Abstract. Stroke is the second most common cause of death worldwide, and thus, it imposes great financial burdens on both individuals and society. Mesenchymal stem cell (MSC) therapy is a promising approach for ischemic brain injury. However, MSC treatment potential is progressively reduced with age, limiting their therapeutic efficacy for brain repair post-stroke. C1q and tumor necrosis factor-related protein 9 (CTRP9) is a novel cytoprotective cytokine with antioxidant effects, which is highly expressed in brain tissue. The present study tested the hypothesis that CTRP9 might act as an antisenescence factor to promote the rejuvenation of aged MSCs. MSCs were isolated from the bone marrow of young (8-weeks-old) and aged (18-months-old) male C57BL/6 mice. Cell proliferation was measured by Cell Counting Kit-8 assay and cell viability was determined by MTT assay. Gene expression levels of interleukin (IL)-6 and IL-10 were evaluated with reverse transcription-quantitative polymerase chain reaction, and secretion of vascular endothelial growth factor, basic fibroblast growth factor, hepatocyte growth factor, and insulin-like growth factor were measured by ELISA. The expression levels of proteins in the peroxisome proliferator-activated receptor γ coactivator 1α/AMPK signaling pathway were investigated with western blotting. Oxidative stress was evaluated by detecting mitochondrial membrane potential, reactive oxygen species, superoxide dismutase activity and malondialdehyde. MSCs isolated from aged mice exhibited reduced proliferation and viability, and impaired immunoregulatory and paracrine abilities, compared withMSCs from younger mice. CTRP9 had a significant antisenescence effect in aged MSCs by activating PGC-1α/AMPK signaling and decreasing the oxidative response. Silencing either PGC-1α or AMPK abolished the above effects of CTRP9. These results suggest that CTRP9 may have a critical role in cellular senescence by facilitating stem cell rejuvenation, and may therefore have the potential to enhance the efficacy of stem cell therapy.

Introduction

Stroke is a multi-factorial polygenic disease and a major cause of death and adult disability (1). Administration of bone marrow stem cells has been demonstrated to protect ischemic rat brain by facilitating the recovery of neurological functions, reducing lesion size, and improving functional outcomes (2). Transplanted mesenchymal stem cells (MSCs) have also been demonstrated to promote the repair of damaged brain tissue by differentiating into cells of neuronal or glial lineage, regulating the immune response, and by the release of trophic factors that stimulate endogenous repair processes, such as neurogenesis, angiogenesis, and synaptogenesis (3). However, the application of MSCs can be complicated by patient-specific factors, such as age (4). Stem cell function generally declines with age, accompanied by reductions in cell number and in the proliferation and differentiation potential of MSCs, with subsequent impairments in their therapeutic effects in ischemic diseases (5,6). Age is known to be the principal non-modifiable risk factor for stroke (7), and it is therefore essential to identify effective methods for rejuvenating senescent MSCs derived from older donors, in order to improve their therapeutic efficacy for patients with ischemic stroke.

Adiponectin (APN) is an adipocytokine with a collagenous domain and a C-terminal globular domain, which is predominantly secreted by adipose tissues (8). APN has been demonstrated to protect against ischemic injury (9). Among C1q and tumor necrosis factor-related protein (CTRP) family members, CTRP9 displays the highest amino acid identity to APN. CTRP9 is also highly expressed in brain tissue (10), and acts as an adipocytokine with beneficial effects on glucose metabolism, cellular survival, oxidant response regulation, and inhibition of endoplasmic reticulum stress, all of which...
are related to the cellular senescence process (11-13). However, the effect of exogenous CTRP9 on cellular senescence in MSCs, which adversely affects the function of engrafted stem cells, has never been studied.

AMP-activated protein kinase (AMPK) is an evolutionarily conserved kinase that serves a central role in maintaining the cellular metabolic balance (14). AMPK initiates biological changes aimed at restoring cellular energy balance under conditions of energetic stress, which are related to cellular senescence (15,16). In addition, AMPK activation has also been demonstrated to increase the lifespan and to the improve metabolism (17), while activation of the AMPK/mammalian target of rapamycin (mTOR) pathway attenuates age-related dementia (18). As an important target of CTRP9, AMPK exerts a cytoprotective effect by regulating the oxidant response and inhibiting endoplasmic reticulum stress (11,19). However, the involvement of AMPK in CTRP9-induced rejuvenation of aged MSCs remains unclear.

