Long noncoding RNA H19 regulates the therapeutic efficacy of mesenchymal stem cells in rats with severe acute pancreatitis by sponging miR-138-5p and miR-141-3p

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Research

Keywords: Long noncoding RNA H19, mesenchymal stem cells, severe acute pancreatitis, autophagy, cell proliferation

DOI: https://doi.org/10.21203/rs.3.rs-34046/v1
Abstract

Background: Patients with severe acute pancreatitis (SAP), which is characterized by high morbidity and mortality, account for an increasing medical burden worldwide. We previously found that mesenchymal stem cells (MSCs) could attenuate SAP and that expression of long noncoding RNA H19 (LncRNA H19) was upregulated in rats receiving MSCs. In the present study, we investigated the mechanisms of LncRNA H19 regulating the therapeutic efficacy of MSCs in the alleviation of SAP.

Methods: MSCs transfected with LncRNA H19 overexpression and knock down plasmids were intravenously injected into rats 12 h after sodium taurocholate (NaT) administration to induce SAP.

Results: Overexpressing LncRNA H19 in MSCs significantly enhanced the anti-inflammatory capacity of the MSCs, inhibited autophagy via promotion of focal adhesion kinase (FAK)-associated pathways, and facilitated cell proliferation by increasing the level of β-catenin in rats with SAP. LncRNA H19 functioned as a competing endogenous RNA by sponging miR-138-5p and miR-141-3p. Knocking down miR-138-5p in MSCs increased the expression of protein tyrosine kinase 2 (PTK2, encoding FAK) to suppress autophagy, while downregulating miR-141-3p enhanced the level of β-catenin to promote cell proliferation.

Conclusions: In conclusion, LncRNA H19 effectively increased the therapeutic efficacy of MSCs in rats with SAP via the miR-138-5p/PTK2/FAK and miR-141-3p/β-catenin pathways.

Introduction

Acute pancreatitis (AP) is an inflammatory disease of the pancreas that causes considerable morbidity and mortality[1]. The global incidence of AP is approximately 30 cases per 100,000 persons, and has been increasing universally[2]. In addition, about 20% of AP cases evolve into severe acute pancreatitis (SAP), with a mortality rate of 15%[3]. The incidence of SAP and its complications has rapidly increased[4].

While, the underlying mechanisms of SAP are still not completely known, there is some consensus regarding its initiation. SAP begins with the activation of digestive enzymes in pancreatic acinar cells leading to cell injury[5]. It has been reported that systemic inflammatory responses and anti-inflammatory responses develop in parallel during the progression of SAP[6]. Some of the proinflammatory cytokines involved in the inflammatory responses of SAP, are interleukin-1β (IL-1β), IL-6, IL-8 and tumor necrosis factor alpha (TNF-α)[7, 8]. In recent years, increasing evidence has demonstrated that impaired autophagy has a crucial effect on the pathogenesis of SAP[9-11]. In addition, maintaining the normal action of adhesion molecules (such as β-catenin) and restricting their abnormal activation are beneficial in blocking the development of AP[12]. Therefore, investigations seeking to develop novel therapeutic tactics for SAP should concentrate on how to inhibit inflammatory responses, regulate autophagy and maintain levels of adhesion molecules.
Treatment of patients with SAP requires synergistic action from various hospital departments, including gastroenterology, surgery and critical care medicine[4]. Unfortunately, these treatments are often invasive and result in more complications. Hence, a noninvasive and effective therapy is required, and we suggest that mesenchymal stem cells (MSCs) be considered as a treatment option. MSCs have been applied in a variety of difficult diseases including musculoskeletal tissue injuries[13], myocardial infarction[14] and Crohn's disease[15]. Hong previously confirmed that human bone marrow-derived clonal MSCs could suppress inflammatory responses in rats with AP[16]. Our team has elucidated some of the important mechanisms by which MSCs attenuate SAP[17-24]. We found that MSCs can ameliorate SAP-associated multiple-organ injury via suppression of autophagy[25], and we also detected statistically significant differences in the expression of long noncoding RNA H19 (LncRNA H19). LncRNAs > 200 bp in length are involved in many biological processes, and in fact LncRNA H19 is an imprinting IncRNA which can facilitate tamoxifen resistance in breast cancer by upregulating autophagy[26]. Based on our previous findings, we hypothesized that LncRNA H19 may also play an important role in SAP. The current study was carried out to clarify the possible mechanisms by which LncRNA H19 may regulate the therapeutic efficacy of MSCs attenuating SAP.

