Long-chain non-coding RNA *GAS5* promotes cell autophagy by modulating the miR-181c-5p/*ATG5* and miR-1192/*ATG12* axes

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Abstract. The main aim of the present study was to explore the role of long-chain non-coding RNA (lncRNA) growth arrest-specific transcript 5 (*GAS5*) in macrophage autophagy. Firstly, the expression of lncRNA *GAS5* during cell starvation or following treatment with 3-methyladenine was determined using reverse transcription-quantitative PCR (RT-qPCR). Additionally, fluorescent *in situ* hybridization (FISH) assay was utilized to determine the localization of the expression of lncRNA *GAS5* in RAW264.7 cells. *In vitro* cell models were established through the transfection of LV5-lncRNA *GAS5* (LV5-*GAS5*) or LV3-shRNA-lnc *GAS5* (sh-*GAS5*), in order to overexpress or knockdown lncRNA *GAS5* expression in RAW264.7 cells. The potential target microRNAs (miRNAs/miRs) of lncRNA *GAS5* were analyzed using bioinformatics. The formation of autophagic bodies was detected with the use of laser confocal and transmission electron microscopy. Dual-luciferase reporter assay was performed to determine the target specificities of miR-181c-5p or miR-1192 to lncRNA *GAS5* and autophagy-related gene (*ATG*) or *ATG12*. The mRNA levels of miR181c-5p, miR-1192, as well as *ATG5* and *ATG12* were detected using RT-qPCR. The protein levels of microtubule-associated proteins 1A/1B light chain 3B (LC3), p62, *ATG5* and *ATG12* were measured using western blot analysis. It was revealed that lncRNA *GAS5* expression in RAW264.7 macrophages increased significantly during starvation-induced autophagy, and that lncRNA *GAS5* overexpression was able to markedly promote the formation of autophagic bodies. Bioinformatics analysis demonstrated that miR-181c-5p and miR-1192 were potential targets of lncRNA *GAS5*, which was further confirmed by RT-qPCR, western blot analysis and the dual-luciferase reporter assay. Finally, it was confirmed that lncRNA *GAS5* promoted autophagy by sponging miR-181c-5p and miR-1192, and upregulating the expression levels of the key autophagic regulators, *ATG5* and *ATG12*. On the whole, the present study demonstrates that total, lncRNA *GAS5* promotes macrophage autophagy by targeting the miR-181c-5p/*ATG5* and miR-1192/*ATG12* axes.

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Introduction

Autophagy is a cellular metabolic process in which cells are stimulated by relevant signals to produce an autophagic membrane. Subsequently, the membrane encapsulates cellular materials, including misfolded/aggregated proteins or damaged organelles, inducing their degradation inside autophagic bodies. Subsequently, autophagic bodies fuse with lysosomes to form autolysosomes, which are sites of degradation (1,2). Several studies have reported that autophagy, which is initiated and regulated by complex gene transcription control networks and post-translational modifications, is implicated in the occurrence and progression of a number of diseases (3-6). Long-chain non-coding RNAs (lncRNAs) have gained increasing attention in autophagy-associated disease research (7-10). However, the elucidation of the role of lncRNAs in autophagy remains at an initial early stage. The macrophage, a type of immune cell that functions as the body's 'scavenger' through the phagocytosis and digestion of cellular contents, is involved in various cellular and molecular immune processes (11). It is well known that autophagy plays a critical role in macrophage-mediated digestion. Therefore, it is of great value to explore the regulatory mechanisms employed by lncRNAs in macrophage autophagy.

The lncRNA growth arrest-specific transcript 5 (*GAS5*) is 2,554 bp in length (GeneBank no. NR_002840.2, of mouse origin) and located on chromosome 1 (12). Although the homology between human and mouse lncRNA *GAS5* is relatively low, its function as a tumor suppressor is highly conserved (13). It has been recently reported that lncRNA *GAS5* also plays an important role in autoimmune disease, inflammation and autophagy (14-17). Although several studies

have demonstrated that lncRNA *GAS5* is able to shift macrophage polarization towards an M1 phenotype (18,19), the role of lncRNA *GAS5* in macrophage autophagy remains largely unknown. In a previously published study, it was revealed that the expression of lncRNA *GAS5* in the RAW264.7 macrophage cell line increased significantly during starvation-induced autophagy (20). However, the specific role of lncRNA *GAS5* in autophagy regulation remains unclear. It has been demonstrated that lncRNAs can participate in the regulation of cell biological processes through a number of mechanisms. A generally recognized theory is that lncRNAs can adsorb microRNAs (miRNAs/miRs) by functioning as molecular sponges (21). Of note, in the present study, bioinformatics analysis revealed that miR-181c-5p used the same binding sequence to interact with lncRNA *GAS5* and autophagy-related gene (*ATG*)*5*. Similarly, the binding site sequence used by miR-1192 to interact with autophagy related gene 12 (*ATG12*) was the same as the one used to bind lncRNA *GAS5*. According to this theory, lncRNAs can regulate gene expression through the competitive sponging of its downstream target miRNAs (22,23). Thus, it was hypothesized that miR-181c-5p and miR-1192 may competitively bind to lncRNA *GAS5*, and directly target the 3'-UTRs of *ATG5* and *ATG12*, respectively, having the same binding sequence.

In the present study, it was demonstrated for the first time, to the best of our knowledge, that lncRNA *GAS5* expression was significantly enhanced during starvation-induced autophagy, and that lncRNA *GAS5* overexpression markedly increased the formation of autophagic bodies and the expression of autophagic markers in the RAW264.7 macrophage cell line. It was also confirmed that lncRNA *GAS5* may promote autophagy by sponging miR-181c-5p and miR-1192, as well as through the upregulation of *ATG5* and *ATG12* expression. Thus, a novel role of lncRNA *GAS5* in autophagy regulation was defined and a theoretical basis was provided concerning the need to further study the effects of lncRNA *GAS5* on autophagy-associated disease through macrophages.

Materials and methods

Cells and cell culture. The mouse peritoneal macrophage cell line, RAW264.7 (cat. no. TCM13), was purchased from the Cell Bank of Chinese Academy Science and cultured in Dulbecco's modified Eagle's medium (DMEM; Biological Industries), supplemented with 10% fetal bovine serum (FBS; Biological Industries). The RAW264.7 cells were incubated at 37˚C with 5% CO_2. For the determination of macrophage autophagy *in vitro*, RAW264.7 cells were cultured in Earle's balanced salt solution (EBSS; Gibco; Thermo Fisher Scientific, Inc.) for 12 h, in order to induce macrophage autophagy (starvation induction). In addition, RAW264.7 cells cultured in either DMEM containing 10 nM 3-methyladenine (3-MA; Sigma-Aldrich; Merck KGaA) for 12 h or in DMEM supplemented with 50 nM rapamycin (RAPA; Sigma-Aldrich; Merck KGaA) for 2 h, were used as cell models of autophagy inhibition.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total cellular RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using a reverse transcription kit (cat. no. K1622; Thermo Fisher Scientific, Inc.). The RT-qPCR detection was performed using the SYBR Select Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) using 500 ng cDNA and 10 pM of each primer and quantified with the Step-One Plus PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following amplifying conditions were used: 95˚C for 4 min; 40 cycles of 95˚C for 25 sec, 60˚C for 25 sec, and 70˚C for 25 sec. lncRNA *GAS5*, miR-181c-5p/miR-1192 and *ATG5*/*ATG12* relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (24). The mouse *GAPDH* gene was used for lncRNA *GAS5* and *ATG5*/*ATG12* normalization, and the small nuclear RNA U6 was utilized for miR-181c-5p/miR-1192 normalization. All primers were synthesized by Sangon Biotech Co., Ltd. The lncRNA *GAS5*-1 primers were used for non-transfected cell detection, whereas lncRNA *GAS5*-2 primers were used for the detection of cells transfected with exogenous lncRNA *GAS5* or its short hairpin RNA (shRNA) form. The primer sequences used in the present study are listed in Table I.

Fluorescence in situ hybridization assay. The distribution of lncRNA *GAS5* in RAW264.7 cells was detected using the fluorescence *in situ* hybridization (FISH) assay. The lncRNA *GAS5* detection probe and detection kit were purchased from Guangzhou RiboBio Co., Ltd. FISH assay was performed according to the manufacturer's instructions. Briefly, RAW264.7 cells were seeded in 24-well plates. Following a 24-h incubation, at 37˚C, cells were treated with EBSS medium or 10 nM 3-MA for a further 12 h to induce or inhibit autophagy. Subsequently, all cells were fixed, pre-hybridized and immersed in the hybridization solution containing the lncRNA *GAS5* probe marked with cyanine 3 (Cy3) and were incubated at 37˚C overnight in the dark. After washing, the cells were stained with DAPI (Beyotime Institute of Biotechnology) and viewed using the LSM 710 laser scanning confocal microscope (Carl Zeiss AG).

