

# World Journal of *Stem Cells*

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Editorial Board Member of *World Journal of Stem Cells*, Ratan K Choudhary, PhD, Assistant Professor, College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141004, Punjab, India. [ratanchoudhary@gadvasu.in](mailto:ratanchoudhary@gadvasu.in)

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## Basic Study

# Mammalian Ste20-like kinase 1 inhibition as a cellular mediator of anoikis in mouse bone marrow mesenchymal stem cells

Tao Zhang, Qian Zhang, Wan-Cheng Yu

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## Abstract

### BACKGROUND

The low survival rate of mesenchymal stem cells (MSCs) caused by anoikis, a form of apoptosis, limits the therapeutic efficacy of MSCs. As a proapoptotic molecule, mammalian Ste20-like kinase 1 (Mst1) can increase the production of reactive oxygen species (ROS), thereby promoting anoikis. Recently, we found that Mst1 inhibition could protect mouse bone marrow MSCs (mBMSCs) from H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis by inducing autophagy and reducing ROS production. However, the influence of Mst1 inhibition on anoikis in mBMSCs remains unclear.

### AIM

To investigate the mechanisms by which Mst1 inhibition acts on anoikis in isolated mBMSCs.

### METHODS

Poly-2-hydroxyethyl methacrylate-induced anoikis was used following the silencing of Mst1 expression by short hairpin RNA (shRNA) adenovirus transfection. Integrin (ITGs) were tested by flow cytometry. Autophagy and ITG $\alpha$ 5 $\beta$ 1 were inhibited using 3-methyladenine and small interfering RNA, respectively. The alterations in anoikis were measured by Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling and anoikis assays. The levels of the anoikis-related proteins ITG $\alpha$ 5, ITG $\beta$ 1, and phospho-focal adhesion kinase and the activation of caspase 3 and the autophagy-related proteins microtubules associated protein 1 light chain 3 II/I, Beclin1 and p62 were detected by Western blotting.

### RESULTS

In isolated mBMSCs, Mst1 expression was upregulated, and Mst1 inhibition significantly reduced cell apoptosis, induced autophagy and decreased ROS levels. Mechanistically, we found that Mst1 inhibition could upregulate ITG $\alpha$ 5

and ITG $\beta$ 1 expression but not ITG $\alpha$ 4, ITG $\alpha$ v, or ITG $\beta$ 3 expression. Moreover, autophagy induced by upregulated ITG $\alpha$ 5 $\beta$ 1 expression following Mst1 inhibition played an essential role in the protective efficacy of Mst1 inhibition in averting anoikis.

### CONCLUSION

Mst1 inhibition ameliorated autophagy formation, increased ITG $\alpha$ 5 $\beta$ 1 expression, and decreased the excessive production of ROS, thereby reducing cell apoptosis in isolated mBMSCs. Based on these results, Mst1 inhibition may provide a promising strategy to overcome anoikis of implanted MSCs.

**Key Words:** Mouse bone marrow mesenchymal stem cell; Mammalian sterile 20-like kinase 1; Anoikis; Integrin; Autophagy; Reactive oxygen species

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**Core Tip:** In isolated mouse bone marrow mesenchymal stem cell (mBMSCs), Mammalian sterile 20-like kinase 1 (Mst1) inhibition could ameliorate not only autophagy formation but also upregulate integrin (ITG)  $\alpha$ 5 $\beta$ 1 expression (but not ITG $\alpha$ 4, ITG $\alpha$ v, or ITG $\beta$ 3). In addition, Mst1 inhibition-induced autophagy could scavenge the excessive production of ITG $\alpha$ 5 $\beta$ 1-triggered ROS. Therefore, Mst1 inhibition-based infusion may improve the survival of MSCs, thereby serving as an ideal candidate for clinical transplantation in pulmonary arterial hypertension.

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## INTRODUCTION

Mesenchymal stem cell (MSC) therapy is characterized by anti-inflammatory, immunomodulatory, and regenerative properties, providing an attractive therapeutic approach for pulmonary arterial hypertension (PAH)[1]. Despite the therapeutic potential of MSCs for improving the outcomes of PAH patients[2,3], no more than 5% of cells survive after transplant[4]. Thus, the low survival rate of the grafted cells is widely perceived as the major hindrance for an MSC-based therapy for PAH.

Anoikis occurs when cells detach from the extracellular matrix and subsequently undergo apoptosis, and potentially acts as a major enabling factor for the apoptosis of transplanted cells[5,6]. Indeed, after isolation from the extracellular matrix (ECM) and injection into the circulatory system for transplantation, MSCs will undergo anoikis, also referred to as cell isolation-induced apoptosis, leading to a series of alterations in anoikis signalling pathways[3,7,8]. Anoikis can be induced by destruction of integrin (ITGs) signalling or deletion of ITGs genes[9]. After isolation, focal adhesion kinase (FAK), a key downstream target of ITGs, is recruited to focal adhesion sites, consequently activating cell survival signals, such as blocking caspase 3 expression[10]. However, it remains unknown whether ITGs signalling is involved in the process of anoikis in MSCs.

Autophagy is a dynamic process that maintains homeostasis by preventing the accumulation of excessive biomolecules and impaired cells and organelles. There is accumulating evidence of a link between autophagy and anoikis[11]. Previously, we demonstrated that mammalian Ste20-like kinase 1 (Mst1) inhibition could reduce H<sub>2</sub>O<sub>2</sub>-induced apoptosis of mBMSCs by inducing autophagy formation [12]. Mst1 is a serine/threonine kinase, known as a key mediator in cellular processes, including mediating the apoptosis[13]. However, the molecular mechanism by which Mst1 inhibition mediates autophagy and anoikis in isolated mBMSCs remains to be clarified.

In this study, we investigated the potential regulatory effect of Mst1 inhibition on ITGs signalling, autophagy and anoikis in isolated mBMSCs.

## MATERIALS AND METHODS

### Cell culture

The mBMSCs were obtained as previously described[12]. Cultured mBMSCs between passages 3 and 5 were selected for subsequent experiments.

### **Adenovirus infection**

Adenovirus harbouring Mst1 short hair RNA (Ad-sh-Mst1) and the control vector for Mst1 shRNA (Ad-NC-Mst1) were purchased from WZ Biosciences (China). Vector details have been previously described [12]. The shRNA sequence targeting Mst1 in mice was GCCCTCACGTA GTCAAGTATT.

### **siRNA transfection**

The small interfering RNAs (siRNAs) were obtained from GenePharma (China). The sense and antisense strand sequences of siRNA are as follows: Mouse siRNA-ITG $\alpha$ 5, 5'-GCAGGGAGA-UGAAGAUCUACC' (sense) and 5'-UAGAUCUUAUCUCCUGCAG' (antisense); mouse siRNA-ITG $\beta$ 1, 5'-GGAGAACCACAGAAGUUUACA-3' (sense) and 5'-UAAACUUCUGUGGUUCUCCUG-3' (antisense); and siRNA-negative control (NC), 5'-UUCUCCGAACGUGUCACGUTT' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (antisense). Subsequently, 24 h after infection with Ad-sh-Mst1, mBMSCs at 75% confluence were transfected with ITG $\alpha$ 5, ITG $\beta$ 1 or NC siRNA (50 nM) using Lipofectamine RNAi MAX (13778500, Invitrogen) according to the manufacturer's instructions. The expression of ITG $\alpha$ 5 or ITG $\beta$ 1 was substantially blocked by the transfected siRNA.

### **Cell treatment**

Petri dishes coated with polyhydroxyethyl methacrylate [Poly-HEMA, 529257, Sigma, United States of America (USA)] were used to prevent cells from adhering to the tissue culture plates. Briefly, poly-HEMA stock material was dissolved in 95% ethanol at a concentration of 12 mg/mL, and 1 mL of 12 mg/mL poly-HEMA was added to each well of a 6-well plate and then dried overnight on a clean bench. Cells were transfected as previously described. Cells ( $5 \times 10^5$ ) were coated with 12.5 mg/mL poly-HEMA in each well for a certain period of time.