**Materials And Methods**

**Cell culture**

Isolation, culture and identification of MSCs were consistent with our previous description[23]. 293T cells were obtained from the Chinese Academy of Sciences (Shanghai, China) and seeded in DMEM media (Gibco, Middleton, WI, USA) with 10% fetal bovine serum (Gibco). All cells were incubated in a humidified atmosphere at 37°C with 5% CO₂.

**Plasmid, siRNA, and transfection**

The overexpression full-length plasmid of LncRNA H19 (pcDNA-H19) was acquired by inserting LncRNA H19 into the pcDNA3.1 vector (Invitrogen, Shanghai, China). Small interfering RNA (siRNA) targeting rat LncRNA H19 (si-H19), miRNA negative control (miR-NC), miRNA mimics and inhibitors for mo-miR-138-5p and mo-miR-141-3p were purchased from GenePharma (Shanghai, China). short hairpin RNAs (shRNA) of PTK2 (sh-PTK2) and β-catenin (sh-β-catenin) were constructed using pGPU6 plasmids (GenePharma). PTK2 and β-catenin overexpression plasmids were constructed with pEX2 plasmids (GenePharma). siRNA, miR-NC, miRNA mimics and inhibitor were transfected into cells using HiPerFect Transfection Reagent (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Plasmids were transfected into cells by using Lipofectamine 3000 (Invitrogen) following the manufacturer's protocol.

**In vivo protocol**

Healthy male Sprague-Dawley rats (4-6 weeks old) were obtained from Shanghai Laboratory Animal Co., Ltd. (Shanghai, China). The 30 rats were randomly divided into five groups (n = 6 per group) for animal experiments as follows: (1) Negative control (NC) group; (2) SAP group: animals were administered NaT
as previously described[23]; (3) MSC group: MSCs (1 × 10^7 cells/kg body weight) suspended in 300 μL of phosphate-buffered saline (PBS) were transplanted into SAP model rats via the tail vein 12 h after treatment with NaT; (4) pcDNA-H19-MSC group: pcDNA-H19-MSCs (1 × 10^7 cells/kg body weight) were injected into SAP rats as described for the MSC group; (5) si-H19-MSC group: si-H19-MSCs were treated as described above. In addition, different MSC-treated groups (n = 6 per group) were administered various MSCs that had been transfected with miR-NC, miRNA mimics, miRNA inhibitor or expression plasmids.

**Histopathologic examination**

After euthanization, fresh rat pancreatic tissues were excised and preserved in 4% paraformaldehyde. Tissues were embedded in paraffin and stained with H&E for histological evaluation. The severity of pancreatic injuries was scored by two experienced and blinded pathologists as previously described[23].

**Biochemical inspection of serum and tissues**

The levels of serum amylase activity were analyzed using colorimetric assay kits (BioVision, Milpitas, CA, USA), according to the manufacturer’s instructions. The levels of mediators in serum and pancreatic tissues (LDH, IL-1β, IL-6, IL-8 and TNF-α) were detected using ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s protocol.

**Flow cytometry**

The survival rate of MSCs was detected using a flow cytometry assay, as previously described[23]. Briefly, collected MSCs were washed twice with PBS, and incubated with FITC-Annexin V (BD Pharmingen, San Diego, CA, USA) and propidium iodide (BD Pharmingen). The percentages of live MSCs were determined by flow cytometry (BD Biosciences, San Jose, CA, USA). The Q4 quadrant represents live MSCs.

**Quantitative real-time polymerase chain reaction**

Total RNA was extracted from frozen pancreatic tissue by means of TRIzol reagent (Invitrogen). According to the instructions of the PrimeScript Reverse Transcriptase Reagent Kit (Kapa Biosystems, Boston, MA, USA), RNA was reverse-transcribed to cDNA. The qRT-PCR assays were conducted using a KAPA qPCR Kit (Kapa Biosystems). GAPDH and U6 were utilized as the endogenous controls. The primer sequences are listed in Table 1. Relative expression of various RNAs and genes was determined using the comparative 2^−ΔΔCT method as previously described[23].

**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[23]. Equal amounts of proteins were transferred to nitrocellulose membranes, which were imaged on an Odyssey scanner (LI-COR Biosciences, USA) after incubation with primary and secondary antibodies. Primary antibodies, purchased from CST (Danvers, MA, USA), were as follows: focal adhesion kinase (FAK), phosphoinositide-dependent kinase 1
(PDK1), protein kinase B (AKT), phosphorylated AKT (p-AKT), mammalian target of rapamycin (mTOR),
phosphorylated mTOR (p-mTOR), P62, Beclin-1, microtubule-associated protein 1 light chain 3 (LC3), β-
catenin, c-Myc and Cyclin D1. The dilution of primary antibodies was 1:1000. Secondary antibodies used
were anti-rabbit IgG (CST) or anti-mouse IgG (CST). The dilution of secondary antibodies was 1:2000.

