

# Mitophagy promotes the stemness of bone marrow-derived mesenchymal stem cells

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## Impact statement

How to maintain the stemness of bone marrow-derived mesenchymal stem cells (BMSCs) in the culture system is a long-standing question. The present research revealed that mitophagy is critically involved in mitochondrial fission promotion of the stemness of BMSCs. This finding thus provides a critical insight into stem cell propagation in cultures for their clinical application since maintenance of the stemness of stem cells is among the most important issues in stem cells and regenerative medicine. Therefore, manipulation of mitochondrial dynamics in the preparation of stem cells for clinical application would be an important undertaking for both experimental and clinical studies.

## Abstract

Previous studies demonstrated that mitochondrial fission arguments the stemness of bone marrow-derived mesenchymal stem cells (BMSCs). Because mitophagy is critical in removing damaged or surplus mitochondrial fragments and maintaining mitochondrial integrity, the present study was undertaken to test the hypothesis that mitophagy is involved in mitochondrial fission-enhanced stemness of BMSCs. Primary cultures of rat BMSCs were treated with tyrphostin A9 (TA9, a potent inducer of mitochondrial fission) to increase mitochondrial fission, which was accompanied by enhanced mitophagy as defined by increased co-staining of MitoTracker Green for mitochondria and LysoTracker Deep Red for lysosomes, as well as the increased co-localization of autophagy markers (LC3B, P62) and mitochondrial marker (Tom20). A mitochondrial uncoupler, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was used to promote mitophagy, which was confirmed by an increased co-localization of mitochondrial and lysosome biomarkers. The argumentation of mitophagy was associated with enhanced stemness of BMSCs as defined by

increased expression of stemness markers Oct4 and Sox2, and enhanced induction of BMSCs to adipocytes or osteocytes. Conversely, transfection of BMSCs with siRNA targeting mitophagy-essential genes *Pink1/Prkn* led to diminished stemness of the stem cells, as defined by depressed stemness markers. Importantly, concomitant promotion of mitochondrial fission and inhibition of mitophagy suppressed the stemness of BMSCs. These results thus demonstrate that mitophagy is critically involved in mitochondrial fission promotion of the stemness of BMSCs.

**Keywords:** Bone marrow-derived mesenchymal stem cells, stem cell stemness, mitophagy, mitochondrial fission

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## Introduction

Bone marrow-derived mesenchymal stem cells (BMSCs) are adult stem cells that can proliferate and differentiate into mature stromal cells, such as osteocytes,<sup>1</sup> neurocytes, and cardiomyocytes.<sup>2,3</sup> Studies have shown that BMSCs participate in bone repairing, neurogenesis, and myogenesis under given pathological conditions either by differentiating into functional cells or through paracrine signaling.<sup>4–7</sup> A number of animal studies have confirmed BMSCs transplantation is a safe and efficient method to

promote tissue repair.<sup>6</sup> Over the last decade, BMSCs have been used in many clinical trials for the treatment of diverse diseases, such as nonunion,<sup>8</sup> stroke,<sup>9</sup> and cardiac infarct,<sup>10</sup> showing good prospects for clinical applications.

The expansion of BMSCs is necessary for their clinical applications, since they are rare in bone marrow (BM).<sup>11</sup> Nevertheless, BMSCs that cultured *in vitro* are prone to differentiate spontaneously.<sup>12–14</sup> The reduction of the stemness of stem cells not only reduces the effectiveness of stem cell-based cell therapies, but also presents a new

challenge in the safety of their use. Therefore, maintaining the stemness of BMSCs is of great significance for their clinical applications. In our previous study, we found mitochondrial fission, a process refers to the breakage of a single mitochondrion into multiple mitochondria, was crucial in maintaining the stemness of BMSCs.<sup>15</sup> The underlying mechanisms are worth to be explored.

Mitophagy is an autophagic process by which impaired or surplus mitochondria are engulfed in autophagosomes and then degraded in lysosomes. This process is important in controlling mitochondrial numbers as well as mitochondrial quality.<sup>16,17</sup> Studies in adult cardiomyocytes and HeLa cells have showed that mitochondrial fission promotes the translocation of Parkin to impaired mitochondria to initiate mitophagy, and inhibition of DRP1-mediated mitochondrial fission prevents mitophagy.<sup>18,19</sup> Some studies have found that mitophagy-driven mitochondrial rejuvenation is closely related to the regulation of the fate of stem cells. For example, mitophagy in BMSCs was shown to play a vital role in protecting cells against the harshness of oxidative stress and irradiation, thus increasing the viability of stem cells.<sup>20–22</sup> In addition, mitophagy was significantly enhanced during cell reprogramming into iPSCs.<sup>23,24</sup> With inhibition of mitophagy, the adipogenic differentiation of MSCs was abolished.<sup>25</sup> Here, we propose that mitophagy is involved in maintenance of the stemness of BMSCs.