To inhibit autophagy, cells were pretreated with 5 mmol/L 3-MA (189490, Selleck, USA) for 1 h and then cultured in poly-HEMA-precoated plates for a certain period of time.

### **Assay of intracellular ROS**

As mentioned above, cellular ROS were assessed using the ROS probe 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA), S0033, Beyotime Biotechnology, China [12]. The mean fluorescence intensity was detected *via* flow cytometry.

### **Isolation-induced anoikis assay**

Anoikis was analysed using an in situ Direct DNA Fragmentation Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling (TUNEL) Assay Kit [ab66108, Abcam, The United Kingdom of Great Britain and Northern Ireland (UK)]. After incubation in poly-HEMA-coated plates, the cells were collected and added to 70% ice ethanol for 30 min. Ethanol was then removed, and the cells were resuspended in washing buffer and then stained with a staining solution for 60 min. Prior to the addition of the PI/RNase A solution, the cells were washed twice with rinse buffer. Quantification analysis was performed by Becton Dickinson Fluorescence Activating Cell Sorter (BD FACSDiva) software, [Ex/Em = 488/520 nm for fluoresceine isothiocyanate and 488/623 nm for propidium iodide].

Anoikis was also detected by a CytoSelect™ 24-Well Anoikis Assay (XY-CBA-080, Cell Biolabs, USA) according to the manufacturer's instructions. Briefly, cells ( $1 \times 10^6$  cells/well) were cultured in each well of 24-well plate for 36 h before staining with ethidium homodimer (EthD-1) at 37°C for 1 h. The presence of red EthD-1 fluorescence in dead cells was observed by a fluorescence microscope, and cell viability was determined using a thiazolyl blue tetrazolium bromide (MTT) assay.

### **Flow cytometry**

Cells were incubated in poly-HEMA-coated petri dishes for 36 h, centrifuged at  $300 \times g$  for 5 min and cultured in antibodies (ITG $\alpha$ 4 [1/500 dilution, 553157, BD], ITG $\alpha$ 5 [1/500 dilution, 557447, BD], ITG $\alpha$ v [1/300 dilution, 740946, BD], ITG $\beta$ 1 [1/500 dilution, 561796, BD], ITG $\beta$ 3 [1/100 dilution, 740677, BD]) for 1 h according to the operation manual.

### **Cell adhesion**

After culture in poly-HEMA-coated petri dishes, the collected cells were resuspended in complete  $\alpha$ -MEM and then plated in triplicate ( $5 \times 10^4$  cells/well) onto wells coated with fibronectin (10 g/mL), which was previously blocked with 1% BSA for 1 h. After 6 h, the cells were washed with phosphate balanced solution (PBS) and stained with crystal violet. Unbound dye was removed with PBS before adding a 10% acetic acid solution. The absorbance was read at 630 nm using a Multiskan MK3 microplate reader. The experiment was repeated three times. Cell adhesion was calculated according to the proportion of adhered cells in the control group.

### **Cytokine levels**

The supernatants in each group were collected after culture in poly-HEMA-coated petri dishes for 36 h. The levels of anti-inflammatory cytokines were measured using a BD™ Cytometric Bead Array (CBA)

Mouse Th1/Th2/Th17 Cytokine Kit (561665, BD, USA) in accordance with the instruction manual. The levels of interleukin (IL)-4 (IL-4), IL-10, IL-17A and IL-6 in cell supernatants were measured using flow cytometry. Data analysis was performed as previously described[14].

### **Nude mouse tumorigenicity**

All animal procedures were approved by the Animal Care and Use Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University (IACUC protocol, Approval No. 2020-333). A total of 10 female nude mice (4 wk old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and raised in a specific pathogen-free environment. Mice were placed at a standard room temperature at a normal day-night cycle with free access to standard diet and water. Afterwards,  $5.0 \times 10^6$  mBMSCs ( $n = 3$ ), mBMSC/NC-Mst1 ( $n = 3$ ), and mBMSC/sh-Mst1 ( $n = 4$ ) were injected into the right flank near the hind legs of each nude mouse. The tumours were measured with a Vernier calliper every 4 d. Sixty days after cell inoculation, all mice were anaesthetized with ether, and tissues were collected.

### **Quantitative real-time polymerase chain reaction (qPCR)**

qPCR was performed as previously reported[12]. mBMSCs were differentiated *via* 21-d exposure to osteogenic or adipogenic conditions, and total mRNA from mBMSCs subjected to these conditions and siRNA-transfected cells was isolated using TRIzol Reagent (15596026, Thermo Fisher Scientific, USA). The RNA was subsequently reverse transcribed into cDNA and amplified using the SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> II kit (RR420, Takara, JPN) and d ABI 7500 real-time PCR system (Applied Biosystems). Each experiment was repeated three times. Data were normalized through the  $2^{-\Delta\Delta CT}$  method using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The primer sequences are shown in Supporting Information [Supplementary Table 1](#).

### **Western blot analysis**

To determine protein expression, Western blot analysis was performed. After culture in poly-HEMA-coated plates, whole-cell protein extracts were prepared in radio-immunoprecipitation assay lysis buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride membranes. The membranes were then blocked with 5% skimmed milk or Bovine serum albumin in Tris-Buffered Saline Tween-20 for 1 h and incubated overnight at 4 °C with the following primary antibodies (diluted by Western Primary Antibody Buffer, P0023A, Beyotime): Mst1 (1:1000, ab51134, Abcam), ITG $\alpha$ 5 (1:1000, ab150361, Abcam), ITG $\beta$ 1 (1:1000, ab179471, Abcam), phospho-FAK (Tyr397) [1:500, 3283S, Cell Signaling Technology (CST)], FAK (1:1,000, 3285S, CST), activated caspase 3 (1:1000, ab214430, Abcam), and caspase 3 (1:1000, ab18297, Abcam). GAPDH (1:1000, 5174S, CST) served as the loading control. Anti-rabbit IgG and HRP-linked antibodies (1:1000, 7074S, CST) were used. The relative protein expression levels were compared with GAPDH using ImageJ software.

### **Statistical analysis**

All results are expressed as the mean  $\pm$  SD. One-way Analysis of Variance was used for data analysis.  $P < 0.05$  was considered statistically significant.

## **RESULTS**

### **The loss of attachment to ECM increased the rate of aberrant cell apoptosis, ROS levels, and Mst1 expression and inhibited autophagy in mBMSCs**

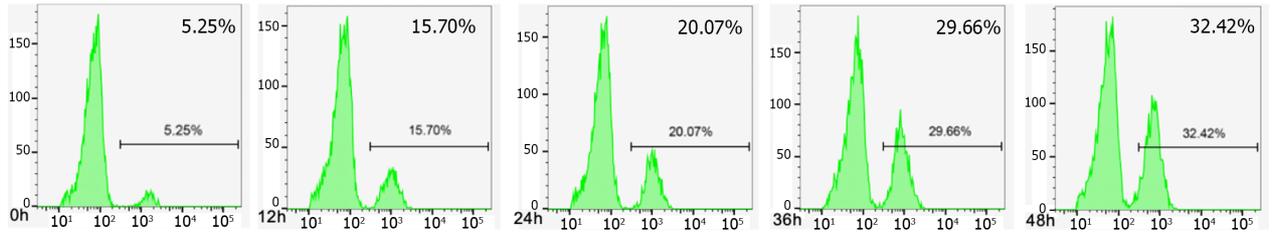
As the ability to reduce cell adhesivity to culture plates, Poly-HEMA was used to simulate an anchorage-independent culture condition. In present study, the sensitivity of mBMSCs to anoikis in Poly-HEMA-pre-coated condition were tested.

Using the TUNEL and Anoikis Assay Kit, the results showed an increased rate of mBMSC apoptosis in a time-dependent manner under poly-HEMA-induced isolated conditions (Figures 1A, B, D and E), suggesting that anoikis of mBMSCs could be induced in precoated poly-HEMA plates. In addition, the cell adhesion decreased at 24 h, 36 h, and 48 h compared with that at 0 h (Figure 1G).

