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Down-regulation of the autophagy gene, *ATG7*, protects bone marrow-derived mesenchymal stem cells from stressful conditions

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Background
Mesenchymal stem cells (MSCs) are valuable for cell-based therapy. However, their application is limited owing to their low survival rate when exposed to stressful conditions. Autophagy, the process by which cells recycle the cytoplasm and dispose of defective organelles, is activated by stress stimuli to adapt, tolerate adverse conditions, or trigger the apoptotic machinery. This study aimed to determine whether regulation of autophagy would affect the survival of MSCs under stress conditions.

Methods
Autophagy was induced in bone marrow-derived MSCs (BM-MSCs) by rapamycin, and was inhibited via shRNA-mediated knockdown of the autophagy specific gene, *ATG7*. *ATG7* expression in BM-MSCs was evaluated by reverse transcription polymerase chain reaction (RT-PCR), western blot, and quantitative PCR (qPCR). Cells were then exposed to harsh microenvironments, and a water-soluble tetrazolium salt (WST)-1 assay was performed to determine the cytotoxic effects of the stressful conditions on cells.

Results
Of 4 specific *ATG7*-inhibitor clones analyzed, only shRNA clone 3 decreased *ATG7* expression. Under normal conditions, the induction of autophagy slightly increased the viability of MSCs while autophagy inhibition decreased their viability. However, under stressful conditions such as hypoxia, serum deprivation, and oxidative stress, the induction of autophagy resulted in cell death, while its inhibition potentiated MSCs to withstand the stress conditions. The viability of autophagy-suppressed MSCs was significantly higher than that of relevant controls ($P<0.05$, $P<0.01$ and $P<0.001$).

Conclusion
Autophagy modulation in MSCs can be proposed as a new strategy to improve their survival rate in stressful microenvironments.

Key Words Autophagy, Mesenchymal stem cells, Oxidative stress, Cell survival, shRNA

INTRODUCTION

Mesenchymal stem cells (MSCs) are valuable cells in cell-based therapy and have been extensively investigated as a new modality for treatment of several diseases [1]. Different tissue-derived MSCs were characterized and expanded *in vitro* [2] to be utilized for cell-therapy proposes [3]. However, they demonstrate low proliferative and survival rates during long *in vitro* expansion or after *in vivo* transplantation [4]. The microenvironments surrounding MSCs contain several hazardous stressors such as poor nutrition [5] and oxidative stress, mainly mediated by reactive oxygen species (ROS) [6].

Practical strategies should therefore be employed to improve the underlying mechanisms of MSC resistance to harsh stress conditions, thus leading to higher survival rates and consequently higher engraftment rates after transplantation [7].

Autophagy (self-digestion), also described as the garbage disposal system of the cell, involves lysosomal-mediated catabolism of long-lived proteins and damaged organelles,

which are sequestered by autophagosomes or specific autophagy vacuoles [8]. Basal autophagy regulates a wide range of physiological and pathophysiological processes, from starvation adaptation and homeostasis to anti-aging and cell death [9]. Autophagy was initially described as a survival mechanism that recycles degraded intracellular components to serve as new building blocks for the synthesis of macromolecules and metabolites, which were then used as the cellular energy source for cell survival [10]. However, autophagy seems to play dual roles depending on the cell type and context. In the context of cellular death, the persistent and extensive occurrence of autophagy leads to the catabolism of cytoplasmic materials, to an extent that causes metabolic and bio-energetic cell collapse. Recent studies have shown that autophagy might act as a double-edged sword, leading to either cell death or survival by inactivation of key autophagy genes, particularly *ATG7* [11, 12].

ATG7 is an indispensable gene for starvation-induced autophagy [13]. Its function in the autophagy process is well known, and its inactivation leads to a significant decrease in the cellular autophagy phenomenon, regardless of any interference from other cellular pathways [14].

Interestingly, few studies have investigated autophagy in stem cells [15], including MSCs, and its role in this cell type is still controversial. This study therefore aimed to address whether autophagy protects or exacerbates MSCs following exposure to various types of stress conditions.

