

# Conditioned medium from tonsil-derived mesenchymal stem cells promotes adiponectin production

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**Abstract.** Mesenchymal stem cells (MSCs) are often considered to be a good source for the development of regenerative medicine. Previously, we reported that tonsil-derived MSC conditioned medium (T-MSC CM) produces visceral fat reducing effects. As reduced visceral adiposity is closely associated with an increase in circulating adiponectin, the present study investigated the effects of T-MSC CM on adiponectin production. T-MSC CM was collected from previously isolated and characterized T-MSCs and injected into senescence-accelerated mouse prone 6 mice, which exhibit characteristics of aging and obesity. The results demonstrated a reduction in mouse weight and epididymal adipose tissue (eAT) mass following injection of T-MSC CM. Significant increases in adiponectin expression in the eAT, and total and high molecular weight (HMW) adiponectin in the circulation were observed in the T-MSC CM-injected mice compared with control mice using reverse transcription-quantitative polymerase chain reaction, western blot analysis and ELISA. In 3T3-L1 adipocytes, T-MSC CM treatment increased adiponectin secretion and multimerization, as detected using western blotting under non-reducing and non-heat-denaturing conditions. Furthermore, glucose oxidase was used to induce oxidative stress in 3T3-L1 adipocytes and it was observed that T-MSC CM reduced reactive oxygen species production and the expression of certain oxidative stress markers. In addition, the results also demonstrated that the production of HMW adiponectin was increased, which indicates that T-MSC CM may enhance adiponectin multimerization via amelioration of oxidative stress. Further studies are required to elucidate

anti-oxidant molecules secreted from T-MSCs, and these results highlight the potential therapeutic relevance of T-MSC CM for the treatment of obesity or obesity-associated diseases.

## Introduction

Mesenchymal stem cells (MSCs) are often considered to be a good source for the development of regenerative medicine due to the self-renewal and multipotent properties of these cells. The MSC secretome is another area of research that will ultimately contribute to the development of cell-free therapy (1,2). From soluble factors to extracellular vesicles, MSCs produce a variety of biologically active secreted proteins. As only a small percentage of transplanted MSCs are successfully engrafted, differentiated and functional in the target tissue (3,4), it was suggested that endocrine and immunomodulatory effects of the MSC secretome may have important roles in tissue regeneration (5,6).

MSCs can be obtained from various tissues, including the bone marrow, adipose tissue, placenta and umbilical cord. In 2008, human tonsils were described as a novel source of MSCs and these MSCs are termed tonsil-derived MSCs (T-MSCs) (7). The characteristics and potential of MSCs to be used for cell banking have been previously reported (8). The therapeutic potential of T-MSCs in the treatment of liver (9,10), pancreas (11), parathyroid gland (12), skeletal muscle (13), and bone (14) diseases, as well as the mechanisms that underlie their immunomodulatory effects (15-18), have been described previously.

Adiponectin, which is primarily produced and secreted by adipocytes, evokes several beneficial metabolic functions via insulin-sensitizing, anti-inflammatory and cardiovascular protective effects (19-21). Although adiponectin is secreted from adipocytes, the levels of this protein in circulation are not always correlated with body mass index (BMI). Instead, weight reduction paradoxically increases adiponectin in circulation (22,23). Adiponectin circulates in different multimer forms: Low molecular weight form (LMW; trimer), medium molecular weight form (hexamer) and high molecular weight form (HMW; octadecamer or higher). LMW and HMW adiponectin promote glucose and lipid metabolism in hepatocytes, while LMW primarily acts on myocytes (19,21).

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Thus, promotion of adiponectin multimerization rather than an increase and/or decrease of total adiponectin affects the function of adiponectin.

In a previous study, we observed that T-MSC CM injection reduces the body weight of senescence-accelerated mouse prone 6 (SAMP6) mice (14). In addition, a selective decrease in visceral adiposity was observed in the previous study, and we also reported that T-MSC CM has the potential to inhibit adipogenesis *in vitro*. Given that weight reduction is closely associated with the improvement of whole body metabolism due, in part, to increased levels of adiponectin in circulation, the present study aimed to determine whether T-MSC CM regulates adiponectin expression, secretion, and/or multimerization in SAMP6 aging mice and 3T3-L1 adipocytes.

## Materials and methods

**T-MSC culture and T-MSC CM preparation.** Previously isolated and characterized human T-MSCs (8,24) were cultured in Dulbecco's modified Eagle's medium (DMEM) high-glucose medium (Welgene, Inc., Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS; Welgene, Inc.), 100 IU/ml penicillin and 100 µg/ml streptomycin (Welgene, Inc.). Cells were maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. When cells reached 80% confluency, fresh culture medium was added and the cells were incubated for an additional 48 h. T-MSC CM was harvested and concentrated 10-fold using 3-kDa cut-off Amicon Ultra centrifugal filter units (Merck KGaA, Darmstadt, Germany) at a speed of 3,800 x g for 30 min at room temperature.

