Supplementary Information for

Original article

MicroRNA-34c-5p provokes isoprenaline-induced cardiac hypertrophy by modulating autophagy *via* targeting ATG4B

Yuhong Zhang[a], Yanqing Ding[a], Min Li[a], Jing Yuan[b], YouhuiYu[b], Xueying Bi[a], Huiqi Hong[a], Jiantao Ye[a,*], Peiqing Liu [a,*]

[a]*Department of Pharmacology and Toxicology, School of Pharmaceutical Sciences, National and Local United Engineering Lab of Druggability and New Drugs Evaluation, Sun Yat-sen University, Guangzhou 510006, China*

[b]*State Key Laboratory of Oncology in Southern China, Collaborative Innovation Center for Cancer Medicine, Sun Yat-sen University Cancer Center, Guangzhou 510006, China*

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[*]Corresponding authors. Tel.: +86 20 39943116; fax: +86 20 39943026.

E-mail addresses: liupq@mail.sysu.edu.cn (Peiqing Liu), yejt@mail.sysu.edu.cn (Jiantao Ye).

1. Supporting tables

Table S1 Sequences of mmu-miR-34c-5p agomir, antagomir and their negative control.

Name	Sequence
mmu-miR-34c-5p agomir	Sense 5′-AGGCAGUGUAGUUAGCUGAUUGC-3′ Antisense 5′-AAUCAGCUAACUACACUGCCUUU-3′
NC agomir	Sense 5′-UUCUCCGAACGUGUCACGUTT-3′ Antisense 5′-ACGUGACACGUUCGGAGAATT-3′
mmu-miR-34c-5pantagomir	5′- GCAAUCAGCUAACUACACUGCCU-3′
NC antagomir	5′-CAGUACUUUUGUGUAGUACAA-3′

Table S2 Primer sequences for qRT-PCR.

Gene	Primer sequence (5′–3′)
β-Actin	Forward: GCAGATGTGGATCAGCAAGC Reverse: GCAGCTCAGTAACAGTCCGC
Anf	Forward: CTGGGACCCCTCCGATAGAT Reverse: CACTCTGGGCTCCAATCCTG
Bnp	Forward: GGCTGTAACGCACTGAAGTT Reverse: CACTTCAAAGGTGGTCCCAG
β-Mhc	Forward: TTACTTGCTACCCTCAGGTGG Reverse: CTCCTTCTCAGACTTCCGCA
Atg4b	Forward: GGCTCATGGGAGTGTTCTCA Reverse: GGCCCTGCACAATCTTCTGAT
Atg9a	Forward: GCGAGGCTGGTAACTGGAAT Reverse: TGGTTGGACAGCTGTTGGG
Lc3b	Forward: AGAGCGATACAAGGGGGAGA Reverse: CTGCAAGCGCCGTCTGATTA

