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Bone marrow-derived mesenchymal stem cells alleviate severe acute pancreatitis-induced multiple-organ injury in rats via suppression of autophagy

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ABSTRACT

Patients with severe acute pancreatitis (SAP) represent a substantial challenge to medical practitioners due to the high associated rates of morbidity and mortality and a lack of satisfactory therapeutic outcomes. In a previous study, our group demonstrated that bone marrow-derived mesenchymal stem cells (BMSCs) can ameliorate SAP; however, the mechanisms of action remain to be fully understood. BMSCs were intravenously injected into SAP rats 12 h after experimental induction of SAP using sodium taurocholate (NaT). Histopathological changes and the levels of pro-inflammatory mediators were assessed by hematoxylin and eosin (H&E) staining and ELISA, respectively. Autophagy levels were assessed using qRT-PCR, western blotting, immunohistochemistry, immunofluorescence, and transmission electron microscopy. AR42J cells and human umbilical vein endothelial cells (HUVECs) were administered BMSC-conditioned media (BMSC-CM) after NaT treatment, and cell viability was measured using a Cell Counting Kit-8 (CCK-8) and flow cytometry. *In vivo*, BMSCs effectively reduced multiple systematic inflammatory responses, suppressed the activation of autophagy, and improved intestinal dysfunction. *In vitro*, BMSC-CM significantly improved the viability of injured cells, promoted angiogenesis, and decreased autophagy. We therefore propose that the administration of BMSCs alleviates SAP-induced multiple organ injury by inhibiting autophagy.

1. Introduction

The morbidity of acute pancreatitis (AP) has risen in recent years, representing a massive medical and social burden [1]. A total of 20% of patients suffering from AP develop severe AP (SAP), which requires advanced medical intervention and care, with mortality ranging from 15 to 39% [2].

Multiple organ dysfunction syndrome (MODS) is considered the leading cause of mortality in SAP [3]. In addition, the associated inflammatory responses exacerbate the severity of SAP, with the involvement of damage-associated molecular patterns (DAMPs) including interleukin-1β (IL-1β), IL-6, IL-8, tumor necrosis factor alpha (TNF-α), and myeloperoxidase (MPO) [4–6]. Thus, research seeking to identify novel therapeutic strategies for SAP requires a focus on how to reduce the release of DAMPs, and attenuate SAP-associated multiple system organ damage, rather than focusing on single organ injuries.

Recently, increasing evidence has suggested that impaired autophagy plays an important role in the evolution of SAP [7–9]. Autophagy is the lysosomal degradation pathway that is fundamental in the differentiation, development, homeostasis, and survival of cells [10]. Recent studies have confirmed that autophagy can be regulated by mammalian target of rapamycin (mTOR), protein P62/sequestosome 1 (SQSTM1), Beclin-1, microtubule-associated protein 1 light chain 3 (LC3), as well as multiple additional factors [11,[12]]. Moreover, a recent study has demonstrated that autophagy is detrimental to pancreatic acinar cells during early stages of SAP, via delivery of

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trypsinogen to the lysosome [13]. Therefore, regulating autophagy as a form of treatment for SAP may lead to beneficial outcomes.

Mesenchymal stem cells (MSCs) are a subtype of vital stem cells, with low immunogenicity, which have been utilized in the treatment of various diseases such as, autoimmune encephalomyelitis [14], myocardial infarction [15], and systemic lupus erythematosus [16]. Jung and colleagues were the first to demonstrate that human bone marrow-derived MSCs (BMSCs) can inhibit inflammation and reduce AP in rats [17]. Our group has previously identified some possible mechanisms by which BMSCs may ameliorate SAP, including by promoting angiogenesis [18,19], reducing oxidative stress [20,21], and inhibiting necroptosis [22]. However, these findings do not explain the protective function of BMSCs on multiple organ injury in SAP, and the underlying mechanisms of action remain unclear. Additionally, some autophagy-inhibiting drugs have been found to attenuate cerulein-induced AP in mice [23]. Therefore, the current study was conducted to elucidate the mechanisms by which BMSCs alleviate SAP, and ascertain the role of autophagy in this process.

2. Materials and methods

2.1. Animal models

Healthy male Sprague-Dawley rats (weighing 150–200 g) were purchased from Shanghai Laboratory Animal Co., Ltd. (Shanghai, China). All animal experiments complied with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All experimental protocols were approved by the Institutional Animal Ethics Committee of the Shanghai Tenth People's Hospital, affiliated to Tongji University School of Medicine (Included in the Supplementary Material is a text file named “Animal ethical guidelines,” SHDSYY-2018-2330, August 8, 2018). The SAP rat model was established by retrograde injection of 3% NaT (Sigma-Aldrich, St. Louis, MO, USA), as previously described [22].

2.2. Cell isolation, culture, identification, and administration

BMSCs were isolated, cultured, and identified as described in our previous study [21]. AR42J cells were purchased from Zeye Biology (Shanghai, China) and incubated with DMEM/F-12 media (Gibco, Middleton, WI, USA) containing 20% fetal bovine serum (Gibco) in a humidified atmosphere at 37 °C and 5% CO_2. HUVECs (EA.hy926 cells) were cultured and identified as previously described [18]. When cells reached approximately 80–90% confluence, they were digested and passaged. AR42J cells and HUVECs were treated with BMSC-CM or control media after administration of NaT (10 nM).

2.3. Extraction of conditioned media from rat BMSCs

Primary rat BMSCs (5×10^5 cells) were incubated in a 100 mm Petri dish, and media were changed to pure DMEM media without serum (Gibco) when cells reached confluence. Next, BMSCs were cultured in a humidified atmosphere at 37 °C and 5% CO_2 for 24 h. The consequent BMSC-CM were gathered, centrifuged for 5 min at 300 × *g*, filtered via a 0.22 μm syringe filter, and stored at −80 °C, as previously described [24].

2.4. Experimental animal protocol

Thirty rats were randomly divided into five groups (n = 6 per group) for *in vivo* experiments as follows: (1) Normal control (NC) group; (2) sham group: rats were anesthetized and subjected to opening and closing of the skin; (3) SAP group: rats were treated with NaT as described above; (4) phosphate-buffered saline (PBS) group: SAP model with PBS administered via the tail vein (1500 μL/kg body weight); (5) BMSC group: rat BMSCs (1×10^7 cells/kg body weight) dissolved in 300 μL of PBS, injected into SAP rats via the caudal vein 12 h after administration of NaT.

2.5. Histopathological analysis

After euthanization, rat pancreatic, small intestine, and lung tissues were obtained and conserved in 4% paraformaldehyde. Tissues were embedded in paraffin and stained with H&E for histological examination. The severity of pancreatic damage was judged by pathological scoring, as previously described [22]. The intestinal histological scores (0–5) were determined based on previously described methods [25]. Pulmonary histological injury was also scored according to previously described methods [26]. The evaluation of tissue sections was performed by two separate and experienced pathologists blinded to experimental group.

2.6. Biochemical examination in serum and tissues

The levels of serum amylase and lipase activity were detected using colorimetric assay kits (BioVision, Milpitas, CA, USA), following the manufacturer's protocols. The levels of cytokines in serum and various tissues (lactate dehydrogenase (LDH), MPO, IL-1β, IL-6, IL-8, and TNF-α) were determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

2.7. Quantitative real-time polymerase chain reaction

Total RNA was extracted from frozen pancreatic tissue using TRIzol reagent (Invitrogen, Carlsbad, California, USA). The cDNA was synthesized using a PrimeScript Reverse Transcriptase Reagent Kit (Kapa Biosystems, Boston, MA, USA). The qRT-PCR assays were carried out using a KAPA qPCR Kit (Kapa Biosystems). GAPDH was used as the endogenous control. The detailed primer sequences are included in Table 1. Relative expression of various target genes was analyzed using the comparative $2^{-\Delta\Delta CT}$ method, as previously described [22].