**Animals.** A total of 12 male SAMP6 mice (age, 4 months; weight, 47.04±2.87 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and maintained at 21–23°C with 51–54% humidity and a 12-h light/dark cycle under conventional conditions, with food and water supplied *ad libitum*. At 7 months of age (weight, 52.64±4.66 g), mice were divided into two groups (n=6 mice/group) and injected via tail vein with either culture medium alone (control) or culture supernatant that was collected and concentrated after a 48 h incubation of 0.8x10<sup>6</sup> T-MSCs (T-MSC CM). Treatments were performed twice a week for 2 weeks using freshly prepared CM. Mice were sacrificed at 9 months of age by cervical dislocation, and whole blood and organs were harvested for further analysis. Experiments and procedures were approved by the Animal Ethics Committee at Ewha Womans University School of Medicine (Seoul, Korea; no. ESM 14-0278), and all experiments were performed in accordance with relevant guidelines and regulations.

**Hematoxylin and eosin (H&E) staining.** Mouse epididymal adipose tissue (eAT) was isolated and fixed with a 4% paraformaldehyde solution overnight at 4°C. Paraffin-embedded eAT was sectioned at 4 µm and subjected to H&E staining. Briefly, cells were stained with 0.7% hematoxylin for 2 min and then with 5% eosin for 1.5 min at room temperature. Stained sections were observed using an Olympus BX50 light microscope (Olympus Corporation, Tokyo, Japan) and images were captured under x100 magnification.

**Adiponectin ELISA.** Blood samples (~200 µl) were collected in a tube containing heparin sodium (JW Pharmaceutical, Seoul, Korea) from the facial vein of 7- and 9-month-old mice following 6 h of fasting. Plasma proteins were separated by centrifugation at 1,000 x g for 10 min at room temperature. The adiponectin concentration was measured using the Adiponectin Total, HMW ELISA kit (cat. no. 47-ADPMS-E01; Alpco Diagnostics, Salem, NH, USA), according to the manufacturer's protocol.

**Cell culture.** The mouse preadipocyte cell line 3T3-L1 was purchased from the Korean Cell Line Bank (Seoul, Korea) and maintained in DMEM high-glucose medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. After reaching 100% confluency, the medium was changed and cells were maintained for an additional 3 days. Cells were subsequently induced to differentiate to adipocytes (day 0). To induce adipocyte differentiation, high-glucose DMEM containing 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin supplemented with 2 mg/ml insulin, 0.25 µM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine (all from Sigma-Aldrich; Merck KGaA) was used. On day 3, the medium was replaced with medium containing insulin only and cells were cultured for another 4 days at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Concentrated culture medium or T-MSC CM was added between days 7 and 10. On day 10, the cells were rinsed with PBS and incubated in serum-free medium overnight. Culture supernatants and cells were harvested the next day for further analyses. To induce oxidative stress in mature adipocytes, cells were pretreated with 50 mU/ml glucose oxidase (Sigma-Aldrich; Merck KGaA) for 2 h, followed by overnight treatment with T-MSC CM at 37°C.

**RNA extraction and reverse transcription (RT).** To harvest adipocytes, the eAT was weighed and finely minced. Minced tissue was transferred to a 4-times greater volume of Hank's Balanced Salt Solution buffer (Welgene, Inc.) containing 1 mg/ml collagenase I (Sigma-Aldrich; Merck KGaA) and 0.28 M fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA). Tissue digestion was performed by incubating the tissue sample for 2 h at 37°C in a shaking incubator. Digested tissue was filtered through a cell strainer and centrifuged at 100 x g for 15 min at room temperature. The floating adipocyte fraction was transferred to a new tube for RNA extraction.

To extract RNA from mouse eAT or 3T3-L1 adipocytes (9.5x10<sup>5</sup>), TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used. Tissue homogenization was performed using a TissueRuptor (Qiagen GmbH, Hilden, Germany), and following phase separation by centrifugation at 12,000 x g for 15 min at 4°C, RNA was isolated using NucleoSpin® RNA Clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), according to the manufacturer's protocol. After determination of the concentration and purity of the RNA samples using BioPhotometer D30 (Eppendorf, Hamburg, Germany), 1 µg RNA was used for cDNA synthesis using a ReverTra Ace-α-kit (Toyobo Co., Ltd., Osaka, Japan). Reverse transcription was performed by incubation at 30°C for 10 min, at 42°C for 20 min and at 99°C for 5 min.

