CD157 mediates mitochondrial production and transfer from bone marrow mesenchymal stem cells (BMSCs) to improve neuronal apoptosis and functional recovery after spinal cord injury

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Research

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Abstract

Background: Recent studies demonstrated that autologous mitochondria derived from bone marrow mesenchymal stem cells (BMSCs) might be valuable in the treatment of spinal cord injury (SCI). However, the mechanisms of mitochondrial transfer from BMSCs to injured neurons are not fully understood.

Methods: We modified BMSCs by CD-157, a cell surface molecule as a potential regulator mitochondria transfer, then transplanted to SCI rats and co-cultured with OGD injured VSC4.1 motor neuron. We detected extracellular mitochondrial particles derived from BMSCs by transmission electron microscope and measured the CD157/cyclic ADP-ribose signaling pathway related protein expression by immunohistochemistry and Western blotting assay. The CD157 ADPR-cyclase activity and Fluo-4 AM was used to detect the Ca^{2+} signal. All data were expressed as mean $\pm$ SEM. Statistical analysis was analyzed by GraphPad Prism 6 software. Unpaired t-test was used for the analysis of two groups. Multiple comparisons were evaluated by one-way ANOVA or two-way ANOVA.

Results: CD157 on BMSCs was upregulated when co-cultured with injured VSC4.1 motor neurons. Upregulation of CD157 on BMSCs could raise the transfer extracellular mitochondria particles to VSC4.1 motor neurons, gradually regenerate the axon of VSC4.1 motor neuron and reduce the cell apoptosis. Transplantation of CD157 modified BMSCs at the injured sites could significantly improve the functional recovery, axon regeneration and neuron apoptosis in SCI rats. The level of Ca^{2+} in CD157 modified BMSCs dramatically increased and when objected to high concentration cADPR, ATP content and MMP of BMSCs also increased.

Conclusion: This study evidences that CD157 can regulate the produce and transfer of BMSCs-derived extracellular mitochondrial particles, enriching the mechanism of the extracellular mitochondrial transfer in BMSCs transplantation and providing a novel strategy to improve the stem cell treatment on SCI.

Backgroud

Spinal cord injury (SCI) is a devastating disease with the complex secondary pathological complications, and there is no effective therapy yet. SCI causes a series of complex pathological changes, including ischemia [1, 2], oxidative stress [3], neuroinflammation [4], axonal demyelination [5], apoptosis [6] and even spinal cord injury-induced immune deficiency syndrome after initial trauma [7, 8]. Furthermore, SCI-induced excessive inflammatory responses [9], loss of ionic homeostasis [10], the up-regulated release of excitatory amino acids and the excessive activation of excitatory amino acid receptors may lead to the cascade of secondary injury [11]. Neuron recovery and axon regeneration are the main goals of research and treatment of spinal cord injury.

Mitochondrion, as one of the essential subcellular organelles, its morphology and function were strongly impaired by the secondary injury after SCI [12]. Irregular shape, enlarged size, disordered cristae, disordered fusion and fission, reduced membrane potential and changed expression of related proteins of mitochondrion occurred in the acute phase after SCI. Meanwhile, resultant morphological and functional

changes of mitochondrion regulated the underlying secondary injury, such as necrosis, apoptosis and autophagy [13]. Mitochondrial dysfunction was critical for the development of secondary injury and neuronal cell death [12, 14]. Therefore, restoring the mitochondria function is a potential therapeutic strategy for SCI.

Bone marrow mesenchymal stem cells (BMSCs), isolated from bone marrow, has been an ideal tool cell for our recent studies on SCI [15, 16]. It is easy to harvest, culture, expand and modulate *in vitro*, BMSCs have more frequently applied to transplantation studies recently [17, 18]. The multiple characteristics of BMSC account for the versatility of the mechanisms of injured tissue' repairmen [19]. For example, BMSCs can improve bone regeneration to repair lost bone [20] but also can differentiate into cardiomyocytes [21]. Our previous studies found that BMSCs transplantation after SCI improved locomotor function on rats and alleviated pathological deterioration by mitochondria transfer and decreasing ER stress-induced neuronal apoptosis and related factors [22]. However, the mechanisms of mitochondrial transfer from BMSCs to injured neurons are not fully understood.

CD157 (bst-1, bone marrow stromal antigen-1), a cell surface molecule expressed on myeloid, endothelial, mesothelial cells, plays roles both as an ectoenzyme and signaling receptor [23]. And it is also capable of signal transduction [24]. Studies have shown that CD157 can support the pre-T cell expansion [25], regulate leukocyte trafficking [26] and ovarian cancer progression [22]. Also, it is involved in humoral immune responses [27], neutrophil transmigration [28] and haematopoietic stem cell support [29]. In addition, as a member of the NADase/ADP-ribosyl cyclase family, CD157 might induce the catalysis of cyclic ADP-ribose in embryonic and adult nervous systems [30]. In the present study, we purposed the hypothesis that CD157 may regulate mitochondria transfer from BMSCs to injured neurons after SCI and promote recovery of motor function and axon regeneration via CD157/cADPR/calcium signaling pathway.