2. Supporting figures

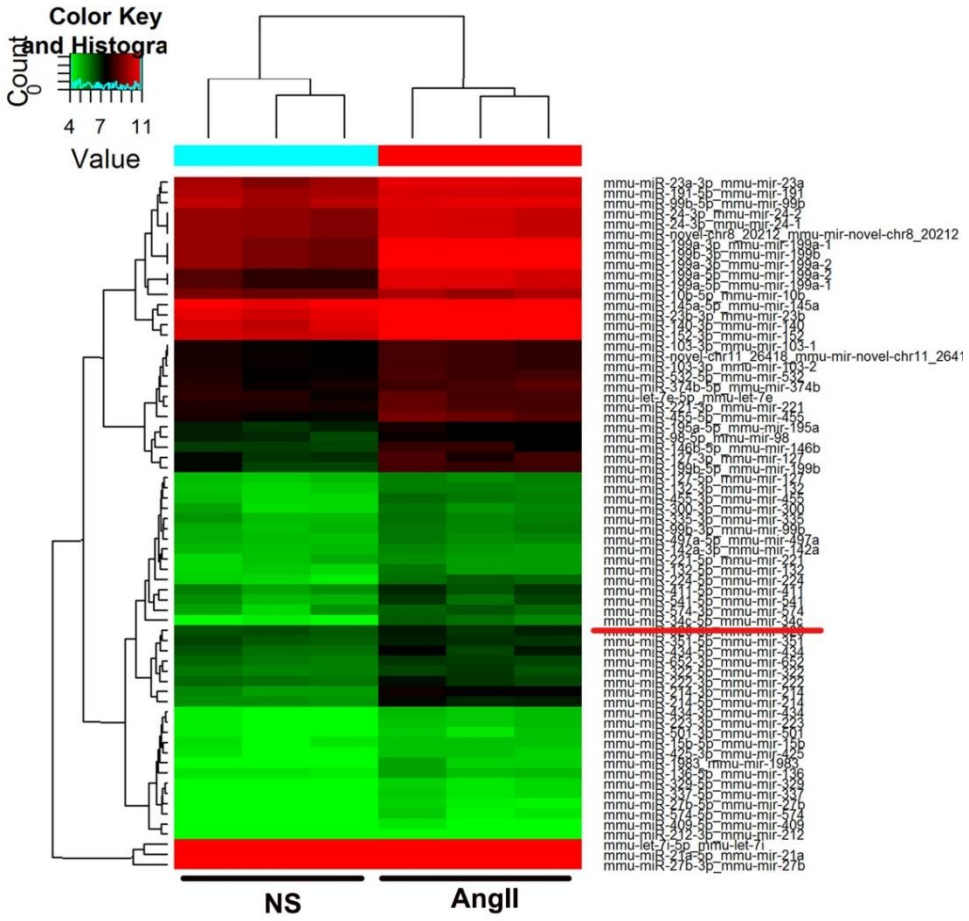

Figure S1 miRNA expression profile in mice cardiac tissues following Ang II treatment. Mice were subcutaneously infused with 2 mg/kg/day Ang II for 2 weeks. The control animals received normal saline (NS). Illumina deep sequencing was performed to detect miRNA expression profiles in left ventricular tissues (3 samples/group). Illumina deep sequencing was performed to determine miRNA expression profile in left ventricular tissues of mice. Heatmap showed the differentially expressed miRNAs between NS and Ang II group including miR-34c-5p.

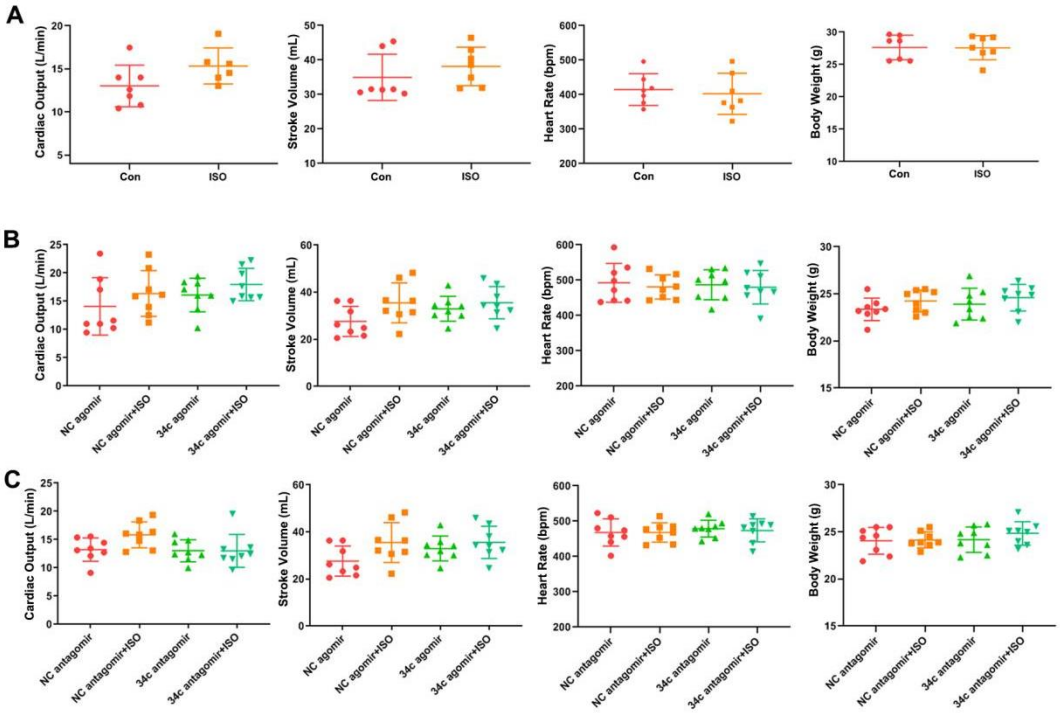

Figure S2 Heart rate, body weight and echocardiography parameters CO and SV in mice with ISO and miR-34c-5p agomir/antagomir treatment. (A) C57B/L6 mice were subcutaneously infused with 2 mg/kg/day ISO for two weeks (n = 7). **(B–C)** Mice with ISO treatment were submitted to miR-34c-5p agomir (5 OD) and antagomir (8 OD) injection *via* tail vein once every two days to mice (n = 8). The heart rate and body weight of mice were measured. The echocardiography parameters including cardiac output (CO) CO and stroke volume (SV) were determined by echocardiography. Data are shown as mean ± SD.

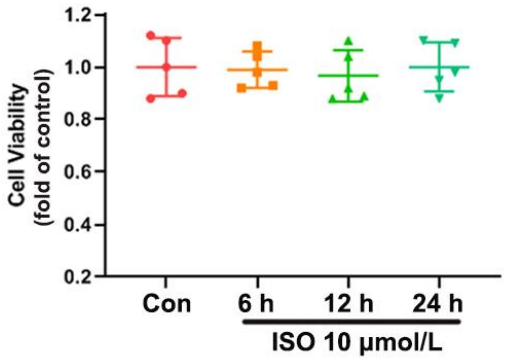

Figure S3 The effect of ISO on cell viability of cultured cardiomyocytes. NRCMs were incubated with ISO for the indicated time durations, and the cell viability was evaluated by CCK-8 assay. Data are shown as mean ± SD (n = 5).