**RT-quantitative polymerase chain reaction (qPCR).** To determine the expression of target genes, a primer pair (0.4  $\mu$ M) and SYBR-Green Real-time PCR Master Mix (Toyobo Co., Ltd.) were mixed with the prepared cDNA. The primer sequences are listed in Table I. Amplification was performed in duplicate by 40 cycles of 15 sec denaturation step at 95°C and a 1 min amplification and signal acquisition step at 60°C using StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Cycle threshold (Cq) values were obtained, and the relative expression level of a target gene was determined as  $2^{-(\text{cyclophilin Cq} - \text{target gene Cq})}$  (25).

**Western blot analysis.** Whole protein lysates were isolated from mouse eAT or 3T3-L1 adipocytes ( $9.5 \times 10^5$ ) using PRO-PREP Protein Extraction Solution (Intron Biotechnology, Inc., Seongnam, Korea). Protein concentrations were determined using the Bradford assay with the Bio-Rad Protein assay solution (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Samples (4  $\mu$ g for tissue lysates and 10  $\mu$ g for cell lysates) were resolved by 10% SDS-PAGE and transferred to Immobilon-P polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk in TBS containing 0.1% Tween-20 (TBS-T) solution for 1 h at room temperature and were subsequently incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: Adiponectin (1:1,000, diluted in 5% skim milk containing TBST; cat. no. ab25891; rabbit; Abcam, Cambridge, UK); and  $\beta$ -actin [1:2,000, diluted in 2% BSA (Bovogen Biologicals, Pty, Ltd., East Keilor, Victoria, Australia) containing TBST; cat. no. A1978; mouse; Sigma-Aldrich; Merck KGaA]. The membranes were washed 3 times for 10 min in TBST and incubated with anti-rabbit (cat. no. BR170-6515; Bio-Rad Laboratories, Inc.) or anti-mouse (cat. no. BR170-6516; Bio-Rad Laboratories, Inc.) horseradish peroxidase-conjugated secondary antibodies (1:3,000, diluted in TBST) for 1 h at room temperature. Following incubation, membranes were washed 3 times for 10 min in TBST and developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce; Thermo Fisher Scientific, Inc.). Images were obtained using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Little Chalfont, UK).

To detect secreted adiponectin in cell culture medium, conditioned medium was prepared by incubating differentiated 3T3-L1 adipocytes ( $3.8 \times 10^5$ ) in serum-free DMEM (500  $\mu$ l) for 18 h. Conditioned medium was concentrated 10-fold using 3-kDa cut-off Amicon Ultra centrifugal filter units (Merck KGaA) and centrifugation at 14,000  $\times$  g for 15 min at room temperature. Concentrated medium (50  $\mu$ l) was prepared in sample buffer (0.5 M Tris/HCl pH 6.8, 25% glycerol, 10% SDS, 500 mM DTT, 1% bromophenol blue; Sigma-Aldrich; Merck KGaA) and 12  $\mu$ l samples were separated on 10% SDS-PAGE for the detection of total secreted adiponectin. To detect adiponectin in multimer forms, concentrated medium was prepared in non-reducing and non-heat-denaturing conditions using sample buffer lacking reducing agents, and 12  $\mu$ l samples were subsequently separated on 4-15% precast polyacrylamide gradient gels (Bio-Rad Laboratories, Inc.). Blots were semi-quantified by densitometric analysis using UN-SCAN-IT gel analysis software version 6.1 (Silk Scientific, Inc., Orem, UT, USA).

**Reactive oxygen species (ROS) and reactive nitrogen species (RNS) measurement.** ROS and RNS production in total cell lysates or culture medium was measured using OxiSelect™ *In Vitro* ROS/RNS assay kit (Cell Biolabs, Inc., San Diego, CA, USA), according to the manufacturer's protocol. Cellular ROS/RNS levels were normalized to total protein measured by the BCA Protein assay kit (Pierce; Thermo Fisher Scientific, Inc.).

**Statistical analysis.** Student's t-test or one-way analysis of variance with Dunnett's multiple comparisons test was performed for statistical analysis using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean  $\pm$  standard error of the mean from 3-4 independent experiments.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**T-MSC CM injection reduces mouse body weight and eAT tissue mass.** In our previous study, we demonstrated that T-MSC CM produces anti-adipogenic effects. In the present study, these *in vitro* findings were further evaluated in a mouse model of accelerated senescence (SPAM6 mice), as these mice have been reported to exhibit an obese phenotype (26). Control culture medium or T-MSC CM was injected into 7-month-old SAMP6 mice, and body weight changes were examined 2 months later. A significant decrease in the weight of mice was observed at 9 months in mice injected with T-MSC CM, whereas the mice injected with the control medium did not exhibit alterations in weight (Fig. 1A). Isolated organs were weighed and normalized to the body weight. T-MSC CM injection led to reduced eAT mass compared with control mice, whereas the inguinal adipose tissue, liver and muscle mass were not affected by injection of T-MSC CM (Fig. 1B). Histological analysis of eAT demonstrated a reduction in the size of adipocytes in the T-MSC CM-injected mice compared with control mice (Fig. 1C). To determine the mRNA expression of adipogenic markers in eAT adipocytes, collagen digestion was performed, followed by floating adipocyte fraction separation. Together with the reduction in eAT mass and adipocyte size, the mRNA expression of the adipogenic markers peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), CCAAT/enhancer-binding protein  $\alpha$  (C/EBP $\alpha$ ) and leptin were significantly decreased, while adiponectin expression was increased in eAT adipocytes, compared with control-treated mice (Fig. 1D).