Materials And Methods

Animals

Male Sprague-Dawley rats sacrificed for this study. All experimental procedures were approved by the Animal Ethics Committee of Zhejiang University and followed the National Institutes of Health guidelines strictly. Animals were housed under a 12-hour light/dark cycle with free access to food and water. All efforts were made to minimize the number of animals used and their suffering. The individual mouse was considered the experimental unit within the studies.

Primary BMSCs isolation, culture and characterization

Primary BMSCs were isolated from the femurs of 3-4 week old Sprague-Dawley male rats following our previous study. BMSCs were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) with 10% fetal bovine serum, 100U/ml penicillin and 100U/ml streptomycin, and medium

were changed every two days. The fluorescence-activated cell sorting (FACS) analysis was adopted to characterize BMSCs in our previous study [31].

Construction of adenovirus and infection of BMSCs

The CD157 over expression vector was constructed with pHBAD-EF1-MCS-3flag-CMV-GFP vector. The MCS segment was inserted bst-1 gene, and the EF1 promoter regulated the expression of bst-1 gene and the CMV promoter regulated EGFP. Meanwhile, pHBA-U6-MCS-CMV-GFP was used to construct CD157 interference vector that contained a U6 promoter regulated the expression of shRNA of bst-1 inserted in the EcoR I and BamH I sites and a CMV promoter regulated the expression of GFP gene. A vector only expressing GFP gene was used to be the control. All vectors were synthesized by the Han Bio Co. LTD (Shanghai, China).

The BMSCs were infected at a 50 multiplicity of infection with the adenovirus vectors after the polybrene (5 μ g/ml) treatment for 30 min. 48 h later, the infected BMSCs were observed with fluorescence microscope (Olympus Corp., Tokyo, Japan) and the Western blotting analysis was applied to detect the expression of CD157.

Oxygen-glucose deprivation (OGD) and re-oxygenation of VSC4.1 motor neurons

The ventral spinal cord 4.1 (VSC4.1) motor neuron cells were cultured in RPMI 1640 medium with 10% (V/V) fetal bovine serum and 100U/m penicillin and streptomycin at 37°C with 5% CO₂ in a fully humidified incubator. OGD and re-oxygenation models were used to mimic ischaemia and hypoxia in SCI. In brief, VSC4.1 motor neurons were cultured in D-Hanks' balanced salt solution without glucose in a sealed hypoxic GENbag fitted with a AnaeroPack (MGC, Japan) to scavenge free oxygen and the Non-OGD group was cultured in Hanks' balanced salt solution containing the normal concentration of glucose with 5% CO₂ for 8 hours. Later, all cells were re-oxygenated and cultured in normal complete medium or were co-cultured with BMSCs. All duration were determined by our previous study [32].

SCI model and BMSCs transplantation

24 Sprague-Dawley male rats weighing 200-220g were divided into four groups randomly, by using the standard = RAND() function in Microsoft Excel. SCI was performed with Allen's method in accordance with our previous study. In brief, all rats were anesthetized with pentobarbital (40 mg/kg, i.p.). Then, their vertebral columns were exposed, and laminectomy were operated at the T10 spinal vertebra. A weight of 10g was dropped from a height of 50mm on the exposed spinal cord. The impounder was left for 20 s to produce a moderate contusion. Immediately, the 10 μ l culture medium containing 10⁶ BMSC+MOCK, BMSC+Over or BMSC+shRNA were injected into the epicenter of the injured spinal cord using an electrode microneedle as the SCI +MOCK group, SCI+Over group, and SCI+shRNA group respectively. Meanwhile, the control rats received the sham operation with the same surgical procedure without injury, while the SCI group received the same dose of DMEM.

Collection of BMSCs-conditioned medium (BCM)

BMSCs were planted at 1×10^5 cells/dish and cultured in DMEM/F12 complete medium. When attached, the cells were washed with phosphate-buffered saline (PBS) for three times and incubated in high glucose Dulbecco's Modified Eagle Medium (DMEM) without serum to stimulate the production of extracellular mitochondria particles. The medium was collected 24 h later. BMSCs-conditioned medium (BCM) was treated by filtering through a 1.2 μm syringe filter or by spinning cell debris down with centrifuging at 2000g for 10 min. Meanwhile, BCM was filtrated through a 0.22 μm syringe filter to prepare mitochondria deleted medium (Md-BCM) that contains no extracellular mitochondria particles.

MitoTracker Red staining

BMSCs were stained with 200 nM MitoTracker Red CMXRos (Molecular Probes, M7512, Invitrogen, USA) for 30 min at 37°C to label the intracellular mitochondria. The cells were washed three times with PBS to exclude the interference of excessive dye.