2.8. Western blotting

Total protein was extracted from tissues and cells using RIPA lysis buffer (Invitrogen) with PMSF (1:100; Beyotime, Nantong, Jiangsu, China), as previously described [22]. Proteins were transferred to nitrocellulose membranes, which were imaged on an Odyssey scanner (LI-COR Biosciences, USA) after incubation with corresponding primary and secondary antibodies. Primary antibodies, purchased from CST (Danvers, MA, USA), were as follows: P62, Beclin-1, LC3, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), protein kinase B (AKT), phosphorylated AKT (*p*-AKT), mTOR, phosphorylated mTOR (*p*-mTOR), Zonula occludens-1 (ZO-1), Claudin-1, and GAPDH. Secondary antibodies used were anti-rabbit IgG (CST) or anti-mouse IgG (CST).

2.9. Immunohistochemistry

Immunohistochemistry was performed as previously described [22]. Tissue sections were incubated with the following primary antibodies (all obtained from Abcam, Cambridge, UK): P62, Beclin-1, LC3, PI3K, AKT, *p*-AKT, mTOR, and *p*-mTOR. Immunohistochemical analysis was

Table 1
Primer sequences for qRT-PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
P62	GAGGCACCCCGAAACATGG	ACTTATAGCGAGTTCCCACCA
Beclin-1	ATGGAGGGGTCTAAGGCGTC	TGGGCTGTGGTAAGTAATGGA
LC3	GACCGCTGTAAGGAGGTGC	AGAAGCCGAAGGTTTCTTGGG
GAPDH	CGCTAACATCAAATGGGGTG	TTGCTGACAATCTTGAGGGAG

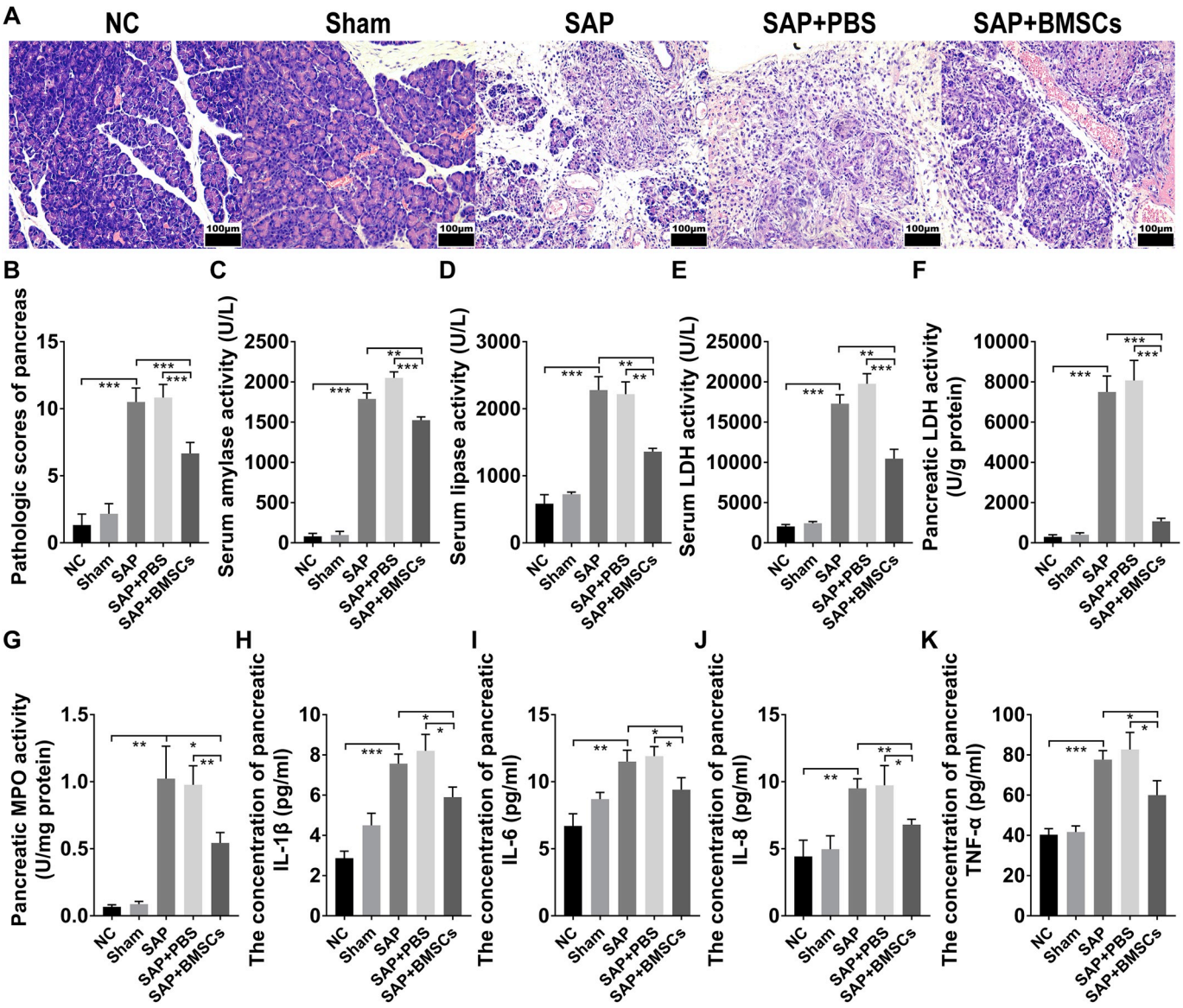

Fig. 1. BMSCs ameliorate NaT-induced SAP. (A) Histopathological assessment of pancreatic tissue by H&E staining (scale bar = 100 μm). (B) Pathological scores of H&E-stained pancreatic tissue (including edema, infiltration, and acinar necrosis). (C) Levels of serum amylase activity. (D) Levels of serum lipase activity. (E) Levels of serum LDH activity. (F) Levels of pancreatic LDH activity. (G) Levels of pancreatic MPO activity. (H–K) Concentration of pancreatic IL-1β, IL-6, IL-8, and TNF-α. Data represent the means ± SD from at least three independent experiments. N = 6/group. *p < 0.05, **p < 0.01, and ***p < 0.001.

performed using Image-Pro Plus software 6.0 (Media Cybernetics, Silver Spring, MD, USA).

2.10. Immunofluorescence

After sacrifice, rat pancreases were excised and preserved in paraformaldehyde, followed by paraffin embedding. Immunofluorescence staining was performed on 5 μm tissue sections, as previously described [27]. Primary antibody LC3 (Abcam) was incubated according to the manufacturer's protocols.

2.11. Transmission electron microscopy

Cells in pancreatic tissue were detected by TEM (JEM 1230, JEOL, Tokyo, Japan), after conservation in glutaraldehyde buffer and fixation in osmium tetroxide, as previously described [23].

2.12. Cell Counting Kit-8 (CCK-8) assay

Cell viability was measured using a Cell Counting Kit-8 (CCK-8) assay, as previously described [22]. AR42J cells and HUVECs of different treatment groups were seeded into 96-well plates (2×10^4 cells/well). After 12, 24, 36, and 48 h of growth, 10 μL of CCK-8 reagent (Dojndo, Kumamoto, Japan) was added to each well, and incubated for 2 h at 37 °C. The absorbance at 450 nm was then detected on a microplate reader (BioTek, Winooski, VT, USA).

2.13. Flow cytometry

The survival percentage of AR42J cells and HUVECs from different treatment groups was measured using a flow cytometry assay, as previously described [22]. Cells were collected and washed twice with PBS, followed by incubation with 100 μL of binding buffer and 5 μL of FITC-Annexin V (BD Pharmingen, San Diego, CA, USA) at 4 °C in the dark for 15 min. Cells were then incubated with 5 μL of propidium iodide in the dark for 5 min. After staining, the rates of cell survival were

analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA). The Q4 region represents live cells.