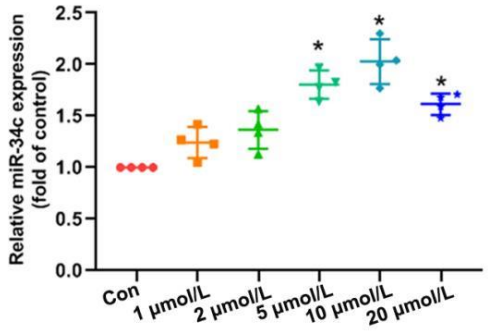

Figure S4 The concentration-dependent effect of ISO on miR-34c-5p expression. Cultured NRCMs were submitted to ISO treatment at different concentrations from 1 to 20 μmol/L for 24 h. The expression of miR-34c-5p was measured by qRT-PCR. Data are shown as mean ± SD ($n = 4$). *$P < 0.05$ *vs.* control group.

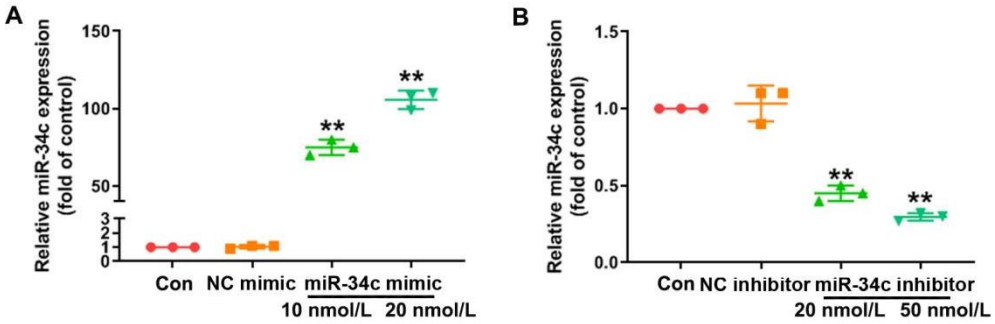

Figure S5 The expression of miR-34c-5p following mimic and inhibitor treatments. (A) NRCMs were treated with miR-34c-5p mimic (10 and 20 nmol/L) or negative control mimic (NC mimic) for 24 h. **(B)** NRCMs were treated with miR-34c-5p inhibitor (20 and 50 nmol/L) or negative control inhibitor (NC inhibitor) for 24 h. The expression of miR-34c-5p was measured by qRT-PCR. Data are shown as mean ± SD ($n = 3$). **$P < 0.01$, *vs.* NC mimic group or group NC inhibitor group.

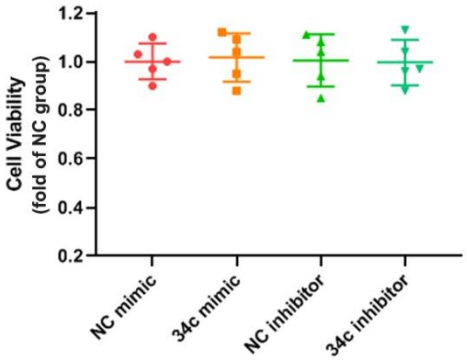

Figure S6 Effect of miR-34c on cell viability of cardiomyocytes. Cultured NRCMs were transfected with miR-34c-5p mimic, negative control mimic (NC mimic), miR-34c-5p inhibitor or negative control inhibitor (NC inhibitor) for 24 h. The cell viability was evaluated by CCK-8 assay. Data are shown as mean ± SD (n =5).

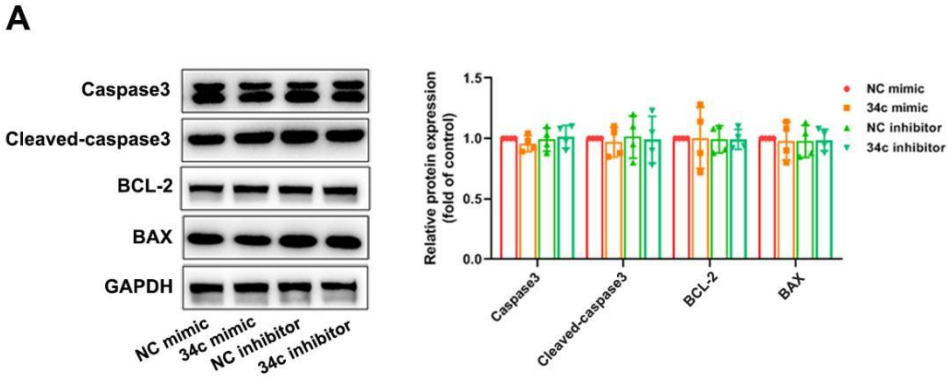

Figure S7 Effect of miR-34c-5p **on cardiomyocyte apoptosis.** Cultured NRCMs were transfected with miR-34c-5p mimic, negative control mimic (NC mimic), miR-34c-5p inhibitor or negative control inhibitor (NC inhibitor). The protein expression of caspase3, cleaved caspase3, BCL-2 and BAX was measured by Western blot. Data are shown as mean ± SD (n = 4)