Carboxyfluorescein succinimidyl ester (CFSE)-fluorescent label

Attached VSC4.1 motor neurons were stained by 10 μM carboxyfluorescein succinimidyl ester (CFSE, #C1031, green colour, Beyotime Institute of Biotechnology, China) for 30 min in 37°C and washed by PBS for three times.

Co-culture of post-OGD VSC4.1 motor neurons with BMSCs

VSC4.1 motor neurons were cultured directly with BMSCs in 10 cm dishes or in the 6-well 8 μm transwell system (Corning, USA) in a 1:1 ration. All co-culture system last for 24 h.

Microscope observation

The observation of extracellular mitochondria particles derived from BMSCs was carried out by transmission electron microscopy in accordance with previous study [33]. VSC4.1 motor neurons were cultured in 1×10^5 cell/well with round coverslips and fixed with 0.5% paraformaldehyde after co-culturing. 4',6-diamidino-2-phenylindole (DAPI, blue colour, #C1002, Beyotime Institute of Biotechnology, China) was applied to all cells to label nucleus after fixing. The internalization of extracellular mitochondria (red colour) derived from BMSCs in co-cultured VSC4.1 motor neurons (green colour) was captured by fluorescence microscope. To detect the regeneration of motor neuron axons, optical microscope was used to observe the length and the number of axons.

Western blot analysis

Western blot was performed to determine the transfection efficiency of vectors and the expression level of proteins related to cellular apoptosis and mitochondrial apoptosis. Each sample was detected concentration and loaded onto 4–12% Bis-Tris gels (M00653, GeneScript, China). After the

electrophoresis and transferred to PVDF membranes, the membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% skim milk (232100, BD, USA) for 90 min at room temperature. Membranes were washed with TBST and then incubated overnight at 4°C with anti-GAPDH (1:2000, 10494-1-AP, Proteintech, USA) and anti-Bone marrow stromal cell antigen 1 (CD157) antibody (1:1000, ab208442, Abcam, USA). After washed with TBST, membranes were incubated with infrared-labelled peroxidase-conjugated secondary antibodies for 1 h at room temperature. Bands were captured and quantificated by Odyssey CLx Image Studio (Gene Ltd, USA).

Immunofluorescence staining

0.5 % paraformaldehyde fixed cells or frozen spinal cord sections were washed 3 times in 1×PBS and blocked in blocking buffer for 60 min. Then cells or spinal cord sections were incubated overnight at 4°C with primary antibody as following: Rabbit anti-Grp 78 antibody (1:200, ER40402, HuaBio, CN), Rabbit Anti-NF-κB p65 antibody (1:200, ab16502, Abcam, USA), GAP 43 (D9C8) Rabbit mAb (1:200, #8945, CST, USA), Bcl-xL (54H6) Rabbit mAb (1:200, #2764, CST, USA), AKT (phospho Thr308) antibody (1:50, om238718, Omnimabs, USA). After washing by PBS 3 times, fluorescent secondary antibodies incubated for 2 hours at room temperature. Finally, DAPI was added to visualize the nucleus, and coverslips were placed

Potential of mitochondrion measurement

The mitochondrial membrane potential (MMP) assay kit with JC-1 (C2006, Beyotime Institute of Biotechnology, China) was used to assess mitochondrial membrane potential to detect whether the extracellular mitochondrion still had function. BCM and Md-BCM were collected and mixed with JC1 (5 μM) for 30 min at 37 °C. When the membrane potential of mitochondria completely lost, green fluorescence (Ex 485 nm Em 516 nm) would be observed and the normal cells stained should show red fluorescence (Ex 579 nm/Em 599 nm). MMP was determined by the Varioskan Flash microplate reader.

ATP measurement

ATP level was determined by CellTiter-Glo luminescence (G7570, Promega, USA). For intracellular ATP content, cells were incubated by 200 μl reagent buffer each well, standing for 30 min at room temperature for lysing cells. For extracellular ATP, the culture medium was collected and centrifuged at 2,000 g for 10 min. Then the supernatant was collected and centrifuged at 20,000 g for 20 min at 4 °C, remaining the lower half to use. CellTiter-Glo luminescence test solution (50 μl) was added into culture media (50 μl) and incubated for 30 min at room temperature in opaque-walled 96-well plates. Luminescent signal was determined by the microplate reader.

FACS analysis on the content of extracellular mitochondria particles

FACS analysis were performed by BD Fortessa or CytoFLEX LX. BCM were prepared and collected as described as before. 200 nm and 300 nm diameter calibration Particles, DMEM media incubating

unstained BMSCs and DMEM media were used to control for determining appropriate gates, voltages, and compensations required in multivariate flow cytometry.

Determination of CD157/ADPR-cyclase activity

ADPR cyclase activity was determined with nicotinamide guanine dinucleotide (NGD⁺) (N5131, Sigma, USA) as the substrate as described before [34]. Briefly, BMSCs were cultured in 96-well plate with 200 µl complete DMEM/F12 medium. After attaching, BMSCs were incubated with 500 µM NGD⁺ in 0.1 M PBS (pH 7.2) at 37 °C for 10 min, and the production was determined at excitation/emission wavelengths of Ex 290 nm/Em 410 nm with the microplate reader.