Bioinformatics analysis. The UCSC database (http://genome.ucsc.edu/) was analyzed lncRNA GAS5 transcripts. Multiple Experiment Viewer version 4.9.0 was used to perform clustering and functional annotation for autophagy-related lncRNAs and miRNAs. Significantly differentially expressed lncRNA GAS5 and miRNAs were screened out. microRNA.org (http://www.microrna.org/microrna/home.do) was used to predict the autophagy-related miRNAs that may be targeted by GAS5. The potential target genes of miRNAs were predicted using miRDB (http://www.mirdb.org/miRDB/), miRWalk2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) and TargetScan (http://www.targetscan.org/). The GAS5/miRNAs/autophagy protein interaction network was drawn using cytoscape 3.4 (https://cytoscape.org/download.html); miR-181C-5p and miR-1192 that overlapped with sequencing and were significantly differentially expressed were screened out.

Cell transfection. For lncRNA *GAS5* overexpression, the *GAS5* sequence containing miR-181c-5p and miR-1192 MRE of *GAS5* complementary DNA with the *Not*I and *Bam*HI restriction sites was subcloned into the LV5 lentivirus vector 12 μg (GenePharma Co., Ltd.) and co-transfected with pGag/Pol

Table I. Sequences of the primers used in the present study.

Name	Sequence
U6-F	5'-CTCGCTTCGGCAGCACA-3'
U6-R	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH-F	5'-GTCAACGGATTTGGTCTGTATT-3'
GAPDH-R	5'-AGTCTTCTGGGTGGCAGTGAT-3'
lncRNA *GAS5*-1 F	5'-ATTGGGTTTTGGTCTGGACA-3'
lncRNA *GAS5*-1 R	5'-GCTCTGCCATCAGAATCGTT-3'
lncRNA *GAS5*-2 F	5'-CAATGGCAAATGAGCACTAA-3'
lncRNA *GAS5*-2 R	5'-TCCTCAGATACGCAGAAACA-3'
miR-181c-5p Stem-loop primer	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACTCACCG-3'
miR-181c-5p F	5'-ACACTCCAGCTGGGAACATTCAACCTGTCG-3'
miR-181c-5p R	5'-CTCAACTGGTGTCGTGGA-3'
miR-1192 Stem-loop primer	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAATTTGGT-3'
miR-1192 F	5'-ACACTCCAGCTGGG AAACAAACAAACAGAC-3'
miR-1192 R	5'-CTCAACTGGTGTCGTGGA-3'
ATG5 F	5'-TTGGAGACTCCTGCTGTTGA-3'
ATG5 R	5'-TCATCTTTTAGCATACTCAGATGGG-3'
ATG12 F	5'-CTCCCAACCCTCACTTCTCG-3'
ATG12 R	5'-GGAGAGATGCAGCTCAGCAA-3'
lncRNA *GAS5* wt/mut F	5'-CAAGCTTGTTCTGTGGCAAAGGAGGAT-3'
lncRNA *GAS5* wt R	5'-GACGCGTTTCCCACCCACTCCTCTATC-3'
lncRNA *GAS5* mut (miR-181c-5p) R	5'-GACGCGTTTACATGTTGTGTGGGTTGAGGGATCTT-3'
lncRNA *GAS5* mut (miR-1192) R	5'-GACGCGTTACTATACCTAGTTAAAGCTGCCCGGTTA-3'
ATG5 wt/mut F	5'-CAAGCTTGTATGCCAAGTATCTGTCTATG-3'
ATG5 wt R	5'-GACGCGTTAGCATACTCAGATGGGTTG-3'
ATG5 mut R	5'-GACGCGTAATGTACATGTGGACAGCAAGCTAGCTC-3'
ATG12 wt/mut F	5'-CAAGCTTCATTGTGATCCATACCTGCT-3'
ATG12 wt R	5'-GACGCGTCTACATAGTGAGACCCAGCTT-3'
ATG12 mut R	5'-GACGCGTTATTGAACAAAAAAAGCATAACAAAAC-3'

F, forward; R, reverse; wt, wild-type; mt, mutant; ATG, autophagy-related gene.

10 μg (GenePharma Co., Ltd.), pRev 4 μg (GenePharma Co., Ltd.) and pVSV-G 6 μg (GenePharma Co., Ltd.) into 293T cells (cat. no. GNHu17, The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences) to generate LV5-*GAS5* lentivirus, with the use of Lipofectamine 3000® (Thermo Fisher Scientific, Inc.). Following a 72-h incubation period at 37˚C, the cell supernatant was collected at 4˚C, 3,000 x g for 4 min, and filtered with a 0.45 μm filter. The filtrate was collected at 4˚C, 48,000 x g for 2 h. The infection concentration was $3x10^8$ TU/ml (MOI, 100/1) for LV5-*GAS5* and $5x10^8$ TU/ml (MOI, 100/1) for sh-*GAS5*. LV5-*GAS5*-NC and sh-*GAS5*-NC were used as controls, at 37˚C for 72 h. To determine the potential function of miR-181c-5p and miR-1192, the miR-181c-5p mimic (50 nM) or miR-181c-5p inhibitor (50 nM) (GenePharma Co., Ltd.), miR-1192 mimic (50 nM) or miR-1192 inhibitor (50 nM) (GenePharma Co., Ltd.), or their negative controls (GenePharma Co., Ltd.) were transfected into RAW264.7 cells using Lipofectamine 3000® (Invitrogen; Thermo Fisher Scientific, Inc.). Following a 48-h incubation period at 37˚C, the infection efficiency was detected. Target gene sequences are shown in Table II.

Confocal microscopy. To evaluate the role of lncRNA *GAS5* in autophagy regulation, laser confocal microscopy was performed to measure the dot-like aggregation of the microtubule-associated proteins 1A/1B light chain 3B (LC3) protein in the cytoplasm, which is known to be relatively increased during autophagy (25). The detection procedure was performed as follows: Cells mounted on a slide were fixed with 4% paraformaldehyde for 25 min and permeabilized using 0.2% Triton X-100. The slides were then immersed in blocking buffer containing 5% BSA at room temperature for 30 min and were incubated with an anti-LC3 rabbit polyclonal antibody (1:500 dilution; cat. no. 43566; Cell Signaling Technology, Inc.) overnight at 4˚C. After washing, the slides were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000; cat. no. SA00006-1; ProteinTech Group, Inc.) for 2 h at room temperature. Finally, the nuclei were stained with DAPI (Beyotime Institute of Biotechnology); 150 μl DAPI were added to each slide, stain for 1 h, and soaked in PBS 4 times for 4 min each at room temperature. Cell morphology was observed and photographed under a disc scanning unit (DSU) spinning disk confocal microscope (Olympus Corporation).

Table II. Synthetic sequences of target genes.

Name	Sequence
sh-*GAS5*	5'-GGUGUUUCUUUCGCGAUCATT-3'
sh-*GAS5*-NC	5'-UUCUCCGAACGUGUCACGUTT-3'
miR-181c-5p mimics	5'-AACAUUCAACCUGUCGGUGAGU-3'
miR-1192 mimics	5'-AAACAAACAAACAGACCAAAUU-3'
Mimics-NC	5'-UUCUCCGAACGUGUCACGUTT-3'
miR-181c-5p inhibitor	5'-ACUCACCGACAGGUUGAAUGUU-3'
miR-1192 inhibitor	5'-AAUUUGGUCUGUUUGUUUGUUU-3'
Inhibitor-NC	5'-CAGUACUUUUGUGUAGUACAA-3'

NC, normal control.

Transmission electron microscopy (TEM). TEM was carried out to determine morphology and autophagosome quantity in RAW264.7 cells. Cell collection, specimen preparation and image acquisition were performed as previously described (26). For each experimental group, at least 20 cellular cross-sections were examined using ITEM digital imaging software (Hitachi, Ltd.).

