focus hub gene. Recent report had confirmed that NEDD4 participated in killing of intracellular bacterial pathogens via promoting autophagy by sustaining the stability of BECN1 (Pei et al. 2017). However, NEDD4 was also found to promote Japanese encephalitis virus replication by suppressing autophagy in human neuroblastoma cells (Xu et al. 2017). In our study, angiotensin II–induced cardiomyocyte autophagy could be attenuated after NEDD4 expression inhibition, suggesting NEDD4 could promote cardiomyocyte autophagy.

In the present study, inhibition of NEDD4 could significantly promote the PAX3 expression, but reduce the expression of UBQLN2, PTEN, and IRS2. The result indicated that the function of NEDD4 on cardiomyocyte autophagy might be mediated by these related genes. Recent study had reported that ATM-mediated PTEN phosphorylation promotes PTEN nuclear translocation and autophagy in response to DNA-damaging agents in cancer cells (Chen et al. 2015). Knockout of IRS2 resulted in a reduction of p62 protein levels and promoted autophagy in the heart of mice (Riehle et al. 2013). However, the role of UBQLN2 and PAX3 on autophagy had not been explained clearly so far.

In conclusion, we identified the differentially expressed genes in angiotensin II–treated cardiomyocytes. By bioinformatics analysis, several genes were predicted to be involved in autophagy. The role of NEDD4 on cardiomyocyte autophagy might provide valuable clues to finding therapeutic targets for heart diseases. Further studies are needed to explore their regulatory mechanisms, which would help to develop novel therapeutic targets for heart disease.

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Compliance with ethical standards

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

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