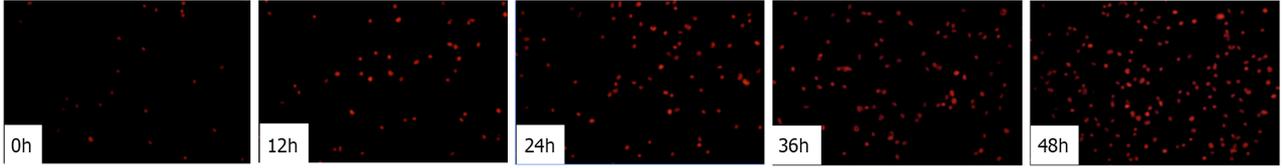
Moreover, staining of intracellular ROS with the ROS probe DCFH-DA showed increased ROS levels at 24, 36, and 48 h compared with 0 h (Figures 1C and F), demonstrating the production of ROS in poly-HEMA-induced isolated mBMSCs.

To determine the alterations in Mst1 expression, autophagy and the FAK/Caspase 3 pathway in mBMSCs under isolated conditions, the protein level of Mst1, autophagy-related proteins (LC3 II/I, Beclin1, p62), p-FAK, and activated caspase 3 was detected by Western blot analysis. The data suggested that Mst1 was upregulated in isolated mBMSCs (Figure 1H). Moreover, the expression of p-FAK decreased, and the activation of caspase 3 increased in a time-dependent manner (Figure 1I). Similarly, LC3 II/I and Beclin1 expression was downregulated, and p62 expression was upregulated in a time-dependent manner (Figure 1H).

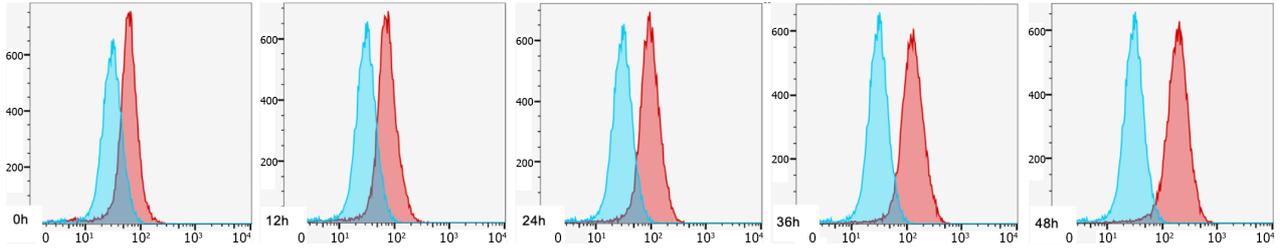
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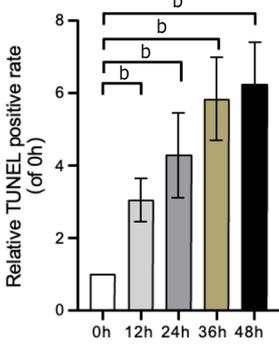
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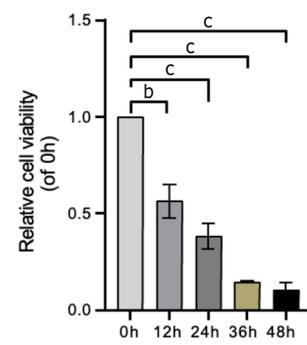
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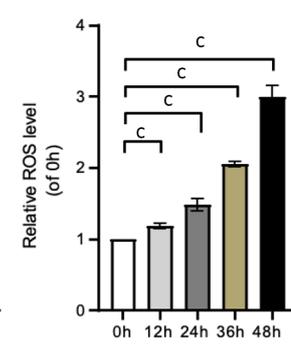
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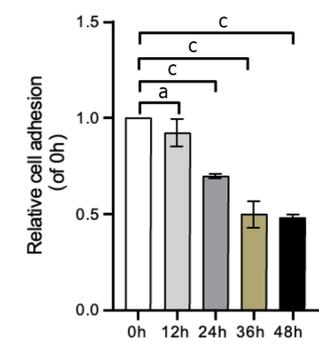
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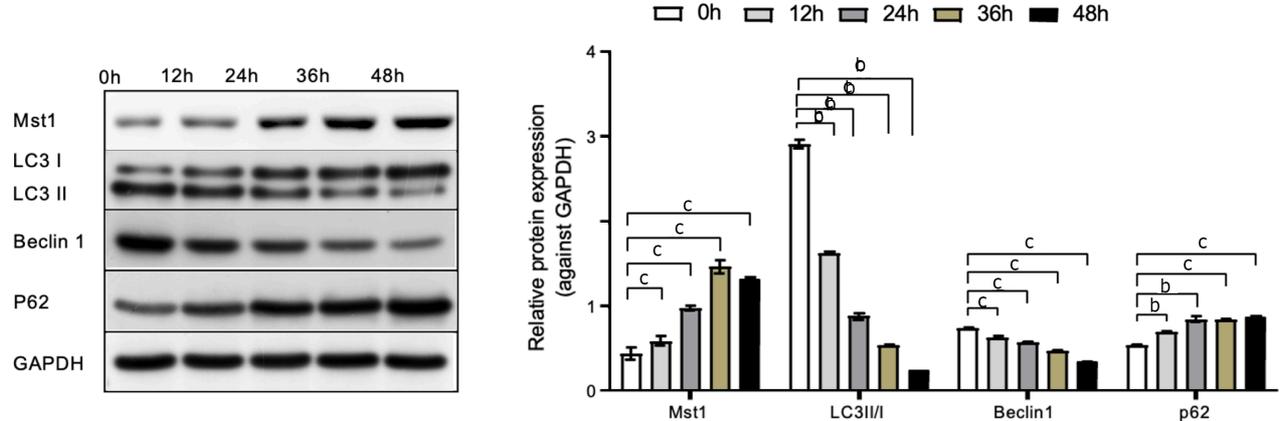
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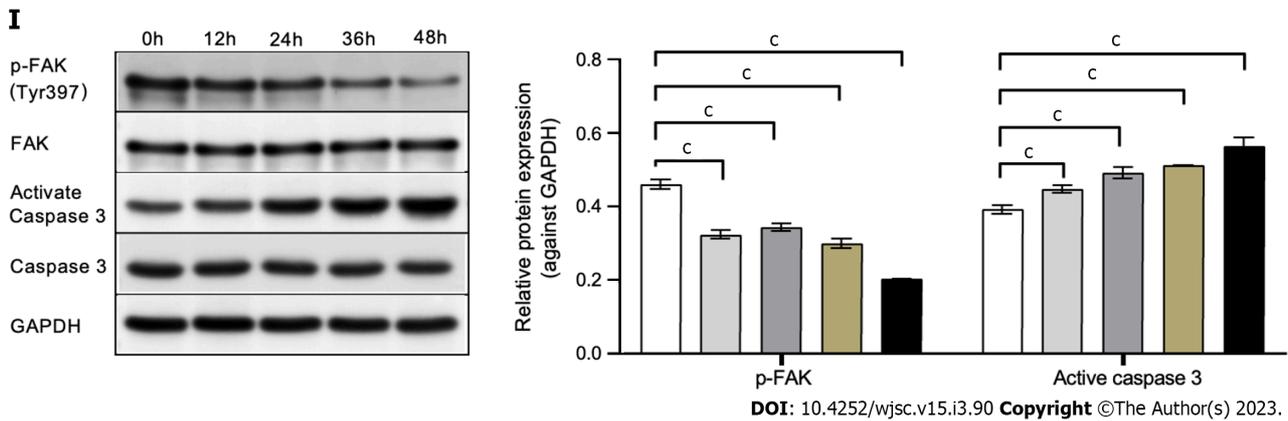


**G**



**H**





**Figure 1 Mouse bone marrow mesenchymal stem cells exhibit susceptibility to anoikis under detachment conditions.** A: Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling (TUNEL)-positive cells (apoptotic cells) of mouse bone marrow mesenchymal stem cells (mBMSCs) cultured in precoated Poly-2-hydroxyethyl methacrylate conditions for 0 h, 12 h, 24 h, 36 h and 48 h using flow cytometry; B: Fluorescence staining images of dead cells; C: Reactive oxygen species (ROS) levels were measured using the ROS probe 2,7-Dichlorodihydrofluorescein diacetate by flow cytometry at 0 h, 12 h, 24 h, 36 h and 48 h; D: Quantitative analysis of the rate of TUNEL-positive cells; E: Quantification of live cells using a thiazolyl blue tetrazolium bromide assay; F: Quantitative analysis of the intracellular ROS level; G: Cell adhesion was expressed as fold changes between 0 h groups; H and I: Mammalian Ste20-like kinase 1 (Mst1), microtubules associated protein 1 light chain 3 II/I, p62, Beclin1, phospho-focal adhesion kinase, and activated caspase 3 expression levels at 0 h, 12 h, 24 h, 36 h and 48 h were evaluated by Western blot analysis; The expression of protein was expressed as the fold change relative to glyceraldehyde-3-phosphate dehydrogenase. Values are the mean  $\pm$  SD of three independent experiments in each case. TUNEL: Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling; ROS: Reactive oxygen species; Mst1: Mammalian Ste20-like kinase 1; LC3-II/I: Microtubules associated protein 1 light chain 3 II/I; p-FAK: Phospho-focal adhesion kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. <sup>a</sup> $P < 0.01$ ; <sup>b</sup> $P < 0.05$ ; <sup>c</sup> $P > 0.05$ .