Dual-luciferase reporter assay. To ascertain the target specificity of miR-181c-5p or miR-1192 to lncRNA *GAS5* and *ATG5* or *ATG12*, a dual-luciferase reporter assay was performed. Briefly, the target sequences of lncRNA *GAS5* and *ATG5* or *ATG12* to miR-181c-5p or miR-1192, and their mutant sequences (prsented in Table I), were amplified and sub-cloned into the pMIR-Luc reporter plasmid (Promega Corporation) to generate the corresponding recombinant plasmids. All the recombinant plasmids were verified by sequencing. The recombinant plasmids, including pMIR-wt-lncRNA *GAS5* (100 ng), pMIR-mut-lncRNA *GAS5* (100 ng), pMIR-wt-*ATG5* (100 ng), or pMIR-mut-*ATG5* (100 ng), were then co-transfected with miR-181c-5p mimics (50 nM), miR-181c-5p (50 nM) inhibitors or their negative controls into RAW264.7 cells, with the use of Lipofectamine 3000® (Thermo Fisher Scientific, Inc.). Similarly, pMIR-wt-lncRNA *GAS5* (100 ng), pMIR-mut-lncRNA *GAS5* (100 ng), pMIR-wt-*ATG12* (100 ng), or pMIR-mut-*ATG12* (100 ng), were co-transfected with miR-1192 mimics (50 nM), miR-1192 inhibitors (50 nM) or their negative controls into RAW264.7 cells, with the use of Lipofectamine 3000®. Following a 48-h incubation period at 37˚C, the luciferase activities in the cells in each group were determined using a dual-luciferase reporter assay kit (TransGen Biotech Co., Ltd.) according to the manufacturer's instructions. Firefly luciferase activity was normalized to *Renilla* luciferase gene activity with a dual-luciferase reporter assay.

Western blot analysis. Protein was extracted using RIPA lysis buffer (Nanjing KeyGen Biotech Co., Ltd.) and the protein concentration was measured with a protein BCA assay kit (Nanjing KeyGen Biotech Co., Ltd.). Subsequently, 30 μg protein was separated on a 12% SDS-PAGE gel by electrophoresis and transferred to 0.45 μm PVDF membranes (Merck KGaA). The membranes were immersed in a blocking solution containing 5% non-fat dry milk at room temperature for 2 h and then incubated overnight at 4˚C with an antiserum containing antibodies against LC3 (1:1,000; cat. no. 2775; Cell Signaling Technology, Inc.), p62 (1:1,000 dilution; cat. no. 5114; Cell Signaling Technology, Inc.) ATG5 (1:500 dilution; cat. no. 10181-2-AP; ProteinTech Group, Inc.) and ATG12 (1:500 dilution; cat. no. 10088-2-AP; ProteinTech Group, Inc.). Subsequently, the membranes were washed and incubated with a peroxidase-conjugated secondary antibody (1:3,000 dilution; cat. no. TA130023; OriGene Technologies, Inc.) at room temperature for 2 h. Finally, the protein bands were visualized with an ECL detection reagent (Thermo Fisher Scientific, Inc.) and the results were quantified with the use of ImageJ software J2x (Rawak Software Inc.). An anti-GAPDH antibody (1:1,000 dilution; cat. no. 2118; Cell Signaling Technology, Inc.) was used as a control and the results were presented as the ratio of density of target protein to the GAPDH value.

Statistical analysis. All data in the present study were analyzed and plotted using SPSS 19.0 (IBM Corp.) and GraphPad Prism 7.0 (GraphPad Software, Inc.). The results are presented as the mean ± SD of independent experiments. The differences between the groups were tested using one-way analysis of variance (ANOVA), followed Tukey's post hoc test.

Results

lncRNA GAS5 expression is associated with macrophage autophagy. In a previous study using high-throughput sequencing and cluster analysis, it was observed that lncRNA *GAS5* was selectively upregulated when the RAW264.7 cells were subjected to starvation treatment (20). Of note, up to 25 transcripts of lncRNA *GAS5* could be identified by means of UCSC database analysis (http://genome.ucsc.edu/), with lncRNA *GAS5*-003 being the longest one (2,554 bp) and containing almost all of the key transcript regions (Fig. 1A). Taking this into account, lncRNA *GAS5*-003 was used to explore the role of lncRNA *GAS5* in macrophage autophagy. Firstly, it was validated through RT-qPCR that lncRNA *GAS5* expression was significantly upregulated following cell starvation. As was initially hypothesized, lncRNA *GAS5* expression was downregulated when autophagy was inhibited by 3-MA (Fig. 1B). Additionally, FISH assay was utilized to localize lncRNA *GAS5* expression in the RAW264.7 cells; it

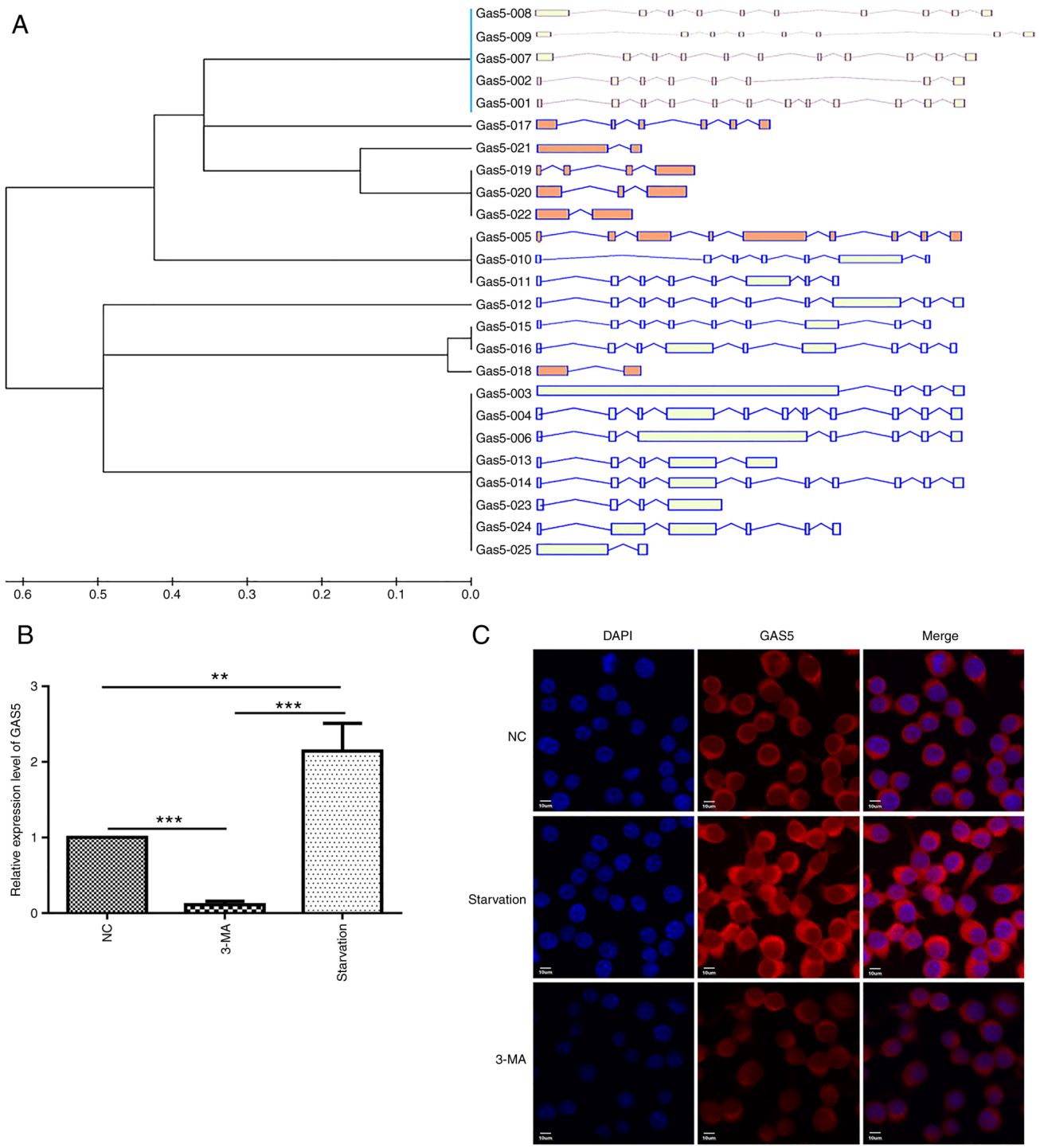

Figure 1. Expression and analysis of lncRNA *GAS5* in relation to macrophage autophagy. (A) Comparative analysis of lncRNA *GAS5* transcripts. (B) Expression of lncRNA *GAS5* in starvation-induced autophagy. Data represent the mean ± SD from three independent experiments. **P<0.01 and ***P<0.001. (C) Fluorescent *in situ* hybridization analysis of lncRNA *GAS5* expression. lncRNA, long-chain non-coding RNA; *GAS5*, growth arrest-specific transcript 5; 3-MA, 3-methyladenine; NC, negative control.