**Immunohistochemistry**

Immunohistochemistry was conducted as previously described[23]. Tissue sections were incubated with
primary antibodies (all purchased from Abcam, Cambridge, UK) as follows: FAK, PDK1, AKT, p-AKT,
mTOR, p-mTOR, P62, Beclin-1, LC3, β-catenin, c-Myc and Cyclin D1.

**Immunofluorescence**

Immunofluorescence staining was performed on 5-μm pancreatic tissue sections as previously
described[25]. Primary antibody LC3 (Abcam) was utilized following the manufacturer's introductions.

**Transmission electron microscopy**

Cells in the pancreas were observed by transmission electron microscopy (JEM 1230, Tokyo, Japan) after
preservation in glutaraldehyde buffer and fixation in osmium tetroxide, as previously described[25].

**Dual luciferase reporter assay**

Fragments of the 3’-UTR of LncRNA H19, PTK2 and β-catenin were synthesized with binding sites for mo-
miR-138-5p or mo-miR-141-3p containing wild type or mutant sequence. These fragments were extended
by PCR and inserted downstream from the luciferase gene in the psiCHECK-2 vector (Promega, Madison,
WI, USA). For luciferase reporter assays, 293T cells were plated in 6-well plates at a density of 5 × 10^5
cells/well, then co-transfected with mo-miR-138-5p mimics, mo-miR-141-3p mimics or negative control
(miR-NC) and luciferase reporter vectors using Lipofectamine 3000. Luciferase activity was measured
using a luciferase reporter assay kit (Promega) 48 h after transfection according to the manufacturer's
instructions.

**RNA pull-down assay**

Biotinylated probes, which contained reverse complementary sequences to LncRNA H19 black-splice
junction sequences (LncRNA H19 probe), were provided by Sangon Biotech (Shanghai, China). MSCs (1 ×
10^7) were washed using cold PBS, then lysed in RIP lysis buffer (Invitrogen) and incubated for 2 h at 25°C
with specific RNA probes, labelled with high-affinity biotin. Next, the suspension and streptavidin
magnetic beads (Thermo Fisher Scientific, Shanghai, China) were mixed and incubated for 1 h at 25°C
and washed twice with wash buffer. In the end, the RNA was analyzed via agarose gel electrophoresis
and quantified using qRT-PCR assays as previously described[27].

**FISH**
FISH reveals the abundance and positioning of nucleic acid sequences in cells or tissues[28]. MSC nuclei staining was performed using 4,6-diamidino-2-phenylindole (DAPI, Beyotime, Shanghai, China). Fluorescein isothiocyanate (FITC) probes were specific to LncRNA H19. All procedures were performed following the manufacturer's protocol (Biofavor, Wuhan, Hubei Province, China).

**Statistical analysis**

All data are presented as means ± standard deviation (SD) from at least three independent experiments. Statistical analysis was conducted using one-way analysis of variance (ANOVA) and unpaired Student's *t*-tests. A *P* value < 0.05 was considered statistically significant.

**Results**

**LncRNA H19 enhanced the therapeutic efficacy of MSCs on SAP**

To investigate the specific lncRNAs involved in the pathological process of SAP, we detected the expression of lncRNAs using quantitative real-time PCR (qRT-PCR) after treating normal rats with sodium taurocholate (NaT) to induce SAP. Zheng has demonstrated that lncRNAs uc.308-, BC158811, BC166549, BC166474 and BC161988 were significantly suppressed by NaT administration[29], but we also found a novel lncRNA, LncRNA H19, which was prominently inhibited in the SAP group. Detailed information about LncRNA H19 (rat species) can be obtained from the website (https://www.ncbi.nlm.nih.gov/gene/309122) (Figure 1a). We determined the survival rate of MSCs under different treatments using flow cytometry and found that overexpressing or knocking down LncRNA H19 did not affect the survival rate of MSCs *in vitro* (Figures 1b and 1c). qRT-PCR was performed to analyze the expression of LncRNA H19 among the MSC, pcDNA-H19-MSC and si-H19-MSC groups. As shown in Figure 1d, the expression of LncRNA H19 in MSCs was significantly upregulated by transfection of pcDNA-H19, but was inhibited after si-H19 treatment.