### Mst1 inhibition upregulated ITG $\alpha$ 5 and ITG $\beta$ 1 expression in isolated mBMSCs

The mBMSCs were infected with adenovirus containing Mst1 shRNA. The effect of shRNA on inhibiting Mst1 expression were measured by qPCR and Western blot (Supplementary Figure 1).

Evidence has shown that ITGs, the heterodimeric cell surface adhesion receptors, mediates anoikis. In this study, the alterations of ITGs in isolated mBMSCs/sh-Mst1 were tested.

The expression profiles of ITG $\alpha$ 5, ITG $\alpha$ v, ITG $\alpha$ 4, ITG $\beta$ 1, and ITG $\beta$ 3 in poly-HEMA-treated mBMSCs were compared by flow cytometry. Compared with the control mBMSC levels, the poly-HEMA-treated isolated mBMSC levels of ITG $\alpha$ 5, ITG $\alpha$ v, ITG $\alpha$ 4, ITG $\beta$ 1, and ITG $\beta$ 3 were significantly decreased (Figure 2). Compared with isolated mBMSCs, isolated mBMSCs/sh-Mst1 show an upwards trend in ITG $\alpha$ 5 and ITG $\beta$ 1 expression. However, there was no difference of the expression profiles of ITG $\alpha$ v, ITG $\alpha$ 4, and ITG $\beta$ 3 between isolated mBMSCs and isolated mBMSCs/sh-Mst1 (Figure 2). This study suggested that the inhibition of Mst1 could reactivate the expression of ITG $\alpha$ 5 and ITG $\beta$ 1.

### Suppression of Mst1 expression protected mBMSCs from anoikis by activating autophagy.

mBMSCs were cultured in precoated poly-HEMA plates for 36 h. A significant decrease in cell apoptosis was observed in mBMSCs/sh-Mst1 (Figures 3A, B, D and E). Similar to the above results, cell adhesion was ameliorated by silencing Mst1 expression (Figure 3G). These results indicated that Mst1 inhibition suppressed ECM-isolated-induced anoikis in mBMSCs.

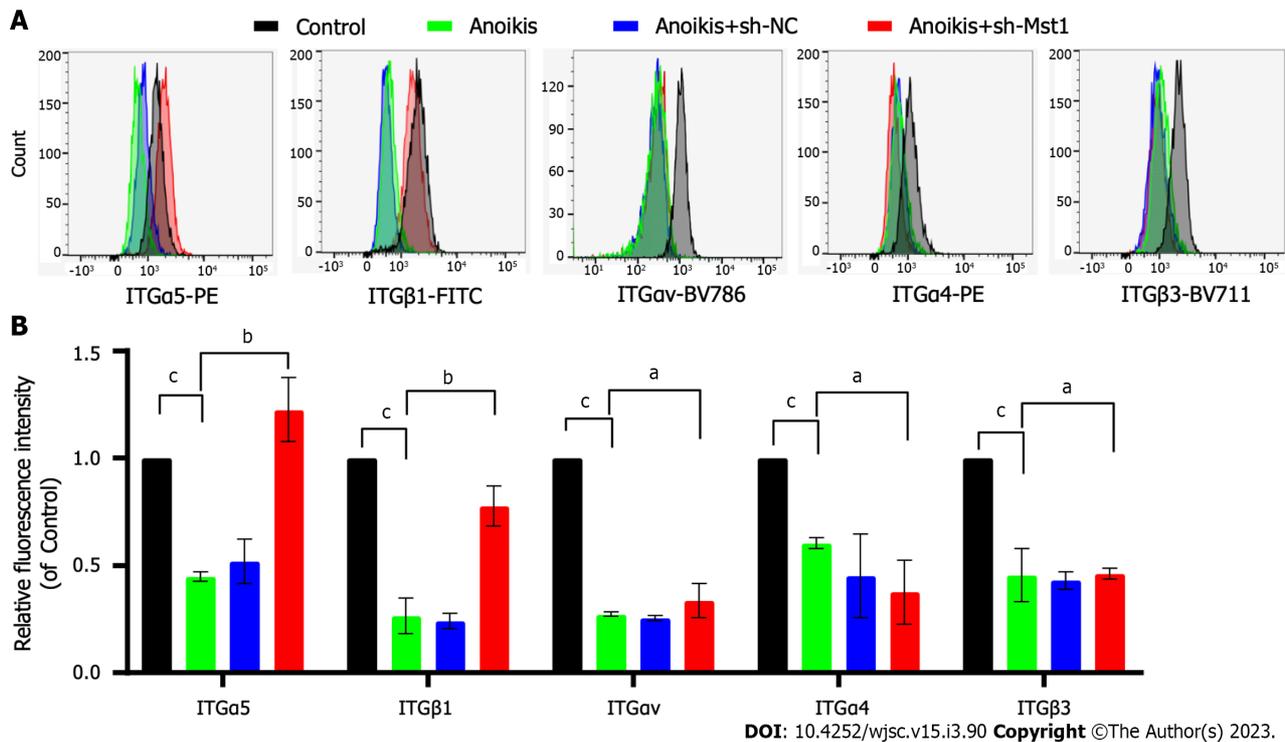
In addition, flow cytometric analysis confirmed decreased ROS levels in isolated mBMSCs/sh-Mst1 compared with those of isolated mBMSCs, whereas ROS levels were re-elevated by the autophagy inhibitor 3-MA (Figures 3C and F).

Western blot assay further suggested the above conception. FAK, has been recognised as the key mediator of cell-substrate adhesion. Western blot analysis results showed that mBMSC/sh-Mst1 exhibited robust FAK activation (Figure 3I). Similar to apoptosis, the activation of caspase can induce anoikis. Thus, we tested effect of Mst1 inhibition on the activation of caspase 3 by Western blotting. In Figure 3I, silencing Mst1 expression significantly inhibited caspase 3 activation in suspension-grown mBMSCs. This study indicated that silencing Mst1 expression could reactivate the FAK/Caspase3 pathway in anchorage-independent mBMSCs. However, 3-MA, an autophagy inhibitor, had no effect on the expression of ITG $\alpha$ 5 and ITG $\beta$ 1 or on cell adhesion (Figure 3I).

Consistent with the previous results, Mst1 inhibition reactivated autophagy in mBMSCs under isolated conditions, which can be demonstrated by the upregulated expression of LC3 II/I and Beclin1 and downregulated expression of p62 (Figure 3H). Furthermore, the number of mBMSCs/sh-Mst1 undergoing anoikis was increased after pretreatment with 3-MA (Figure 3H). In conclusion, the protective effect of Mst1 knockdown on anoikis in mBMSCs is associated with autophagy.