was observed that lncRNA *GAS5* was confined to the cytoplasm, and when autophagy occurred, its levels were markedly increased, and this effect was significantly reversed with the use of 3-MA (Fig. 1C).

lncRNA GAS5 promotes macrophage autophagy. To explore the role of lncRNA *GAS5* in macrophage autophagy, cell models were established *in vitro* by transfecting LV5-lncRNA *GAS5* (LV5-*GAS5*) or LV3-shRNA-lnc *GAS5* (sh-*GAS5*) into RAW264.7 cells in order to overexpress or knockdown lncRNA *GAS5* expression (Fig. 2A and B). Furthermore, FISH assay confirmed that the overexpression or knockdown of lncRNA *GAS5* expression through LV5-*GAS5* or sh-*GAS5* maintained the same intracellular localization pattern with cells containing wild-type (wt) lncRNA *GAS5* (Fig. 2C). Subsequently, the effects of lncRNA *GAS5* on cell autophagy were examined. lncRNA *GAS5* silencing reduced the LC3II to LC3 ratio, a marker of autophagy, while lncRNA *GAS5* overexpression

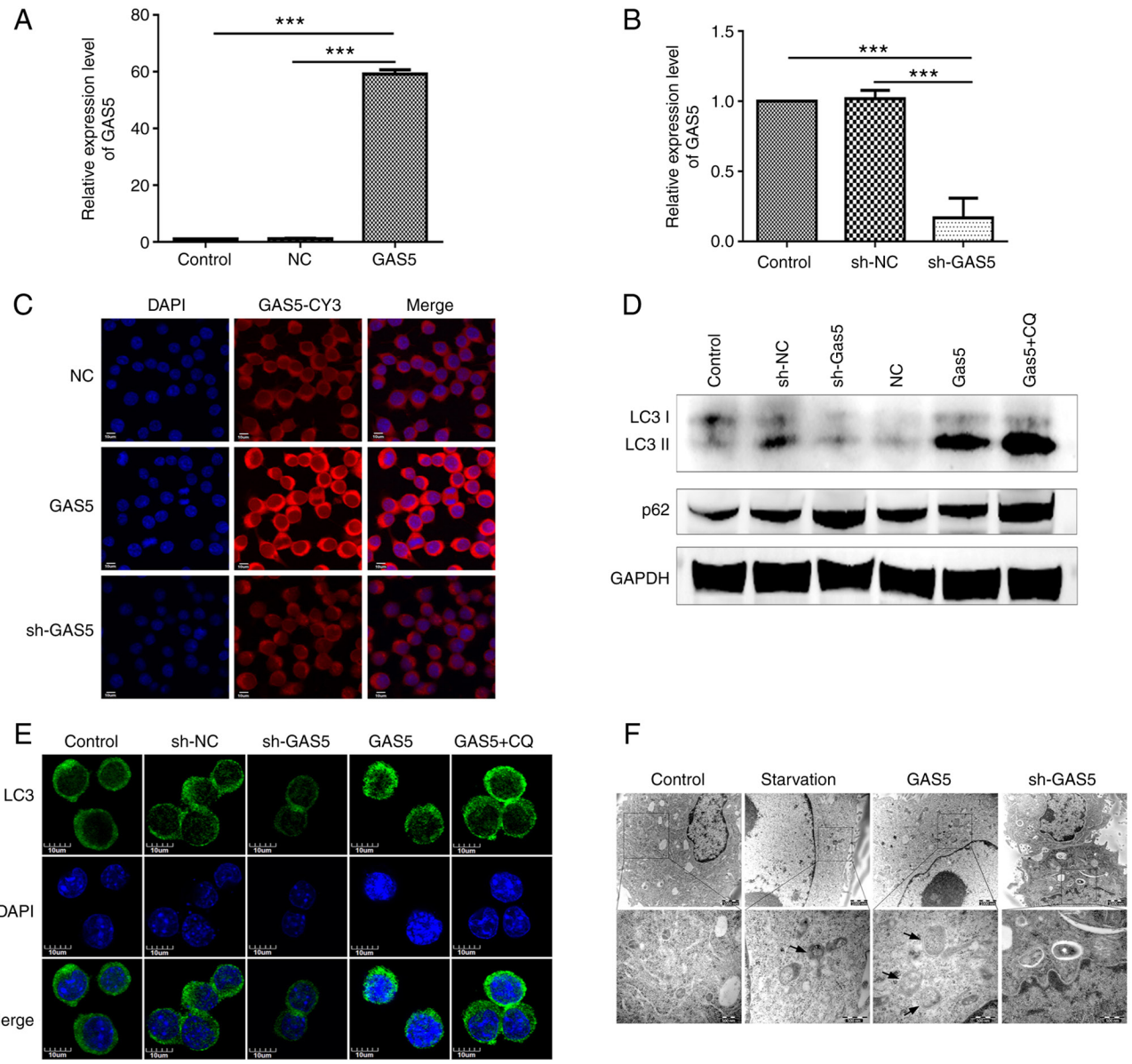

Figure 2. Effect of lncRNA *GAS5* expression on macrophage autophagy. (A) RT-qPCR validation of the lncRNA *GAS5* overexpression cell model. Data represent the mean ± SD from three independent experiments. ***P<0.001. (B) RT-qPCR validation of the lncRNA *GAS5* inhibition cell model. Data represent the mean ± SD from three independent experiments. ***P<0.001. (C) Validation of the lncRNA *GAS5* cell model through fluorescent *in situ* hybridization. (D) Western blot analysis of autophagy-associated protein expression. (E) Laser confocal microscopic analysis of the effect of lncRNA *GAS5* on macrophage autophagy. (F) Transmission electron microscopic analysis of lncRNA *GAS5* effect on autophagic body formation. lncRNA, long-chain non-coding RNA; *GAS5*, growth arrest-specific transcript 5; NC, negative control; LC3, light chain 3.

exerted the opposite effect (Fig. 2D). The effect of lncRNA *GAS5* on autophagy was also verified through laser confocal microscopy, in order to confirm that the level of lncRNA *GAS5* was associated with LC3 aggregation, a typical autophagy activation marker (Fig. 2E). In addition, the role of lncRNA *GAS5* in the formation of autophagic bodies was further explored, using TEM. The overexpression of lncRNA *GAS5* exerted the same effect as that observed with starvation, increasing the numbers of autophagic bodies in the cytoplasm, whereas lncRNA *GAS5* silencing exerted the opposite effect (Fig. 2F).

lncRNA GAS5 targets miR-181c-5p and miR-1192. To further explore the molecular mechanisms through which lncRNA *GAS5* promotes autophagy, its potential miRNA binding partners were analyzed using bioinformatics software, including StarBase V3.0 (http://starbase.sysu.edu.cn/), and TargetScan (http://www.targetscan.org/vert_71/). The analysis identified eight miRNAs selectively targeted by lncRNA *GAS5*. Of note, two of these miRNAs, miR-181c-5p and miR-1192, can also target key proteins of the autophagy pathway (Fig. 3A-C). RT-qPCR was then used in order to confirm the association between lncRNA *GAS5* and miR-181c-5p or miR-1192. lncRNA *GAS5* overexpression significantly downregulated miR-181c-5p and miR-1192 expression in RAW264.7 cells (Fig. 3D), whereas the silencing of lncRNA *GAS5* markedly increased the expression of both miRNAs (Fig. 3E). Since the regulation of lncRNA/miRNAs/autophagy interaction is a regulatory network, miRNA expression involves a time-course of events, and the same lncRNA can simultaneously regulate multiple miRNAs. At the same time, a miRNA can regulate