2.14. HUVEC angiogenesis

A tube formation assay was used to determine the effect of BMSC-CM on the angiogenic activity of HUVECs *in vitro*, as previously described [18]. We coated each well of a 96-well plate with 50 μL of matrigel matrix (Becton, Dickinson and Company, New Jersey, USA), and allowed wells to incubate for 30 min at 37 °C. HUVECs were seeded onto the concretionary gels (8×10^3 cells/well) in 50 μL of culture media. With the exception of the control group, 50 μL of NaT (10 nM) with or without 50 μL of BMSC-CM was added to each well. The resulting tube-like formations were then observed by microscopy after incubation for 6–8 h.

2.15. Statistical analysis

All experimental data represent the mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was carried out using a one-way analysis of variance (ANOVA) and an unpaired Student's *t*-test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. BMSCs attenuate the systemic inflammatory responses to SAP

We induced SAP by retrograde perfusion of NaT in rats, and prepared BMSCs were administered via tail vein injection 12 h post-SAP induction. All groups of rats were euthanatized 72 h after BMSC injection. Simultaneously, blood and multiple organ tissues (including pancreas, small intestine, and lung) were collected for further analysis. Hematoxylin and eosin (H&E) staining of pancreatic tissues indicated that administration of BMSCs was able to decrease pathological scores (including edema, infiltration, and acinar necrosis), which were increased by SAP (Fig. 1A and B). Additionally, compared with the SAP and PBS groups, injection of BMSCs significantly inhibited the serum levels of amylase and lipase, as well as serum LDH, pancreatic LDH, pancreatic MPO, and proinflammatory cytokines including IL-1β, IL-6, IL-8, and TNF-α (Fig. 1C–K).

3.2. BMSCs inhibit pancreatic autophagy in SAP

To evaluate the effect of BMSCs on SAP, we investigated the expression of pancreatic P62/SQSTM1, Beclin-1 and LC3, as they play important roles in the development of autophagy, which is likely related to the process of SAP. The results of quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot revealed that transplanted BMSCs could significantly suppress the expression of Beclin-1 and LC3, which were increased by NaT treatment (Fig. 2B–D, F and G). However, the expression of P62 showed the opposite pattern (Fig. 2A, D and E). In addition, immunohistochemistry and immunofluorescence results also confirmed the above finding (Fig. 2H–L). Moreover, we used transmission electron microscopy (TEM) to observe differences in the ultrastructure of pancreatic cells under different conditions. A significant increase in the number of autophagosomes was detected in the NaT-induced group, while administration of BMSCs decreased the number of autophagosomes (Fig. 2M).

3.3. BMSCs suppress autophagy by up-regulating the PI3K/AKT/mTOR signaling pathway in SAP

The mediation of autophagy is a complex process. Although we have demonstrated that BMSCs can prevent the over activation of autophagy in SAP, the underlying mechanisms involved remain unclear. The

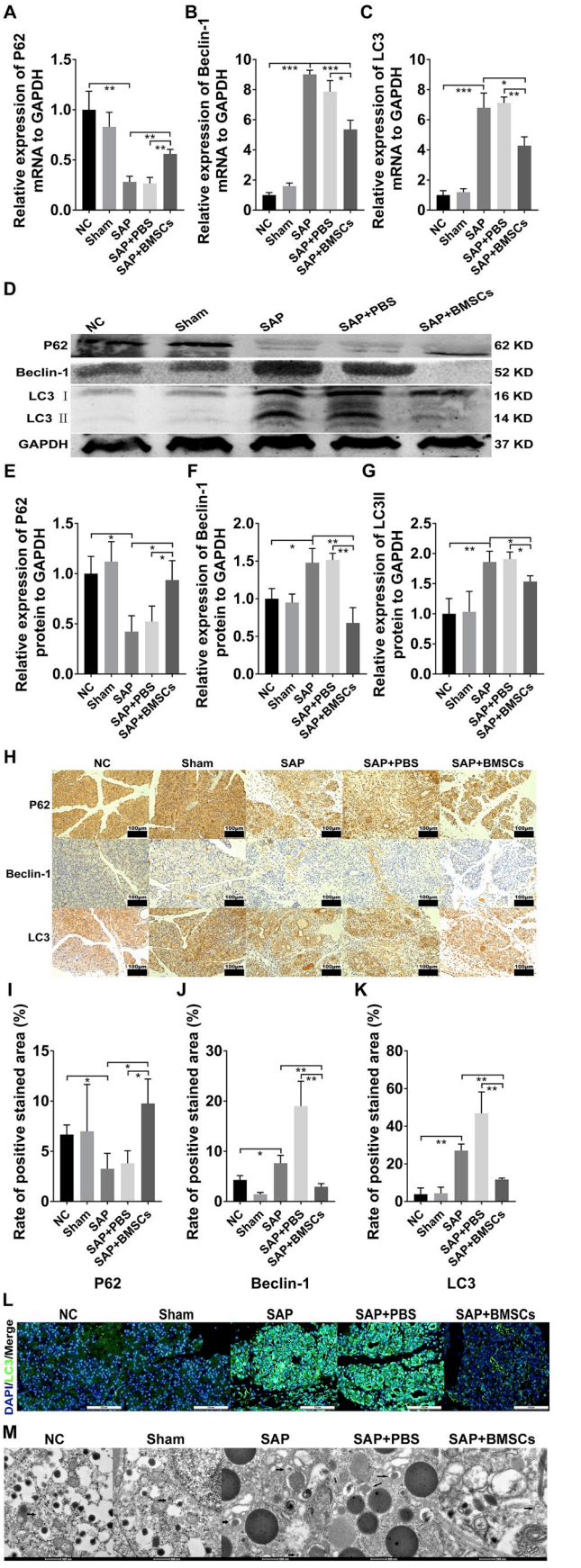

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Fig. 2. BMSCs suppress pancreatic autophagy in SAP. (A–C) Relative mRNA expression levels of pancreatic P62, Beclin-1, and LC3 measured by qRT-PCR. (D–G) Western blot analysis of P62, Beclin-1, and LC3 (including LC3Iand LC3II) protein levels in pancreatic tissue. (H) Immunohistochemical staining of pancreatic P62, Beclin-1, and LC3 protein (scale bar = 100 μm). (I–K) Immunohistochemical analysis of the proportion of tissue positively stained for P62, Beclin-1, and LC3. (L) Immunofluorescence staining of pancreatic LC3 (scale bar = 100 μm). (M) Representative images of autophagosomes in pancreatic tissue observed using TEM (scale bar = 500 μm). Black arrows indicate autophagosomes. Data represent the mean ± SD from at least three independent experiments. N = 6/group. *p < 0.05, **p < 0.01, and ***p < 0.001.

mTOR has been found to play an important role in the regulation of autophagy. This pathway is controlled by AMPK, PI3K/AKT, MAPK/ERK1/2, as well as additional factors. Thus, we examined whether BMSCs could attenuate pancreatic injury by regulating the PI3K/AKT/mTOR pathway. Western blotting results suggested that BMSCs markedly increased the expression of PI3K, *p*-AKT, and *p*-mTOR, which were significantly reduced in the SAP group, while total AKT and total mTOR were unchanged (Fig. 3A–D). In addition, immunohistochemical analyses supported these findings (Fig. 3E–J).