Induction of the oxidative response is implicated in cellular aging (4), and maintaining a delicate oxidative/antioxidative balance has been demonstrated to be critical for MSC maintenance and DNA integrity, as well as for preventing reactive oxygen species (ROS)-mediated cellular senescence (20). MSCs are normally quiescent with an inherently low metabolic rate, and generate low levels of ROS; however, ROS levels accumulate with age, leading to ROS-induced oxidative free radical damage and impaired cellular function, associated with reduced efficacy for treating ischemic diseases (21,22). CTRP9 is an effective antioxidant factor that has been reported to ameliorate endothelial dysfunction via a peroxisome proliferator-activated receptor γ coactivator (PGC)-1α/AMPK-mediated antioxidant effect (19). These results suggested that this antioxidant capacity of CTRP9 may influence MSC aging.

The present study aimed to investigate the effect of CTRP9 on MSC senescence and the role of the PGC-1α/AMPK signaling pathway in CTRP9-induced rejuvenation in relation to age-related cellular impairments in MSCs.

**Materials and methods**

**Reagents.** X-tremeGENE HP DNA transfection reagent was purchased from Roche Diagnostics (Basel, Switzerland). Rabbit monoclonal antibodies against PGC-1α (1:1,000; cat. no. 2178), AMPK (1:1,000; cat. no. 5832), phospho-AMPK (1:1,000; cat. no. 32051), and β-actin (1:1,000; cat. no. 4970) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Vascular endothelial growth factor (VEGF, cat. no. AB1546), basic fibroblast growth factor (bFGF, cat. no. RAB0184), hepatocyte growth factor (HGF, cat. no. RAB0214), and insulin-like growth factor (IGF, cat. no. RAB0229) ELISA kits were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:1,000; sc-2357) were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Small interfering RNAs (siRNAs) targeting PGC-1α and AMPK transcripts were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Superoxide Dismutase (SOD) Activity Colorimetric assay and Lipid Peroxidation (malondialdehyde; MDA) assay kits were purchased from Abcam (Cambridge, UK). Human recombinant CTRP9 was obtained from Aviscera Bioscience Inc. (Santa Clara, CA, USA).

**Animals.** The young (8-weeks-old, n=12) and aged (18-months-old, n=12) male C57BL/6 mice were purchased from the Laboratory Animal Center of Wenzhou Medical University (Wenzhou, China). All procedures were approved by the Laboratory Animal Ethics Committee of Wenzhou Medical University. The mice were housed under a 12 h light/dark cycle at 21±2°C and 30-70% humidity. Food and water were provided ad libitum.

**Cell culture and cell treatment.** Bone marrow MSCs were isolated using a standard protocol, as described previously (23). Briefly, bone marrow was isolated from the femurs and tibias of mice by flushing with PBS. Adherent MSCs were propagated and maintained at 37°C and 5% CO₂ in high glucose DMEM (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences) and 1% penicillin/streptomycin (Beyotime Institute of Biotechnology, Jiangsu, China).

For CTRP9 treatment, cells were cultured with medium containing 2 µg/ml of recombinant CTRP9 and incubated at 37°C for various periods, as described previously (13).

**Cell proliferation assay.** MSCs were plated at 1x10⁴ cells/well in 96-well plates and cell proliferation was determined using a Cell Counting Kit-8 (CCK-8; HaiGene Technology, Harbin, China), according to the manufacturer’s protocol. The absorbance of each well at 450 nm was recorded.

**MTT assay.** Cell viability was determined by MTT assay. Briefly, 300 µl of MTT reagent was added to each well 2 h prior to harvesting. The supernatant was then removed and the cells were incubated with 400 µl of dimethyl sulfoxide for 10 min. Absorbance at 540 nm was recorded using an ELISA plate reader.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** The expression levels of several genes were analyzed by RT-qPCR. RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific, Inc.) and reverse transcribed using a First Strand cDNA Synthesis kit (Roche Diagnostics). qPCR was performed using Fast Start Universal SYBR Master reagent (Sigma-Aldrich; Merck KGaA). The samples were subjected to 40 cycles of amplification at 95°C for 15 sec followed by 63°C for 20 sec and 71°C for 25 sec by using specific primers. The quantification number of cycles (Cq) was set within the exponential phase of the PCR. The ΔCq value for each target gene was calculated by subtracting the Cq value for the GAPDH gene (internal control). Relative fold changes in mRNA expression were calculated using the formula 2^-ΔΔCq (24). The primer pairs used to detect the mRNA levels of target genes are listed in Table I.