**T-MSC CM injection increases adiponectin in the circulation by upregulating expression in eAT.** As weight reduction is associated with an increase in circulating adiponectin levels, the present study investigated total and HMW adiponectin in mouse plasma prior to (7 months) and following (9 months) treatment with T-MSC CM. A significant increase in total adiponectin following T-MSC CM treatment was observed, while levels of adiponectin were significantly decreased in the mice injected with control medium (Fig. 2A). In addition, levels of HMW adiponectin were increased following T-MSC CM injection, although this was not statistically significant (Fig. 2B). Total and HMW adiponectin levels in mouse plasma were normalized to

Table I. Sequences of primers used for quantitative polymerase chain reaction.

Gene	GeneBank accession	Primer sequence	
		Forward	Reverse
Cyclophilin	NM_021130.4	5'-CGTTTTGGGTCCAGGAATGG-3'	5'-TACAGGACATTGCGAGCAGA-3'
PPAR $\gamma$	NM_011146.3	5'-GGAAGACCACTCGCATTCCTT-3'	5'-GTAATCAGCAACCATTGGGTCA-3'
C/EBP $\alpha$	NM_007678.3	5'-CAAGAACAGCAACGAGTACCG-3'	5'-GTCAGTGGTCAACTCCAGCAC-3'
Leptin	NM_008493.3	5'-GTGGCTTTGGTCCTATCTGTC-3'	5'-CGTGTGTGAAATGTCATTGATCC-3'
Adiponectin	NM_009605.4	5'-GTTCCCAATGTACCCATTTCG-3'	5'-TGTTGCAGTAGAACTTGCCAG-3'
p40 <sup>phox</sup>	NM_008677.2	5'-GCCGCTATCGCCAGTTCTAC-3'	5'-GCAGGCTCAGGAGGTTCTTC-3'
p47 <sup>phox</sup>	NM_010876.4	5'-GATGTTCCCCATTGAGGCCG-3'	5'-GTTTCAGGTCATCAGGCCGC-3'
P67 <sup>phox</sup>	NM_010877.5	5'-CTGGCTGAGGCCATCAGACT-3'	5'-AGGCCACTGCAGAGTGCTTG-3'
gp91 <sup>phox</sup>	NM_007807.5	5'-TTGGGTCAGCACTGGCTCTG-3'	5'-TGGCGGTGTGCAGTGCTATC-3'

PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; C/EBP $\alpha$ , CCAAT/enhancer-binding protein  $\alpha$ .