Ca²⁺ signal detection

To explore whether the CD157/cADPR mechanism was Ca²⁺ dependent, the level of Ca²⁺ in BMSCs was determined by Fluo-4 AM (S1060, Beyotime Institute of Biotechnology, China). BMSCs were incubated in 96-well plates with 2 µM Fluo-4 AM for 30 min at room temperature. After washing with PBS for three times, fluorescence intensity was determined by the Varioskan Flash microplate reader at excitation/emission wavelengths of Ex 488 nm/Em 516 nm.

Assessment of motor function

Basso, Beattie, and Bresnahan (BBB) locomotor scales were used to assess the recovery of rats' motor function. The scores were obtained by two independent examiners who were blind to the four groups. All rats were observed and assessed the relevant indicators of hind limb motor function and physical control function in an open field for 3 min at 1, 7, 14, 21 and 28 days post-surgery.

Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was analyzed by GraphPad Prism 6 software. Unpaired t-test was used for the analysis of two groups. Multiple comparisons were evaluated by one-way ANOVA or two-way ANOVA. P<0.05 was considered to be statistically significant.

Result

Production and transfer of BMSC-derived mitochondria

MitoTracker Red CMXRos efficaciously visualized mitochondrion in BMSCs (Fig. 1a). BMSCs conditioned-medium (BCM) were collected and concentrated after 24 hours of pre-stained. We used transmission electron microscopy to capture the extracellular mitochondria in BCM. The results showed that partial extracellular mitochondria still maintain their normal morphology and structure (Fig. 1b). Previous study showed that extracellular mitochondria particles spanned a range of sizes from 300 to 1100 nm. So 0.22 µm filters were used to deplete the mitochondria in the BCM to obtain mitochondria deleted medium (Md-BCM). BCM and Md-BCM were collected, and FACS were used to detect the content of mitochondria

particles in BCM. The result showed that compared to Md-BCM, BCM contained a certain amount of mitochondria particles derived from BMSCs (Fig. 1c-d).

Double-labeled, MitoTracker Red CMXRos and CFSE, BMSCs were co-cultured directly with VSC4.1 motor neurons in a 1:1 ratio for 24 h. Fluorescence microscope observation showed that mitochondrion derived from BMSCs were successfully transferred into VSC4.1 motor neurons (Fig. 1e). Meanwhile, MitoTracker Red CMXRos pre-stained BMSCs were seed at transwell inserts and co-cultured with CFSE pre-stained VSC4.1 motor neurons in a transwell system. The red pots indicated that BMSCs could produce and transfer extracellular mitochondria particles, and the mitochondria particles can be seized and initialized by VSC4.1 motor neurons (Fig. 1f). Furthermore, we co-cultured BMSCs and pre-stained VSC4.1 motor neurons w/o OGD treatment. The result confirmed that the mitochondrial internalization in VSC4.1 motor neurons significantly increased after OGD treatment (Fig. 2g-h).

We detected whether the extracellular mitochondria were still functional by ATP content measurement and JC-1 assay. Notably, when the mitochondria in extracellular particles were deleted, ATP level decreased (Fig. 1i) and MMP lost sharply (Fig. 1j). Both results indicated that the mitochondria were functional in the extracellular particles.

Expression of CD157 on BMSCs and VSC4.1 motor neurons

CD157 expressed both on BMSCs and VSC4.1 motor neurons. We assumed that CD157 involved in the transfer of extracellular mitochondria. However, whether the CD157 on BMSC, VSC or both of them involved should be figured out. We co-cultured BMSCs and VSC4.1 motor neurons w/o OGD directly on round coverslips. Immunofluorescence staining was applied to detect the expression of CD157. BMSCs were pre-stained with CFSE and DAPI marked all cell nuclei. CD157 were visualized by anti-CD157 antibody (Fig. 2a). Image J was applied to the measurement of IOD (Fig. 2b-c). The results showed that the expression of CD157 had no difference between OGD and normal VSC4.1 motor neurons. While, compared to BMSCs co-cultured with normal VSC4.1 motor neurons, CD157 on BMSCs co-cultured with OGD VSC4.1 motor neurons had a significant increase. These manifested that the CD157 on BMSCs might involve in and regulate the production and the transfer of BMSC-derived mitochondria. Thus, we constructed the *bst-1*⁺ and *bst-1*⁻ adenovirus vector. The titers of all vectors were observed by fluorescence microscope (Fig. 2d), and the expression of CD157 were tested by western blotting (Fig. 2e).