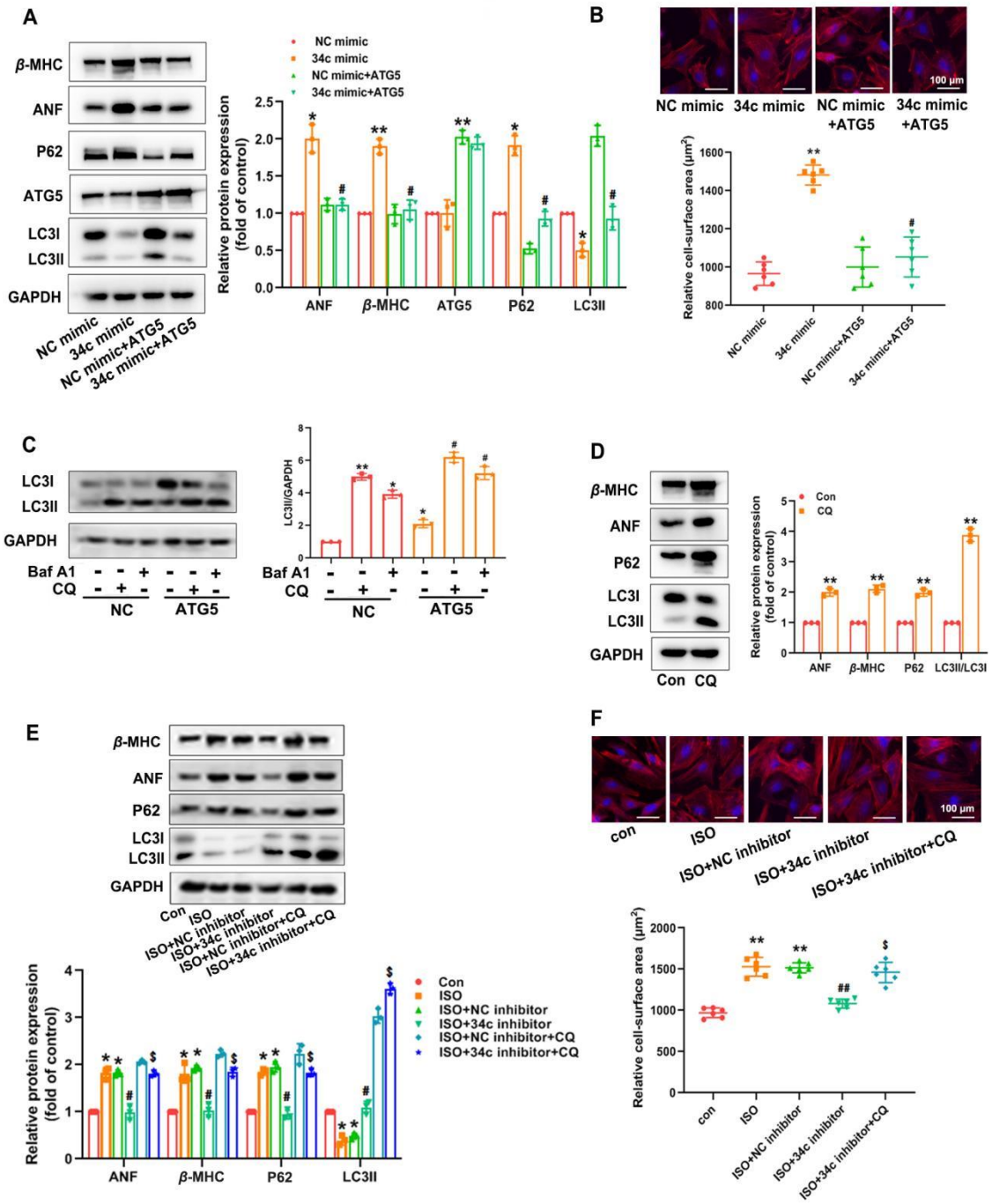

Figure S8 The effects of ATG5 overexpression and CQ treatment on miR-34c-5p-meidated autophagy and cardiomyocyte hypertrophy. (A–B) Cultured NRCMs were submitted to miR-34c-5p mimic treatment and ATG5 overexpression for 24 h. The protein level of ANF, *β*-MHC, ATG5, P62 and LC3-II were measured by Western blot. Data are shown as mean ± SD ($n = 3$); *$P < 0.05$, **$P < 0.01$ *vs.* NC mimic group; $^{\#}P < 0.05$ *vs.* miR-34c-5p mimic group. And the cell surface area was measured ($n = 6$); **$P < 0.01$ *vs.* NC mimic group; $^{\#}P < 0.05$ *vs.* miR-34c-5p mimic group. **(C)** Cultured NRCMs were incubated with chloroquine (CQ) or bafilomycin A1 (Baf A1) at the presence of ATG5 for 24 h. LC3-II expression was measured by Western blot. Data are shown as mean ± SD ($n = 3$); *$P < 0.05$, **$P < 0.01$ *vs.* control group; $^{\#}P <$

0.05, *vs.* ATG5 group without CQ and Baf A1 treatment. **(D)** NRCMs were treated with CQ for 24 h. The protein levels of hypertrophic and autophagic markers were measured by Western blot. Data are shown as mean ± SD ($n = 3$); $^{**}P < 0.01$ *vs.* control group. **(E–F)** NRCMs transfected miR-34c-5p inhibitor were treated with ISO and CQ treatment for 24 h. The expression of autophagic and hypertrophic markers were determined by Western blot ($n = 3$), and the cell surface area was measured ($n = 6$). Data are shown as mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* ISO group; $^{\$}P < 0.05$ *vs.* ISO +34c inhibitor group; $^{\&}P < 0.05$ *vs.* ISO + NC inhibitor + CQ group.