The present study was undertaken to specifically test the hypothesis above. Our results showed that mitochondrial fission promoted mitophagy in BMSCs, and enhanced mitophagy help maintain the stemness of the BMSCs. The inhibition of mitophagy abolished mitochondrial fission maintained stemness of BMSCs. Thus, mitophagy is important in the maintenance of the stemness of BMSCs.

## Materials and methods

### Cell culture and treatment

BMSCs were obtained from four- to five-day-old Sprague-Dawley (SD) rats and isolated as described previously.<sup>15</sup> In brief, after isolating the tibia and femur from the hind limbs of rats carefully, the cells in bone marrow were flushed using a 1 mL syringe containing Dulbecco's modified Eagle's medium-low glucose (DMEM-L) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Natocor, AR). Cells were then seeded on plastic wells or flasks and cultured in the cell incubator (5% CO<sub>2</sub>, 37°C). Media was replaced two or three times a week. When cell colonies grew to 70%–80% confluency, cells were rinsed with phosphate-buffered saline (PBS) and then treated with 0.25% trypsin (Gibco, USA) (Sigma-Aldrich, USA). Afterwards, cells were resuspended in 10% FBS-supplemented media and 5–7 × 10<sup>5</sup> cells were plated to 25 cm<sup>2</sup> flask. Only the third passage was used for further experiments. To promote mitochondrial fission, 1 μM TA9 (Sigma-Aldrich, USA) was added into the media for 24 h. To determine the effects of mitophagy on the stemness of BMSCs, cells were treated with 1 μM FCCP (Sigma-Aldrich, USA) for 4 h to promote mitophagy. Furthermore, for exploration of the role of mitophagy in regulating mitochondrial

fission enhanced stemness, 50 nM *Pink1* siRNA or *Prkn* siRNA was transfected into the cells to inhibit mitophagy.

### Cell infection

*Pink1* siRNA or *Prkn* siRNA was transfected into the cultured cells with jetPRIME (Invitrogen, USA) according to the manufacturer's instructions. A mismatched siRNA (mm siRNA) was used as a negative control. All siRNA were purchased from RUIBO. Cells were used for further studies after transfection for 48 h.

### Immunofluorescence staining

When BMSCs reached a confluence around 70% on coverslips, cells were washed with PBS, fixed with 4% PFA (Thermo Fisher, USA), and then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, USA) at room temperature for 8 min, followed by incubation with 2% BSA (Cell Signaling Technology, USA) at 37°C for 30 min. Afterwards, cells were incubated overnight at 4°C with Oct4 antibody (1: 200; ab27985, Abcam, USA), Sox-2 antibody (1: 100; 6772-100, Bio Vision, USA), Tom20 antibody (1: 200; sc-17764, Santa Cruze), LC3B antibody (1: 200; 2775S, Cell Signaling), or P62 antibody (1: 200; ab56416, Abcam, USA). After washing with PBS for three times, BMSCs were then incubated with an Alexa Fluor 488 conjugated secondary antibody (1: 1000; 1869589, Thermo Fisher, USA) or Alexa Fluor 568 conjugated secondary antibody (1: 1000; 1793903, Thermo Fisher, USA) in the dark for 1 h and then incubated with DAPI at room temperature for 5 min. At last, a confocal microscope (Nikon Ti A1, Japan) was used to observe the fluorescence of cells. The mean fluorescence intensity (MFI) of Oct4 and Sox-2 and the co-localization of mitochondrial marker Tom20 and autophagy marker LC3B or P62 were assessed using ImageJ software.

### Mitochondria and lysosomes imaging

Cells were seeded on glass bottom dishes and co-incubated with MitoTracker Green (Invitrogen, USA) and LysoTracker Deep Red (Invitrogen, USA) according to the manufacturer's instructions. Briefly, freshly prepared MitoTracker Green or LysoTracker Deep Red solution was added to media to reach a final concentration of 1 μM and 0.5 μM, respectively. Cells were kept in the dark and maintained at 37°C for 20 min, then washed with FBS-free DMEM-L for three times. Live cell images of mitochondria and lysosomes were captured using a confocal microscope (Nikon Ti A1, Japan) equipped with oil immersion objective lens 60 × (λ<sub>ex</sub>: 488 nm; λ<sub>em</sub>: 515–530 nm). Pictures were taken in a blinded fashion and at least 30 cells were counted in 3 independent experiments. The co-localization of mitochondria and lysosomes was assessed using ImageJ software. For mitochondrial length measurement, images were extracted to grayscale and inverted to show the specific fluorescence of mitochondria as black pixels. After sharpening the images, unified threshold was set to resolve individual cells and to measure mitochondrial length. Because the vast majority of mitochondrial mass shows an interconnected "network" in cells which makes the image hard to

resolve, the resolvable mitochondrial length was always reported.