Effect of CD157 on production and transfer of extracellular mitochondria derived from BMSCs

BMSCs were transfected with Mock vector, CD157 over-expression vector and CD157 interference vectors, represented by Mock group, Bst-1⁺ group and Bst-1⁻ group respectively. MitoTracker Red CMXRos pre-stained Mock group, Bst-1⁺ group and Bst-1⁻ group, as unstained BMSC group and DMEM blank group as a control. All the BMSCs conditioned-medium was collected and filtered through 1.2 μm filter for subsequent use. FACS results (Fig. 3a-b) and OD value (Fig. 3c) showed that, the production of extracellular mitochondria was up-regulated in the Bst-1⁺ group when compared to Mock group.

Conversely, it was down-regulated in the Bst-1⁻ group. In addition, we co-cultured the pre-stained BMSCs and OGD treated VSC4.1 motor neurons directly (Fig. 3d-e) or in the transwell system (Fig. 3f-g) respectively. The mitochondrial internalization, quantitatively analyzed by the IOD of VSC4.1 motor neurons, were detected by Image J. The results showed that compared to the Mock group, mitochondria derived from the Bst-1⁺ group were increasingly internalized by injured VSC4.1 motor neurons, while the Bst-1⁻ group were opposite. To sum up, CD157 overexpressing on BMSCs can upregulate the production and the transfer of BMSC-derived extracellular mitochondria particles.

Effects of CD157 on OGD VSC4.1 motor neurons *in vitro*

We co-cultured OGD treated VSC4.1 motor neurons and transfected BMSCs in the transwell system, respectively. After 24 h co-culturing, VSC4.1 motor neurons on round coverslips were fixed with 4% paraformaldehyde for 30 min and then washed with PBS for three times. Cells were observed by the optical microscope (Fig. 4a). The length and the number of neurites were measured by Image J. The statistical results showed as the two points as follows: The expression level of CD157 on BMSCs had no effect on the number of neurites (Fig. 4b); CD157 over-expressing could promote the regeneration of neurites on length (Fig. 4c). That is, over-expression of CD157 on BMSCs could improve neurite outgrowth of VSC4.1 motor neurons in length rather than in number.

We also carried out Immunofluorescence staining to detect the expression of apoptosis and inflammation-related proteins, including Grp78, NF-κB and Bcl-xl. Image J measured the IOD of each injured VSC4.1 motor neuron. The results showed that the expression of Grp 78 (Fig. 4d-e) and NF-κB (Fig. 4f-g) decreased in Over group and increased in shRNA group conversely, compared to Mock group. While Grp 78 and NF-κB of the three groups were all downregulated compared to the OGD group. And the expression of anti-apoptotic protein, Bcl-xl, increased in both Mock group and Over group, compared to the OGD group and shRNA group (Fig. 4h-i). In summary, overexpression CD157 modified BMSCs could alleviate apoptosis and inflammation of injured motor neurons.

Effects of CD157 on spinal cord neurons *in vivo*

We operated *in vivo* BMSCs-transplant surgeries after SCI on rats. The secondary injury tends to ease off at 7 d after SCI. At this time, inflammation and neuron regeneration begin to increase. Thus, we sampled and took freezing sections at 7 d after SCI (Fig. 5a). As our previous study already proved that normal BMSCs transplantation could facilitate the recovery of rats' motor function after SCI, we divided the rats only into four groups: sham operation group (control), SCI+MOCK group, SCI+Over group and SCI+shRNA group. BBB scores determined the severity of paralysis caused by SCI. BBB scores were significantly increased in the SCI+Over group and decreased in the SCI+shRNA group respectively compared to the SCI+MOCK group (Fig. 5b).

We also adopted immunofluorescence staining for detection of proteins expression. Images indicated that after transplantation of the transfected BMSCs, Grp 78 (Fig. 5c-d) and NF-κB (Fig. 5e-f) significantly decreased, while p-Akt (Fig. 6a-b) increased in SCI+Mock group and SCI+Over group, compared to the SCI

group and SCI+shRNA group. Meanwhile, the axon regeneration-related protein, GAP 43, was upregulated in SCI+Mock group and SCI+Over group, which evidenced that CD157 overexpression could improve neuron regeneration (Fig. 6c-d).

CD157/ADPR-cyclase activity and Ca^{2+} signal on BMSCs

CD157 is a member of the NADase/ADP-ribosyl cyclase family. We supposed that CD157-cADPR- Ca^{2+} signaling pathway was involved in the production and the transfer of extracellular mitochondria derived from BMSCs. We applied two groups of BSMCs to detect enzymatic activity of BMSCs. 500nM NGD⁺ was added as the substrate into the experimental group, and the same volume 1×PBS was added into the control group. The result confirmed that CD157 did have NADase activity (Fig. 7a). Then we confirmed whether cADPR could regulate the production of extracellular mitochondria. Pre-stained BMSCs were subjected to cADPR at different concentrations for 24 hours. The mitochondrion measurement (Fig. 7b) and ATP content detection (Fig. 7c) of BMSCs conditioned-medium demonstrated that ADPR-cyclase activity regulated the production of extracellular mitochondria. In final, we confirmed the cADPR- Ca^{2+} signaling pathway and detected the level of calcium in the transfected BMSCs. The result showed a higher level of calcium with a higher concentration (Fig. 7d). Consistent with this result, Ca^{2+} signal of Over group was the highest among the four groups (Fig. 7e). Together, CD157-cADPR-calcium was most likely a mechanism for the production of extracellular mitochondria derived from BMSCs.