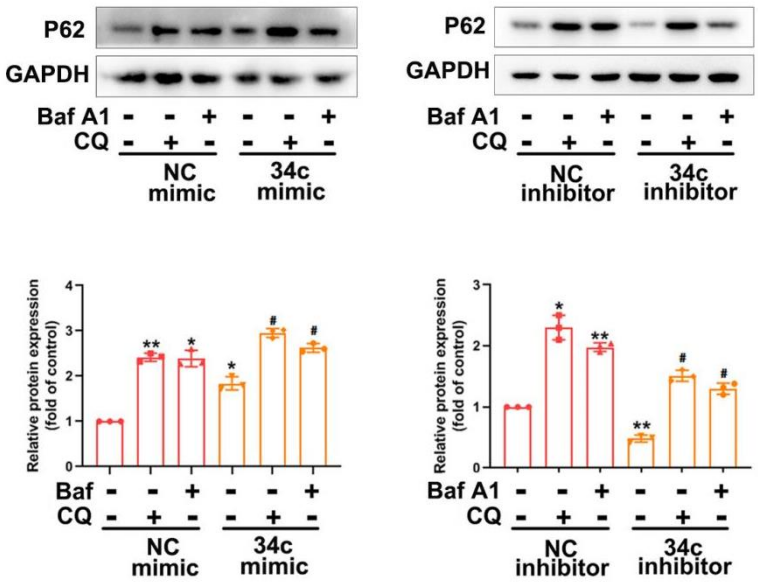

Figure S9 MiR-34c-5p suppresses P62 in NRCMs. Cultured NRCMs were incubated with chloroquine (CQ) or bafilomycin A1 (Baf A1) at the presence of miR-34c-5p mimic/inhibitor for 24 h. The P62 expression was measured by Western blot. Data are shown as mean ± SD ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* NC mimic or NC inhibitor group; $^{\#}P < 0.05$ *vs.* NC groups + CQ or Baf A1.