MATERIALS AND METHODS

Isolation of MSCs from human bone marrow aspirates

Human MSCs were isolated from the bone marrow of healthy volunteers in the bone marrow transplantation (BMT) center of Shariati hospital under the institutional protocol and consent procedure. These human bone marrow-derived-mesenchymal stem cells (hBM-MSCs) were successfully isolated using their adhesive characteristics as previously described [16]. hBM-MSC surface markers were detected by flow cytometry and their differentiation potentiality for osteogenesis, chondrogenesis, and adipogenesis was analyzed as previously described [16].

Autophagy suppression by gene silencing of *ATG7* using short hairpin RNA

To suppress autophagy in hBM-MSCs, the RNAi (RNA interference) technique was performed, since it is the most specific strategy for knockdown studies. SureSilencing short hairpin RNA (shRNA) plasmids targeting human *ATG7* were purchased from Qiagen Inc. (SABioscience Qiagen, Germany). This product contains a negative control and 4 *ATG7* shRNA plasmids (no. 1-4), of which only one caused a significant knockdown of *ATG7*. *ATG7*-targeting shRNA plasmids were separately cloned into DH5α E. coli (Invitrogen, USA). Competent cells were cultured in Luria-Bertani (LB) broth medium, followed by plasmid extraction.

Transfection of hBM-MSCs

Following plasmid extraction, hBM-MSCs were transfected with the negative control and each of the 4 shRNA vectors using the transfection reagent FuGENE HD (Roche, Germany) according to the manufacturer's protocol. The transfected cells were named MSC-shRNA 1, MSC-shRNA 2, MSC-shRNA 3, MSC-shRNA 4 according to the number of the shRNA vector, and MSC-shRNA Cont for the negative control shRNA transfected MSCs. A plasmid containing EGFP conjugated ubiquitous microtubule-associated protein 1A/1B-light chain 3 (pEGFP-LC3m) was obtained from Dr. Y. Kuwahara, Department of Pathology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Miyagi, Japan as a gift. hBM-MSCs were also transfected with pEGFP-LC3m, and were named MSC-LC3. After transfection for 48 hours, MSC-LC3 cells were observed under a fluorescence microscope. RT-PCR analysis was performed to select proper shRNA vectors, which could effectively knock down *ATG7* mRNA.

Autophagy induction using rapamycin

Rapamycin (Rapa), a well-known inducer of autophagy, was used to activate the autophagy pathway. To optimize rapamycin (Invitrogen, USA) concentration, hBM-MSCs were cultured in 96-well plates (Orange Scientific, Belgium), and different rapamycin doses (10, 200, and 500 nM) were added and incubated for 24 hours in a standard cell-culturing incubator. The rapamycin-treated hBM-MSCs were named MSC-10 nM Rapa, MSC-200 nM Rapa, and MSC-500 nM Rapa according to the rapamycin dose applied. MSC-LC3, MSC-shRNA Cont, and MSC-shRNA 3 treated with the optimized rapamycin dose were named MSC-LC3-Rapa, MSC-shRNA Cont-Rapa and MSC-shRNA 3-Rapa, respectively. These rapamycin-treated hBM-MSCs were then subjected to western blot analysis.

Western blot analysis

Ubiquitous microtubule-associated protein 1A/1B-light chain 3 (LC3) is a soluble protein in mammalian cells. During the autophagy process, the cytosolic form of LC3 (LC3-I) is converted to a lipid bound form (LC3-II) [17]. Western blot analysis was performed to evaluate these autophagy markers (LC3-I and LC3-II) at the translational level. Whole cell extract was isolated from different rapamycin-treated cells as well as the untreated control. Samples were run on 16% SDS-polyacrylamide gel, and then, transferred to a PDVF membrane. Samples were then incubated with diluted rabbit anti-LC3-I and LC3-II (1 : 1,000) (Cell Signaling Technology, USA) primary antibodies for 3 hrs. After primary antibody incubation, the membrane was washed 3 times, and then incubated with an HRP-conjugated goat anti-rabbit secondary antibody for 1.5 hrs. Finally, enhanced chemiluminescence (ECL) (Abcam, UK) substrate solutions were used to visualize the proteins on the PVDF membrane using a chemiluminescence imaging system. β-actin served as an internal loading control protein.