### Oil red O and alizarin red S staining

When rat BMSCs reached a confluence around 95% on 6-well plates, cells were induced to adipocytes or osteocytes by culturing cells in the conditioned media. After two or three weeks, cells were rinsed twice with PBS and then fixed with freshly prepared 4% PFA at room temperature for 15 min. After three additional washes with PBS, cells were incubated with 0.5% Oil Red O solution or 0.1% Alizarin Red S (Cyagen, USA) at room temperature for 20–30 min. Then, cells were rinsed again with PBS for two to three times. Finally, the inverted phase-contrast microscope (Nikon, Tokyo, Japan) was used to capture images. The total area of lipid droplets or calcium nodus was assessed using ImageJ software.

### qRT-PCR

Total RNA was extracted from BMSCs with the TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. RNA concentration was quantified with a Gene Quant pro (Amersham Biosciences, GE healthcare, USA). Complementary DNAs (cDNAs) were synthesized with a SYBR Premix Ex Taq (TaKaRa, Japan) in MJ Mini personal thermal Cycler (Bio-Rad, USA). The amount of

cDNA corresponding to 100 ng of RNA was amplified in triplicate with a total of 40 cycles (15 s at 95°C, 15 s at 56°C, and 60 s at 72°C) using a SYBR green PCR kit (Applied Biosystems) with the following primers: Pink1 forward, TATGAAGCCACCATGCC CAC; Pink1 reverse, GATCCT GCCGAGATATTCCAC; Prkn forward, TGTGTAC AA-GGCTCAACGA; Prkn reverse, CCTGTTGTACTGCTCTT CTCCA;  $\beta$ -actin forward, TGTGTGGATTGGTGGCTCTA;  $\beta$ -actin reverse, GCTCAGTAACAGTCC GCCTA. The relative C values of Pink1 and Prkn were normalized to that of  $\beta$ -actin. Each test was performed in triplicate and repeated three times.

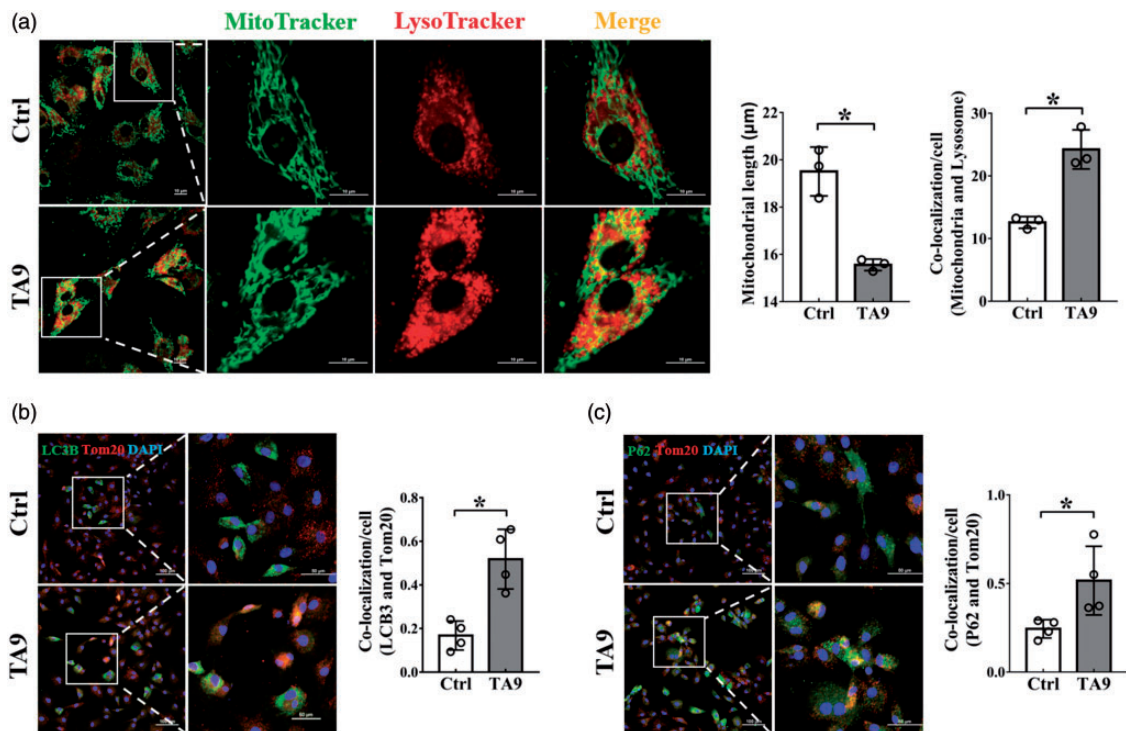
### Statistical analysis

Data were obtained from at least three independent experiments. All data were analyzed by GraphPad prism 7.0 (GraphPad Software, USA) and presented as mean  $\pm$  SEM. One way- or two way-ANOVA followed by Student's *t*-test were used for analysis of differences. A value of  $P < 0.05$  was considered statistically significant.