Discussion

SCI caused devastating disabilities with instance motor dysfunction or paralysis, and effective treatment on SCI are not satisfactory. Due to the secondary injury cascades, an extremely complex destructive pathological process, axonal regeneration and functional recovery are severely restricted in SCI. As many related signaling pathways subsequently intersect and initiate other secondary injury events, targeting on a specific cascade doesn't seem to be an efficient therapeutic strategy. Mitochondrion, the center of cellular energy production and metabolism, can induce apoptosis and the performer of apoptosis [35]. Its trafficking was crucial to neuron survival, especially the axons and dendrites because of their energy demand or calcium flux [36-38]. Studies have reported that SCI causes mitochondria damage and disruption, which deteriorates oxidative stress reaction and cell apoptosis. Mitochondrial dysfunction was the initial step of neuronal injury with indirect and direct involvement in the progression of SCI pathology [39]. Hence, Fang's study and our previous research have focus on the role of mitochondria in the neuron recovery and the treatment in SCI [22]. Mitochondrial transplantation has presented significant benefits, including improving the lower-limb locomotor function, suppressing the regional endoplasmic reticulum stress and inhibiting the mitochondria-dependent apoptosis [39]. In present study, we observed that a portion of extracellular mitochondria derived from the BMSCs were integral. ATP and MMP detection verified that extracellular mitochondria were functional. In the process of co-culture, the functional mitochondria were transferred to VSC4.1 motor neurons and were internalized by the latter. Moreover, the injured neurons tended to internalize more exogenous mitochondria than normal cells for rescue.

Meanwhile, we found that the CD157 protein of BMSCs co-cultured with OGD neurons upregulated. CD157 was reported that as an extracellular mammalian ADP-ribosyl cyclases, it could generate intracellular messengers in response to stimuli, catalyzing the synthesis of cADPR from NAD⁺ [40, 41]. cADPR was an important Ca²⁺-mobilizing cytosolic messenger [42, 43], who provided the prominent Ca²⁺ wave at fertilization [44], regulated dendritic cell functions [45] and contributed to airway disease [46]. Moreover, the cytosolic calcium and mitochondria were interlaced. Ca²⁺ signaling regulated some important mitochondrial activities, for instance, the rise of cytoplasmic Ca²⁺ concentration caused an increase of mitochondrial ATP production [47-49]; in turn, mitochondrial Ca²⁺ uptake regulated the intracellular calcium signaling via buffering cytosolic Ca²⁺ levels [50, 51]. Research has shown that CD38 could mediate extracellular mitochondria transfer via calcium-dependent CD38/cyclic ADP-ribose pathway in astrocytes [33]. Therefore, we proposed that CD157 could regulate the production and the transfer of BMSC-derived extracellular mitochondria particles.

We firstly constructed the expression vectors of CD157 and transfected the BMSCs. The level of the extracellular mitochondrial particles derived from the high expression modified BMSCs was elevated in separate culture, indicating that CD157 could regulate the production of extracellular mitochondria. When we co-cultured modified BMSCs and injured neurons, directly or indirectly, highly internalization of extracellular mitochondria occurred in the CD157 overexpression groups. All above results suggested that the upregulation of CD157 on BMSCs was most likely the cause of high-level internalization in damaged neurons.

We thought that internalization did not necessarily work and, in consequence, the recovery of injured neurons was observed both morphologically and physiologically, as well as *in vivo* and *in vitro*. The results of *in vitro* experiments showed that, compared to the other three groups, in the CD157 high expression group, the length of the regenerative synapses of neurons were longest, signifying that highly internalization of mitochondria was a benefit to the synaptic regeneration. After the primary injury, strong inflammatory response and apoptosis were evoked in which the related proteins actively changed, including Grp 78, NF- κ B, Bcl-xl and p-Akt [52-55]. We detected the expression of these proteins both *in vivo* and *in vitro*. Consistently, the results showed that the expression of Grp78 and NF- κ B decreased and the level of Bcl-xl and p-Akt increased significantly in the high CD157 group. These meant that the injured-cell apoptosis was decreased by upregulating CD157 on BMSCs. Moreover, the BBB scores evidenced that transplantation of the overexpression modified BMSCs did dramatically improve the functional recovery of SCI rats.

To verify whether the cADPR/calcium signaling pathway was involved in this processing, we detected the ADPR-cyclase activity of CD157 and the mitochondria content, ATP content and Ca²⁺ level in BMSCs treated with different concentration cADPR. Results validated that the levels of cytoplasmic Ca²⁺, mitochondria and ATP were cADPR-dependent. Given that the cytoplasmic Ca²⁺ in the CD157 overexpressed BMSCs increased significantly, we might conclude that CD157 regulated the mitochondrial production and transfer via cADPR-Ca²⁺ pathway.