RT-PCR

Total RNA was extracted from transfected hBM-MSCs using the Tripure reagent (Roche, Germany) according to the manufacturer's instructions. The first-strand cDNA was generated from 1 μg of the isolated total RNA using the cDNA synthesis kit (Bioneer, USA) according to the manufacturer's instructions. Appropriate primer sets for *ATG7* and *β-actin I* were designed using the Primer3 software and the primers used are as follows: *ATG7* forward: 5'-ACCCAG AAGAAGCTGAACGA-3' and reverse: 5'-CTCATTTGCTGC TTGTTCCA-3'; *β-actin* forward: 5'-TTCTACAATGAGCTG CGTGTGG-3' and reverse: 5'-GTGTTGAAGGTCTCAAACA TGAT-3').

PCR reactions were performed using Taq DNA polymerase (Roche, Germany). *β-actin* expression was used to normalize the gene expression. Electrophoresis of DNA fragments on 2% agarose gel and ethidium bromide staining was performed to detect *ATG7* expression.

qPCR

For further confirmation of the RT-PCR results, cDNA from MSC-shRNA 3 and MSC-shRNA Cont was subjected to qPCR analysis to quantify the suppression level of *ATG7*. qPCR was performed in triplicate using specific primers (described above) and the SYBR Green Real-Time PCR Master Mix (Takara, Japan) on the Rotor gene RG-3000 system (Corbett, Germany). The cycle threshold (Ct) was automatically calculated, and the *β-actin* Ct value was used for normalization.

Induction of different stress conditions

To investigate the effects of autophagy on cellular survival rate, the different groups of hBM-MSCs including autophagy-suppressed hBM-MSCs (MSC-shRNA 3), Rapamycin-treated hBM-MSCs (MSC-Rapa), MSC-shRNA Cont, and normal h-BM-MSCs without any treatment (MSC-Cont) were cultured under hypoxic, serum-deprived, and oxidative stress conditions. Cells were seeded at a density of 2×10^4 cells/well in a 96-well plate. To induce oxidative stress, cells were exposed to different doses of 1, 2, 3, 4, and 5 mM hydrogen peroxide (H_2O_2) (Sigma, Germany) for 1 hour. For induction of serum deprivation, cells were washed three times with serum-free medium and maintained in this condition for various lengths of time. The hypoxic condition was created by transferring plates to a Galaxy 48 R hypoxic incubator (New Brunswick, Germany). These cells were incubated at 37°C in the presence of 5% CO_2, 1% O_2, and N_2 for different lengths of time. Each test was done in triplicate and the cytotoxic effects of H_2O_2, hypoxia, or serum deprivation on cells were determined by a WST-1 cytotoxicity assay. The viability of the four MSC groups: MSC-shRNA3, MSC-Rapa, MSC-shRNA Cont and MSC-Cont, were also studied under normal conditions *i.e.* in the absence of any stress condition.

WST-1 assay

A WST-1 assay was performed to evaluate the viability of MSCs. The WST-1 reagent (Sigma, Germany) was added to cells at a concentration of 10 μL/well (Sigma, Germany) in 100 μL culture medium and the samples were incubated at 37°C for 4 hours. The optical density (OD) of each sample was measured using a microplate reader at 450 nM.

Statistical analysis

The quantitative results were analyzed using the SPSS statistical software (version 19) (SPSS Inc., Chicago, IL, USA). Statistically significant differences ($P<0.05$) were determined by an analysis of variances (one-way ANOVA) analysis.

RESULTS

Down-regulation of *ATG7* resulted in suppression of autophagy in MSCs

Four different *ATG7* shRNA vectors containing GFP (no. 1-4) were transfected into hBM-MSCs, as well as a negative control shRNA vector. After 48 hours, hBM-MSCs that were transfected with the *ATG7* shRNA clone 3 (MSC-shRNA 3) were observed under a fluorescence microscope (Fig. 1A). RT-PCR analysis of the various transfected cells indicated that MSC-shRNA 3 expressed lower levels of *ATG7* mRNA than MSC-shRNA Cont. and other shRNA MSC transfected cells (1, 2 and 4) (Fig. 1B).

Next, qPCR was performed to evaluate the level of *ATG7* suppression in MSC-shRNA 3 compared to MSC-shRNA Cont. Expression of *ATG7* in MSCs-shRNA 3 was considerably lower than in MSC-shRNA Cont ($P<0.001$) (Fig. 1C). Taken together, the shRNA clone 3 was selected as the best *ATG7* suppressive vector.