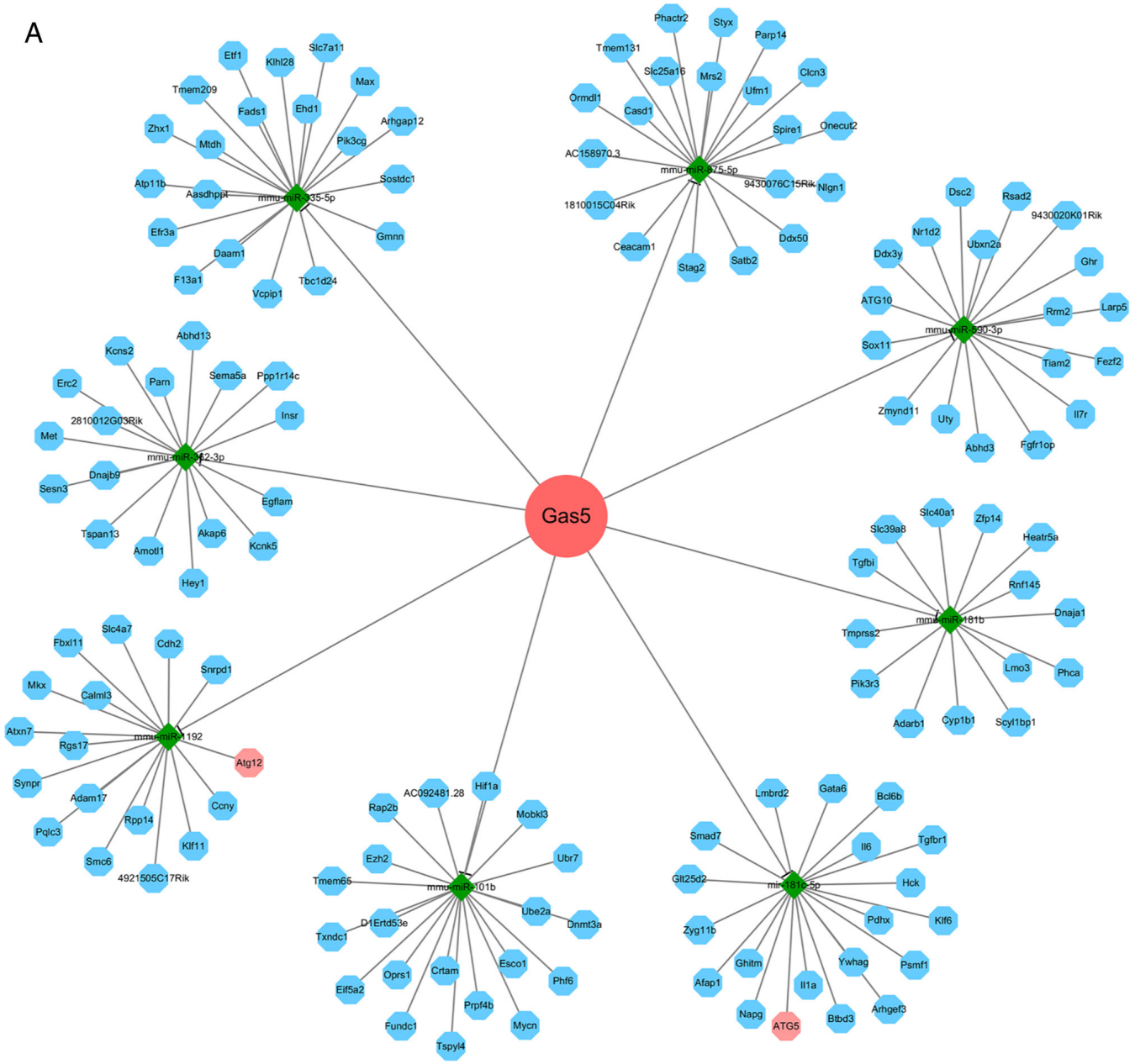

Figure 3. Continued.

multiple genes. Therefore, there are certain differences in the expression of miR-1192, leading to a slight increase in expression with the sh-NC control. Overall, the experimental results revealed that silencing GAS5 promoted the expression of miR-1192, and the increased expression of sh-NC control did not affect the conclusion of the study. In view of the results of both bioinformatics analysis and RT-qPCR, it was preliminarily surmised that lncRNA *GAS5* negatively regulates miR-181c-5p and miR-1192 expression.

Subsequently, the effects of miRNA mimic and inhibitor on RAW 264.7 cells were validated. miR-181c-5p mimic was able to increase miR-181c-5p expression, while miR-181c-5p inhibitor markedly decreased the expression level of miR-181c-5p in RAW 264.7 cells (Fig. 3F). Similarly, the miR-1192 expression level was significantly increased by the miR-1192 mimic and decreased by the miR-1192 inhibitor (Fig. 3G). Using these tools and a luciferase reporter with either a wt or mutated miR-181c-5p or miR-1192 lncRNA-*GAS5* binding site (Fig. 3B and C), the direct interaction between both miRNAs and lncRNA *GAS5* was examined. Dual-luciferase reporter assay revealed a significant decrease in luciferase activity in the cells co-transfected with the miR-181c-5p mimic and report-*GAS5*-wt. By contrast, the luciferase activity was almost unaltered when the cells were co-transfected with report-*GAS5*-mut (miR-181c-5p) and miR-181c-5p mimic (Fig. 3H). Co-transfection with miR-1192 mimic and report-*GAS5*-wt significantly decreased luciferase activity, while the luciferase activity was unaltered following co-transfection with miR-1192 mimic and report-*GAS5*-mut (miR-1192) (Fig. 3I).

miR-181c-5p and miR-1192 inhibit macrophage autophagy by targeting ATG5 and ATG12, respectively. Initially,