We then established an SAP model by retrograde injection of NaT and transplanted MSCs with various treatments into SAP rats via the tail vein as previously described[23]. To explore the effect of LncRNA H19 in MSCs used to treat SAP, we investigated the expression of LncRNA H19 in pancreatic tissues from different treatment groups. The results suggested that MSCs could enhance the expression of LncRNA H19, which was suppressed in the SAP group; meanwhile, the expression of LncRNA H19 in the pcDNA-H19-MSC group was much higher than in the MSC group, but the expression of LncRNA H19 in the SAP and si-H19-MSC groups was not significantly different (Figure 1e). Hematoxylin and eosin (H&E) staining of pancreatic tissues showed that transplantation of pcDNA-H19-MSCs could decrease pathological scores, which were enhanced in the SAP group (Figures 1f and 1g). In addition, compared with the SAP and MSC groups, pcDNA-H19-MSCs clearly suppressed the serum levels of amylase, as well as serum lactate dehydrogenase (LDH), pancreatic LDH and pancreatic pro-inflammatory mediators (IL-1β, IL-6, IL-8 and TNF-α); in contrast, si-H19-MSCs significantly reversed these protective effects in SAP (Figures 1h–1n). Therefore, LncRNA H19 increased the therapeutic efficacy of MSCs on SAP.
LncRNA H19 suppressed autophagy in SAP

Western blot analyses were performed to determine protein expression of the FAK/PDK1/AKT/mTOR pathway, due to its crucial roles in the process of autophagy, which is associated with the development of SAP[25]. The western blot results showed that pcDNA-H19-MSCs enhanced the expression of FAK, PDK1, p-AKT, p-mTOR and P62, but inhibited the expression of Beclin-1 and LC3 II, compared with the SAP and MSC groups (Figures 2a–2h). Simultaneously, there was very little difference between the si-H19-MSC and SAP groups in the protein levels of the FAK/PDK1/AKT/mTOR pathway (Figures 2a–2h). The findings from immunohistochemistry and immunofluorescence assays also supported the western blot results (Figures 2i and 2j). Additionally, transmission electron microscopy was utilized to observe the ultrastructure of cells in pancreatic tissues, and transplanted pcDNA-H19-MSCs significantly reduced the number of autophagosomes (Figure 2k). Thus, LncRNA H19 inhibited autophagy via activation of the FAK/PDK1/AKT/mTOR pathway in rats with SAP.

LncRNA H19 facilitated proliferation in SAP

β-catenin belongs to the adhesion molecule family, and WNT/β-catenin signaling has been shown to promote cell proliferation in human melanomas[30]. To investigate the effect of LncRNA H19 on proliferation, we performed western blots of β-catenin and its targeted genes including c-Myc and cyclin D1. The results suggested that expression of β-catenin, c-Myc and cyclin D1 in the LncRNA H19 group was much higher than in the SAP and MSC groups, while si-H19 MSCs reversed the overexpression of cell proliferation-related proteins (Figures 3a–3d). Moreover, Ki67 staining analysis suggested that LncRNA H19 facilitated proliferation in SAP (Figures 3f and 3g). Therefore, we conclude that LncRNA H19 may benefit treatment of SAP by increasing cell proliferation.

LncRNA H19 acted as a sponge for miR-138-5p and inhibition of miR-138 restrained autophagy in SAP

We previously detected the expression of pancreatic miRNAs using qRT-PCR and discovered that the pcDNA-H19-MSC group had the lowest expression of miR-138 among the five groups (Figure 4a). We determined that LncRNA H19 and rno-miR-138-5p had complementary base sequences by using bioinformatics analysis software (Figure 4b). Luciferase analysis was performed by inserting either the wild-type (wt) LncRNA H19 sequence or a mutant-type (mut) sequence into a luciferase construct, and the results indicated that luciferase activity was decreased after co-transfection of LncRNA H19-wt plasmid and miR-138-5p mimics into 293T cells as compared with the negative control (miR-NC; Figures 4B and 4C). However, the activity of the mutant reporter did not show a significant difference after co-transfection with LncRNA H19-mut plasmid and miR-138-5p mimics as compared with miR-NC (Figures 4b and 4c). Furthermore, less LncRNA H19 was acquired in the biotin-coupled miR-NC group than in the biotin-coupled miR-138-5p group (Figures 4d and 4e), suggesting that LncRNA H19 could bind to miR-138-5p. In addition, fluorescence in situ hybridization (FISH) results showed that LncRNA H19 was localized to the cytoplasm of MSCs (Figure 4f). We then transfected MSCs with miR-NC, miR-138 mimics or miR-138 inhibitor in vitro and discovered using qRT-PCR that the MSCs transfected with miR-138 mimics expressed the highest level of miR-138 among the MSCs (Figure 4g). Next, we transplanted these
transfected MSCs into SAP rats 12 h after NaT induction. The western blot results indicated that miR-138-inhibitor-MSCs significantly upregulated the FAK/PDK1/AKT/mTOR pathway to suppress autophagy in SAP rats, but miR-138-mimic-MSCs exhibited the opposite result (Figures 4h–4o). Therefore, LncRNA H19 could adsorb miR-138-5p as a sponge RNA and downregulating miR-138 could inhibit autophagy in SAP.