## Results

### Promotion of mitophagy by mitochondrial fission

To investigate the effect of mitochondrial fission on mitophagy in BMSCs, we treated the cells with TA9 to specifically promote mitochondrial fission and then observed changes



**Figure 1.** Effects of mitochondrial fission on mitophagy. BMSCs were treated with 1  $\mu$ M TA9 for 24 h and visualized by confocal microscopy. (a) The length of mitochondria and the co-localization of mitochondria and lysosomes. Mitochondria in both groups were stained with freshly prepared MitoTracker Green and LysoTracker Deep Red for 20 min. Scale bars represent 10  $\mu$ m; Original magnification: 1000 $\times$  (left), 3000 $\times$  (right). Mitochondrial length and the co-localization of mitochondria and lysosomes were analyzed by ImageJ software. Data were obtained from three separate experiments and more than 10 cells were analyzed in each experiment; (b, c) The co-localization of fluorescently stained autophagy markers (LC3, P62) and mitochondrial marker (Tom20) in BMSCs. Nucleus was stained with DAPI (blue). Scale bars represent 100  $\mu$ m (left), 50  $\mu$ m (right); Original magnification: 200 $\times$  (left), 600 $\times$  (right). The changes in mean fluorescence intensity (MFI) of LC3B, P62 and Tom20 were analyzed by ImageJ software. Each dot presents one independent experiment. Bar graph values are mean  $\pm$  SEM. Data were compared using unpaired Student's *t*-test. \*Significantly different from control ( $P < 0.05$ ). (A color version of this figure is available in the online journal.)

of mitophagy. An increased mitochondrial fragmentation was observed in cells treated with TA9 for 24 h, resulting in a significant reduction of the length of mitochondria (Figure 1(a)), a typical phenotype of mitochondrial fission. Along with the increased mitochondrial fission, mitophagy was greatly enhanced as indicated by an increased co-localization of MitoTracker Green for mitochondria and LysoTracker Deep Red for lysosomes (Figure 1(a)). Furthermore, an increased co-staining of autophagy markers (LC3B, P62) and mitochondrial marker (Tom20) confirmed the increase in mitophagy (Figure 1(b) and (c)).

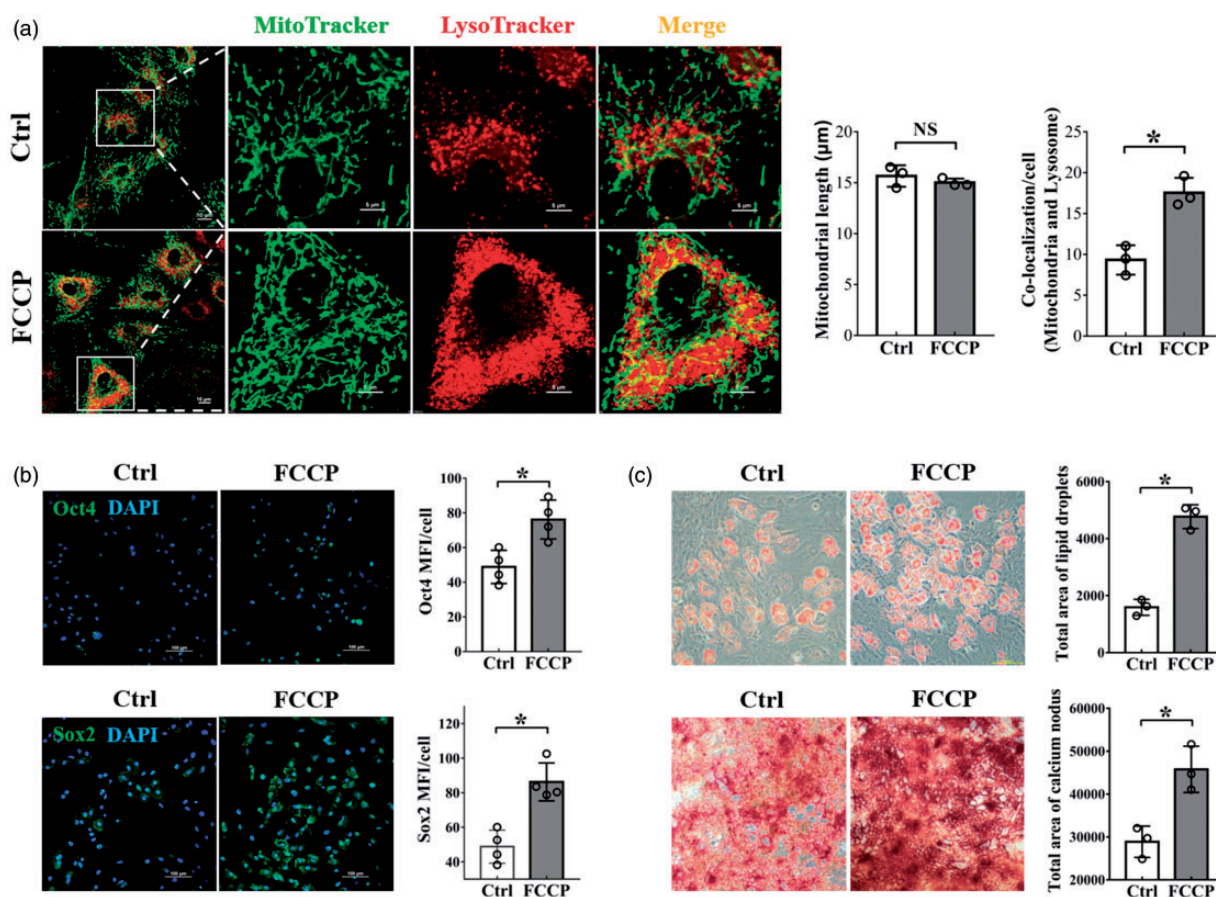
### Maintenance of BMSCs stemness by mitophagy