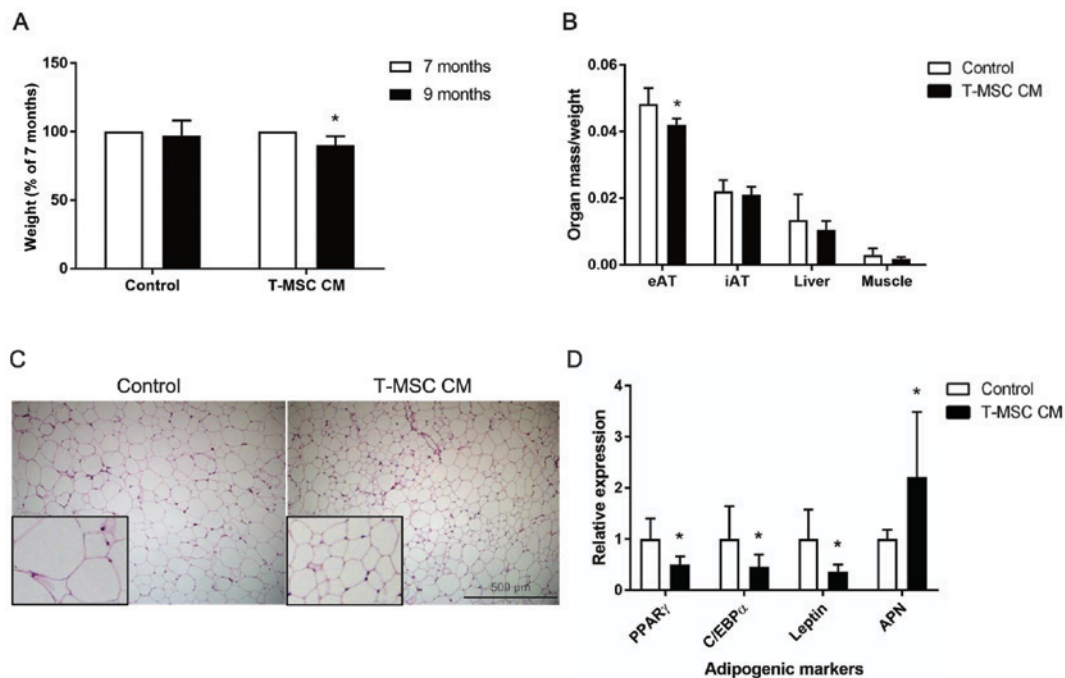


Figure 1. T-MSC CM injection reduced body weight and eAT accumulation in SAMP6 mice. (A) Mouse body weights were measured prior to (7-month-old) and following (9-month-old mice) treatment with control or T-MSC CM. (B) eAT, iAT, liver and skeletal muscle were harvested and weighed upon sacrifice of treated mice. Organ weights were normalized to the body weight of each mouse. (C) Hematoxylin and eosin staining of mouse eAT was performed. Figures are representative of images taken from 10-12 fields of each mouse eAT section. Magnification,  $\times 100$ . Inserts, magnification  $\times 250$ . (D) mRNA expression of the adipogenic genes PPAR $\gamma$ , C/EBP $\alpha$ , leptin and APN in fractionated eAT adipocytes were assessed by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean  $\pm$  standard error of the mean,  $n=6$ . \* $P<0.05$  vs. control. T-MSC CM, tonsil-derived mesenchymal stem cell conditioned medium; eAT, epididymal adipose tissue; SAMP6, senescence-accelerated mouse prone 6; iAT, inguinal AT; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; C/EBP $\alpha$ , CCAAT/enhancer-binding protein  $\alpha$ ; APN, adiponectin.

the eAT mass and the results demonstrated a significant increase in the levels of total and HMW adiponectin following treatment with T-MSC CM, compared with control-treated mice (Fig. 2C). Furthermore, adiponectin protein levels in eAT lysates were significantly increased by T-MSC CM treatment compared with control treatment (Fig. 2D). Non-reduced and non-denatured tissue lysates were used to determine adiponectin multimerization. These experiments indicated that T-MSC CM enhances formation of adiponectin multimers (Fig. 2E). In addition, the present study investigated whether oxidative stress in the

adipose tissue, an established negative regulator of adiponectin expression in aging, is modulated in the presence or absence of T-MSC CM injection (27). The mRNA expression of subunits of NADPH oxidase was investigated by RT-qPCR, and the results demonstrated a significant reduction of p40<sup>phox</sup> and gp91<sup>phox</sup> in eAT from mice injected with T-MSC CM compared with controls (Fig. 2F).

*Adiponectin secretion and multimerization are enhanced by T-MSC CM treatment.* The current study confirmed these