LncRNA H19 directly bound miR-141-3p and repression of miR-141 boosted proliferation in SAP

We previously examined the expression of miRNAs in pancreatic tissues and found that the expression of miR-141 in the pcDNA-H19-MSC group was the lowest (Figure 5a). To investigate how LncRNA H19 mediates MSC treatment of SAP, miRNAs interacting with LncRNA H19 were identified using Target Scan software, and rno-miR-141-3p was predicted to be a potential target of LncRNA H19 (Figure 5b). To further explore whether LncRNA H19 directly regulates miR-141-3p, we performed luciferase reporter assays. miR-NC or miR-141-3p mimics and LncRNA H19-wt or LncRNA H19-mut were co-transfected into 293T cells. Luciferase activity of the LncRNA H19-wt reporter was significantly decreased by miR-141 mimics, but luciferase activity of LncRNA H19-mut did not show any change after transfection with miR-141 mimics or miR-NC (Figure 5c). In addition, more LncRNA H19 was captured in the biotin-coupled miR-138-5p group than in the miR-NC group (Figures 5d and 5e), indicating direct binding of LncRNA H19 to miR-141-3p. The expression of miR-141 in MSCs was visibly increased after transfection of miR-141 mimics, whereas miR-141 inhibitor transfection markedly reduced the expression of miR-141 (Figure 5f). We found that suppression of miR-141 in MSCs significantly enhanced the expression of β-catenin and its targeted gene c-Myc; in contrast, miR-141-mimic-MSCs markedly suppressed the expression of β-catenin, c-Myc and cyclin D1 (Figures 5g–5j). Thus, LncRNA H19 could act as an miRNA sponge for miR-141 and suppression of miR-141 promoted cell proliferation in SAP.

PTK2 was a direct target of miR-138-5p and overexpressing PTK2 repressed autophagy in SAP

Feng has confirmed that protein tyrosine kinase 2 (PTK2), encoding focal adhesion kinase (FAK), acts a candidate synthetic lethal gene[31]. In the present study, we have demonstrated that FAK plays an important role in regulating autophagy in MSCs used to treat SAP. miRNA-138 has been shown to control osteogenic differentiation of human mesenchymal stem cells by targeting PTK2 directly[32], but few studies have focused on rat cells. We predicted the complementary sequences of PTK2 and rno-miR-138-5p using TargetScan (Figure 6a). By performing luciferase reporter assays, we discovered that transfection of rno-miR-138-5p mimics could downregulate PTK2-wt, but not PTK2-mut (Figure 6b). In vitro, short hairpin RNA (shRNA) of PTK2 (sh-PTK2) was constructed using the pGPU6 plasmid (GenePharma) to knock down PTK2, and a PTK2 overexpression plasmid was constructed with pEX2 plasmids (GenePharma; Figure 6c). Western blot analysis suggested that upregulating PTK2 in MSCs could markedly enhance signaling in the FAK/PDK1/AKT/mTOR pathway to restrain autophagy in SAP, whereas, downregulation of PTK2 successfully reversed this suppressive effect (Figures 6d–6k). Thus, miR-138-5p binds to PTK2 directly and overexpressing PTK2 can repress autophagy in SAP.

MiR-141-3p targeted β-catenin directly and upregulation of β-catenin promoted proliferation in SAP
We predicted the complementary sequences of rno-miR-141-3p and β-catenin using TargetScan (Figure 7a). Luciferase assays suggested that co-transfection of miR-141-3p mimics with a β-catenin 3’-untranslated region (3’-UTR)-wt plasmid inhibited luciferase activity markedly compared to transfection of miR-NC or β-catenin 3’-UTR-mut (Figure 7b). The expression of β-catenin in MSCs was upregulated by transfection of a β-catenin overexpression plasmid; in contrast, expression was downregulated by sh-β-catenin plasmid transfection (Figure 7c). We discovered that overexpressing β-catenin in MSCs clearly upregulated the expression of β-catenin and c-Myc, whereas repression of β-catenin significantly suppressed proliferation-related proteins (Figures 7d–7g). Therefore, β-catenin was a direct target of miR-141-3p and upregulation of β-catenin increased cell proliferation in SAP.