Rapamycin was then added to cells at different concentrations in order to induce autophagy. Following activation of the autophagy pathway, the conversion of LC3I to LC3II leads to a higher intensity of the LC3II band and an elevated relative ratio of LC3 II to LC3 I. The induction of autophagy was detected after 24-hour-treatment with 500 nM rapamycin (Fig. 2A).

Autophagy was also investigated in hBM-MSCs transfected with GFP-LC3 plasmids (MSC-LC3). MSC-LC3 were exposed to 500 nM rapamycin (the optimized autophagy inducing dose) for 24 hours (MSC-LC3-Rapa), and then observed under a fluorescence microscope. Rapamycin induced stimulation of autophagy resulted in the formation of shiny green dots in MSC-LC3-Rapa (Fig. 2B.I), which were not observed in untreated MSC-LC3 (Fig. 2B.II). MSC-shRNA 3 and MSC-shRNA Cont were also treated with 500 nM rapamycin (MSC-shRNA 3-Rapa and MSC-shRNA Cont-Rapa, respectively). Induction of autophagy was observed in MSC-shRNA Cont-Rapa as determined by the presence of shiny green dots (Fig. 2B.III). However this was not observed in MSC-shRNA 3-Rapa, indicating that rapamycin treatment could not induce autophagy under *ATG7* suppression.

Western blot analysis of MSC-shRNA Cont-Rapa and MSC-shRNA 3-Rapa also indicated that the down-regulation