3.4. BMSCs alleviate intestinal injury, suppress autophagy in intestinal tissues, and strengthen the intestinal epithelial barrier in SAP

Intestinal epithelial barrier dysfunction leads to multiple organ failure in SAP patients, which aggravates the severity of the inflammatory response. After administration of BMSCs, we found a significant increase in pathological scores (Fig. 4A and B), while the levels of intestinal proinflammatory mediators were significantly reduced compared with the SAP and PBS groups (Fig. 4C–F). We detected the expression of intestinal P62 and Beclin-1 to explore a possible effect of BMSCs on SAP-associated intestinal damage. We found that BMSCs enhanced the expression of P62 and inhibited the expression of Beclin-1 and LC3, as assessed by qRT-PCR, Western blot, and immunohistochemistry (Fig. 4G–P). ZO-1 and Claudin-1 are important elements of tight junctions, which form the intestinal epithelial barrier. Administration of BMSCs markedly enhanced the expression levels of intestinal ZO-1 and Claudin-1, which were decreased by NaT treatment, with or without PBS (Fig. 4Q–S).

3.5. BMSCs ameliorate damage and suppress autophagy in pulmonary tissues in SAP

A significant decrease in the pathological scores of pulmonary tissues was observed in the BMSC group compared with the SAP group (Fig. 5A and B). To determine the expression of proinflammatory mediators in the lungs, the levels of IL-1β, IL-6, IL-8, and TNF-α were evaluated. ELISA results showed that BMSC transplantation reduced the levels of pro-inflammatory cytokines, which were increased in the SAP and PBS groups (Fig. 5C–F). Neutrophil sequestration in pulmonary tissues was quantified by measuring pulmonary MPO activity. Immunohistochemistry and ELISA results indicated that treatment with BMSCs significantly weakened the expression of pulmonary MPO (Fig. 5G and I). Finally, we determined the expression of pulmonary P62, Beclin-1, and LC3 using qRT-PCR and Western blot, and found that BMSCs increased the expression of P62, and reduced the expression of Beclin-1 and LC3 (Fig. 5J–P).

3.6. BMSCs improve viability, up-regulate PI3K/AKT/mTOR pathway, and inhibit autophagy in NaT-treated AR42J cells in vitro

We used NaT to treat AR42J cells as an *in vitro* cellular model of SAP. As shown in Fig. 6A and B, administration of NaT decreased the viability of AR42J cells compared with the control group, whereas this effect was reversed by addition of BMSC-CM. In addition, BMSC-CM treatment significantly enhanced the survival rate of AR42J cells treated with NaT, as assessed by flow cytometry (Fig. 6C and D). Moreover, we detected the expression of PI3K, AKT, *p*-AKT, mTOR, *p*-mTOR, P62, Beclin-1, and LC3 in AR42J cells by western blotting. We found that BMSC-CM increased the expression of PI3K, *p*-AKT, *p*-mTOR, and P62, but reduced the expression of Beclin-1 and LC3 II, compared with the NaT group (Fig. 6E–K). However, there was no marked change in the expression of total AKT and total mTOR (Fig. 6E–K).

3.7. BMSCs promote viability and angiogenesis, and inhibit autophagy in NaT-treated HUVECs in vitro

We next established an *in vitro* model of SAP-associated vascular endothelial injury by stimulating human umbilical vein endothelial cells (HUVECs) with NaT (Fig. 7A). BMSC-CM treatment was able to significantly increase the cellular viability of NaT-treated HUVECs (Fig. 7B). Furthermore, flow cytometry results indicated that BMSC-CM increased the survival rate of injured HUVECs (Fig. 7C and D). To investigate the effect of BMSC-CM on the angiogenic activity of HUVECs, we used a tube-formation assay to demonstrate that BMSC-CM effectively promoted the angiogenesis of damaged HUVECs *in vitro* (Fig. 7E and F). Additionally, compared with the NaT group, BMSC-CM significantly enhanced the expression of P62, but inhibited the expression of Beclin-1 and LC3 II in HUVECs *in vitro* (Fig. 7G–J).

4. Disscusion

AP is one of the leading causes of gastrointestinal-related hospitalizations globally. The incidence of AP ranges from 13 to 45 in 100,000 people annually, and the burden of pancreatic disorders is believed to be increasing [28,29]. Furthermore, 20% of AP cases may evolve into SAP, which is characterized by high mortality, MODS, and systemic inflammatory response syndrome [30]. Even with patient management by an interdisciplinary team, including gastroenterologists, interventional radiologists, and surgeons, the mortality rates of SAP have failed to substantially decrease [31]. Hence, new effective therapeutic interventions are urgently needed. Increasing research, including from our group, has confirmed that BMSCs can attenuate SAP via regulating immune responses and secreting cytokines or microRNA [17,32,33]. However, the underlying mechanism by which BMSCs exert their protective effects remains to be fully understood. In the present study, we investigated the possible mechanisms involved in the BMSC-induced alleviation of SAP. Our findings suggested that BMSCs suppressed autophagy in multiple organs (including the pancreas, small intestine, and lungs) to protect against SAP-induced multiple-organ injury.

SAP is characterized by a large release of various pro-inflammatory cytokines, including IL-1β, IL-6, IL-8, TNF-α, and MPO [4,34]. In the present study, we found that the concentration of pancreatic pro-inflammatory cytokines was much lower in the BMSC group than in the SAP group, along with the levels of serum amylase and lipase, as well as the pathological scores of pancreatic tissue. LDH is a crucial marker in predicting the severity of AP, and in particular SAP [35]. The measurements of serum and pancreatic LDH suggested that BMSC transplantation decreased the severity of SAP. Our findings indicated that administration of BMSCs resulted in a clear anti-inflammatory effect, which is essential and indispensable for the treatment of SAP.

Autophagy is a double-edged sword, as it is known to protect against various diseases such as aging, cancer, and neurodegeneration, yet it may also be harmful in some pathologies [10]. Increasing evidence indicates that autophagy in pancreatic acinar cells plays a significant role in the progression of AP [36]. Moreover, suppression of autophagy is beneficial in the treatment of cerulein-induced AP [23]. MSCs have the potential to regulate autophagy and treat a variety of diseases. MSCs effect autophagy in a context-dependent manner; for example, in Alzheimer's disease, MSCs induce autophagy to enhance β-