**Western blot analysis.** MSCs were lysed with ice-cold lysis buffer (Beyotime Institute of Biotechnology) to obtain total protein. Expression levels of PGC-1α, AMPK, phospho-AMPK
and β-actin were evaluated by western blotting. Cellular extracts were prepared according to the manufacturer's instructions. Protein samples were quantified and separated by SDS-PAGE. Western blot assays were performed as described previously (22).

ELISA. Total concentrations of VEGF, bFGF, HGF and IGF proteins secreted by MSCs were assessed by ELISA, according to the manufacturer's protocol, as described previously (23). The absorbance of each well was quantified at 450 nm. Samples were analyzed in triplicate.

siRNA knockdown. MSCs were transfected using X-tremeGENE HP DNA Transfection Reagent, according to the manufacturer's protocol. Briefly, MSCs at 80% confluence were transfected with siRNA for 6 h at 37˚C. The transfection reagent-siRNA mixture was then replaced with fresh growth medium and the cells were harvested for further experiments at 72 h post-transfection. The siRNA sequences were: siRNA-PGC-1α, AAGACGGATTGCTTGCAGT; siRNA-AMPK, CCAGCUACAGUACACCUCUGAU; and siRNA-NT (non-targeting control), TTCTCGGACAGTGTACGT.

Relative telomere length measurement. Relative telomere length quantification in U87 cells was performed using a qPCR approach, as previously described (25). GAPDH was used as the normalizing gene. The primer pairs used to detect the telomere length are listed in Table I.

Relative telomerase activity measurement (RTA). Telomerase activity of whole cell lysate was measured by a Telo TAGGG telomerase PCR ELISA PLUS kit (Roche Diagnostics GmbH, Penzberg, Germany). Cell lysates were centrifuged at 12,000 x g (20 min at 4˚C) and 3 µl of cell extract was used for each telomeric repeat PCR amplification reaction and 3 µl of inactivated cell lysate was used for Telomeric Repeat Amplification Protocol (TRAP) reaction, according to the manufacturer's recommendations. The amount of TRAP products was determined with ELISA by measurement of absorbance at 450 nm using a microplate reader, as previously reported (26).

Mitochondrial membrane potential. Cells were grown in a 96-well microtiter plate at 37˚C for 1 day in complete culture medium to a density of 1x10^5 cells per well. The cells were then washed with PBS and incubated at 37˚C for 15 min with 5 µg/ml JC-1. After two wash cycles with PBS, the time-dependent JC-1 fluorescence was recorded using an ELISA plate reader. The fluorescent probe was excited at 490 nm and emission was measured at 530 and 590 nm.

SOD activity. SOD activity in cells was determined using a colorimetric assay kit (Abcam), according to the manufacturer's protocol. Briefly, protein was isolated from MSCs using lysis buffer, and SOD activity was measured in 10 µg of total protein extract. Absorbance was measured at 450 nm.

ROS measurement. Levels of intracellular ROS were determined using 2,7-dichlorodihydrofluorescein diacetate (Beyotime Institute of Biotechnology), following the manufacturer's instructions. The fluorescence intensity of the cells was measured using a fluorescence spectrophotometer, with excitation and emission wavelengths of 488 and 525 nm, respectively.

Lipid peroxidation assays. Lipid peroxidation was monitored using an assay kit (Abcam) to measure the formation of MDA, according to the manufacturer's protocol. Briefly, MSCs (1x10^6 cells) were homogenized on ice in 300 µl of MDA lysis buffer (with 3 µl of 100X butylated hydroxytoluene), then centrifuged (13,000 x g, 10 min) to remove insoluble material. The supernatant (200 µl) was added to 600 µl of thiobarbituric acid and incubated at 95˚C for 60 min. The samples were cooled to room temperature in

### Table I. Sequences of primers used in the study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>TCTATACACTTCACAAGTGGGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAATGCCCATTGCAACAATCTTTT</td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward</td>
<td>GGAACATCGAGGCTAATCTCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTGGAGGTGTTTCTGTTG</td>
</tr>
<tr>
<td>PGC1-α</td>
<td>Forward</td>
<td>CTCTATGTGTGTTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTCTGTTG TTGTTGTTG</td>
</tr>
<tr>
<td>AMPK</td>
<td>Forward</td>
<td>TGAAGTAGGAGATGGCCACTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGCCGAGAACGAGAAGTACG</td>
</tr>
<tr>
<td>Telomere length</td>
<td>Forward</td>
<td>AATCTCCACTTTGCACTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATGGTGAAAGTCGGTACG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
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IL, interleukin; PGC1-α, peroxisome proliferator-activated receptor coactivator-1α; AMPK, AMP-activated protein kinase.
an ice bath for 10 min, and the absorbance at 532 nm was measured spectrophotometrically.