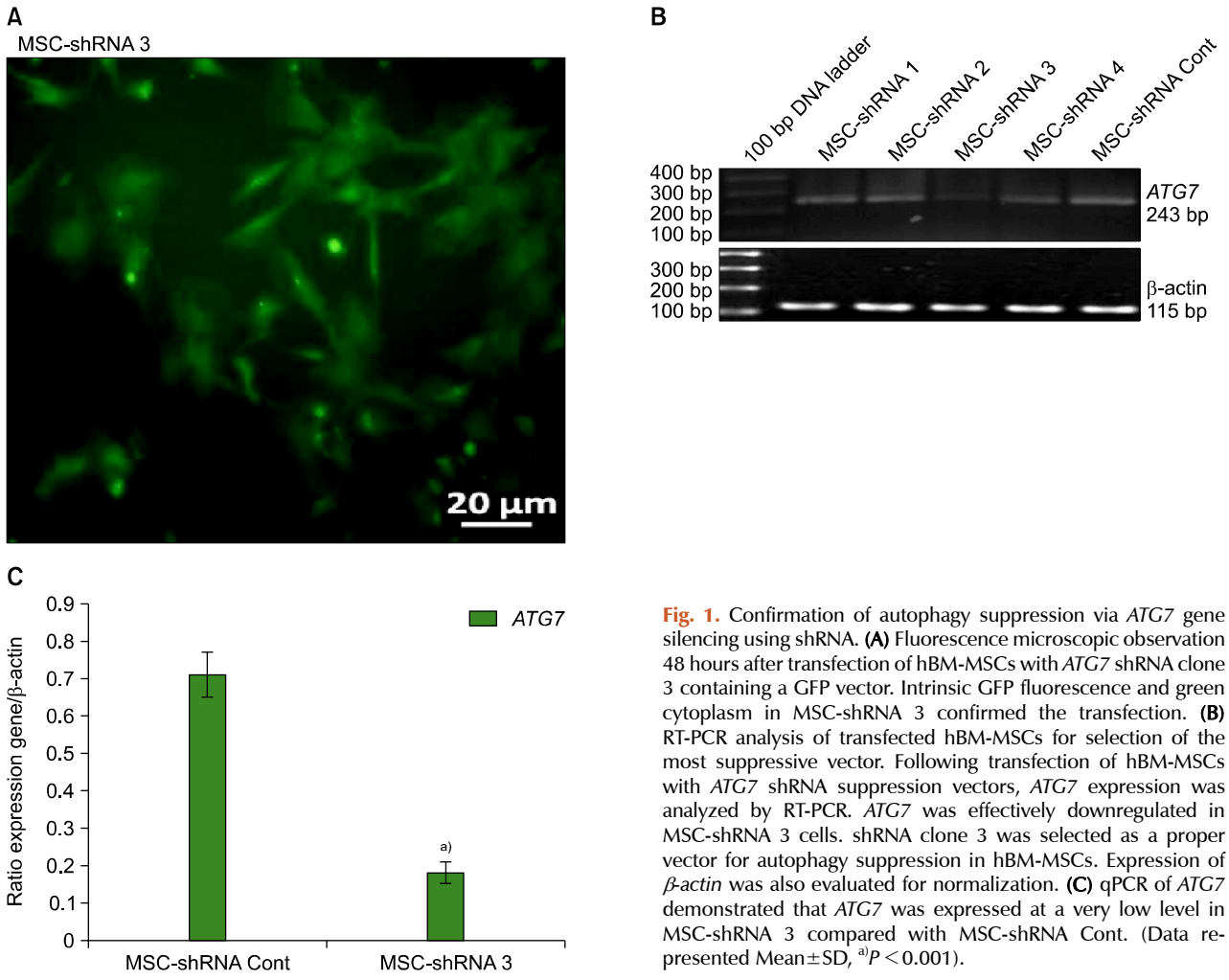

Fig. 1. Confirmation of autophagy suppression via *ATG7* gene silencing using shRNA. **(A)** Fluorescence microscopic observation 48 hours after transfection of hBM-MSCs with *ATG7* shRNA clone 3 containing a GFP vector. Intrinsic GFP fluorescence and green cytoplasm in MSC-shRNA 3 confirmed the transfection. **(B)** RT-PCR analysis of transfected hBM-MSCs for selection of the most suppressive vector. Following transfection of hBM-MSCs with *ATG7* shRNA suppression vectors, *ATG7* expression was analyzed by RT-PCR. *ATG7* was effectively downregulated in MSC-shRNA 3 cells. shRNA clone 3 was selected as a proper vector for autophagy suppression in hBM-MSCs. Expression of *β-actin* was also evaluated for normalization. **(C)** qPCR of *ATG7* demonstrated that *ATG7* was expressed at a very low level in MSC-shRNA 3 compared with MSC-shRNA Cont. (Data represented Mean±SD, $^{a)}P<0.001$).

of *ATG7* prevented autophagy induction even after rapamycin treatment (Fig. 2C). This was also further confirmed by transfection of hBM-MSCs with GFP-LC3 plasmids (data not shown).

Suppression of autophagy protects MSCs from hypoxic, serum-deprived, and oxidative stress conditions

To determine the effects of autophagy induction or inhibition on the survival rate of MSCs, cells were cultivated under normal conditions for five days, and cell viability was evaluated using the WST-1 assay. Survival of MSCs increased with the induction of autophagy and decreased with autophagy inhibition, however the differences were not significant ($P>0.05$) (Fig. 3A).

Next, all hBM-MSCs groups including autophagy-suppressed MSCs (MSC-shRNA 3), autophagy-induced MSCs (MSC-Rapa) and their relevant controls, MSC-shRNA Cont, and normal MSCs without any treatment (MSC-Cont) were exposed to hypoxic, serum-deprived, and oxidative stress conditions. As shown in Fig. 3B, the cells with *ATG7* knockdown demonstrated lower cell death rates at higher H_2O_2 doses ($P<0.01$; $P<0.001$). This observation indicates that autophagy suppression results in cytoprotection against strong oxidative stress (Fig. 3B). MSC-Rapa cells demonstrated high cell viability compared to MSC-shRNA3 after 24 hours of serum deprivation. This finding demonstrates the protective effects of autophagy on the survival of hBM-MSCs against serum deprivation-induced toxicity 24 hours after starvation. Interestingly, after 48 or 72 hours of serum deprivation, MSC-shRNA 3 demonstrated stronger resistance to cell death compared to other groups (Fig. 3C).

Following 24-hour exposure to hypoxic conditions, the viability of MSC-shRNA 3 was higher than that of MSC-Rapa and the control group. This indicates that *ATG7* knockdown protected hBM-MSCs against hypoxia-induced cytotoxicity (Fig. 3D). Overall, these findings suggest that inhibition of *ATG7* plays a critical role in the protection of MSCs from hypoxia, serum deprivation, and oxidative stress.