Discussion

The incidence rate of AP in the USA and Europe is increasing about 5% per year, and approximately 20% of these AP cases will progress to SAP (necrotizing pancreatitis), which is characterized by 10–30% mortality, mainly due to infection of pancreatic necrotic tissue and infectious complications[33]. Hence, patients with SAP require multidisciplinary treatment from a team of gastroenterologists, interventional radiologists and surgeons. Nevertheless, SAP mortality increases yearly[34]. In view of current SAP therapeutic strategies, it is imperative to explore more effective treatment. Our group has demonstrated that MSCs transplantation can successfully alleviate SAP by suppressing autophagy[25], but the underlying mechanisms remain unknown. We unexpectedly discovered that expression of LncRNA H19 was much higher in the MSC group than that in the SAP group, but there have been no studies of the role of LncRNA H19 in SAP. Therefore, this important finding motivated us to further explore the underlying mechanisms by which LncRNA H19 regulated the therapeutic efficacy of MSCs in rats with SAP.

The levels of serum amylase is a typical biological marker of SAP[35], as is LDH[36]. SAP is associated with the release of abundant pro-inflammatory mediators, including IL-1β, IL-6, IL-8 and TNF-α[37]. As shown in Figure 1, pcDNA-H19-MSCs exhibited a stronger anti-inflammatory effect than did MSCs, while downregulation of LncRNA H19 in MSCs markedly weakened their inhibition of systemic inflammatory responses. Thus, LncRNA H19 effectively increased the anti-inflammatory efficacy of MSCs on SAP.

As mentioned earlier, we previously confirmed that MSCs could repress autophagy to attenuate SAP by promoting the PI3K/AKT/mTOR signaling pathway[25]. LncRNA H19 has been shown to induce autophagy activation via the H19/SAHH/DNMT3B axis[26]. Meanwhile, LncRNA H19 overexpression suppressed autophagy of vascular smooth muscle cells in atherosclerosis[38]. These two examples illustrate that LncRNA H19 may regulate autophagy bidirectionally. Our results using a variety of methods demonstrated that overexpression of LncRNA H19 in MSCs significantly restrained activated autophagy in rats with SAP by upregulating the FAK/PDK1/AKT/mTOR signaling pathway, compared with the MSC and si-H19-MSC groups (Figure 2).

β-catenin, a type of transmembrane protein, is likely to play a crucial role in the progression and severity of AP[12]. Our previous research has determined that MSCs enhance the expression of β-catenin in
SAP[20]. In the current study, the expression of the β-catenin in the pcDNA-H19-MSC group was much higher than in the MSC and si-H19-MSC groups, as well as c-Myc and cyclin D1 (Figure 3). The Wnt/β-catenin signaling pathway has been confirmed to increase cell proliferation and differentiation[39]. Thus, it is likely that LncRNA H19 can promote proliferation in SAP via this pathway.

The function of IncRNAs is closely correlated with their localization within the cell[40]. LncRNAs can function as competing endogenous RNA when they are mainly located in the cytoplasm[41, 42]. We performed RNA FISH assays and found that LncRNA H19 was mainly localized to the cytoplasm of MSCs (Figure 4). Therefore, to investigate the mechanism of LncRNA H19 in SAP, we utilized bioinformatics prediction to seek other miRNAs that could interact with LncRNA H19. Our research illustrated that LncRNA H19 could directly bind to rno-miR-138-5p and rno-miR-141-3p (Figures 4, 5). There have been no previous investigations to our knowledge of miR-138 in AP or SAP, but the expression of serum miR-141 was upregulated in AP[43]. Here, suppression of miR-138-5p in MSCs significantly promoted the FAK/PDK1/AKT/mTOR pathway to repress autophagy in SAP (Figure 4). In addition, knock down of miR-141-3p in MSCs increased proliferation by upregulating the expression of β-catenin, c-Myc, and cyclin D1 (Figure 5).

Furthermore, we explored the potential target genes of rno-miR-138-5p and rno-miR-141-3p using TargetScan. Our results indicated that miR-138-5p and miR-141-3p directly targeted PTK2 (Figure 6) and β-catenin (Figure 7), respectively, and suppressed their expression. PTK2, encoding FAK, has been confirmed to mediate protective autophagy in anoikis-resistant glioma stem cells[44]. In our study, upregulation of PTK2 in MSCs restrained autophagy via activation of the FAK/PDK1/AKT/mTOR signaling pathway in SAP (Figure 6). Overexpression of β-catenin in MSCs effectively facilitated cell proliferation in SAP, along with enhanced expression of c-Myc (Figure 7).

There are some weaknesses in our study, which we do not believe compromise our conclusions. We neglected the potential and meaningful consequences that concentration gradients and time may bring out, and we also ignored the possible side effects of cell therapy which may occur in the future. We will address these issues in future studies.