### Inhibition of ITG $\alpha$ 5 $\beta$ 1 reversed the protective effects of Mst1 inhibition against anoikis in mBMSCs

To determine whether ITG $\alpha$ 5 or ITG $\beta$ 1 contributes to anoikis resistance in mBMSC/sh-Mst1 cells,



**Figure 2** Changes in integrin protein expression in detached mouse bone marrow mesenchymal stem cells/sh-Mammalian Ste20-like kinase 1. Cells were cultured in detached conditions for 36 h. A: Flow cytometry analysis investigating the expression of integrin $\alpha$ 5 (ITG $\alpha$ 5), ITG $\alpha$ v, ITG $\alpha$ 4, ITG $\beta$ 1 and ITG $\beta$ 3; B: Data are expressed as the fold change compared to the control groups. Values are expressed as the mean  $\pm$  SD,  $n = 3$ . ITG: Integrin. <sup>c</sup> $P < 0.01$ ; <sup>b</sup> $P < 0.05$ ; <sup>a</sup> $P > 0.05$ .

siRNA was used to knock down ITG $\alpha$ 5 or ITG $\beta$ 1 expression, respectively (Supplementary Figure 2).

In isolated mBMSC/sh-Mst1, cell apoptosis was increased, and cell adhesion was blocked by siRNA-mediated ablation of ITG $\alpha$ 5 or ITG $\beta$ 1 (Figures 4A, B, D, E and G). Similarly, p-FAK expression was downregulated and caspase3 activation was upregulated using ITG $\alpha$ 5 or ITG $\beta$ 1 siRNA (Figure 4I). In addition, LC3 II/I, Beclin1 and p62 expression was also reversed by ITG $\alpha$ 5 or ITG $\beta$ 1 siRNA (Figure 4H). In addition, the results in Figures 4C and F suggested that the ROS level was reduced by ITG $\alpha$ 5 or ITG $\beta$ 1 siRNA.

### The properties and biological safety of mBMSCs/sh-Mst1

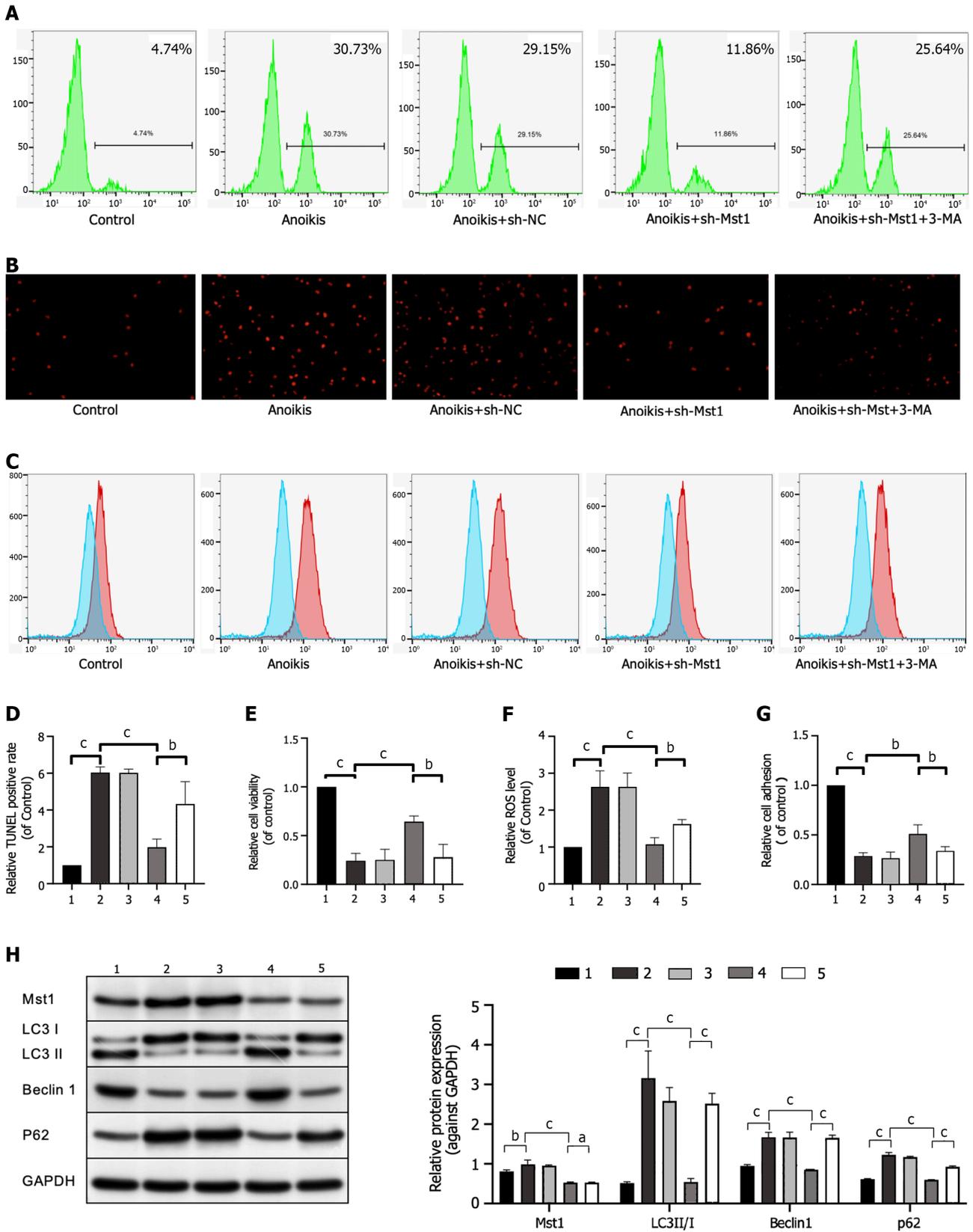
In isolated conditions, the levels of anti-inflammatory cytokines IL-4, IL-10 and IL-17A increased, while the level of pro-inflammatory cytokine IL-6 decreased in mBMSCs/sh-Mst1 compared with those of other mBMSCs (Figure 5A).

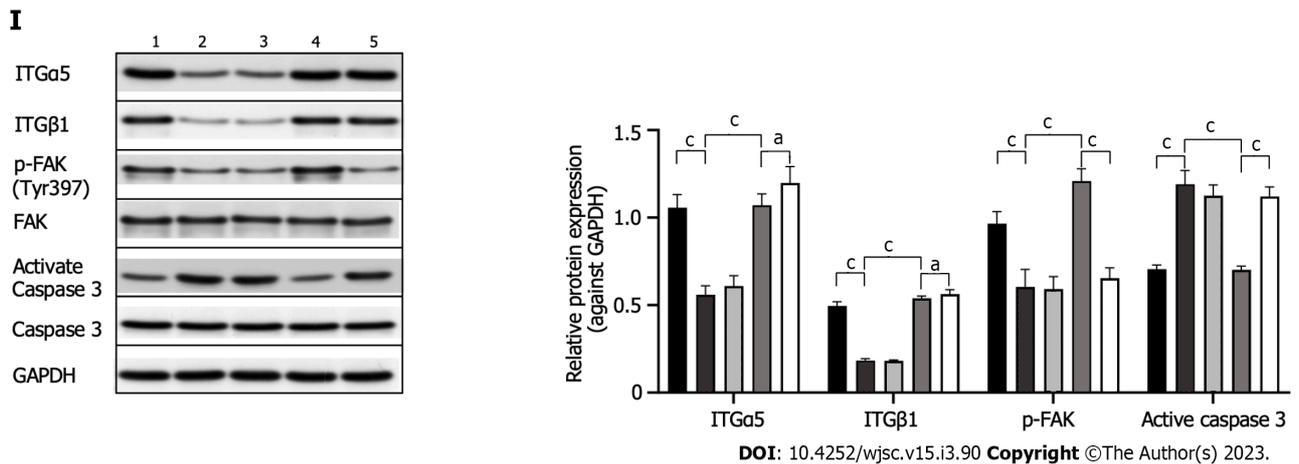
We assessed the effect of silencing Mst1 expression on the osteogenic differentiation of mBMSCs. In Figure 5B, Mst1 inhibition was correlated with increased osteogenic differentiation of mBMSCs. Subsequently, qPCR was performed to accurately determine the role of Mst1 inhibition on osteogenic differentiation in mBMSCs. As known as the markers of osteoblast differentiation, we tested the mRNA levels of runt-related transcription factor 2 (Runx2) and alkaline phosphatase (ALP). We found that the expression of Runx2 and ALP were both increased in mBMSC/sh-Mst1 (Figure 5B)[15].