Treatment of BMSCs with a mitochondrial uncoupler, FCCP, for 4 h remarkably increased mitophagy detected by an increased co-localization of mitochondrial and lysosome biomarkers (Figure 2(a)). It was noticed that FCCP

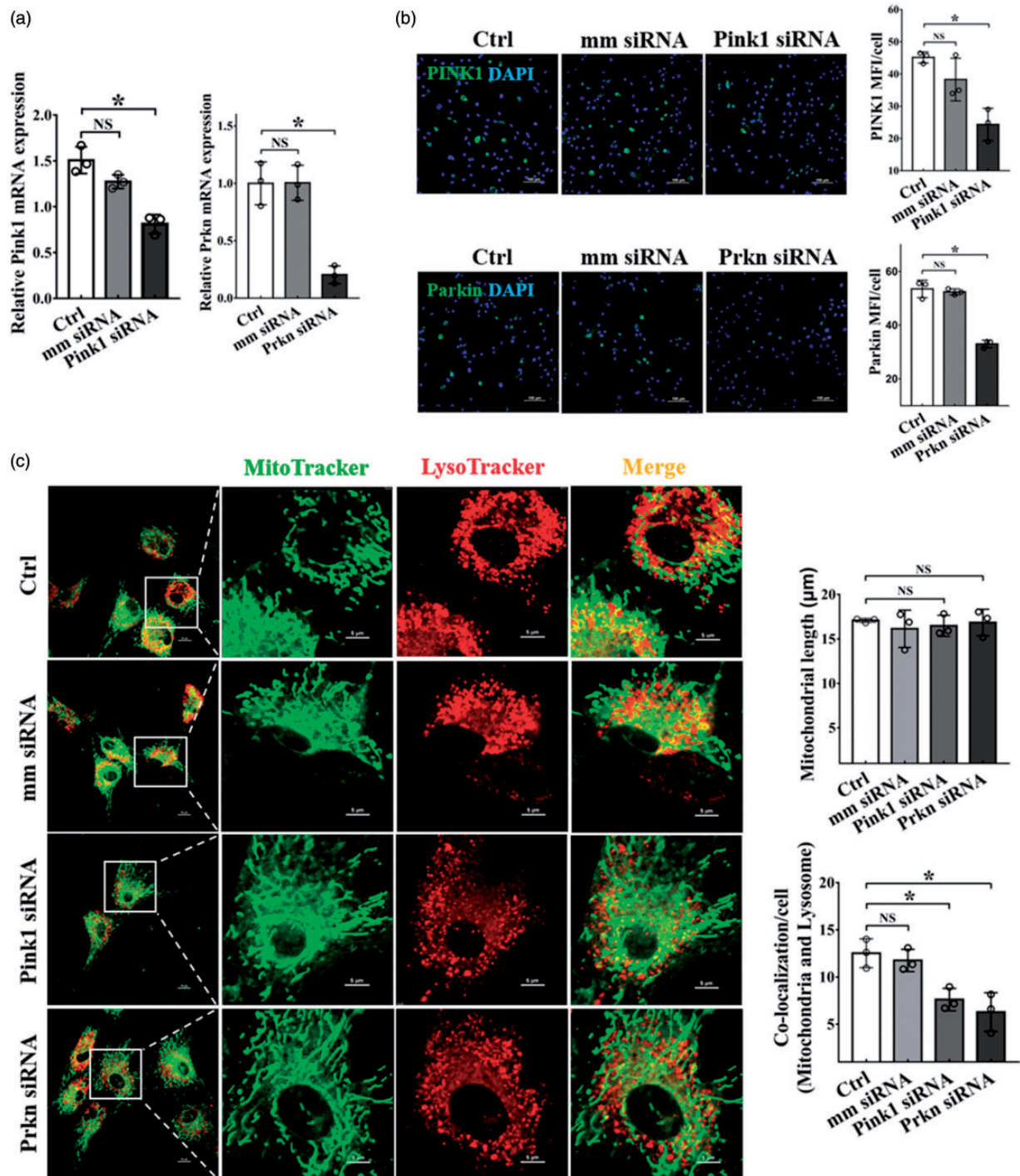
treatment did not change the length of mitochondria (Figure 2(a)), indicating FCCP-induced mitophagy was mitochondrial fission-independent. Under this condition of enhanced mitophagy without a concomitant mitochondrial fission, an enhanced expression of Oct4 and Sox2 (Figure 2(b)), as well as an increase in the potency of BMSCs induction to adipocytes or osteocytes were observed (Figure 2(c)), indicating a role of mitophagy in maintaining the stemness of BMSCs.