In conclusion, the present study demonstrated that up-regulation of CD157 on BMSCs could raise the transfer extracellular mitochondria particles to VSC4.1 motor neurons, gradually regenerate the axon of VSC4.1 motor neuron and reduce the cell apoptosis by increasing the level of Ca^{2+} . The results of animal models also found that upregulation of CD157 on BMSCs could significantly improve the functional recovery, axon regeneration and neuron apoptosis in SCI rats. However, the clinical application of CD157 modified BMSCs on SCI requires further investigation.

Conclusion

The present study for the first time demonstrated that CD157 on BMSCs might be stimulated by the microenvironment with injured neurons. In turn, upregulation of CD157 on BMSCs could increase the produce and transfer of mitochondria from BMSCs to injured neurons, improving the neuroregeneration and cell apoptosis. The calcium-dependent CD157/cyclic ADP-ribose signaling pathway may be involved in the mitochondria transfer from BMSCs to injured neurons of SCI rats and OGD treated VSC4.1 motor neurons. The results suggested a novel approach for the efficiency of stem cell therapy strategies on SCI.

Abbreviations

SCI: spinal cord injury; BMSCs: bone marrow mesenchymal stem cells; ATP: adenosine triphosphate; OGD: oxygen glucose deprivation; MMP: mitochondrial membrane potential; NAD: nicotinamide adenine dinucleotide; NGD: nicotinamide guanine dinucleotide; ADP: adenosine–diphosphate; cADPR: cyclic ADP-ribose; ER: endoplasmic reticulum; BBB: Basso, Beattie and Bresnahan; BCM: BMSCs conditioned-medium; Md-BCM: mitochondria deleted BMSCs conditioned-medium; FACS: fluorescence-activated cell sorting; CFSE: carboxyfluorescein succinimidyl ester; DAPI: 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride; IOD: integrated optical density

Declarations

Ethics approval and consent to participate

All experimental procedures were approved by the Animal Ethics Committee of Zhejiang University and followed the National Institutes of Health guidelines strictly.

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZX and HC contributed to the experimental conceptualization and design. JL contributed to the experimental conceptualization and design, performed the most experiments and the statistics, and was a major contributor in writing the manuscript. HL performed the observation of extracellular mitochondria particles by transmission electron microscopy and motor functional detection. SC and SB performed partial experiments and provided considerable manuscript review. All authors read and approved the final manuscript.

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Figures

Figure 1

BMSCs produce functional extracellular mitochondria particles transferred into neurons. **a** Mitochondria in BMSCs visualized by Mito Tracker Red CMXRos. The red dots are mitochondria. Scale bar, 200 μm. **b** Representative TEM images of extracellular mitochondria derived from BMSCs. Scale bar, 200 nm. **c** FACS results of extracellular mitochondria particles pre-stained by Mito Tracker Red CMXRos. Control group, the medium of BMSCs without staining. Md-BCM group, the mitochondria depleted medium. BCM group, the normal pre-stained medium. **d** The ratio of extracellular mitochondria in medium (n=5).

* $p < 0.05$. e Representative fluorescence images of internalized mitochondria in VSC4.1 motor neurons co-cultured with BMSCs directly. BMSCs were pre-stained by CFSE. The purple arrows indicate VSC4.1 motor neurons. The white arrows indicate co-cultured BMSCs. Scale bar, 50 μm . f Representative fluorescence images of internalized mitochondria in VSC4.1 motor neurons co-cultured with BMSCs in the transwell system. VSC4.1 motor neurons were pre-stained by CFSE. Scale bar, 100 μm 100 μm . g Representative fluorescence images of VSC4.1 motor neurons. All VSC4.1 motor neurons were co-cultured with BMSCs in the transwell system. Control group, normal VSC4.1 motor neurons. OGD group, VSC4.1 motor neurons with oxygen and glucose deprivation treatment. Scale bar, 50 μm . h Quantification of internalized mitochondria in VSC4.1 motor neurons w/o OGD (fold of control). Image J was applied to the measurement of IOD. ** $p < 0.01$. i Quantification of ATP content in medium ($n=6$, fold of Md-BCM). ** $p < 0.01$. j Statistics for extracellular mitochondria membrane potential ($n=8$). *** $p < 0.001$.