There was no tumour-like mass in animals injected with mBMSC/sh-Mst1 after 60 d post-injection. After 60 d post-injection, we collected the subcutaneous tissue and the lung, liver, kidney and heart. There were no difference of the weights of the lung, liver, kidney and heart among each group (Figures 5C and Supplementary Table 2). It also showed that no stromal structures appeared in subcutaneous tissue of mBMSCs/sh-Mst1 groups (Figure 5C).

## DISCUSSION

Convincing suggestion has confirmed that anoikis limits the therapeutic efficacy of MSC transplantation for tissue repair[16]. Herein, this study has proven that mBMSC/sh-Mst1 could survive after isolation from the ECM, and this response was mediated by the effect of Mst1 inhibition-induced autophagy on ITG $\alpha$ 5 $\beta$ 1-modulated production of ROS.





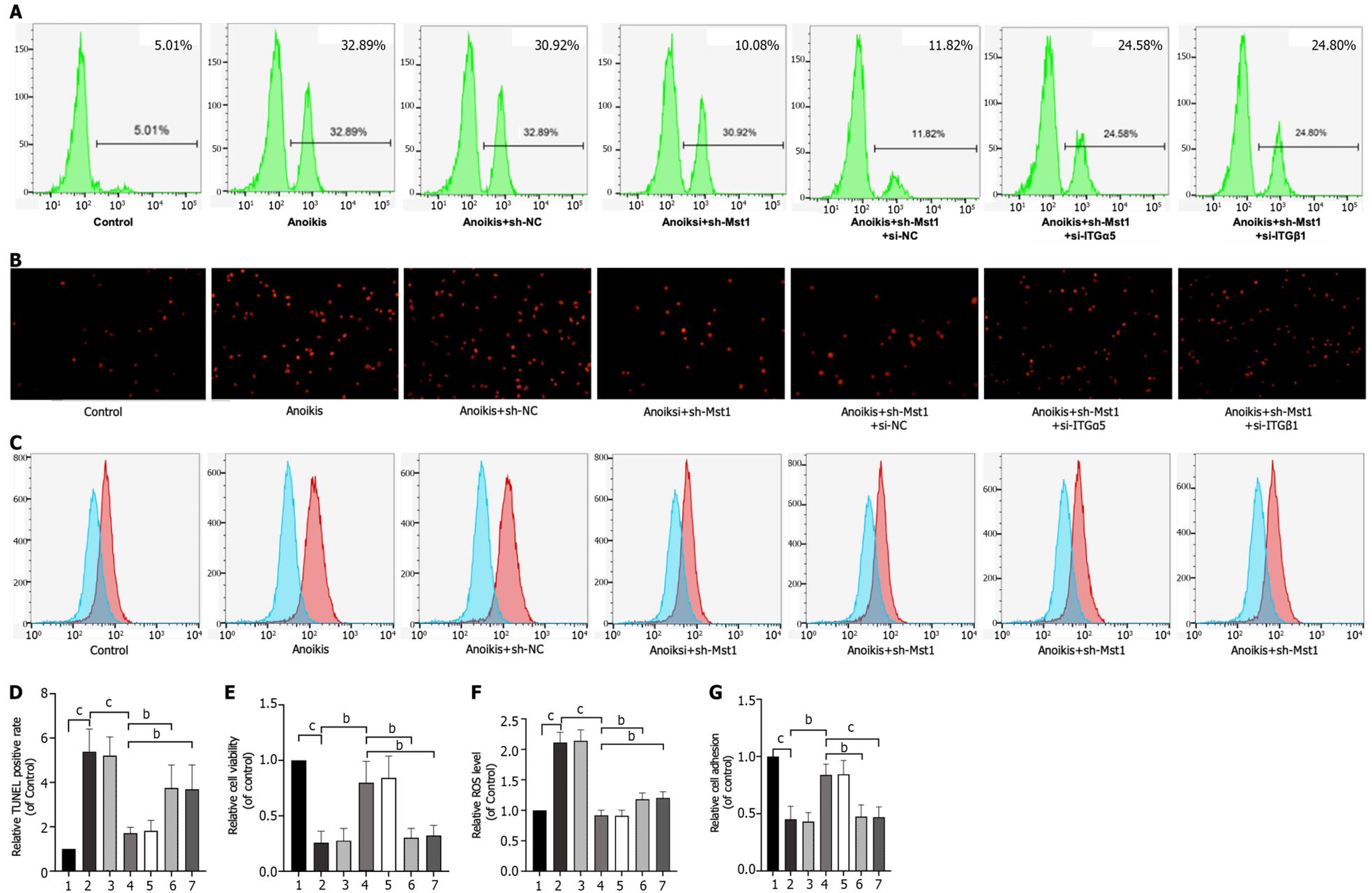
**Figure 3 Mammalian Ste20-like kinase 1 inhibition-induced autophagy reduced cell apoptosis in detached mouse bone marrow mesenchymal stem cells.** A: Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling (TUNEL)-positive cells analysed by flow cytometry; B: Representative images and quantification of fluorescence staining of dead cells; C: Reactive oxygen species (ROS) levels were measured using the ROS probe 2,7-Dichlorodihydrofluorescein diacetate by flow cytometry. D: Quantitative analysis of the rate of TUNEL-positive cells. E: Quantification of live cells using a thiazolyl blue tetrazolium bromide assay. F: Quantitative analysis of the intracellular ROS level. G: Cell adhesion was evaluated as the fold change compared back to the control groups; H and I: Mammalian Ste20-like kinase 1 (Mst1), microtubules associated protein 1 light chain 3 III/I, p62, Beclin1, phospho-focal adhesion kinase, and activated caspase 3 expression were evaluated by Western blot analysis. glyceraldehyde-3-phosphate dehydrogenase served as a control. Values are the mean  $\pm$  SD, and experiments were completed in triplicate. 3-MA: 3-methyladenine; TUNEL: Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling; ROS: Reactive oxygen species; Mst1: Mammalian Ste20-like kinase 1; LC3-III/I: Microtubules associated protein 1 light chain 3 III/I; p-FAK: Phospho-focal adhesion kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. 1. Control group; 2. Anoikis group; 3. Anoikis + sh-NC group; 4. Anoikis + sh-Mst1 group; 5. Anoikis + sh-Mst1 + 3-MA group. \* $P < 0.01$ ; <sup>b</sup> $P < 0.05$ ; <sup>a</sup> $P > 0.05$ .