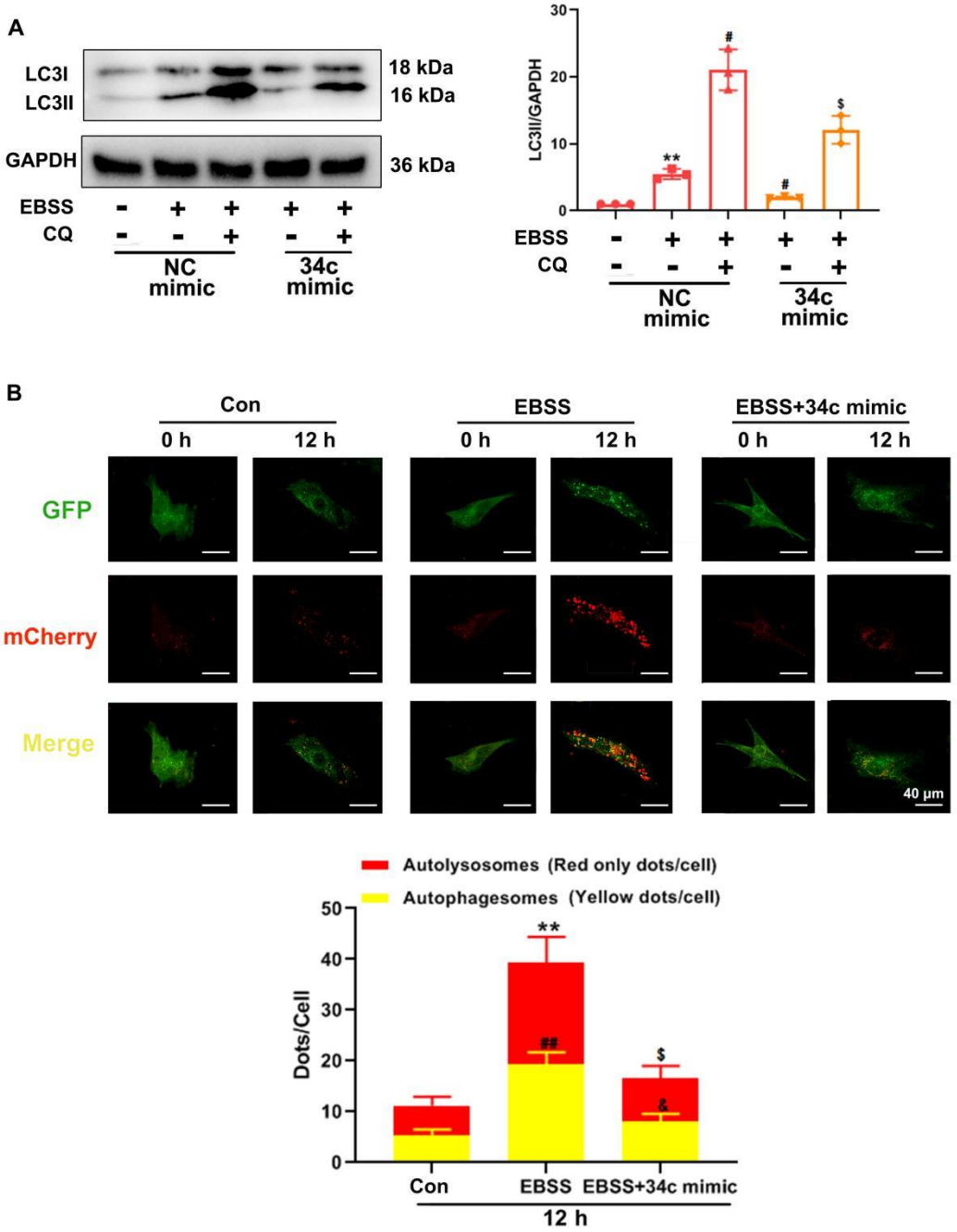

Figure S10 miR-34c-5p mimic suppresses nutrient starvation-induced autophagic activity in NRCMs. (A) Cultured NRCMs were treated with miR-34c-5p mimic and subsequently incubated in nutrient-deprived medium (EBSS without serum) in the presence or absence of chloroquine (CQ). LC3-II expression was measured by Western blot. Data are shown as mean ± SD ($n = 3$); $^{**}P < 0.01$ *vs.* NC mimic group without CQ and EBSS; $^{\#}P < 0.05$ *vs.* NC mimic + EBSS group; $^{\$}P < 0.05$ *vs.* NC mimic + CQ + EBSS group. **(B)** NRCMs were transfected with adenovirus harboring mCherry-GFP-LC3 and further submitted to nutrient starvation. The fluorescent LC3 dots were detected under a confocal laser scanning microscopy. Data are shown as mean ± SD ($n = 5$); $^{**}P < 0.05$ *vs.* control group (red only dots /cell); $^{\#\#}P < 0.05$ *vs.* control group (yellow dots/cell); $^{\$}P < 0.05$ *vs.* EBSS group (red only dots/cell); $^{\&}P < 0.05$ *vs.* EBSS group (yellow dots /cell).

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile
Position 325-332 of ATG4B 3' UTR hsa-miR-34c-5p	5' ...AGAGUGUUCUCUCGACACUGCCA... 3' CGUUAGUCGAUUGAUGUGACGGA	8mer	-0.60	99

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile
Position 286-292 of ATG9A 3' UTR hsa-miR-34c-5p	5' ...CAGCCCAGCCCCAGU-ACUGCCAC... 3' CGUUAGUCGAUUGAUGUGACGGA	7mer-A1	-0.25	84

Figure S11 Bioinformatic prediction of putative targets of microRNA-34c-5p. Snap-shots from of TargetScan showed both ATG4B and ATG9A as the potential targets of miR-34c-5p.