DISCUSSION

The advancement of MSC-based cell therapy is hindered by the low viability of infused MSCs. Harsh microenvironments such as those induced by hypoxia, serum deprivation,

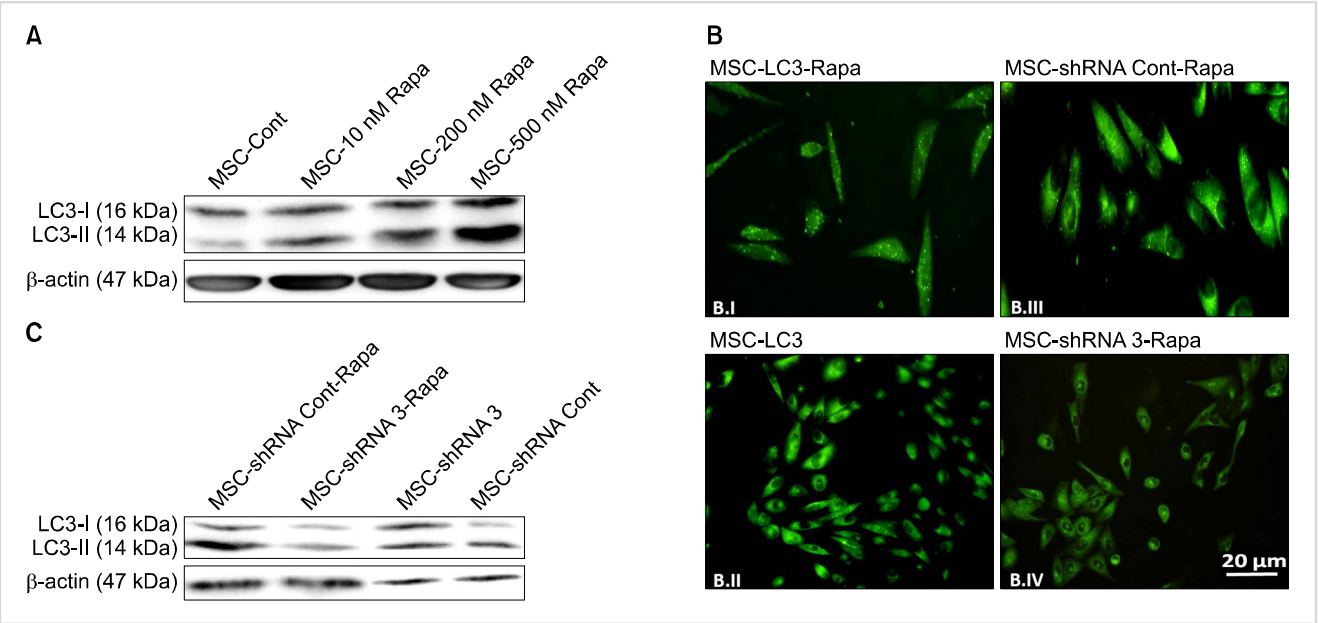

Fig. 2. Confirmation of autophagy induction with rapamycin. **(A)** Western blot analysis of 24 hour-rapamycin-treated hBM-MSCs exposed to 10, 200, and 500 nM rapamycin. LC3-I induction and conversion of LC3-I to LC3-II were determined by western blot analysis. Expression of LC3-II and its significantly increased level compared to LC3-I in MSCs-500 nM Rapa indicated induction of autophagy in these cells. β-actin served as the loading control. **(B)** Microscopic observation of transfected hBM-MSCs before and after rapamycin treatment. hBM-MSCs were transfected with GFP-LC3 containing a GFP vector (MSC-LC3), and 48 hours later, MSC-LC3 was treated with 500 nM rapamycin for 24 hours (MSC-LC3-Rapa). Fluorescence microscopic observations indicated that MSC-LC3-Rapa manifested green dots (punctate signal) (B.I), which confirmed effective autophagy induction in MSC-LC3-Rapa compared to MSC-LC3 (B.II). Autophagy was observed in MSC-shRNA Cont-Rapa cells, which were transfected with a negative control shRNA vector and treated with rapamycin (shiny green dots) (B.III). Very few green dots were detected in MSC-shRNA 3-Rapa cells, which were treated with rapamycin after suppression of autophagy (B.IV). **(C)** Western blot analysis of shRNA 3-transfected hBM-MSCs before and after rapamycin treatment. hBM-MSCs were treated with 500 nM rapamycin for 24 hours following transfection with *ATG7*-shRNA clone 3 (*ATG7* suppressive vector) and the negative control shRNA, and subjected to western blot analysis.

and oxidative stress are well-known causes of MSC death *in vivo* [18, 19].