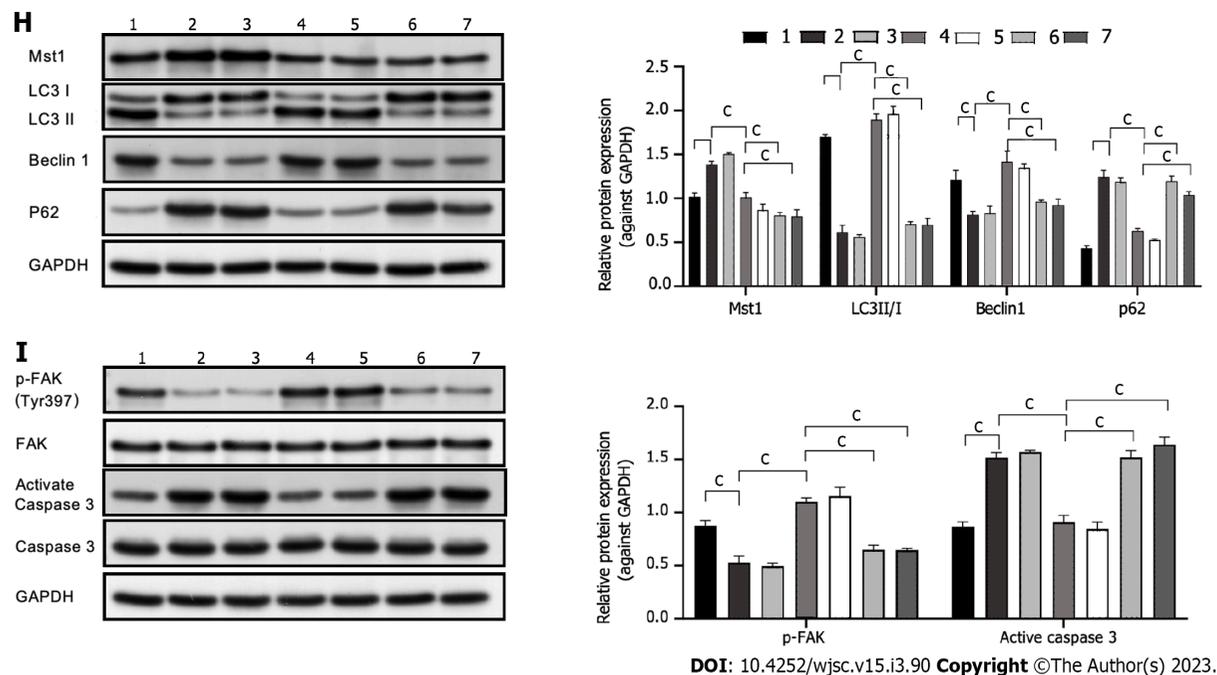
Corresponding alterations in cell-ECM isolation and autophagy also exist[17]. As a special type of apoptotic cell death, anoikis contributes to the loss of cell attachment to the ECM[18,19]. In the present study, we observed increased cell apoptosis and inhibited autophagy, as well as upregulated Mst1 expression in isolated mBMSCs. One hypothesis derived from a combination of previous studies is that Mst1 inhibition can not only overcome anoikis but also induce autophagy in isolated mBMSCs. In this study, we confirmed that mBMSCs averted anoikis by Mst1 inhibition-induced autophagy. Autophagy promotes cell survival or apoptosis in a stimulus-dependent manner. A series of experiments have elucidated the role of autophagy in promoting cell survival during anoikis[20]. Accordingly, our results established Mst1 inhibition-induced autophagy as a survival mechanism in isolated mBMSCs.

ITGs are transmembrane  $\alpha\beta$  heterodimers, with at least 18 well-known  $\alpha$  and 8  $\beta$  subunits. An increasing amount of experimental data has demonstrated that cells can overcome anoikis by changing ITGs expression[21]. In addition, ITGs-mediated cell adhesion to ECM is critical for maintaining appropriate cellular function and survival[22]. Therefore, the upregulation of ITGs allows cells to survive during anoikis[9,22,23]. This study has proved that the expression of ITG $\alpha$ 5 and ITG $\beta$ 1 were increased in cultured mBMSCs/sh-Mst1 under cell isolation conditions. Furthermore, upregulated ITG $\alpha$ 5 and ITG $\beta$ 1 expression may be the underlying mechanism of anoikis resistance in mBMSCs/sh-Mst1. These results suggested the role of ITG $\alpha$ 5 $\beta$ 1 downstream of Mst1, as well as a collaboration between ITG $\alpha$ 5 and ITG $\beta$ 1, in anoikis-resistant mBMSCs/sh-Mst1.

ITGs relay signals from the ECM to initiate intracellular signalling through intracellular ROS production[24], by which p-FAK expression is mediated[25]. Moreover, a recent study confirmed that excessive or persistent increases in ROS levels might promote the process of anoikis[26]. However, high ROS levels may also promote the formation of autophagy, which could contribute to reducing ROS accumulation[27]. Despite the essential role of increased ROS levels in anoikis resistance reported in several studies[28], we still hypothesized the necessity of appropriate cellular regulation of ROS levels for anoikis inhibition. As a result, we speculated that a negative-feedback loop was formed among Mst1 inhibition-induced autophagy, Mst1 inhibition-triggered ITG $\alpha$ 5 $\beta$ 1 and ROS levels. Mst1 inhibition increased ITG $\alpha$ 5 $\beta$ 1 expression, thereby facilitating cell adhesion. In addition, Mst1 inhibition-induced autophagy reduced the level of ITG $\alpha$ 5 $\beta$ 1-triggered ROS in isolated mBMSCs, which contributed to the evasion of anoikis, elucidating why 3-MA did not affect the expression of ITG $\alpha$ 5 or ITG $\beta$ 1.

Mst1 has been known to play a key role in the signalling pathway that controls manifold cellular processes[29]. In the present study, silencing Mst1 expression was found to ameliorate the anti-inflammatory cytokine production, osteogenic differentiation capability and cell proliferation of mBMSCs, thereby making mBMSCs/sh-Mst1 an attractive target for anti-inflammatory, immunomodulatory, and regenerative therapies and potentially improving the curative efficacy of mBMSCs in PAH[1-3].





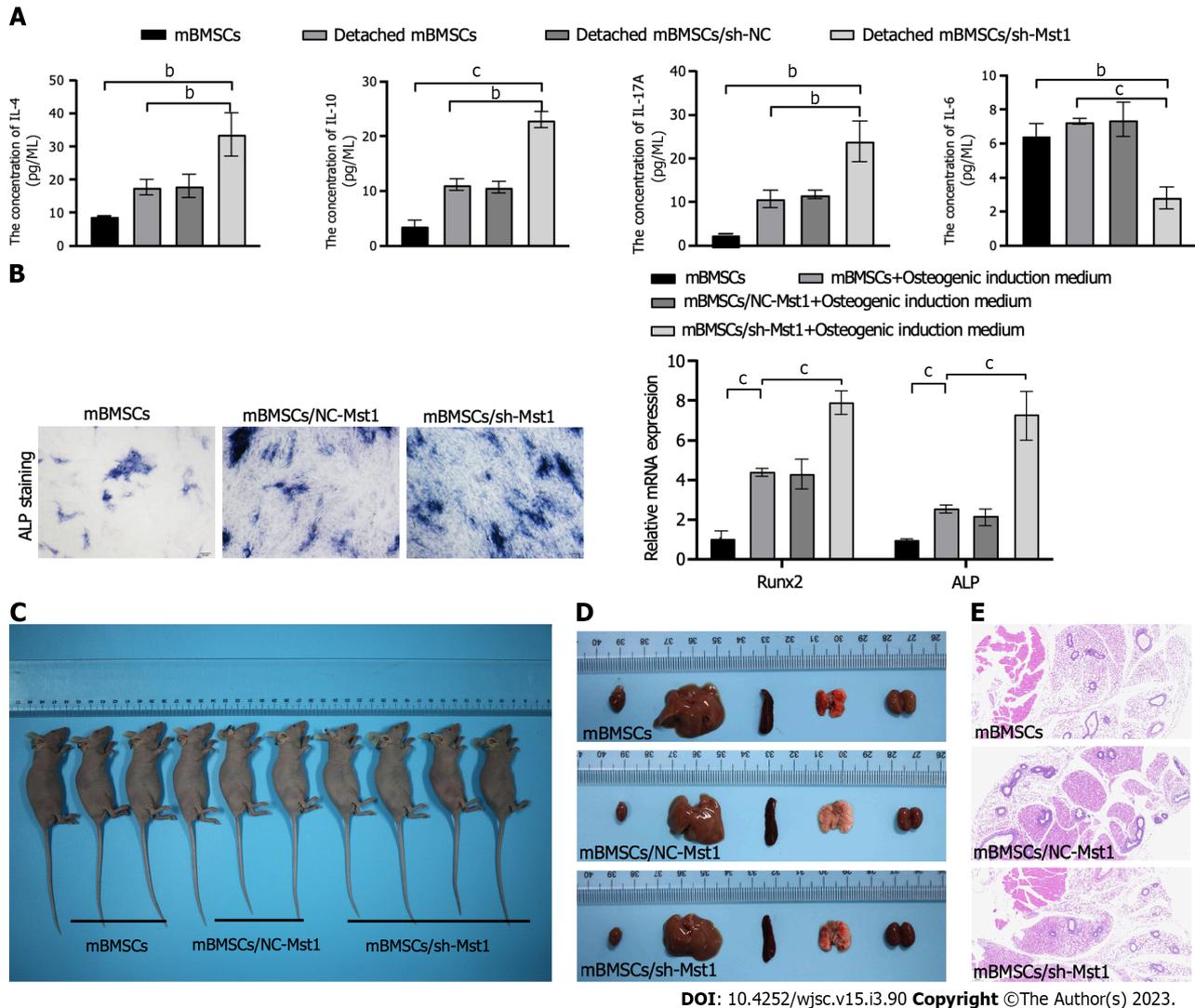
**Figure 4 Upregulated integrin $\alpha$ 5 $\beta$ 1 expression triggered by Mammalian Ste20-like kinase 1 inhibition protected mouse bone marrow mesenchymal stem cells from anoikis.** A: Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling (TUNEL)-positive cells analysed by flow cytometry; B: Representative images and quantification of fluorescence staining of dead cells; C: Reactive oxygen species (ROS) levels were measured by flow cytometry; D: Quantitative analysis of the rate of TUNEL-positive cells; E: Quantification of live cells using a thiazolyl blue tetrazolium bromide assay; F: Quantitative analysis of the intracellular ROS level; G: Cell adhesion was evaluated as the fold change compared back to the control groups; H and I: Western blotting was used to measure the expression levels of Mammalian Ste20-like kinase 1 (Mst1), microtubules associated protein 1 light chain 3 II/I, p62, Beclin1, phospho-focal adhesion kinase, and activated caspase 3. glyceraldehyde-3-phosphate dehydrogenase was used as the loading control. Values are expressed as the mean  $\pm$  SD. Measurements were performed in three replicates. 3-MA: 3-methyladenine; TUNEL: Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling; ROS: Reactive oxygen species; Mst1: Mammalian Ste20-like kinase 1; LC3-II/I: Microtubules associated protein 1 light chain 3 II/I; p-FAK: Phospho-focal adhesion kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. 1. Control group; 2. Anoikis group; 3. Anoikis + sh-NC group; 4. Anoikis + sh-Mst1 group; 5. Anoikis + sh-Mst1 + si-NC group; 6. Anoikis + sh-Mst1 + si-ITG $\alpha$ 5; 7. Anoikis + sh-Mst1 + si-ITG $\beta$ 1. <sup>a</sup> $P$  < 0.01; <sup>b</sup> $P$  < 0.05; <sup>a</sup> $P$  > 0.05.