### Inhibition of mitophagy by *Pink1/Prkn* gene silencing in BMSCs

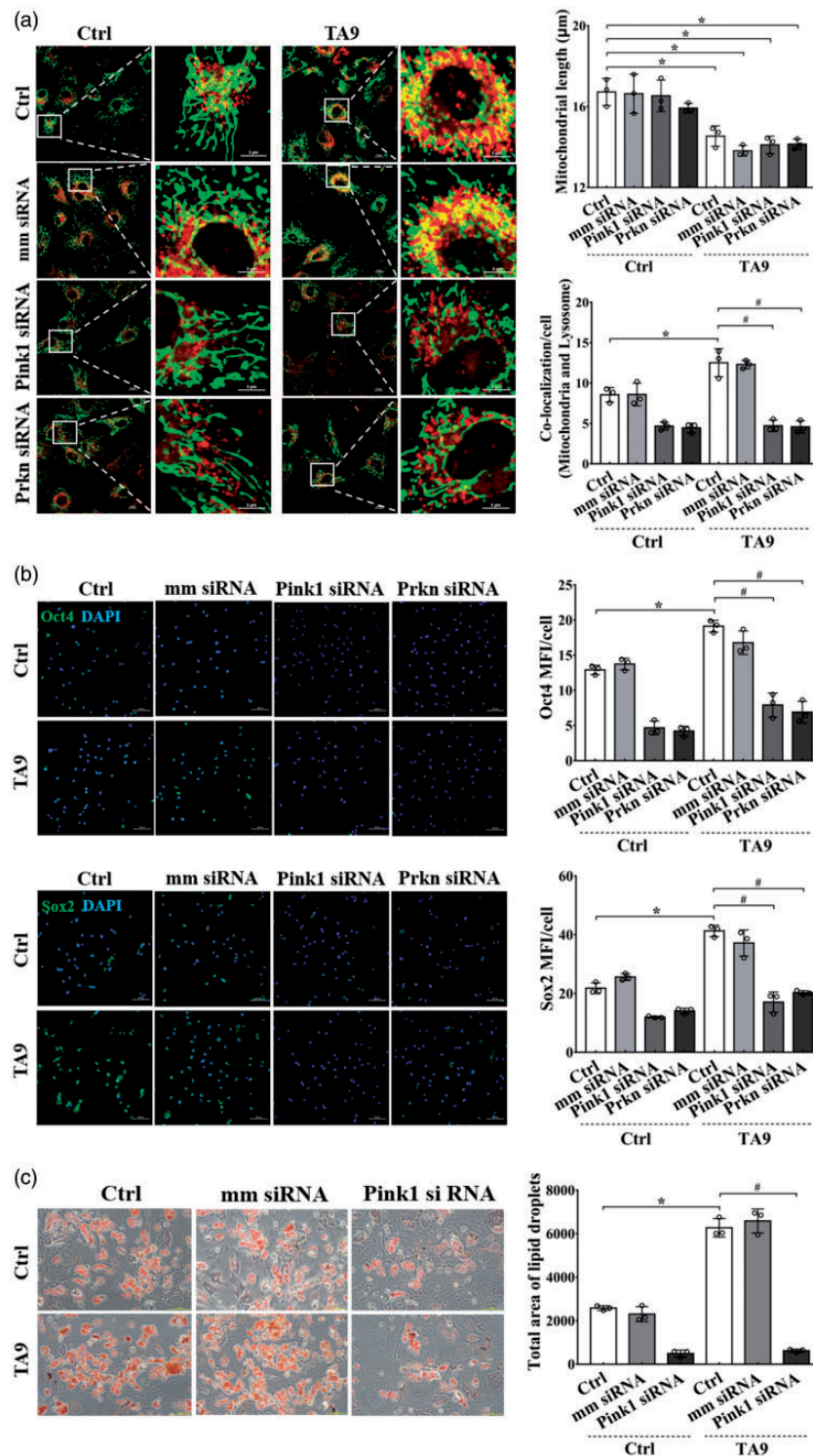
Transfection of BMSCs with siRNA targeting mitophagy essential genes *Pink1/Prkn* led to depression of *Pink1/Prkn* mRNA (Figure 3(a)) and protein levels (Figure 3(b)) in BMSCs. As expected, PINK1/Parkin reduction led to a decreased co-localization of mitochondrial and lysosome