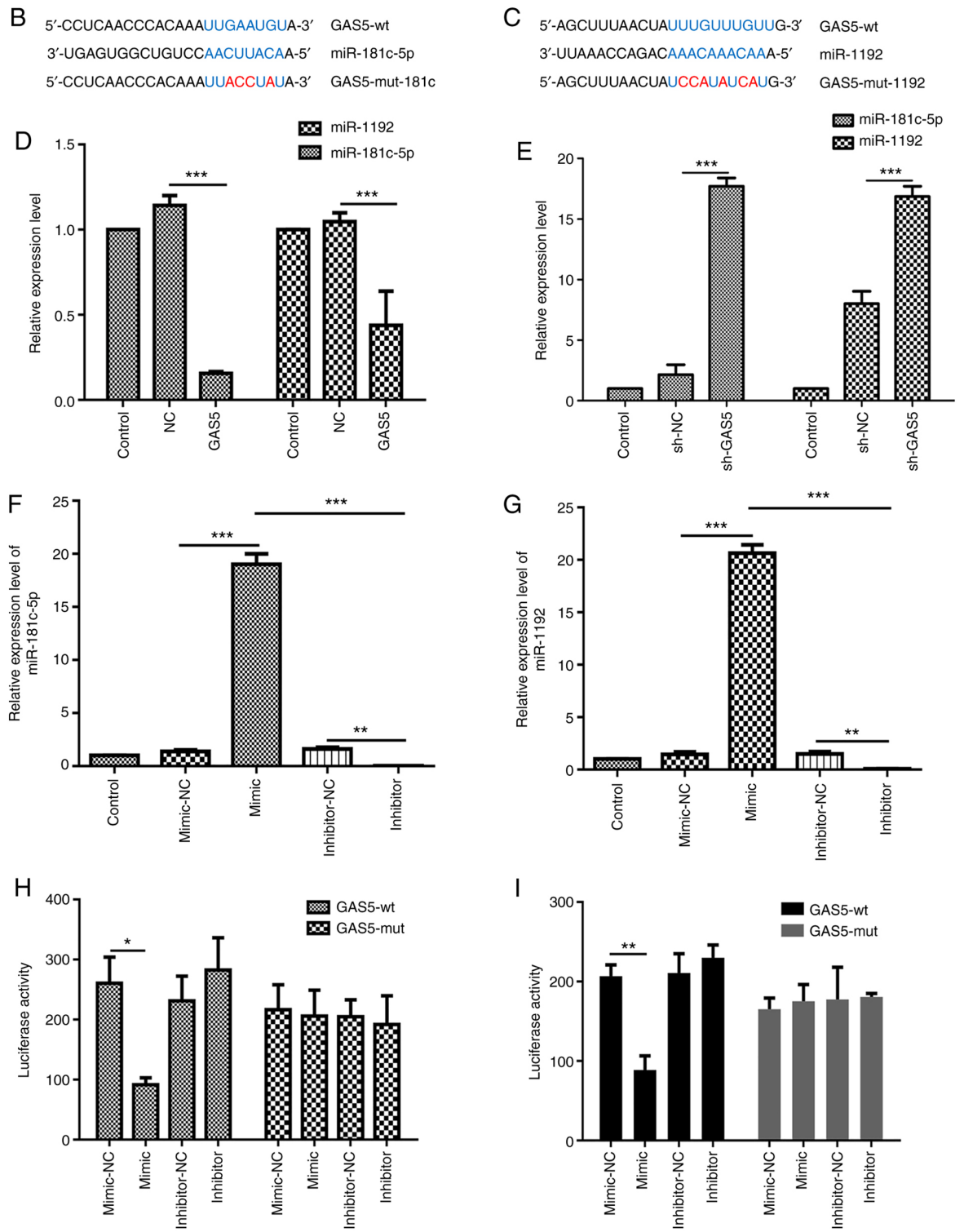

Figure 3. lncRNA *GAS5* targets miR-181c-5p and miR-1192. (A) Regulatory network diagram of lncRNA *GAS5*/miRNAs/autophagy (red circles represent *GAS5* or *ATG*; blue circles represent genes; green circles represent miRNAs). (B) Binding sequence analysis of the lncRNA *GAS5*/miR-181c-5p interaction. (C) Binding sequence analysis of the lncRNA *GAS5*/miR-1192 interaction. (D) RT-qPCR verification of the effect of lncRNA *GAS5* overexpression on miR-181c-5p and miR-1192 levels. Data represent the mean ± SD from three independent experiments. ***P<0.001. (E) RT-qPCR verification of the effect of lncRNA *GAS5* attenuation on miR-181c-5p and miR-1192 expression. Data represent the mean ± SD from three independent experiments. ***P<0.001. (F) RT-qPCR verification of the expression of miR-181c-5p. Data represent the mean ± SD from three independent experiments. **P<0.01 and ***P<0.001. (G) RT-qPCR verification of miR-1192 expression of. Data represent the mean ± SD from three independent experiments. **P<0.01 and ***P<0.001. (H) Target validation between lncRNA *GAS5* and miR-181c-5p using dual-luciferase reporter assay. Data represent the mean ± SD from three independent experiments. *P<0.05. (I) Target validation between lncRNA *GAS5* and miR-1192 using dual-luciferase reporter assay. Data represent the mean ± SD from three independent experiments. **P<0.01. lncRNA, long-chain non-coding RNA; *GAS5*, growth arrest-specific transcript 5; RT-qPCR, reverse transcription-quantitative PCR.

bioinformatics analysis revealed that *ATG5* was a potential target gene of miR-181c-5p (Fig. 4A and B), while *ATG12* was a potential target gene of miR-1192 (Fig. 4A and C). Subsequently, it was demonstrated that miR-181c-5p and

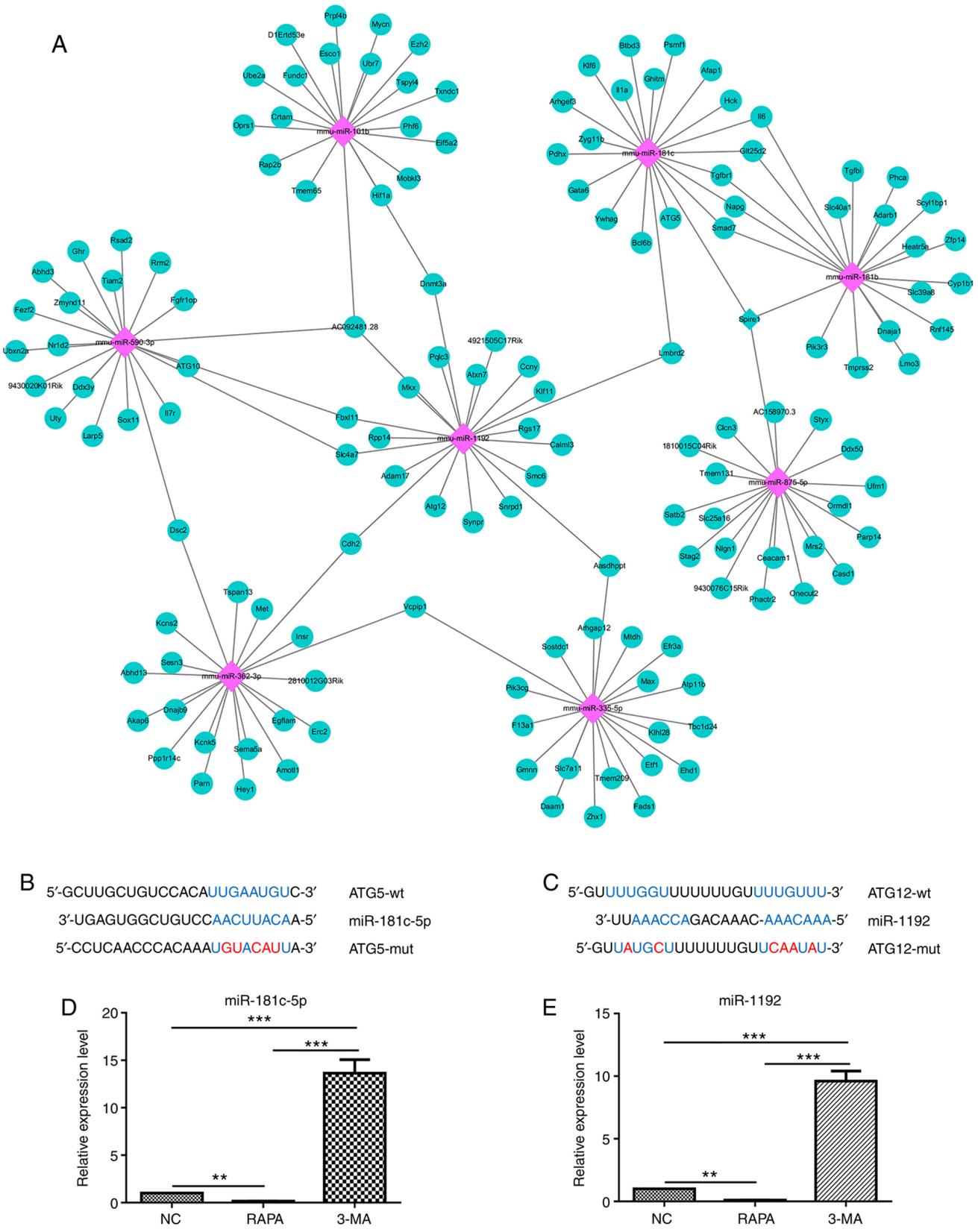

Figure 4. Roles of miR-181c-5p and miR-1192 in macrophage autophagy. (A) Regulatory network diagram illustrating the association of miRNAs with autophagy (green circles represent miRNAs; pink circles represent genes). (B) Binding sequence analysis of the miR-181c-5p/*ATG5* interaction. (C) Binding sequence analysis of the miR-1192/*ATG12* interaction. (D) Expression of miR-181c-5p in macrophage autophagy. Data represent the mean ± SD from three independent experiments. **P<0.01 and ***P<0.001. (E) Expression of miR-1192 in macrophage autophagy. Data represent the mean ± SD from three independent experiments. **P<0.01 and ***P<0.001. *ATG*, autophagy-related gene; 3-MA, 3-methyladenine; RAPA, rapamycin.

miR-1192 expression was significantly decreased following treatment with RAPA (a potent autophagy stimulus), whereas it was significantly increased as a result of 3-MA autophagy inhibitor treatment (Fig. 4D and E). These results thus