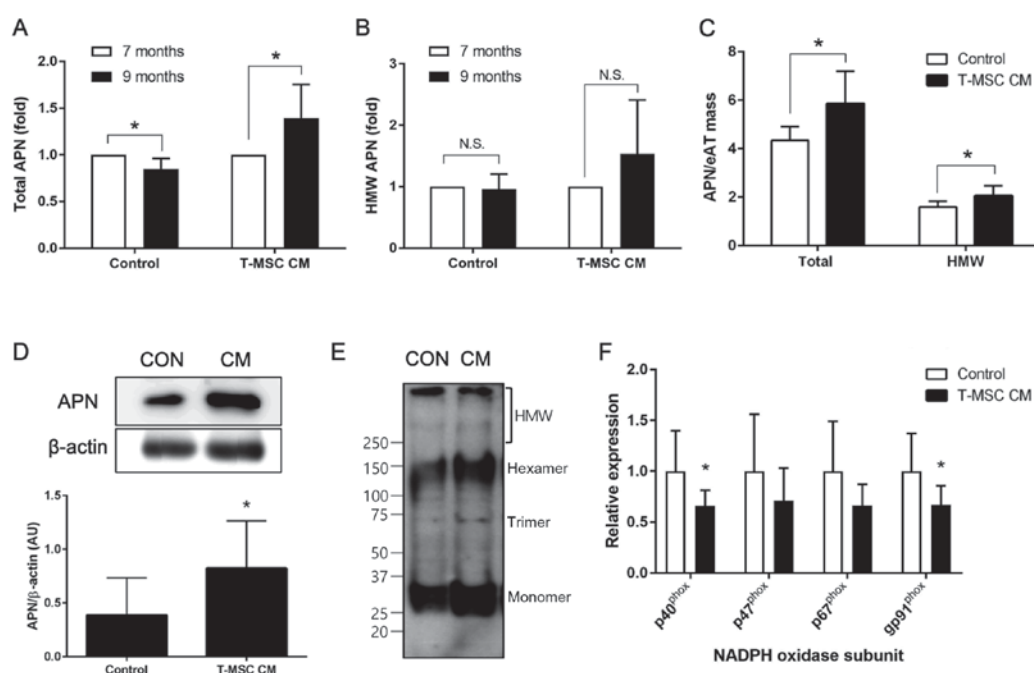


Figure 2. T-MSC CM injection increases APN in the plasma and eAT of SAMP6 mice. (A) Total and (B) HMW APN in mouse plasma prior to (7-month-old) and following (9-month-old) treatment with T-MSC CM or control medium were measured by ELISA. Results were expressed as a fold-change compared to the levels measured in 7-month-old mice. (C) Total and HMW adiponectin in +/- T-MSC CM-treated mouse plasma was normalized to the eAT mass of each mouse. (D) Adiponectin expression levels in eAT lysates were determined by western blot analysis. (E) Adiponectin multimer composition in eAT lysates was analyzed by SDS-PAGE on gradient gels under non-reducing and non-heat-denaturing conditions. (F) mRNA expression of oxidative stress markers p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup> and gp91<sup>phox</sup> in eAT were examined by reverse transcription-quantitative polymerase chain reaction. Blots are representative images and graphs present quantification for 6 mice per group. Data are presented as the mean  $\pm$  standard error of the mean, n=6. \*P<0.05 vs. control. T-MSC CM, tonsil-derived mesenchymal stem cell conditioned medium; APN, adiponectin; eAT, epididymal adipose tissue; SAMP6, senescence-accelerated mouse prone 6; HMW, high molecular weight; CON, control.

*in vivo* findings *in vitro* by performing similar experiments with the murine adipocyte cell line 3T3-L1. Preadipocytes were induced to differentiate to adipocytes for 7 days and subsequently treated with control medium or T-MSC CM for an additional 3 days. Total RNA or whole cell lysates were collected, and adiponectin expression was examined by RT-qPCR and western blot analysis. No significant changes in the cellular levels of adiponectin mRNA or protein were observed following treatment with T-MSC CM, compared with controls (Fig. 3A and B), however, adiponectin secretion into the culture medium was significantly increased in cells treated with T-MSC CM compared with controls (Fig. 3C). Adiponectin multimer formation was also investigated under non-reduced and non-heat-denatured conditions. T-MSC CM treatment slightly increased the formation of HMW adiponectin by differentiated 3T3-L1 adipocytes (Fig. 3D).

*T-MSC CM treatment ameliorates oxidative stress and restores adiponectin multimerization.* In order to extend the *in vivo* findings of the reduction in NADPH oxidase subunit transcription, we induced oxidative stress in mature 3T3-L1 adipocytes using glucose oxidase (28). Cells were differentiated for 10 days, challenged with glucose oxidase for 2 h and treated with control medium or T-MSC CM for the following 24 h. Cell culture medium and cells were collected and ROS/RNS production was measured. The results demonstrated that enhanced ROS/RNS generation in glucose oxidase-treated cells was significantly ameliorated

in cells incubated with T-MSC CM, in culture medium and cells (Fig. 4A and B). Adiponectin expression was also determined, and T-MSC CM treatment was not able to reverse reduced mRNA expression induced by glucose oxidase (Fig. 4C), however, HMW adiponectin formation was restored to a certain extent in cells treated with T-MSC CM (Fig. 4D).

## Discussion

The present study has demonstrated the effects of T-MSC CM treatment on weight reduction and the promotion of adiponectin secretion from adipocytes using SAMP6 aging mice and 3T3-L1 adipocytes. Obesity increases inflammation and insulin resistance in organs. This pathophysiology is associated with a reduction in the anti-inflammatory adipokine adiponectin the formation of HMW adiponectin in adipocytes. The results of the current study indicated that T-MSC CM infusion in mice reduces eAT accumulation and increases adiponectin synthesis, secretion and multimerization.

T-MSC CM injection resulted in weight loss in mice via a reduction in visceral adiposity and adipocyte size. Investigation of the expression of adipogenic markers in eAT adipocytes revealed downregulation of the transcription factors PPAR $\gamma$  and C/EBP $\alpha$  following T-MSC CM injection, and these results are consistent with our previous findings that T-MSC CM treatment inhibits adipocyte differentiation *in vitro* (14). The reduced expression of adipogenic regulators