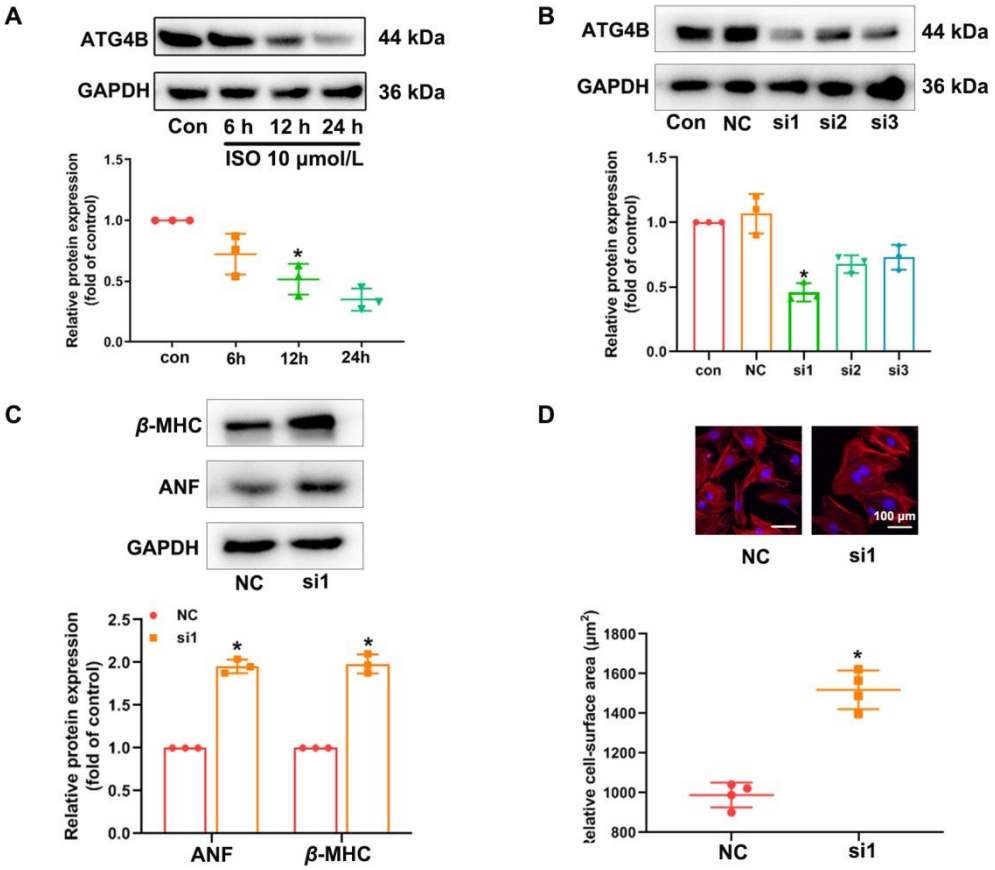

Figure S12 Knockdown of ATG4B provokes cardiac hypertrophy. (A) Cultured NRCMs were transfected respectively with three different siRNAs for *Atg4b* (si1, si2, si3) and negative control (NC) for 24 h. The silencing efficacy was validated by Western blot. Data are shown as mean ± SD ($n = 3$); *$P < 0.05$ *vs.* control group. **(B–C)** NRCMs were transfected with *Atg4b* siRNA (si1) and negative control for 24 h. The levels of hypertrophic markers were detected by Western blot ($n = 3$). The cell surface area was determined ($n = 4$). Data are shown as mean ± SD; *$P < 0.05$ *vs.* NC group.

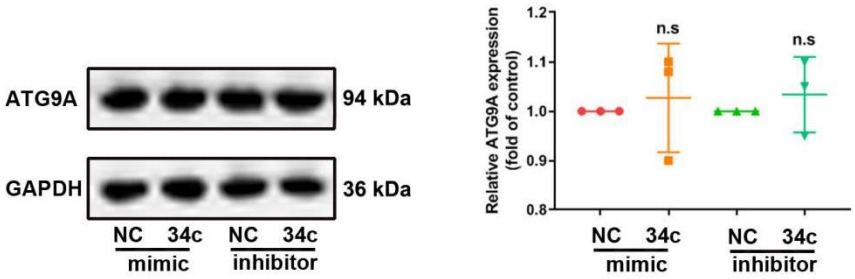

Figure S13 The protein level of ATG9A following miR-34c-5p mimic and inhibitor treatments. NRCMs were treated with miR-34c-5p mimic (20 nmol/L), inhibitor (50 nmol/L) or relevant negative control (NC mimic and NC inhibitor) for 24 h. ATG9A protein expression was measured by Western blot. Data are shown as mean ± SD (n =3); n,s: no significance *vs.* NC mimic group or NC inhibitor group.