Figure 2

CD157 expression of BMSCs is upregulated but there is no change in VSC4.1 motor neurons. a Representative immunofluorescence images of CD157 protein. BMSCs were pre-stained by CFSE. Normal group, cells without any treatment. OGD group, VSC4.1 motor neurons were treated with oxygen and glucose deprivation for 8 hours before co-culture with BMSCs. The purple arrows indicate VSC4.1 motor neurons and the white arrow indicates BMSCs. Scale bars, 100 μm . b-c Quantification of CD157 expression (fold of normal) of VSC4.1 motor neurons b and BMSCs c. Image J was applied to the measurement of IOD. n.s., non-significant, ** $p < 0.01$. d Transfection effects of mock, Bst-1+, and Bst-1- adenovirus vectors, alphabetically. All vectors were recombined with the GFP gene. Scale bar, 100 μm 100 μm . e Western blotting of CD157 protein expression of transfected BMSCs.

Figure 3

CD157 overexpression of BMSCs enhances the production and transfer of extracellular mitochondria. a FACS results of extracellular mitochondria derived from different transfected BMSCs. Control, BMSCs without pre-staining by Mito Tracker Red CMXRos. b The ratio of extracellular mitochondria particles detected by FACS (n=4). *p<0.05, **p<0.01. c Quantification of extracellular mitochondria particles detected by Varioskan Flash (n=5). *p<0.05, ***p<0.001. d and f Representative confocal images of internalized mitochondria of VSC4.1 motor neurons. All VSC4.1 motor neurons were subjected to OGD

treatment before direct co-culture b or co-cultured in transwell system d with BMSCs. Transfected BMSCs were marked by GFP d and VSC4.1 motor neurons were pre-stained by CFSE f. The white arrows indicate VSC4.1 motor neurons. Scale bars, 100 μ m. e and g Quantification of mitochondria internalization of VSC4.1 motor neurons (fold of Mock group). e for direct co-culture and g for transwell co-culture. Image J was applied to the measurement of IOD. * p <0.05, *** p <0.001, **** p <0.0001.

Figure 4

CD157 overexpression of BMSCs improves the injured VSC4.1 motor neurons in vitro. a Representative images of recovered VSC4.1 motor neurons. The four groups are in alphabetical order: OGD group, Mock group, Over group, and shRNA group. The black arrows show the axons. Scale bar, 50 μ m. b Statistics for the number of neurites of each recovered VSC4.1 motor neurons. n.s., non-significant. c Statistics for the length of neurites of each recovered VSC4.1 motor neurons (fold of OGD group). * $p < 0.05$, ** $p < 0.01$. d and f and h Respectively, representative immunofluorescence images of Grp 78 protein, NF- κ B protein, and Bcl-xl protein of VSC4.1 motor neurons. GFP marked BMSCs. The white arrows show VSC4.1 motor neurons. Scale bars, 100 μ m. e and g and i Respectively, quantification of Grp 78, NF- κ B, and Bcl-xl of VSC4.1 motor neurons (fold of OGD group). n.s., non-significant, **** $p < 0.0001$ vs OGD group. # $p < 0.05$, ## $p < 0.01$, #### $p < 0.0001$ vs Mock group.

Figure 5

Expression of related proteins in vitro. a Schematic diagram of the spinal cord. b BBB scores of injured rats (n=6). * $p < 0.05$, & $p < 0.05$ vs SCI+Mock group. #### $p < 0.001$ vs SCI+shRNA group. c and e Representative immunofluorescence images and quantification of Grp 78 and NF-κB of neurons at the injured site. The white arrows indicate the positive cells. Scale bars, 100 μm. d and f Respectively, quantification of Grp 78 and NF-κB of neurons. * $p < 0.05$, **** $p < 0.0001$, n.s., non-significant vs SCI group. # $p < 0.05$, ## $p < 0.01$, #### $p < 0.0001$ vs Mock group.

Figure 6

Expression of related proteins in vitro. a and c Representative immunofluorescence images and quantification of p-Akt and Gap 43 of neurons at the injured site. The white arrows indicate the positive cells. Scale bars, 100 μ m. b and d Respectively, quantification of p-Akt and Gap 43 of neurons. *** p <0.001, **** p <0.0001, n.s., non-significant vs SCI group. # p <0.05, ## p <0.01, ### p <0.001, #### p <0.0001 vs Mock group.

Figure 7

CD157/ADPR-cyclase activity and Ca²⁺ signal of BMSCs. a Confirmation of CD157/ADPR-cyclase activity (n=5, fold of 0 nM). *p<0.05. b Statistics for the mitochondria content of BMSCs pre-stained by Mito Tracker Red CMXRos (n=5, fold of 0 nM). **p<0.01, ***p<0.001. c Statistics for the ATP content of BMSCs subjected to cADPR at different concentrations (n=3, fold of 0 nM). *p<0.05, ***p<0.001, ****p<0.0001. d Statistics for the Ca²⁺ signal of BMSCs subjected to cADPR at different concentrations (n=3, fold of 0 nM). *p<0.05, **p<0.01. e Statistics for the Ca²⁺ signal of transfected BMSCs when co-cultured with OGD VSC4.1 motor neurons (n=4, fold of OGD group). n.s., non-significant, **p<0.01, ****p<0.0001 vs OGD group. #p<0.05, ###p<0.001 vs Mock group.