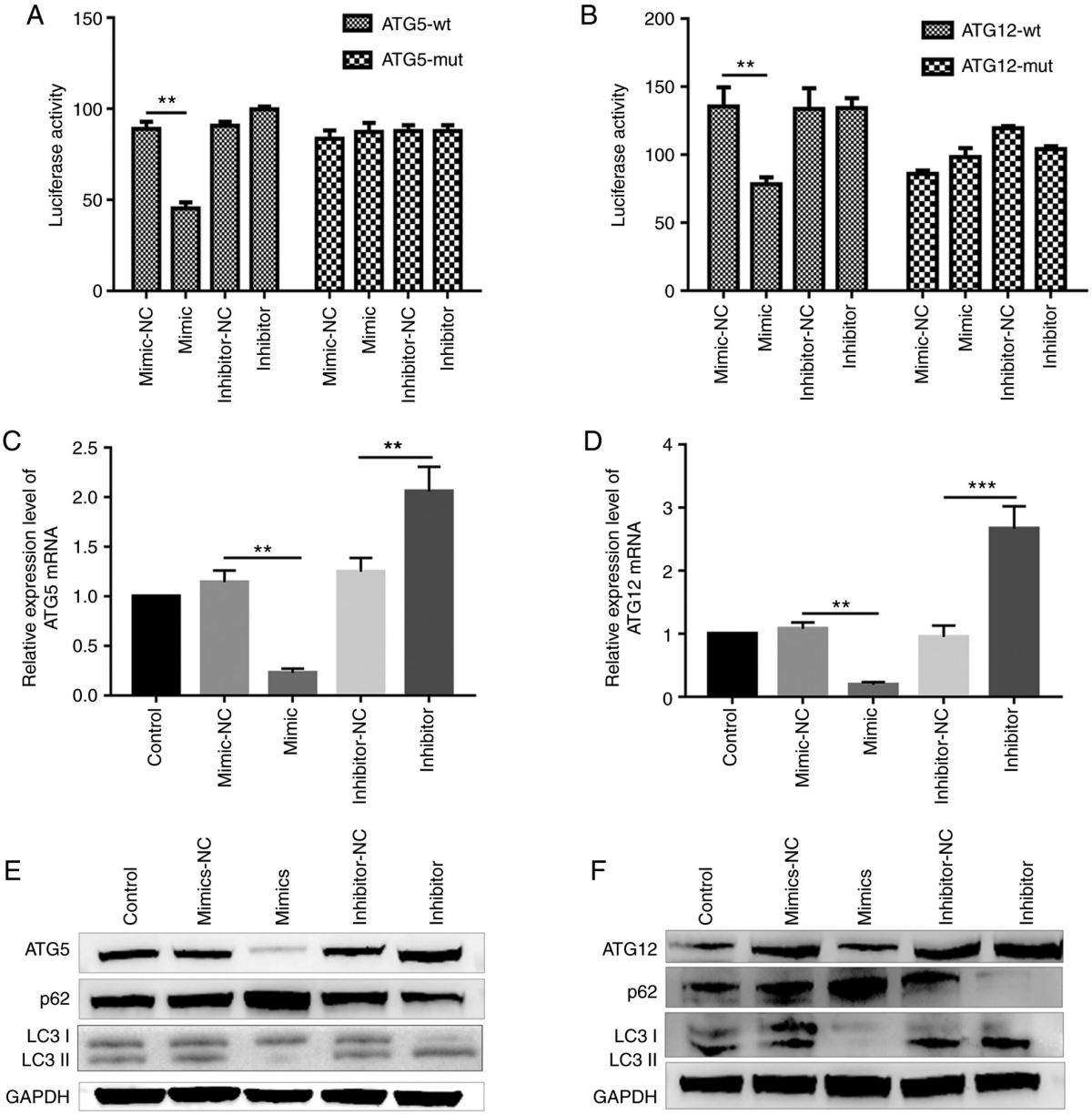

Figure 5. miR-181c-5p and miR-1192 targeting inhibits *ATG5* and *ATG12* expression, respectively, in RAW264.7 cells (A) Target validation between miR-181c-5p and *ATG5* by dual-luciferase reporter assay. Data represent the mean ± SD from three independent experiments. **P<0.01. (B) Target validation between miR-1192 and *ATG12* by dual-luciferase reporter assay. Data represent the mean ± SD from three independent experiments. **P<0.01. (C) RT-qPCR verification of the effect of miR-181c-5p on the expression of *ATG5*. Data represent the mean ± SD from three independent experiments. **P<0.01. (D) RT-qPCR verification of the effect of miR-1192 on the expression of *ATG12*. Data represent the mean ± SD from three independent experiments. **P<0.01 and ***P<0.001. (E) Western blot analysis of the effect of miR-181c-5p on the expression of *ATG5*. (F) Western blot analysis of the effect of miR-1192 on the expression of *ATG12*. *ATG*, autophagy-related gene; LC3, light chain 3; RT-qPCR, reverse transcription-quantitative PCR.

suggested that miR-181c-5p and miR-1192 were involved in macrophage autophagy.

To confirm the roles of these miRNAs in the regulation of autophagy, dual-luciferase reporter assay was performed. It was revealed that the luciferase activity decreased significantly in the cells co-transfected with the miR-181c-5p mimic and report-*ATG5*-wt; however, luciferase activity remained unaltered in the mutation control group (Fig. 5A). As was initially anticipated, miR-1192 mimic transfection resulted in a decrease in luciferase activity when wt *ATG12* vector was used. However, this inhibitory effect was abolished following transfection with the mutant miR-1192-targeting sequences in their 3'-UTR (Fig. 5B). Thus, miR-181c-5p and miR-1192 could directly target the key autophagy factors *ATG5* and *ATG12*, respectively. In addition, *ATG5* expression was significantly decreased in the cells transfected with the miR-181c-5p mimic, both at the mRNA and protein level. By contrast, the mRNA and protein expression of *ATG5* was increased in the miR-181c-5p inhibitor-transfected group (Fig. 5C and E). Furthermore, the LC3II/LC3I ratio decreased and p62 expression increased in the miR-181c-5p mimic-transfected group. The opposite effect was observed in the miR-181c-5p inhibitor-treated group, indicating that miR-181c-5p plays an inhibitory role in macrophage autophagy by targeting ATG5, a key autophagic protein. Simultaneously, RT-qPCR analysis revealed that *ATG12* was negatively regulated by miR-1192

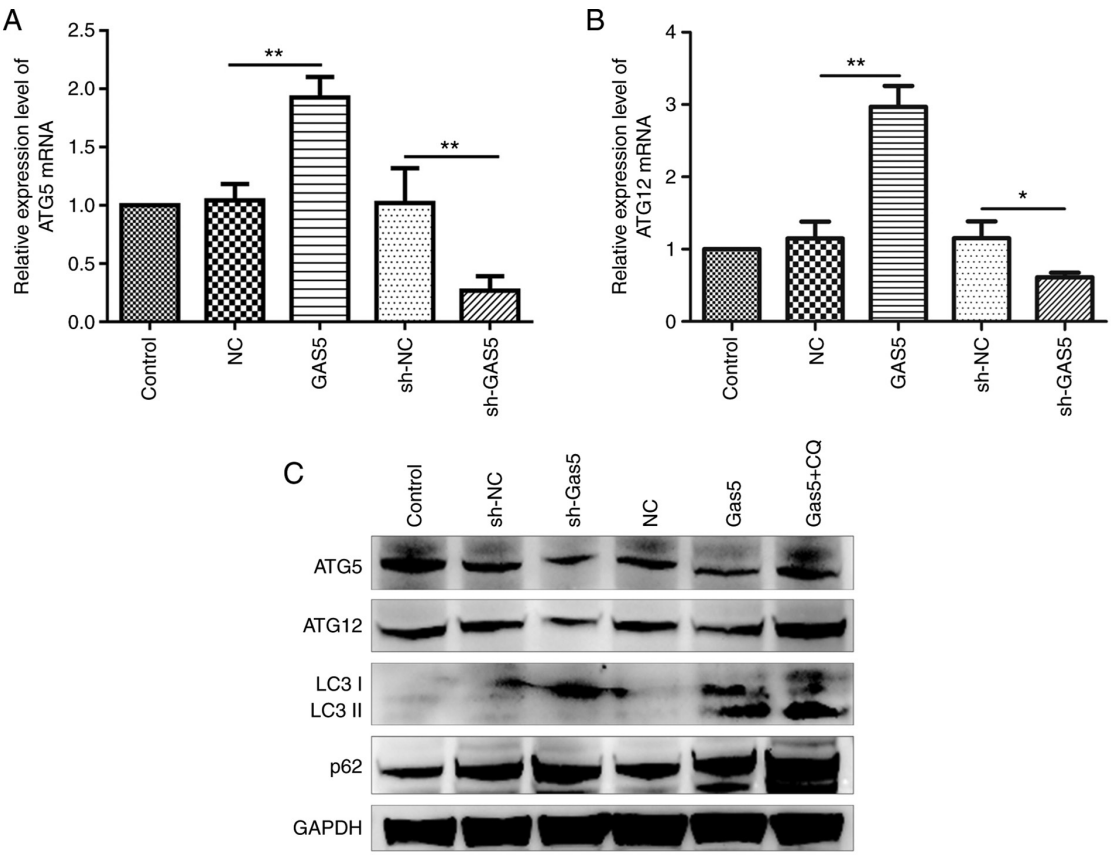

Figure 6. Effect of lncRNA *GAS5* on the expression of *ATG5* and *ATG12*. (A) Effect of lncRNA *GAS5* on *ATG5* mRNA expression. Data represent the mean ± SD from three independent experiments. **P<0.01. (B) Effect of lncRNA *GAS5* on *ATG12* mRNA expression. Data represent the mean ± SD from three independent experiments. *P<0.05 and **P<0.01. (C) Western blot analysis of the effect of lncRNA *GAS5* on the expression of *ATG5* and *ATG12*. *ATG5*, autophagy related 5; *ATG*, autophagy-related gene; lncRNA, long-chain non-coding RNA; *GAS5*, growth arrest-specific transcript 5.