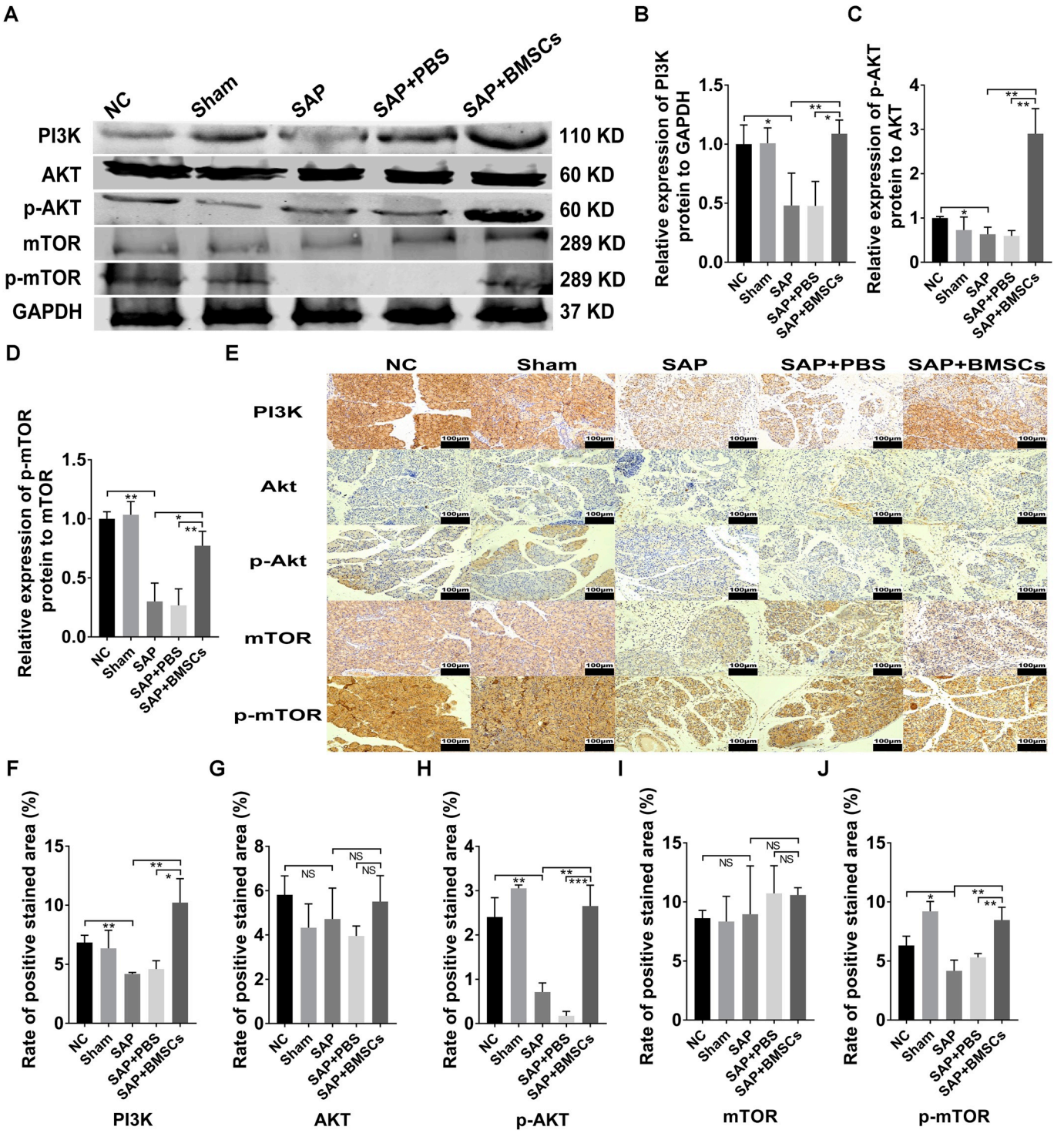

Fig. 3. BMSCs promote PI3K/AKT/mTOR signaling to inhibit pancreatic autophagy in SAP. (A–D) Western blot analysis of PI3K, AKT, *p*-AKT, mTOR, and *p*-mTOR protein levels in pancreatic tissue. (E) Immunohistochemical staining of pancreatic P62, Beclin-1, and LC3 (scale bar = 100 μm). (F–J) Immunohistochemical analysis of the proportion of tissue positively stained for PI3K, AKT, *p*-AKT, mTOR, and *p*-mTOR. Data represent the mean ± SD from at least three independent experiments. N = 6/group. *p < 0.05, **p < 0.01, ***p < 0.001, and NS means not significant.

amyloid clearance [37], while in systemic lupus erythematosus they inhibit autophagy in T cells [38]. In this study, we demonstrated for the first time that BMSCs inhibited autophagy in pancreatic tissues in SAP. The activation of autophagy led to the formation of autophagosomes, which are characterized by certain indicators of autophagy, including P62, Beclin-1, and LC3 [39]. In our study, we determined the expression of P62, Beclin-1, and LC3 using qRT-PCR, Western blot, immunohistochemistry, immunofluorescence, and TEM. We found that BMSCs suppressed the expression of Beclin-1 and LC3, and promoted the expression of P62 in an NaT-induced SAP model (including in the pancreas, small intestines and lungs) and in AR42J cells and HUVECs. This indicated that ubiquitous autophagy in the pathological development of SAP can be reduced by BMSC transplantation. Additionally, we explored the factors that regulate autophagy in order to further identify which pathways were mediated by BMSCs, and confirmed that the PI3K/AKT/mTOR signaling pathway was activated by BMSCs in SAP. In

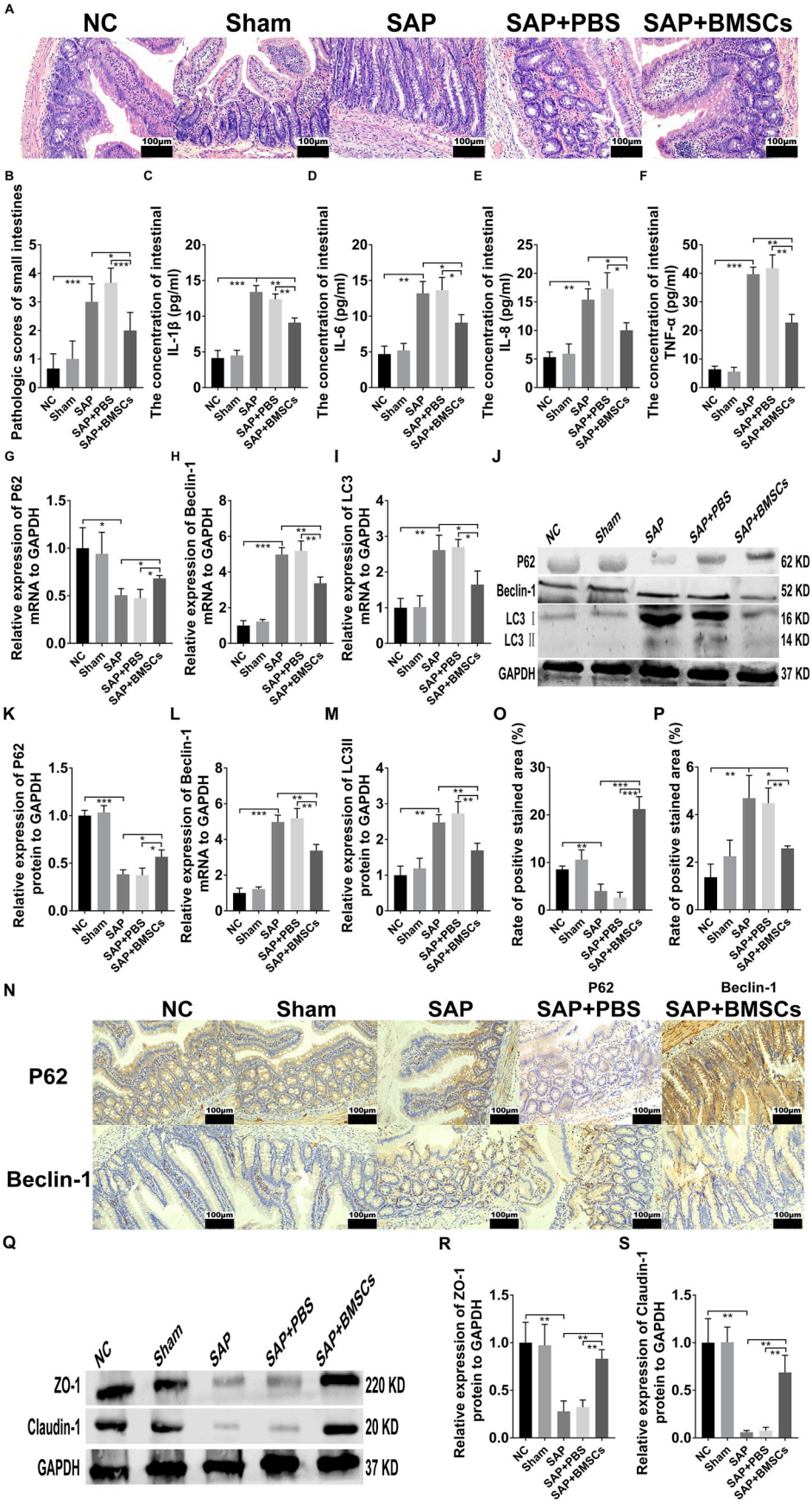

Fig. 4. BMSCs attenuate damage to the small intestine, inhibit autophagy in intestinal tissues, and improve intestinal vascular endothelial dysfunction in SAP. (A) H&E staining of small intestinal tissues (scale bar = 100 μm). (B) Pathological scores for H&E staining in the samll intestine. (C–F) Levels of intestinal IL-1β, IL-6, IL-8, and TNF-α. (G–I) Relative mRNA expression of intestinal P62, Beclin-1 and LC3 determined by qRT-PCR. (J–M) Western blot analysis of P62, Beclin-1, and LC3 protein levels in small intestinal tissues. (N) Immunohistochemistry of intestinal P62 and Beclin-1 protein (scale bar = 100 μm). (O, P) Immunohistochemical analysis of the proportion of small intestinal tissue positively stained for P62 and Beclin-1. (Q–S) Western blot analysis of ZO-1 and Claudin-1 protein levels in small intestinal tissue. Data represent the mean ± SD from at least three independent experiments. N = 6/group. *p < 0.05, **p < 0.01, and ***p < 0.001.