Statistical analysis. Data were expressed as mean ± standard deviation. Differences among groups were assessed by one-way analysis of variance followed by a Tukey's b test, and comparisons between two groups were evaluated with Student's t-tests, using SPSS package v19.0 (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

MSCs display degenerative properties during aging. MSC properties were compared between cells derived from young (8-weeks-old) and aged (18-months-old) male C57BL/6 mice. Cell proliferation rates measured by CCK-8 assay were significantly lower in aged MSCs after 1, 3, 5 and 7 days of culture, compared with the young MSCs (Fig. 1A). MTT assay also demonstrated impaired cellular viability in the aged group (Fig. 1B). The mRNA expression levels of IL-10 and IL-6 were
reduced in aged MSCs compared with young MSCs (Fig. IC and D). Finally, the levels of secreted VEGF, bFGF, HGF and IGF proteins were significantly decreased in aged compared with young MSCs, as evaluated by ELISA (Fig. 1E-H).

CTRP9 restores the degenerative properties of aged MSCs. The reduced proliferative ability of aged MSCs was significantly reversed by addition in the culture of human recombinant CTRP9, according to results from the CCK-8 assay (Fig. 2A), while cell viability was increased by CTRP9 at the given doses (2 µg/ml; Fig. 2B). CTRP9 treatment also increased the immunoregulatory abilities of MSCs accompanied by increased IL-10 and IL-6 mRNA expression (Fig. 2C and D), and promoted the paracrine abilities of aged MSCs,
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as evidenced by increased secretion of VEGF, bFGF, HGF and IGF (Fig. 2E-H). Furthermore, incubation of MSCs with CTRP9 increased the senescence-impaired telomere length (Fig. 2I) and telomerase activity (Fig. 2J).

The PGC-1α/AMPK signaling pathway is involved in the rejuvenating effect of CTRP9. Cellular senescence impeded PGC-1α protein expression, but this effect was significantly reversed by CTRP9 treatment (Fig. 3A and B). CTRP9 also abrogated the age-mediated suppression of AMPK phosphorylation (Fig. 3C and D), suggesting a critical role for CTRP9 in activation of the PGC-1α/AMPK signaling pathway. Inhibition of PGC-1α or AMPK by transient transfection with specific siRNAs (Fig. 3E and F) abolished the ameliorating effects of CTRP9 on age-related decreases in cellular proliferation (Fig. 4A) and viability (Fig. 4B), on the decreased immunoregulatory (Fig. 4C and D) and paracrine abilities (Fig. 4E-H), accompanied by shortened telomere length (Fig. 4I) and impaired telomerase activity (Fig. 4J).

CTRP9 exerts a rejuvenating effect via the antioxidant response. The role of the antioxidant response in CTRP9-induced rejuvenation of MSCs was further investigated by examining the mitochondrial transmembrane potential, activation of SOD, generation of ROS, and lipid peroxidation by MDA assay. Aging significantly decreased the mitochondrial transmembrane potential (Fig. 5A) and

Figure 3. PGC-1α/AMPK signaling pathway activation. (A) Representative blots and (B) quantification of western blot analysis of PGC-1α and β-actin protein expression levels in young MSCs, aged MSCs, and aged MSCs treated with CTRP9 (2 µg/ml). (C) Representative blots and (D) quantification of western blot analysis of AMPK and p-AMPK protein expression levels in young MSCs, aged MSCs, and aged MSCs treated with CTRP9. Data represent mean ± standard deviation from three independent experiments. *P<0.05 vs. aged. (E) PGC-1α mRNA levels in MSCs transfected with siRNA-PCG-1α or siRNA-NT. *P<0.05 vs. siRNA-PCG-1α. (F) AMPK mRNA levels in MSCs transfected with siRNA-AMPK or siRNA-NT. *P<0.05 vs. siRNA-AMPK. PGC-1α, peroxisome proliferator-activated receptor γcoactivator-1α; AMPK, AMP-activated protein kinase; MSCs, mesenchymal stem cells; CTRP9, C1q and tumor necrosis factor-related protein 9; p-, phosphorylated; si, small interfering; NT, non-targeting control.
SOD activation (Fig. 5B), while it increased ROS generation (Fig. 5C) and MDA activation (Fig. 5D). By contrast, CTRP9 treatment significantly increased the mitochondrial transmembrane potential (Fig. 5A) and SOD activation (Fig. 5B), and greatly reduced ROS generation (Fig. 5C) and MDA activation (Fig. 5D). Furthermore, inhibition of PGC-1α or AMPK by siRNA abolished the antioxidant effects of CTRP9, resulting in increased ROS generation and MDA activation.
Discussion