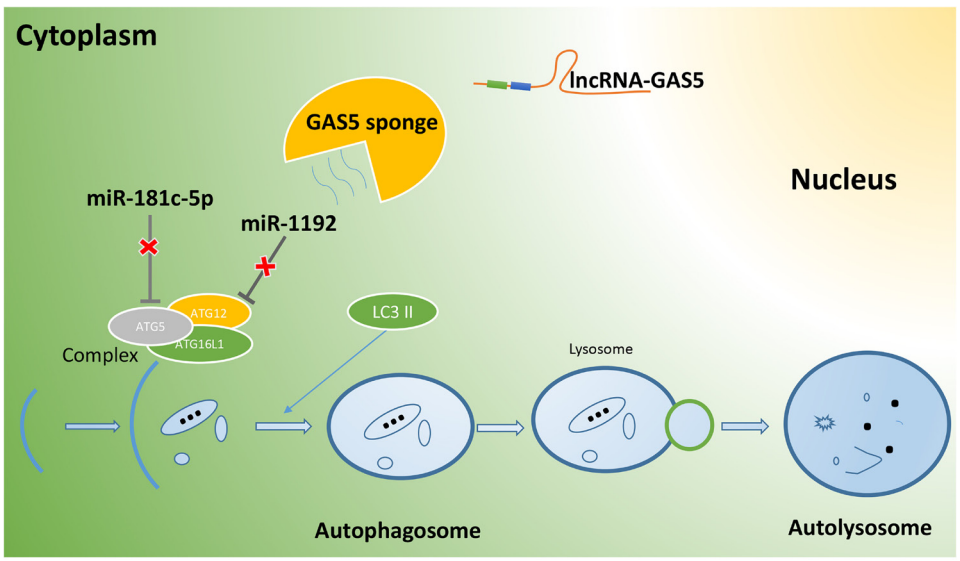

Figure 7. Summary of the role of lncRNA *GAS5* in autophagy. lncRNA, long-chain non-coding RNA; *GAS5*, growth arrest-specific transcript 5.

(Fig. 5D), and western blot analysis confirmed that miR-1192 inhibited *ATG12* expression (Fig. 5F).

lncRNA GAS5 preserves ATG5 and ATG12 expression. It was confirmed lncRNA *GAS5* negatively regulated both miR-181c-5p and miR-1192 and therefore positively affected autophagy through the preservation of *ATG5* and *ATG12* levels, which are two autophagy factors suppressed by the aforementioned miRNAs. To this end, the precise mechanisms of the regulation of autophagy by lncRNA *GAS5* were investigated. It was revealed that *ATG5* and *ATG12* mRNA and protein expression was significantly enhanced as a result

of lncRNA *GAS5* overexpression, whereas this was markedly decreased following the silencing of lncRNA *GAS5* (Fig. 6). On the whole, lncRNA GAS5 functions as a ceRNA in regulating Atg5 and Atg12 expression by binding to miR-181c-5p and miR-1192, thus promoting the autophagy of RAW264.7 cells (Fig. 7).

Discussion

lncRNA *GAS5*, located on chromosome 1, has been reported to participate in biological processes, such as cell proliferation, apoptosis, migration and invasion (27-29). Long regarded as a tumor suppressor, lncRNA *GAS5* has also been found to be involved in autoimmune disease, inflammation and autophagy. It has been revealed that there is a 5'-TOP sequence in exon 1 of the lncRNA *GAS5* gene, whose transcription is regulated by the mTOR signaling pathway. When cell growth is suppressed by starvation, the activity of mTOR signaling decreases and the translation of lncRNA *GAS5* is blocked. Subsequently, the degradation of lncRNA *GAS5* -regulated by the NMD pathway- is reduced, which finally culminates in lncRNA *GAS5* aggregation and increased expression (30).

In the present study, cell models of autophagy and autophagy inhibition were established using the EBSS medium (for starvation induction) and complete medium supplementing with 3-MA, respectively. It was demonstrated that lncRNA *GAS5* expression was significantly higher than that of other lncRNAs in the autophagy group. In addition, the lncRNA *GAS5*-003 contained almost all the key regions of the lncRNA *GAS5* gene within its 25 transcripts. Therefore, lncRNA *GAS5*-003 was selected for use in subsequent experiments. The increased expression of lncRNA *GAS5* in the starvation-induced autophagy group was confirmed through RT-qPCR and FISH assay. lncRNA *GAS5* overexpression markedly increased the autophagy-associated protein LC3 levels and promoted autophagic body formation, while the opposite results were observed in the group which was subjected to lncRNA *GAS5* silencing. Thus, it was hypothesized that lncRNA *GAS5* may be implicated in macrophage-mediated autophagy regulation.

To further explore the autophagy-promoting function of lncRNA *GAS5*, its potential target miRNAs were evaluated using bioinformatics. miR-181c-5p and miR-1192 were revealed as putative targets of lncRNA *GAS5*. Although in a previous study, it was demonstrated that lncRNA *GAS5* antagonized the chemoresistance of pancreatic cancer cells through the regulation of miR-181c-5p expression (31), the role of lncRNA *GAS5* and miR-181c-5p in macrophage autophagy remains unknown. In the present study, it was found that the overexpression of lncRNA *GAS5* in RAW264.7 cells significantly downregulated the expression of miR-181c-5p and miR-1192, via their direct interaction. Therefore, it was confirmed that lncRNA *GAS5* acts as a sponge for miR-181c-5p and miR-1192. Additionally, it has been noted that miR-181c-5p is involved in the regulation of inflammation (32), cell proliferation (33) and apoptosis (34). Moreover, miR-1192 has been proven to be implicated in myocardial infarction (35), inflammation (36) and muscle injury (37).

In the present study, it was demonstrated that miR-181c-5p and miR-1192 expression could be regulated by RAPA and 3-MA. Of note, miR-181c-5p and miR-1192 reduced the LC3II/LC3I ratio and promoted p62 expression. It was hypothesized that these may also be implicated in RAW264.7 cell autophagy. Furthermore, it was revealed that *ATG5* and *ATG12* were potential targets of miR-181c-5p and miR-1192, respectively, as confirmed by luciferase assay, RT-qPCR and western blot analysis. To date, >40 ATGs have been found in yeast and mammals, which are highly conserved among species and participate in the autophagy regulation via complex networks (38). When autophagy is initiated, *ATG5*, a key protein in the initial stage of autophagy, is recruited by ATG16L1 to form the *ATG5-ATG12*-ATG16L1 complex, which provides interaction sites for other proteins involved in the formation of the autophagy membrane, accelerates the extension of the autophagy membrane, and promotes the transformation of LC3I into LC3II (39). Therefore, it was suggested that lncRNA *GAS5* may regulate *ATG5* and *ATG12* expression, by interacting with miR-181c-5p and miR-1192, respectively. However, in the present study, only a small part of the autophagic pathway was analyzed. In order to better define the mechanistic role of lncRNA *GAS5* in macrophage autophagy, a more selective approach may be necessary, including the design of 'Target Protectors' (40) for the definition of the selective role of a particular miRNA-targeted mRNA in a specific phenotypic readout. This may be a future research approach based on currently presented results.

Based on the experimental results presented herein, it can be concluded that lncRNA *GAS5* promotes macrophage autophagy by targeting the miR181c-5p/*ATG5* and miR-1192/*ATG12* axes. In a follow-up study, the authors aim to construct *in vivo* animal models of lncRNA *GAS5* overexpression and interference through lentiviral transduction, and to examine the mechanisms and application of lncRNA *GAS5* in diseases. These findings may provide a theoretical basis for the further exploration of the mechanisms of autophagy-associated diseases, such as intracellular bacterial and virus infections and tumorigenesis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TX and GX participated in the design and coordination of the study. TX, XX and YC performed the experiments. XX and DJ performed the statistical analyses. TX and XX wrote the manuscript. TX, XX and GX reviewed and edited the manuscript. XX and GX confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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