Regardless of the extraordinary safety profile of MSC therapy verified in clinical trial data, several scholarly reviews have proposed that MSCs play a role in tumorigenesis and progression[18,30,31]. Therefore, the enhancement of the anti-anoikis ability of MSCs may promote tumorigenesis. However, in the present study, tumorigenic experiments in nude mice demonstrated the safety profile of mBMSC/sh-Mst1 administration.

## CONCLUSION

In summary, the mechanism by which Mst1 inhibition acts on anoikis in mBMSCs was expounded in

this study. First, Mst1 inhibition was demonstrated to ameliorate not only autophagy formation but also ITG $\alpha$ 5 $\beta$ 1 expression. Second, Mst1 inhibition-induced autophagy could scavenge the excessive production of ITG $\alpha$ 5 $\beta$ 1-triggered ROS. Third, silencing Mst1 expression not only ameliorated the pluripotency of mBMSCs but also retained the safety profile of mBMSCs. Overall, Mst1 inhibition-based infusion may improve the therapeutic efficacy of MSCs, thereby serving as the ideal candidate for clinical transplant therapy in PAH.



**Figure 5 The properties and biological safety of mouse bone marrow mesenchymal stem cells/sh-Mammalian Ste20-like kinase 1.** A: The levels of interleukin-4 (IL-4), IL-10, IL-17A and IL-6 were measured using a Cytometric Bead Array Cytokine Kit in detached mouse bone marrow mesenchymal stem cells (mBMSCs); B: Representative images of alkaline phosphatase (ALP) staining of mBMSCs after culture in osteogenesis induction medium for 21 d; C: Quantitative real-time polymerase chain reaction analysis of the relative mRNA expression levels of runt-related transcription factor 2 (Runx2) and ALP in mBMSCs cultured in adipogenesis induction medium for 21 d; D: Nude mice were subcutaneously implanted with mBMSCs ( $n = 3$ ), mBMSCs/NC-Mst1 ( $n = 3$ ), and mBMSCs/sh-Mst1 ( $n = 4$ ). Representative images of the heart, liver, spleen, lung and kidney in each group 60 d postinjection; E: Paraffin-embedded subcutaneous sections derived from cell-injection sites were stained with haematoxylin and eosin. Data are the mean  $\pm$  SD of three technical replicates. mBMSCs: Mouse bone marrow mesenchymal stem cells; Mst1: Mammalian Ste20-like kinase 1; IL: Interleukin; Runx2: Runt-related transcription factor 2; ALP: Alkaline phosphatase.  $^{\circ}P < 0.01$ ;  $^bP < 0.05$ ;  $^aP > 0.05$ .

## ARTICLE HIGHLIGHTS

### Research background

Anoikis plays a limiting role in the therapeutic efficacy of mesenchymal stem cells (MSCs). As a proapoptotic molecule, mammalian Ste20-like kinase 1 (Mst1) can increase the production of reactive oxygen species (ROS), thereby promoting anoikis. Recently, Mst1 inhibition was found to protect mouse bone marrow MSCs (mBMSCs) from H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis by inducing autophagy and reducing ROS production. However, the influence of Mst1 inhibition on anoikis in mBMSCs remains unclear.

### **Research motivation**

To investigate whether Mst1 inhibition could reduce anoikis in isolated mBMSCs.

### **Research objectives**

To investigate the mechanisms by which Mst1 inhibition acts on anoikis in isolated mBMSCs.

### **Research methods**

Poly-2-hydroxyethyl methacrylate-induced anoikis was used following Mst1 inhibition in mBMSCs. Integrin (ITGs) levels were tested by flow cytometry. Autophagy and ITG $\alpha$ 5 $\beta$ 1 were inhibited using 3-methyladenine and small interfering RNA, respectively. The alterations in anoikis were evaluated by Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling and anoikis assays. The levels of the anoikis-related proteins ITG $\alpha$ 5, ITG $\beta$ 1, and phospho-focal adhesion kinase, which activate caspase 3, and the autophagy-related proteins microtubules associated protein 1 light chain 3 II/I, Beclin1 and p62 were detected by Western blotting.

### **Research results**

In isolated mBMSCs, Mst1 expression was upregulated, and Mst1 inhibition significantly reduced cell apoptosis, induced autophagy and decreased ROS levels. Mechanistically, we found that Mst1 inhibition upregulated ITG $\alpha$ 5 and ITG $\beta$ 1 expression but not ITG $\alpha$ 4, ITG $\alpha$ v, or ITG $\beta$ 3 expression. Moreover, ITG $\alpha$ 5 $\beta$ 1 upregulation and autophagy induction by Mst1 inhibition played an essential role in terms of the protective efficacy of Mst1 inhibition on averting anoikis.

### **Research conclusions**

Mst1 inhibition ameliorated autophagy formation, increased ITG $\alpha$ 5 $\beta$ 1 expression, and decreased the excessive production of ROS, thereby reducing cell apoptosis in isolated mBMSCs. On this basis, Mst1 inhibition may provide a promising strategy to overcome the anoikis of transplanted MSCs.

### **Research perspectives**

In isolated mBMSCs, Mst1 inhibition ameliorated not only autophagy formation but also ITG $\alpha$ 5 $\beta$ 1 expression (not ITG $\alpha$ 4, ITG $\alpha$ v, or ITG $\beta$ 3). Mst1 inhibition-induced autophagy scavenged excessive ITG $\alpha$ 5 $\beta$ 1-triggered ROS. Consequently, Mst1 inhibition-based infusion may improve the therapeutic efficacy of MSCs, thereby serving as an ideal candidate for clinical transplantation in pulmonary arterial hypertension.

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## **FOOTNOTES**

**Author contributions:** Yu WC contributed to the conception and design and manuscript writing; Zhang T contributed to conception and design, collection and assembly of data, data analysis and interpretation. Yu WC, Zhang T, Zhang Q performed the experiments; All authors participated in discussing, revising the manuscript, and approving the final manuscript; All authors have read and approve the final manuscript.

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