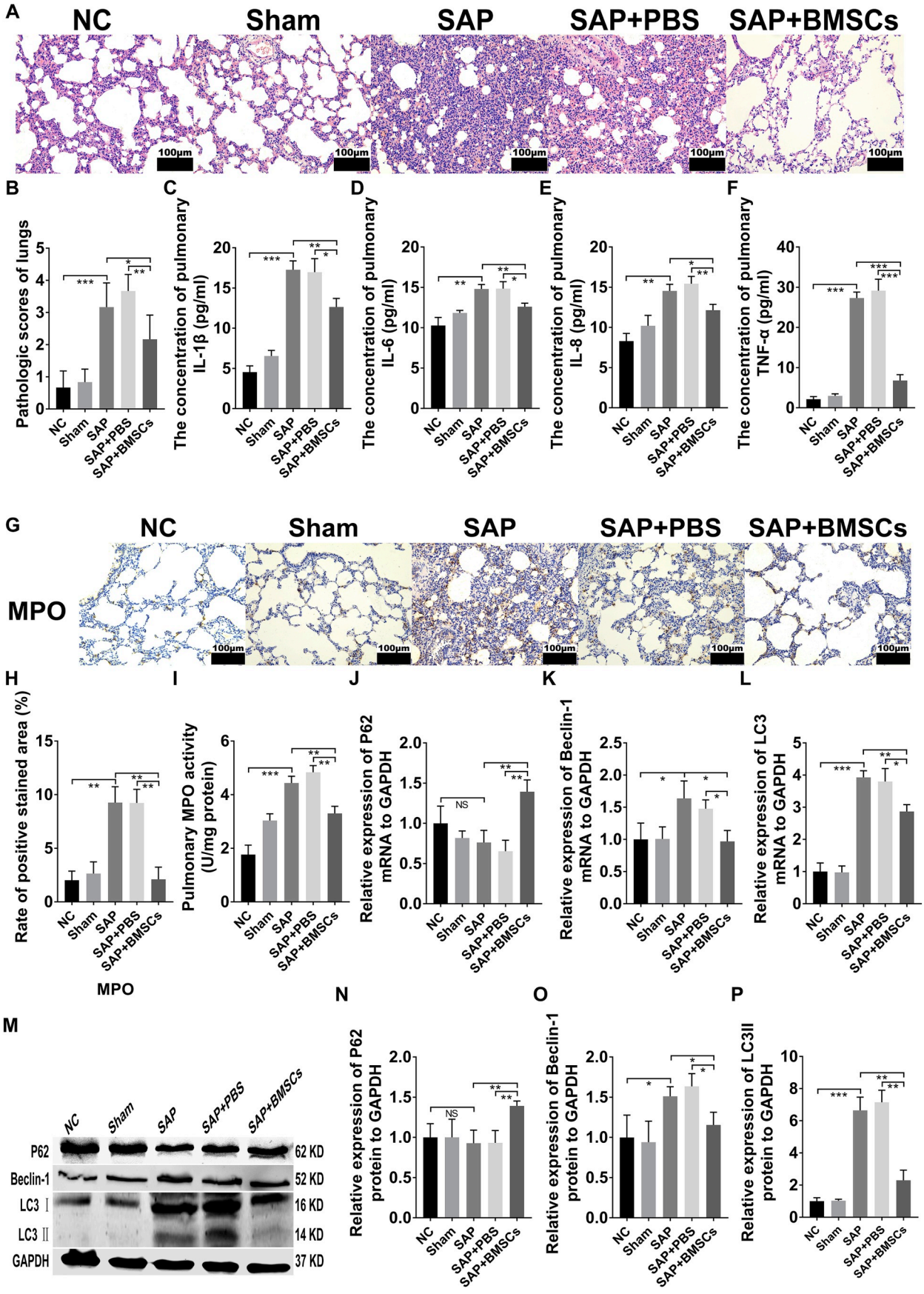

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Fig. 5. BMSCs alleviate lung inflammatory responses and inhibit autophagy in pulmonary tissues in SAP. (A, B) Pathological changes (H&E) and pathologic scores of pulmonary tissue sections (scale bar = 100 μm). (C–F) Concentration of pulmonary IL-1β, IL-6, IL-8, and TNF-α. (G) Immunohistochemical staining for pulmonary MPO protein (scale bar = 100 μm). (H) Immunohistochemical analysis of the proportion of pulmonary tissue positively stained for MPO. (I) Levels of pulmonary MPO activity. (J–L) Relative mRNA expression of pulmonary P62, Beclin-1, and LC3 detected by qRT-PCR. (M − P) Western blot analysis of P62, Beclin-1, and LC3 protein levels in lung tissues. Data represent the mean ± SD from at least three independent experiments. N = 6/group. *p < 0.05, **p < 0.01, ***p < 0.001, and NS means not significant.

chronic pancreatitis, Saiko saponin has been shown to attenuate pancreatic fibrosis by promoting the PI3K/AKT/mTOR signaling pathway, which leads to the suppression of autophagy [40]. Our results demonstrated that BMSCs enhanced the expression of pancreatic PI3K, *p*-AKT, and *p*-mTOR, but have no influence on total AKT and total mTOR. In a previous study, we demonstrated that fewer administered BMSCs reach injured pancreatic tissues, intestines and lungs, compared with liver and spleen tissue [19]. Hence, we hypothesized that BMSCs may predominantly exert their therapeutic effects via paracrine mechanisms, rather than via direct interaction. Therefore, we used BMSC-CM to treat injured AR42J cells or HUVECs *in vitro*, and found that the viability of NaT-treated AR42J cells was significantly improved. Based on these *in vivo* and *in vitro* findings, we speculate that BMSCs upregulated the PI3K/AKT/mTOR pathway to suppress autophagy, leading to an amelioration of pancreatic damage in SAP.

SAP-associated intestinal injury must also be considered in SAP therapy. BMSCs have been found to attenuate small intestinal damage in rats with SAP [41], but the underlying mechanisms, and whether this is related to the regulation of autophagy, remains to be fully described. Our results suggested that transplanted BMSCs markedly suppressed inflammatory responses and prevented the over-activation of autophagy in the small intestine. Intestinal epithelial permeability is mediated by a protein system consisting of tight junctions, including the proteins Claudin-1 and ZO-1 [42]. In the present study, Western blot results indicated that the expression of intestinal Claudin-1 and ZO-1 was much higher in the BMSC group than that in the SAP and PBS