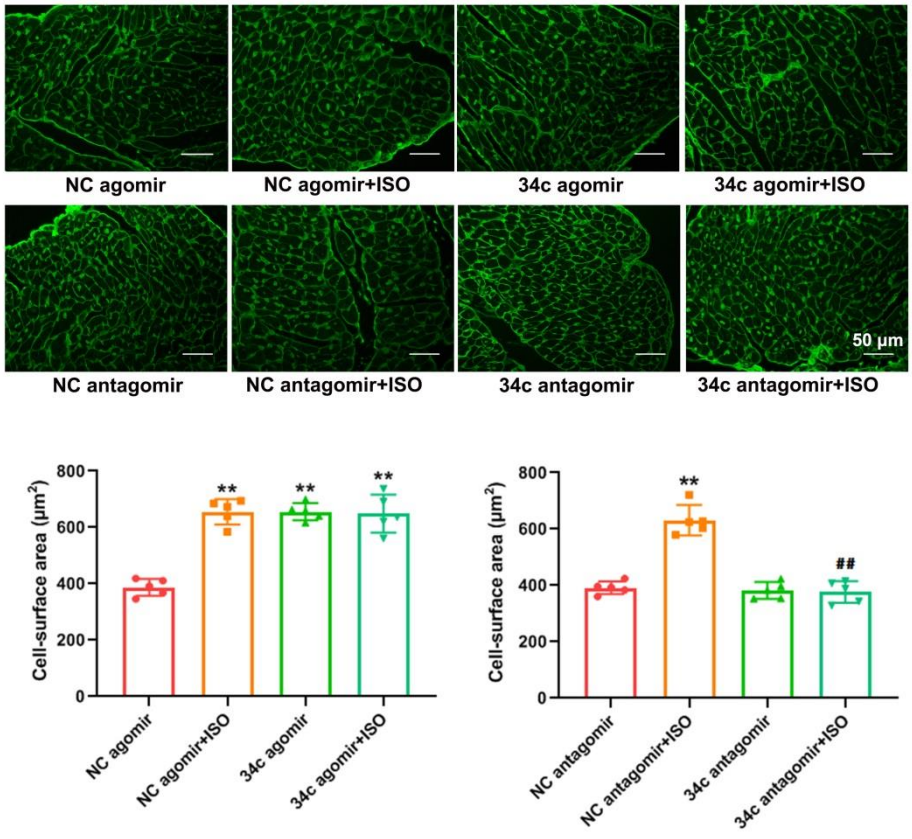

Figure S14 WGA staining of heart sections from mice with miR-34c-5p agomir or antagomir treatment. C57B/L6 mice were subcutaneously infused with 2 mg/kg/day ISO for two weeks. Specific miR-34c-5p agomir (5 OD) and antagomir (8 OD) were administrated via tail vein once every two days to modulate miR-34c-5p *in vivo*. WGA staining was performed to measure cross-sectional areas in heart sections. Scale bar: 50 μm. Data are shown as mean ± SD (n = 5); $^{**}P < 0.01$ *vs.* NC agomir group or NC antagomir group; $^{\#\#}P < 0.01$ *vs.* NC antagomir + ISO group.

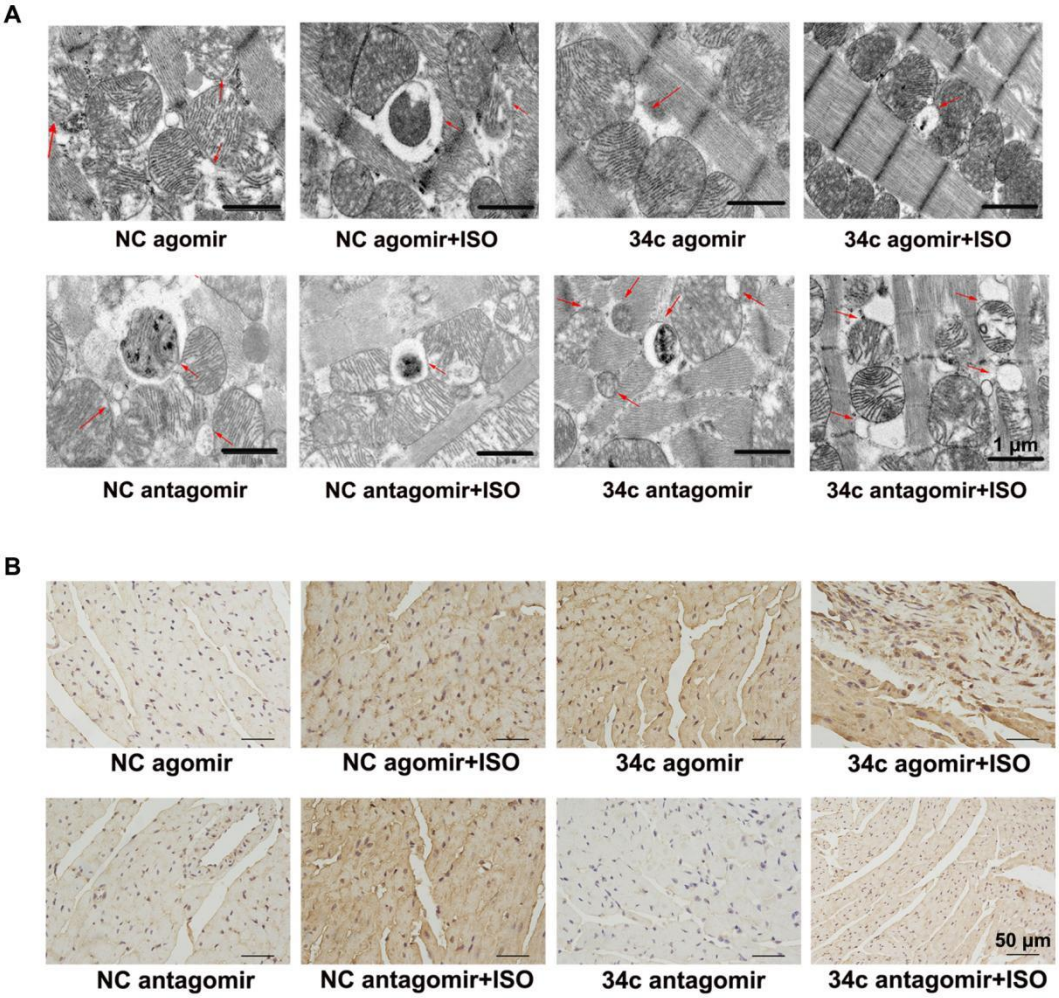

Figure S15 MiR-34c-5p agomir suppressed autophagy in mice hearts and its antagomir attenuated ISO-induced autophagy inhibition. C57B/L6 mice were subcutaneously infused with 2 mg/kg/day ISO for two weeks. Specific miR-34c-5p agomir (5 OD), negative control agomir (NC agomir), antagomir (8 OD) and negative control antagomir (NC antagomir) were administrated via tail vein once every two days to modulate miR-34c-5p *in vivo*. **(A)** TEM observation on autophagosome formation in mice heart tissues. **(B)** Immunohistochemical staining for P62 expression.