In summary, negative control rats treated with NaT developed SAP, along with activated autophagy and suppressed cell proliferation. As a competing endogenous RNA, LncRNA H19 sponges rno-miR-138-5p and rno-miR-141-3p, enhances the expression of PTK2 and β-catenin, upregulates the FAK/PDK1/AKT/mTOR signaling pathway and β-catenin target genes (c-Myc and cyclin D1), all the while inhibiting autophagy and promoting proliferation. Hence, LncRNA H19 may provide us with a novel strategy utilizing MSC therapy for SAP in the future.

**Conclusion**

When the pancreas is critically damaged, AP or even SAP may injure the organism, causing excessive autophagy and suppressed cellular proliferation. MSCs can be utilized to attenuate SAP and LncRNA H19 markedly enhances their therapeutic efficacy. During MSC treatment, upregulated LncRNA H19 can act as
an miRNA sponge to adsorb rno-miR-138-5p and rno-miR-141-3p, facilitating the expression of PTK2 and β-catenin, which in turn increases FAK/PDK1/AKT/mTOR signaling to suppress autophagy and promotes cell proliferation (Additional file 1).

**Declarations**

**Ethics approval**

*In vivo* animal research complied with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). All animal protocols were approved by the Institutional Animal Ethics Committee of the Shanghai Tenth People's Hospital, affiliated to Tongji University School of Medicine.

**Consent for publication**

All authors have reviewed the manuscript and approved the publication.

**Availability of data and materials**

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

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

The current study was supported by the National Natural Science Foundation of China (No. 81670582).

**Authors’ contributions**

Z.S. conceived and designed the experiments. G.S., J.Z., R.S., D.L., W.Y., and W.X. performed the experiments. G.S., Z.M., J.G., H.M., and T.Y. analyzed the data and wrote the paper. All authors have read the manuscript and agreed to submit it in its current form for consideration for publication.

**Acknowledgements**

Not applicable.

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### Table

Table 1. Primer sequences for qRT-PCR.

| Gene/RNA   | Forward (5'-3')                  | Reverse (5'-3')                  |
|------------|----------------------------------|----------------------------------|
| LncRNA H19 | CGTTCCCTTTAGTCTCCTGAC            | AGTCCGTGTTCCAAGTCC               |
| PTK2       | GTGCTCTTGGTTCAAGCTGA             | ACTTGAGTGAAGTCAGCAAGA TGTGT      |
| β-catenin  | TGATAAAGGCAACTGTTGATTGA          | CCGCTGGGTGTCCTGATGT              |
| GAPDH      | CGCTAACATCAAATGGGGTG             | TTGCTGACAATCTTTGAGGGAG           |

| Primers    | Forward                         | Reverse                         |
|------------|---------------------------------|---------------------------------|
| mo-miR-138 | AGCUUGGUUGUGUUGAAUC             | GTAGTCGGCGAAGGTCTCAC            |
| Forward    | GTGCAAGGTCCGAGGT                | ACCGTGGATGCAATGCTAA             |
| Primers    | mo-miR-141 (5'-3')             | U6 (5'-3')                      |
| Forward    | UAACACUGUCUGGUAAAGAUG           | AACGTTTCAGGAATTTCGCT            |
| Reverse    | CAUCUUCCAGUACAGUGUUGA           | CTCGCTTCGCGACAGCAC             |

### Figures
Figure 1

LncRNA H19 upregulated the therapeutic efficacy of MSCs on SAP (a) Information about genomic regions, transcripts and products of LncRNA H19 (Rattus norvegicus). (b) Allocation of necrotic, apoptotic, and live MSCs, detected by flow cytometry. (c) The survival rate of MSCs determined by flow cytometry. (d, e) Relative mRNA expression of LncRNA H19 in MSCs and pancreatic tissues analyzed by qRT-PCR respectively. (f) H&E staining of pancreatic tissues (scale bar = 100 μm). (g) Pathological scores for H&E staining in the pancreas. (h) Levels of serum lipase activity. (i) Levels of serum LDH activity. (j) Levels of pancreatic LDH activity. (k–n) Concentration of IL-1β, IL-6, IL-8 and TNF-α in pancreas. Data represent means ± SD from at least three independent experiments. n = 6/group. *p < 0.05, **p < 0.01, ***p < 0.001, and NS means not significant.
Figure 2

LncRNA H19 repressed autophagy in SAP (a–h) Western blot analysis of FAK, PDK1, AKT, p-AKT, mTOR, p-mTOR, P62, Beclin-1 and LC3 protein levels in pancreas. (i) Immunohistochemical staining of pancreatic FAK, PDK1, AKT, p-AKT, mTOR, p-mTOR, P62, Beclin-1 and LC3 (scale bar = 100 μm). (j) Immunofluorescence staining of LC3 in pancreas (scale bar = 100 μm). (k) Characteristic pictures of autophagosomes observed using TEM in pancreas (scale bar = 1 μm). Red arrows show autophagosomes. Data are demonstrated means ± SD from at least three separate experiments. n = 6/group. *p < 0.05, **p < 0.01, ***p < 0.001, and NS means not significant.