**Figure 2.** The relationship between mitophagy and stemness maintenance. (a) The length of mitochondria and the co-localization of mitochondria and lysosomes. Mitochondria in both groups were stained with freshly prepared MitoTracker Green and LysoTracker Deep Red for 20 min. Scale bars represent 10  $\mu\text{m}$  (left), 5  $\mu\text{m}$  (right); Original magnification: 1000 $\times$  (left), 4000 $\times$  (right). Mitochondrial length and the co-localization of mitochondria and lysosomes were analyzed by ImageJ software. Morphometric analysis of mitochondrial length and the co-localization of mitochondria and lysosomes were performed from data obtained from three separate experiments, and more than 10 cells were analyzed in each experiment; (b) Representative images of fluorescently stained Oct4 and Sox2 (Green). Nucleus was stained with DAPI (blue). Scale bars represent 100  $\mu\text{m}$ , original magnification: 200 $\times$ . The changes in MFI of Oct4 and Sox2 were analyzed by ImageJ software. Data were obtained from four independent experiments; (c) Induction of BMSCs into adipocytes by culturing cells in adipogenic induction media for three weeks. The adipocytes were visualized by Oil Red O staining (top). Scale bars represent 100  $\mu\text{m}$ , original magnification: 200 $\times$ ; Induction of BMSCs into osteocytes by culturing the cells in osteogenic induction media for two weeks. The osteocytes were visualized by Alizarin red S staining (bottom). Scale bars represent 500  $\mu\text{m}$ , original magnification: 40 $\times$ . Total area of lipid droplets and calcium nodus were analyzed by ImageJ software. Data were obtained from three separate experiments. Bar graph values are mean  $\pm$  SEM. Data were compared using unpaired Student's *t*-test. \*Significantly different from control ( $P < 0.05$ ). (A color version of this figure is available in the online journal.)



**Figure 3.** Decreased mitophagy by Pink1/Prkn gene silencing. BMSCs were transfected with 50 nM *Pink1* siRNA or *Prkn* siRNA for 48 h, followed by qRT-PCR analysis and immunofluorescence staining. (a) qRT-PCR determination of the mRNA expression of *Pink1* and *Prkn*. Three independent experiments were carried out; (b) Representative images of fluorescently stained PINK1 and Parkin (Green), nucleus was stained with DAPI (blue). Scale bars represent 100 μm, original magnification: 200×. The changes in MFI of PINK1 and Parkin were analyzed by ImageJ software. Data were obtained from three independent experiments; (c) The length of mitochondria and the co-localization of mitochondria and lysosomes. Scale bars represent 10 μm (left), 5 μm (right); Original magnification: 1000× (left), 4000× (right). Images were analyzed by ImageJ software. Data were obtained from three independent experiments and more than 10 cells were analyzed in each experiment. Bar graph values are mean ± SEM. Data were compared using one way-ANOVA. \*Significantly different from the control group ( $P < 0.05$ ). (A color version of this figure is available in the online journal.)



**Figure 4.** Effects of mitochondrial fission on the stemness of BMSCs with mitophagy inhibition. BMSCs were treated with *Pink1/Prkn* siRNA for 24 h before next treatment with 1  $\mu\text{M}$  TA9 for additional 24 h. Immunofluorescence and adipogenic induction were carried out to detect the stemness of stem cells. (a) The length of mitochondria and the co-localization of mitochondria and lysosomes. Scale bars represent 10  $\mu\text{m}$  or 5  $\mu\text{m}$ ; Original magnification: 1000 $\times$  or 3000 $\times$ . Images were analyzed by ImageJ software. Morphometric analysis of mitochondrial length and the co-localization of mitochondria and lysosomes were performed from data obtained from three separate experiments, and more than 10 cells were analyzed in each experiment; (b) Representative image of fluorescently stained Oct4 and Sox2 (Green), nucleus was stained with DAPI (blue). Scale bars represent 100  $\mu\text{m}$ ; Original magnification 200 $\times$ . Three independent experiments were carried out; (c) Induction of BMSCs into adipocytes by culturing cells in adipogenic induction media for three weeks. The adipocytes were visualized by Oil Red O staining. Scale bars represent 100  $\mu\text{m}$ , original magnification: 200 $\times$ . Images are analyzed by ImageJ software, each dot presents one independent experiment and bar graph values are mean  $\pm$  SEM. Data were compared using two way-ANOVA. \*Significantly different from the control group ( $P < 0.05$ ). (A color version of this figure is available in the online journal.)

biomarkers without affecting mitochondrial length (Figure 3(c)), indicating that PINK1/Parkin reduction suppresses mitophagy in BMSCs.