Aging is associated with reduced organ function and an increased incidence of disease (27), and functional impairment and mortality have been demonstrated to be significantly increased in aged stroke patients compared with relatively young patients (28). Stem cell therapy has shown great potential for repairing and remodeling the neurovascular structure following ischemic brain injury (29); however, functional improvement after stem cell transplantation is impaired by cellular senescence (6). It is therefore crucial to be able to rejuvenate aged MSCs in order to improve their efficacy for the treatment of ischemic stroke. MSC aging driven by both intrinsic and extrinsic factors is linked to impaired MSC self-renewal and regeneration (30), as well as decreased immunoregulatory and angiogenic abilities (31,32), which are important in the treatment of ischemic stroke. The results of the current study indicated that MSC proliferation and viability, immune modulation, and angiogenesis-related paracrine ability were all impaired in MSCs from aged donors.

CTRP9 is a novel adipokine exclusively expressed in adipocytes and also highly expressed in the brain (10). CTRP9 has been suggested to act as a regulator of vascular function, and to exert protective effects in ischemic diseases (12). The cytoprotective effects of CTRP9 have been well documented, through promoting cell proliferation, inhibiting the oxidative response, and modulating endoplasmic reticulum stress (13,33). The present study demonstrated that CTRP9 treatment promoted MSC proliferation and viability and increased their immunoregulatory and angiogenic abilities, accompanied by elongation of telomere length and recovery of telomerase activity.

PGC-1α is a transcriptional coactivator and a fundamental regulator of mitochondrial function (34). Several studies have highlighted an important protective role for PGC-1α in cellular senescence (35), and activation of PGC-1α maintains cellular metabolic homeostasis through regulation of mitochondrial ROS to exert a rejuvenating effect in senescent cells (14). Activation of PGC-1α also enhances the engraftment and angiogenesis of MSCs in ischemia diseases (36). The present study demonstrated that cellular senescence inhibited PGC-1α and that exogenous CTRP9 re-activated PGC-1α in aged MSCs, while PGC-1α inhibition abolished the CTRP9-mediated rejuvenation. These results suggested that CTRP9 may attenuate senescence-stimulated MSC dysfunction through activation of PGC-1α.

AMPK is an energy sensor that is ubiquitously expressed in MSCs and exerts antisenescence effects (31). The protective effects of APNCTRP9 on cellular protection are expected to be partially mediated by the activation of AMPK signaling (13). In addition, AMPK activation has been demonstrated to be responsible for the anti-inflammatory effect of CTRP9 in cells (37). The current results demonstrated that CTRP9 prevented the senescence-mediated suppression of AMPK phosphorylation, while knockdown of AMPK eradicated the inhibitory effects of CTRP9 on MSC dysfunction in response to senescence, implying an essential role for AMPK.

Impaired mitochondrial function and increased oxidative effects lead to DNA damage and are considered to contribute

and decreased mitochondrial membrane potential and SOD activation (Fig. 5).
to cellular senescence in stem cells (4). Accumulating oxidant stress impairs the function of aged MSCs via excessive ROS production (23). A recent study revealed that CTRP9 promotes MSC proliferation and migration, and protects against hydrogen peroxide-induced cellular death through its antioxidative effect (13). Similarly, the present results demonstrated that senescence elicited an oxidant reaction, and this effect was prevented by CTRP9 treatment. Furthermore, this CTRP9-mediated antioxidative effect was suppressed by knockdown of PGC-1α or AMPK expression by siRNA.

In conclusion, CTRP9 treatment inhibited the oxidant effect associated with cell senescence via PGC-1α/AMPK signaling activation, contributing to rejuvenation of MSCs. CTRP9 may thus serve as a candidate for rejuvenating MSCs and for potentially improving the therapeutic efficacy of MSC transplantation in aged patients with ischemic stroke.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions

QL and ZZ were the major contributors in writing the manuscript. WMZ and XBC were involved in conception and design, revising it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures involving the use of animals were approved by the Laboratory Animal Ethics Committee of Wenzhou Medical University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