Autophagy was one of the most ambiguous concepts, due to its dual role in cellular life and death depending on the cell source and the type of stress the cell is exposed to [20].

Our findings indicate that inhibition of *ATG7*, one of the key autophagy genes, exerts protective influence on MSCs, by allowing them to withstand harsh microenvironments. We determined that under mild nutrient deprivation or oxidative stress, autophagy acts in favor of MSC survival; however, under severe stress conditions, it induces cell death. Specifically, under conditions of severe stress, inhibition of autophagy plays an important role in the protection of MSCs. Our findings are consistent with that of Kuwahara *et al.* [21], where they observed an increase in cell survival when autophagy was inhibited in a cancer cell line following radiation exposure [21]. Also in agreement with our results, Han *et al.* reported that time-dependent induction of autophagy in cardiac myocytes leads to cell injury, while its inhibition results in cell viability [22]. Yu *et al.* reported that hippocampal neural stem cells treated with rapamycin followed by insulin withdrawal increased cell death while siRNA knockdown of *ATG7* led to cell survival [23]. In the present study, we applied an autophagy specific *ATG7*-shRNA knockdown method using bacterial plasmids, which are considered transient and safe vectors. However, some studies demonstrate results that are inconsistent with our findings. For example, Hou *et al.* reported that autophagy acts as a preventive factor against ROS generation and irradiation injury in MSCs [24]. Furthermore, Mortensen *et al.* reported that hematopoietic stem cells (HSCs) lacking *ATG7* are unable to survive *in vivo*. In their analyses, *ATG7* was eliminated in the hematopoietic system via *ATG7* gene knockout, which resulted in a decrease in the population of HSCs and progenitors of multiple lineages [25]. However, in our study, we induced autophagy knockdown −not knockout− to suppress the basal autophagy level of MSCs in order to ensure normal cellular homeostasis.

More recently, Song *et al.* suggested that autophagy induction is a survival response to oxidative stress in murine bone marrow-derived mesenchymal stromal cells [26]. In their study, autophagy was inhibited via non-specific genes (*ATG5*) and inhibitors such as 3MA and bafilomycin [27-29]. They also applied sub-lethal and prolonged low doses of an oxidative agent, which ensured the induction of senescence in MSCs [30]; however, our study suppressed *ATG7*, the most specific autophagy-related gene, and also utilized higher concentrations of H_2O_2. It is noteworthy that in the present study, the WST-1 results were similar that of Song *et al.*, although lower concentrations of H_2O_2 were used in their study.