### Mitochondrial fission promotion of BMSCs stemness is mitophagy dependent

A mitochondrial fission promotion but mitophagy inhibition condition was generated by treating BMSCs with TA9 and concomitantly transfected with siRNA targeting *Pink1/Prkn*. As shown in Figure 4(a), the mitochondrial fragmentation and the decreased mitochondrial length occurred without mitophagy in the cells treated with TA9 along with transfection of *Pink1/Prkn* siRNA. In these cells, the expression of Oct4 and Sox2 was significantly depressed, and the induction of these BMSCs to adipocytes was blocked (Figure 4(b) and (c); Supplementary Figure 1(a) and (b)).

### Discussion

The *in vitro* expansion of BMSCs is necessary for obtaining sufficient amounts of cells for clinical applications. However, BMSCs that cultured *in vitro* are prone to differentiate spontaneously, thus it is important to make efforts to maintain the stemness of BMSCs in cultures. Our previous studies found that mitochondrial fission contribute to maintain the stemness of stem cells,<sup>15</sup> but the underlying mechanism is elusive. In the present study, we found that mitophagy is indispensable for mitochondrial fission maintenance of the stemness of BMSCs.

We first explored the relationship between mitophagy and mitochondrial fission in BMSCs. Mitochondrial fission was accelerated by TA9, and under this condition, mitophagy was remarkably augmented in these cells. This accompanied increase in mitophagy would result from the stimulation of accumulated mitochondrial fragments. It was found that mitochondrial fission and mitophagy shared some critical regulatory proteins involved in the two parallel cellular processes. For instance, DRP1 and FIS1, both participating in mitochondrial fission, function together with the LC3 adapters/receptors to promote mitophagy.<sup>19,26,27</sup> Therefore, mitochondrial fission followed by mitophagy is an important sequential process to ensure the removal of damaged or fragmented mitochondria from the cells. The results here demonstrated that this sequential process is also important to maintain the stemness of BMSCs. Mitophagy could occur independent of mitochondrial fission.<sup>28</sup> The result here using FCCP to promote mitophagy also showed that FCCP-augmented mitophagy was independent of mitochondrial fission. Taking an advantage of this distinguished approach, we examined the essential role of mitophagy in maintenance of the stemness of BMSCs. The results indeed showed that the enhancement of mitophagy induced by FCCP, without a concomitant mitochondrial fission, significantly increased the stemness of BMSCs. Conversely, inhibition of mitophagy blocked mitochondrial fission-enhanced stemness of BMSCs. During the process of mitophagy, PINK1 is accumulated at the outer membrane of mitochondria, which recruits E3 ubiquitin ligase Parkin, leading to clearance of

impaired mitochondria by lysosomes.<sup>29–31</sup> Gene silencing *Pink1/Prkn* suppressed mitophagy, and under this condition, stimulation of mitochondrial fission did not promote the stemness of BMSCs. Thus, it clearly demonstrated the critical role of mitophagy in mediating mitochondrial fission-enhanced stemness of BMSCs.

The association of mitophagy with the stemness of stem cells was also reported in other studies. An increase in mitophagy was observed in cells that were programmed to iPSCs,<sup>23</sup> and inhibition of mitophagy impaired the stemness of MSCs.<sup>25</sup> On the other hand, there were studies showing that mitophagy may also play a key role in cell differentiation during some particular developmental stages in mammalian cells. For example, a study in CD34<sup>+</sup> hematopoietic progenitor cells (HPCs) showed that mitophagy would possibly contribute to cell differentiation as enhanced mitophagy was observed in erythroid differentiation in  $\beta$ -thalassemia.<sup>32</sup> If the positive role of mitophagy in maintenance of stem cell stemness versus that in promotion of stem cell differentiation were considered to be contradictory, it would be a misinterpretation. As shown in the present study, augmented mitophagy in BMSCs was associated with enhanced stemness of these cells in cultures, but was also with promoted induction of these cells to adipocytes or osteocytes in response to different stimulatory signals. It thus is important to note that the fate of stem cells is not determined by mitophagy, but rather by its surrounding environment. Mitophagy would only enhance the intracellular machinery for whatever lineages of the stem cells.

The present study demonstrates that mitophagy is important in the maintenance of the stemness of BMSCs in cultures, and is essential in mediating mitochondrial fission-enhanced stemness of BMSCs. This understanding would provide a critical insight into stem cell propagation in cultures for their clinical application since maintenance of the stemness of stem cells is among the most important issues in stem cells and regenerative medicine. Therefore, manipulation of mitochondrial dynamics in the preparation of stem cells for clinical application would be an important undertaking for both experimental and clinical studies.

### AUTHORS' CONTRIBUTIONS

All authors participated in conceptualization and designing of this study. XRF and WY, performed the experimentation, data collection, and manuscript drafting; JLW, performed experimentation and participated writing; LF, provided consultation and supervision; YJK, funding acquisition, data reviewing and validating, and manuscript editing and final approval.

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**DECLARATION OF CONFLICTING INTERESTS**

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**SUPPLEMENTAL MATERIAL**

Supplemental material for this article is available online.

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