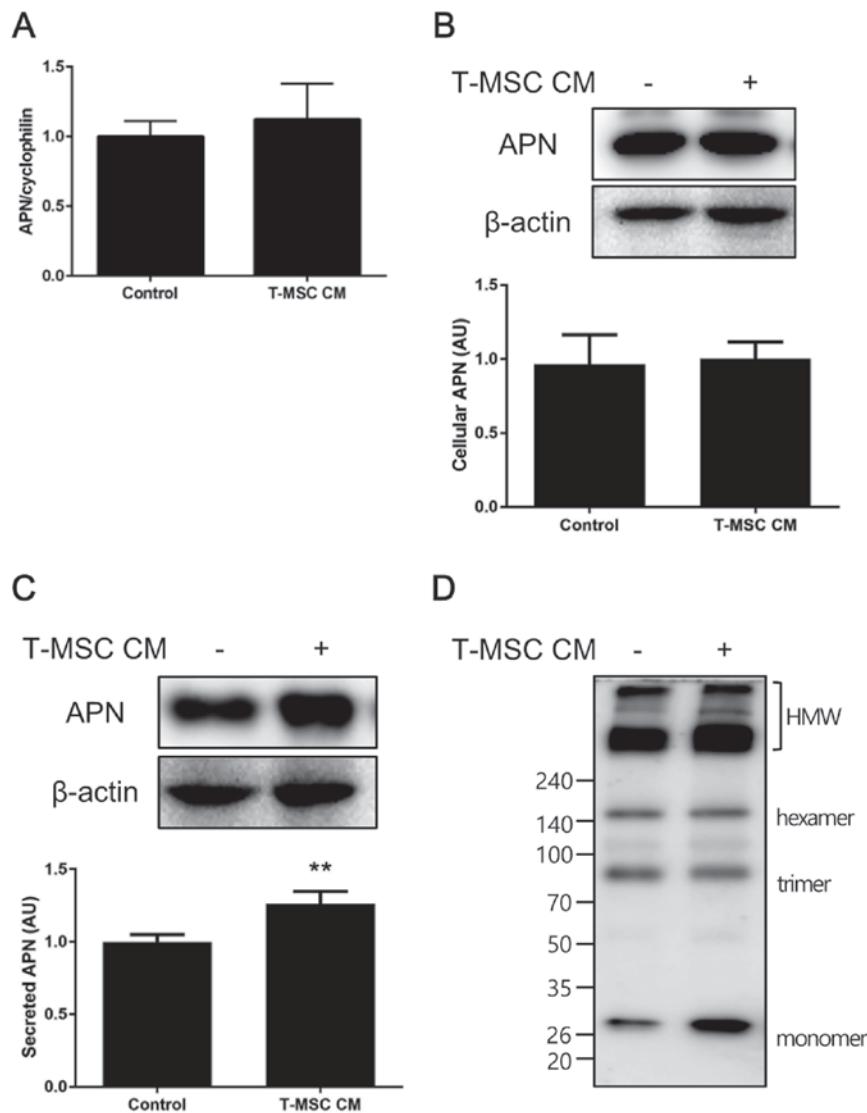


Figure 3. T-MSC CM treatment increases APN secretion in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were induced to differentiate into adipocytes using a standard protocol until differentiation day 7, cells were then incubated with or without T-MSC CM from day 7 to day 10. Cells were incubated in serum-free medium overnight, and culture supernatants and cells were harvested for further analyses. (A) mRNA expression of APN was measured using reverse transcription-quantitative polymerase chain reaction. APN in (B) total cell lysates and (C) culture supernatants was investigated using western blot analysis. (D) APN multimer composition in culture supernatants was analyzed by SDS-PAGE on gradient gels under non-reducing and non-heat-denaturing conditions. Data are presented as the mean  $\pm$  standard error of the mean,  $n=4$ . \* $P<0.01$  vs. control. T-MSC CM, tonsil-derived mesenchymal stem cell conditioned medium; APN, adiponectin; HMW, high molecular weight.

in T-MSC CM-injected mice confirmed the anti-adipogenic effects of T-MSC CM *in vivo*.

An increase in adiponectin expression was observed in T-MSC CM-treated mice, while PPAR $\gamma$  expression was reduced, which is a master regulator of adipogenesis. Regarding promotion or inhibition of adipogenesis, conflicting results currently exist. The promotion of adipogenesis is required for the generation of small and insulin-sensitive adipocytes. This restrains expansion of adipose tissue via adipocyte hypertrophy, which may lead to insulin resistance and ectopic lipid accumulation (29). On the other hand, it has also been demonstrated that blocking adipocyte differentiation using a PPAR $\gamma$  antagonist protected mice from high fat diet-induced adipocyte hypertrophy and insulin resistance (30). The results of the present study demonstrate that anti-adipogenic effect of T-MSC CM involves reduction of adipocyte size and increased

adiponectin synthesis. These effects may yield medical benefits and further studies are required to elucidate whether T-MSC CM may also be able to improve inflammation and insulin sensitivity.