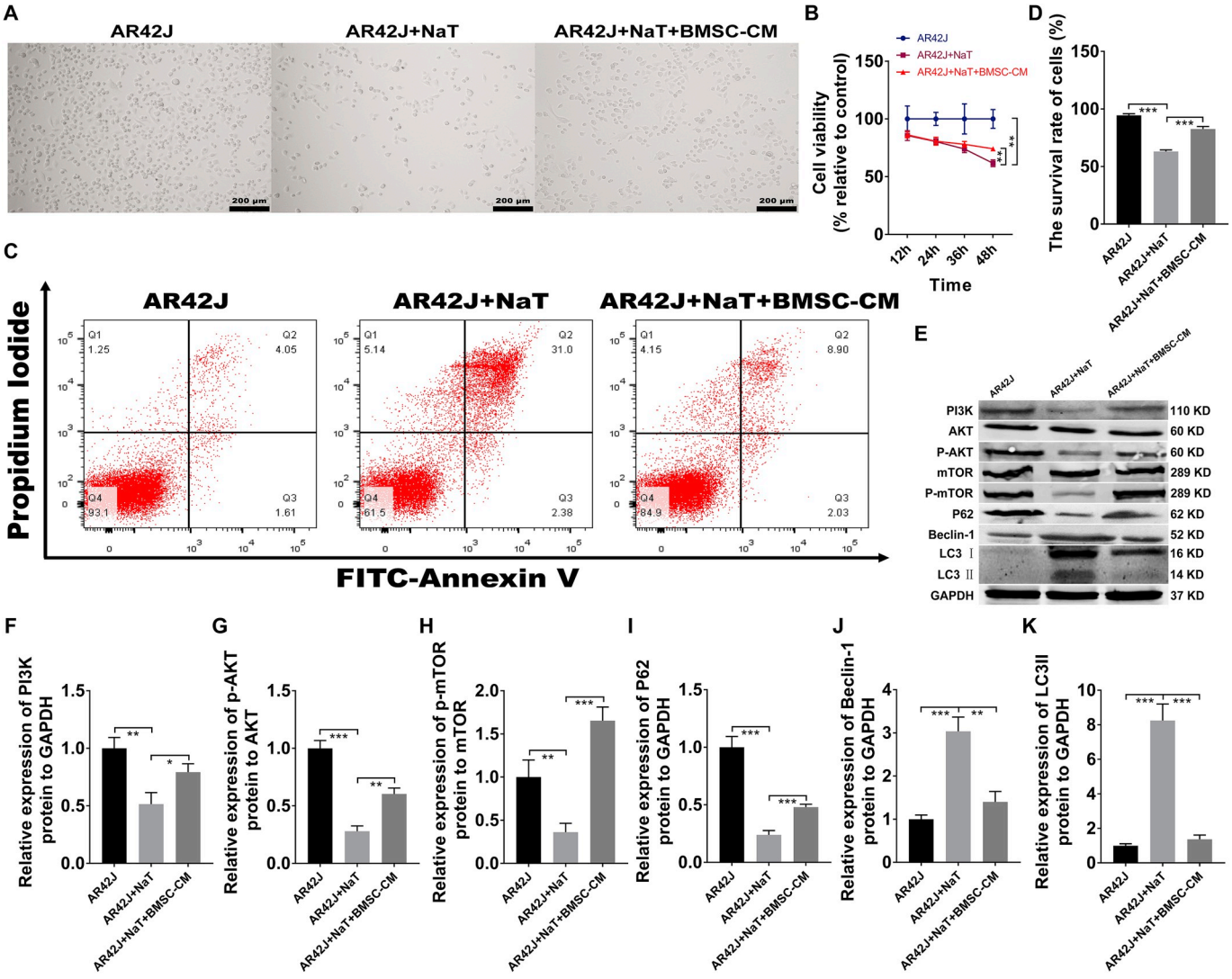

Fig. 6. BMSCs promote viability, up-regulate PI3K/AKT/mTOR signaling, and restrict autophagy in NaT-treated AR42J cells *in vitro*. (A) Representative images of AR42J cells with different treatments (scale bar = 200 μm). (B) Cell viability of AR42J cells was measured using the Cell Counting Kit-8. (C) Distribution of live, apoptotic, and necrotic AR42J cells, determined by flow cytometric analysis of FITC-Annexin V/PI staining. (D) The survival rate of AR42J cells analyzed by flow cytometry. (E–K) Western blot analysis of PI3K, AKT, *p*-AKT, mTOR, *p*-mTOR, P62, Beclin-1, and LC3 protein levels in AR42J cells. Data represent the mean ± SD from at least three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001.

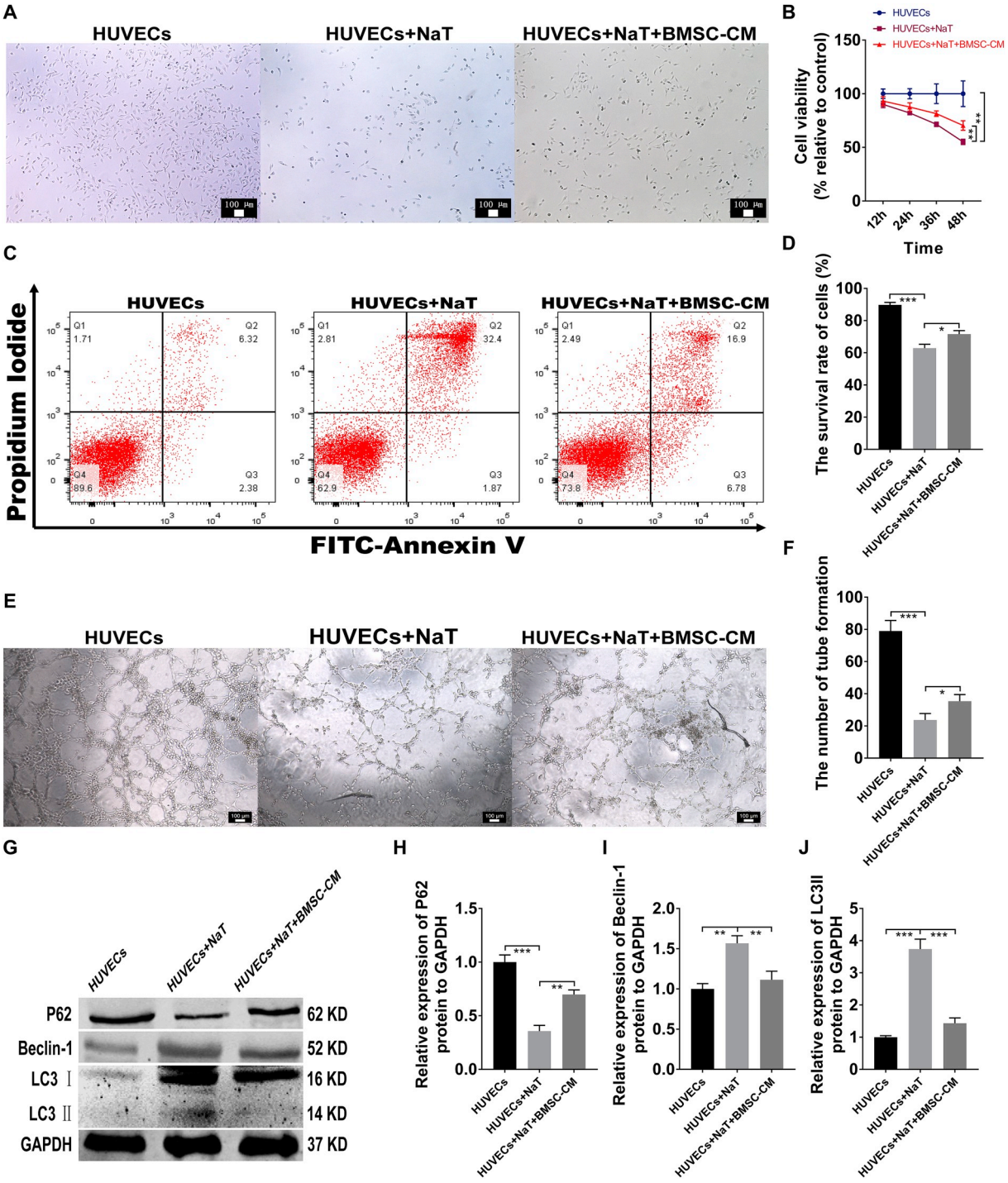

Fig. 7. BMSCs improve viability, promote angiogenesis, and suppress autophagy in NaT-induced HUVECs *in vitro*. (A) Representative images of HUVECs under various conditions (scale bar = 100 μm). (B) Cell viability of HUVECs was determined using the Cell Counting Kit-8. (C) HUVECs with different treatments double stained with FITC-Annexin V/PI, analyzed by flow cytometry. (D) Flow cytometric analysis of the survival rate of HUVECs. (E) Representative images from the tube formation assay in HUVECs (scale bar = 100 μm). (F) Quantification of tube formation. (G–J) Western blot analysis of P62, Beclin-1, and LC3 protein levels in HUVECs. Data represent the mean ± SD from at least three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001.