Figure 3

LncRNA H19 facilitate proliferation in SAP (a–d) Western blot analysis of pancreatic β-catenin, c-Myc and cyclin D1 protein levels. (e) Immunohistochemical assay of pancreatic β-catenin, c-Myc, and cyclin D1 (scale bar = 100 μm). (f) Immunohistochemistry of Ki67 staining in pancreas (scale bar = 100 μm). (g) Proportion of Ki67 positive stained area. Data are shown means ± SD from at least three separate experiments. n = 6/group. *p < 0.05, **p < 0.01, ***p < 0.001, and NS means not significant.
Figure 4

LncRNA H19 directly bonded with miR-138-5p and repression of miR-138 inhibited autophagy in SAP (a) Relative mRNA expression of miR-138 in pancreatic tissues estimated by qRT-PCR. (b) Putative complementary sites within LncRNA H19 and mno-miR-138-5p were predicted by Target Scan software. (c) Co-transfection of the 293T cells with LncRNA H19 and miR-138-5p mimics decreased the luciferase activity. (d) Agarose gel electrophoresis assay for the complex containing LncRNA H19 and biotin-coupled miR-138-5p or biotin-coupled miR-NC. (e) Fold enrichment of LncRNA H19 in capture assays. (f) Observation of localization of LncRNA H19 in cytoplasm of MSCs by FISH assay, and nuclei were stained blue, and LncRNA H19 was stained stained red (scale bar = 50 μm). (g) Relative mRNA expression of miR-138 in MSCs with different treatments analyzed by qRT-PCR. (h–o) Western blot analysis of pancreatic FAK, PDK1, AKT, p-AKT, mTOR, p-mTOR, P62, Beclin-1, and LC3 protein levels. Data represent means ± SD from at least three independent experiments. n = 6/group. *p < 0.05, **p < 0.01, ***p < 0.001, and NS means not significant.
LncRNA H19 functioned as a sponge for miR-141-3p and suppression of miR-141 promote cell proliferation in SAP (a) Relative mRNA expression of pancreatic miR-141 analyzed by qRT-PCR. (b) The schematic diagram illustrated the binding sites of LncRNA H19 with rno-miR-141-3p. (c) Co-transfection of the 293T cells with LncRNA H19 and miR-141-3p mimics reduced the luciferase activity. (d) Agarose gel electrophoresis assay for the complex containing LncRNA H19 and biotin-coupled miR-141-3p or biotin-coupled miR-NC. (e) Fold enrichment of LncRNA H19 in capture assays. (f) Relative mRNA expression of miR-141 in MSCs with various administrations detected by qRT-PCR. (g–j) Western blot analysis of pancreatic β-catenin, c-Myc, and cyclin D1 protein levels. Data are shown means ± SD from at
least three independent experiments. n = 6/group. *p < 0.05, **p < 0.01, ***p < 0.001, and NS means not significant.
PTK2 targeted miR-138-5p directly and upregulation of PTK2 repressed autophagy in SAP (a) Potential bind sites of rno-miR-138-5p in PTK2 3'UTR. (b) PTK2-wt or PTK2-mut plasmid was co-transfected with
miR-NC or miR-138-5p mimics into 293T cells for 24 h, then luciferase activity was detected. (c) Relative mRNA expression of PTK2 in MSCs with different administrations analyzed by qRT-PCR. (d–k) Western blot analysis of pancreatic FAK, PDK1, AKT, p-AKT, mTOR, p-mTOR, P62, Beclin-1, and LC3 protein levels. Data represent means ± SD from at least three separate experiments. n = 6/group. *p < 0.05, **p < 0.01, ***p < 0.001, and NS means not significant.

Figure 7

β-catenin was a direct target of miR-141-3p and overexpressing β-catenin boosted proliferation in SAP (a) Feasible bind sites of rno-miR-141-5p in β-catenin 3’UTR. (b) β-catenin-wt or β-catenin-mut plasmid was co-transfected with miR-NC or miR-141-3p mimics into 293T cells for 24 h, then luciferase activity was examined. (c) Relative mRNA expression of β-catenin in MSCs with various treatments detected by qRT-PCR. (d–g) Western blot analysis of pancreatic β-catenin, c-Myc, and cyclin D1 protein levels. Data represent means ± SD from at least three separate experiments. n = 6/group. *p < 0.05, **p < 0.01, ***p < 0.001, and NS means not significant.

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