Here, we provide evidence that the induction of autophagy

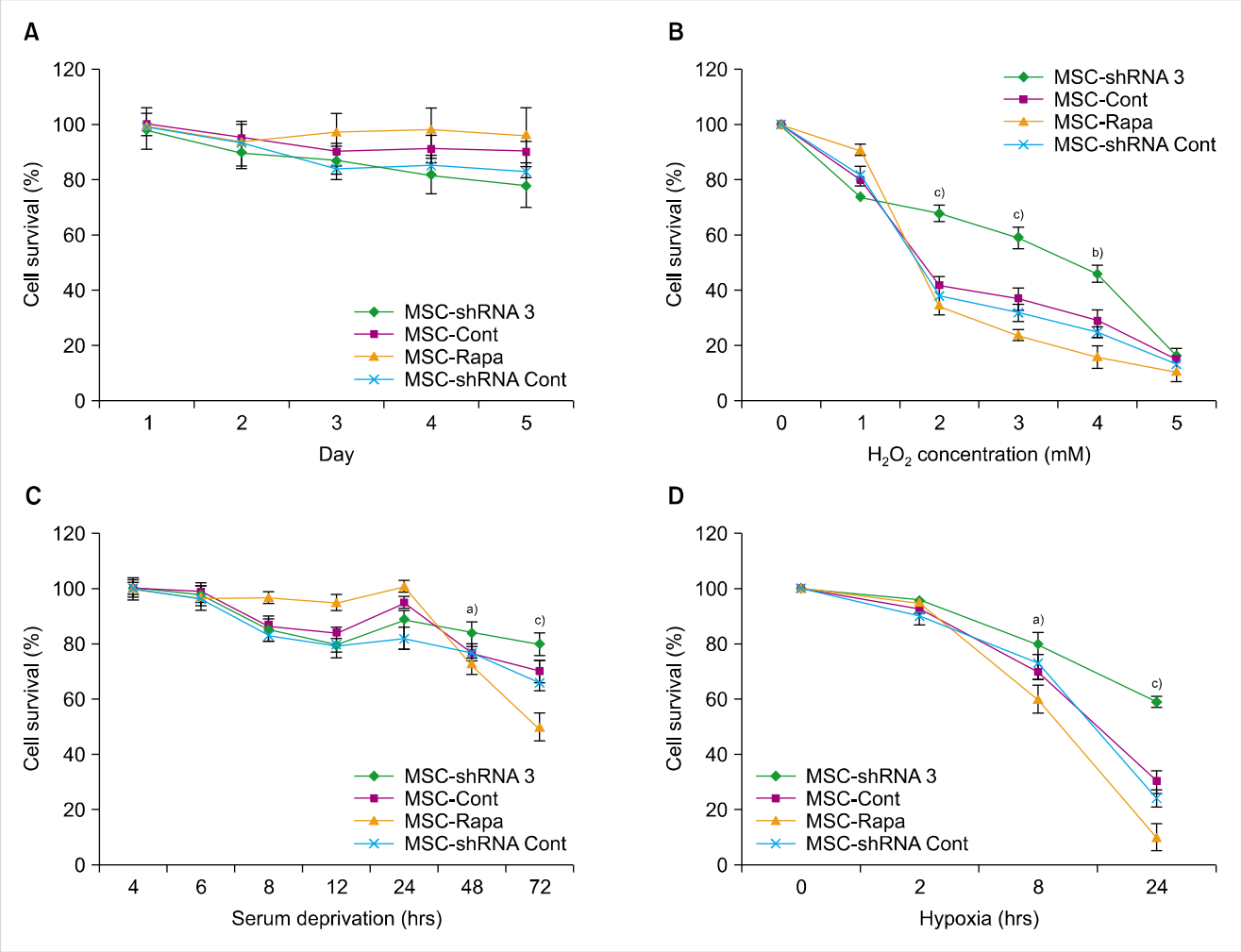

Fig. 3. WST-1 assay to detect the effects of autophagy on hBM-MSC survival. **(A)** Survival rates of MSC-shRNA3 (autophagy-suppressed MSCs), MSC-Rapa (autophagy-induced MSCs) and the relevant control groups (MSC-shRNA Cont and MSC-Cont) were assayed at daily intervals during a five-day treatment. **(B)** Both autophagy modulated and control hBM-MSCs were exposed to different H_2O_2 concentrations for 1 hour. The viability of MSC-shRNA 3 was higher than MSC-Rapa and controls (MSC-Cont and MSC-shRNA Cont) at 2-4 mM H_2O_2. **(C)** After exposing the experimental groups to serum deprivation for different time durations, we found that the suppression of autophagy in MSC-shRNA 3 renders them more resistant to severe SD stress compared to other groups. **(D)** Following 8 and 24-hour-duration of hypoxia, *ATG7*-shRNA knockdown protected the hBM-MSCs from cell death compared to other groups. This demonstrated that autophagy knockdown enhances survival of hBM-MSCs under severe persistent stress conditions (Data represented Mean±SD; $^{a)}P<0.05$, $^{b)}P<0.01$ and $^{c)}P<0.001$).

induces cell death while its inhibition protects MSCs in response to severe stressful microenvironments. Our findings highlight the importance of autophagy regulation in the fate of MSCs, and suggest a possible new strategy to prevent the death of engrafted cells in MSC-based cell therapy. It also emphasizes that management of cellular responses to stress can be harnessed in practical applications.

In conclusion, autophagy modulation can be proposed as a new strategy for improving the efficacy of MSC-based cell therapies as a result of enhanced survival rates. However, further ongoing studies including *in vivo* assessment of the therapeutic potential of autophagy modulated MSCs are required.

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Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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