groups, which suggested that BMSC treatment was able to alleviate intestinal barrier injury. *In vitro*, a HUVEC injury model was established by stimulating cells with NaT. Administration of BMSC-CM markedly enhanced the viability of damaged HUVECs, inhibited the numbers of apoptotic and necrotic cells, and promoted angiogenesis. Taken together, our investigation into SAP-induced intestinal damage *in vivo* and *in vitro* strongly implied that BMSCs have the potential to inhibit autophagy, improve intestinal epithelial dysfunction, and promote angiogenesis.

Acute lung injury is the most likely and most serious systemic complication of SAP, which contributes to approximately 60% of all SAP-associated deaths [43]. Results showed that BMSCs markedly decreased the severity of SAP-induced lung injury, and reduced the levels of MPO, which is related to pulmonary damage [44]. In addition, autophagy in lung tissues was strongly suppressed by BMSCs.

Our study has several limitations. First, we have only determined

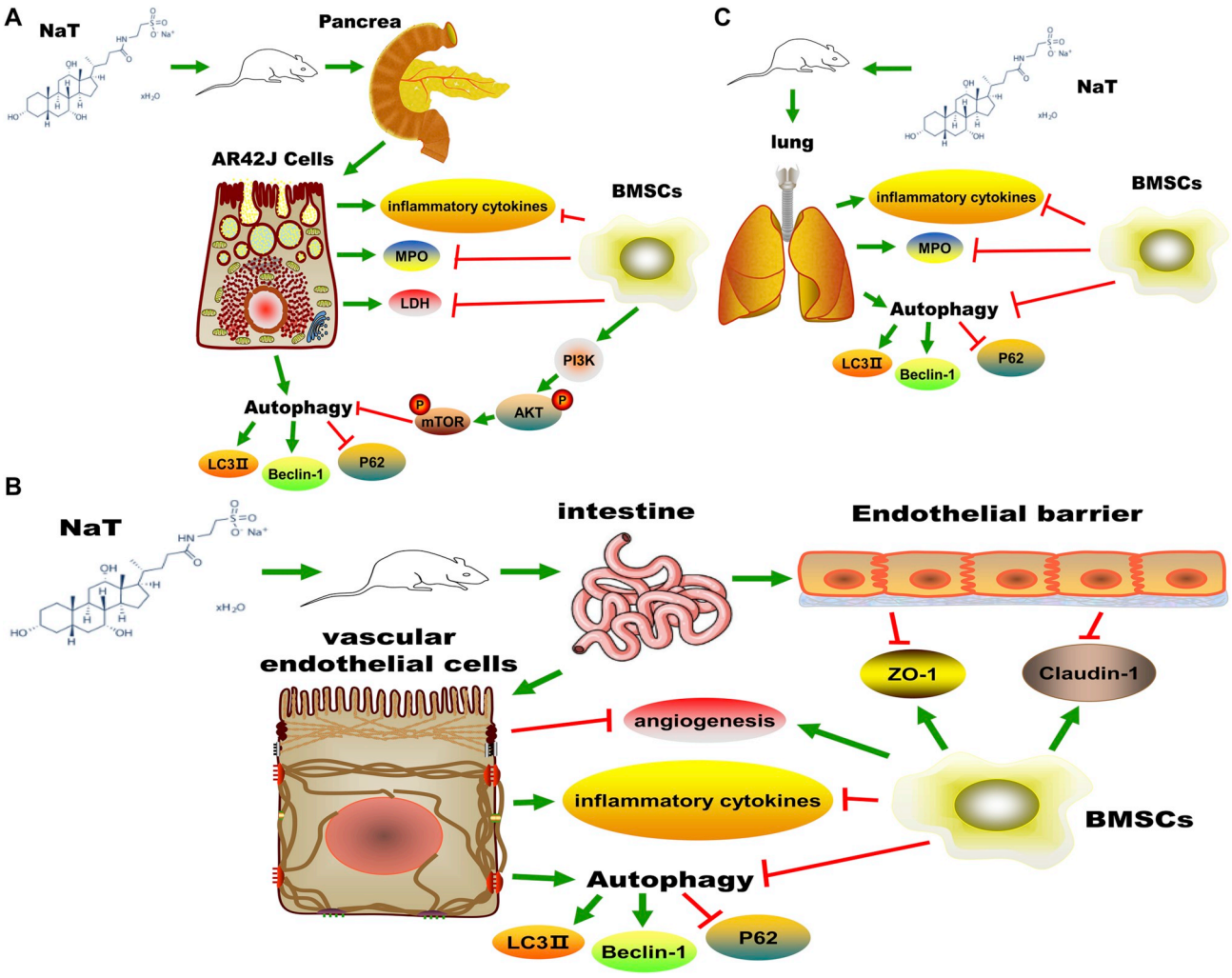

Fig. 8. Schematic diagram showing the possible mechanisms by which BMSCs or BMSC-CM ameliorate NaT-induced SAP-associated multiple organ injury via the inhibition of autophagy. (A) Pancreatic tissues and AR42J cells. (B) Small intestinal tissues and HUVECs. (C) Lung tissues.

the effect of BMSCs on the mediation of autophagy, and have not identified the protein, peptide, hormonal, nucleotide, and miRNA contents of BMSCs and BMSC-CM. Thus, we have not identified the precise molecular mechanisms responsible for our observed outcomes. Second, we have not combined treatment with BMSCs or BMSC-CM with inhibitors or inducers of autophagy, experiments which may be informative. Additional investigation is therefore required in order to identify the underlying mechanisms by which BMSCs attenuate SAP.

In summary, we have revealed that transplanted BMSCs are able to inhibit the activation of autophagy, which led to protection against SAP-associated multiple-organ injury.

5. Conclusion

This study demonstrated that administration of BMSCs significantly ameliorated SAP-induced multiple-organ injury *in vivo*. We also demonstrated that BMSC-CM improved the viability of NaT-treated AR42J cells and HUVECs *in vitro*. BMSCs act by enhancing PI3K/AKT/mTOR signaling, leading to the inhibition of autophagy (we observed an increase in P62, and a decrease in Beclin-1 and LC3 II) in pancreatic tissues. Additionally, we have demonstrated that transplanted BMSCs promote angiogenesis, suppress autophagy, and improve intestinal epithelial barrier dysfunction in small intestinal tissue. Finally, BMSCs significantly reduced the levels of MPO and autophagy in lung tissue (Fig. 8).

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Zhenshun Song: Conceptualization and Supervision; Guodong Song: Data curation and Writing - original draft; Dalu Liu and Xiang Geng: Formal analysis; Zhilong Ma and Yuxiang Wang: Investigation; Wangcheng Xie: Methodology; Daohai Qian: Software; Hongbo Meng and Bo Zhou: Validation. Our study was supported by the National Natural Science Foundation of China (No. 81670582), College Natural Science Foundation of Anhui Province (KJ2017A271), talent introduction fund of Yijishan Hospital of Wannan Medical School (YR201601) and Funding of “Peak” Training Program for Scientific Research of Yijishan Hospital, Wannan Medical College (GF2019G03).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexcr.2019.111674.

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