The SAMP6 mouse strain is useful for the study of obesity in aging as reduced physical performance and energy metabolism lead to the development of obesity in this mouse strain. Furthermore, a previous report demonstrated that SAMP6 mice develop characteristics of obesity (26). A significantly higher BMI and the increase of obesity parameters in the plasma, including glucose, triglyceride, insulin and leptin, were reported in SAMP6 mice compared with age-matched AKR/J or SAMR1 mice. In addition, adiponectin in plasma was reported to be significantly lower in SAMP6 mice compared with control mice, and low adiponectin is an established characteristic of obesity (31,32). However, the

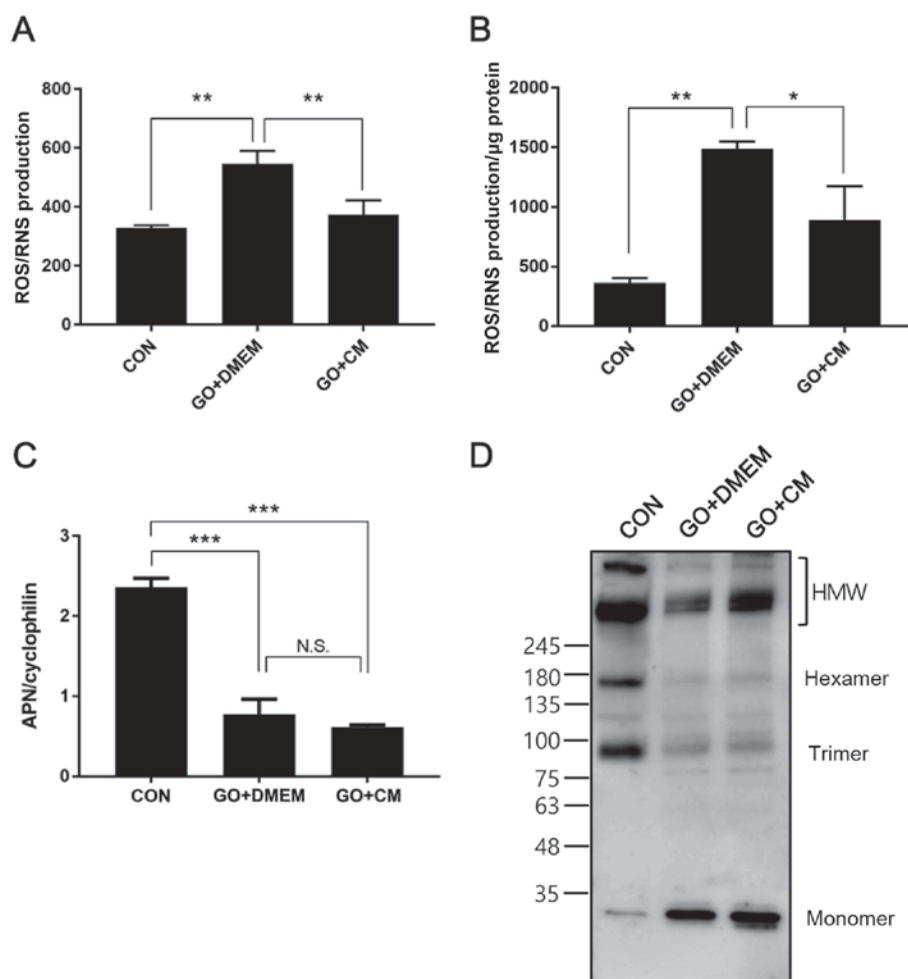


Figure 4. T-MSC CM treatment reduces oxidative stress and restores HMW APN formation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were differentiated for 10 days and challenged with GO for 2 h, followed by incubation in serum-free medium overnight in the presence or absence of T-MSC CM. ROS/RNS production in (A) culture supernatants and (B) cell lysates was measured. (C) mRNA expression of APN was measured using reverse transcription-quantitative polymerase chain reaction. (D) APN multimer composition in culture supernatants was analyzed by SDS-PAGE on gradient gels under non-reducing and non-heat-denaturing conditions. Data are presented as the mean  $\pm$  standard error of the mean,  $n=3$ . \* $P<0.05$ , \*\* $P<0.01$  and \*\*\* $P<0.001$ , as indicated. T-MSC CM, tonsil-derived mesenchymal stem cell conditioned medium; HMW, high molecular weight; APN, adiponectin; GO, glucose oxidase; ROS, reactive oxygen species; RNS, reactive nitrogen species; CM, T-MSC CM; DMEM, Dulbecco's modified Eagle's medium; CON, control; N.S., not significant.

observations in the present study should be confirmed using a diet-induced obesity mouse model. More detailed analyses on the consequences of adiponectin induction on body metabolism are required. The action of T-MSC CM on other metabolic organs, such as the liver or skeletal muscle, needs to be determined. Identification of adiponectin receptors that are involved in T-MSC CM-induced adiponectin function and changes in the signal transduction may explain the association between T-MSC CM and adiponectin. In addition, the association between increases in adiponectin and improvement in metabolic parameters requires further investigation. Measurement of blood glucose, triglyceride and inflammatory markers, as well as performing glucose tolerance and insulin tolerance tests in the presence or absence of T-MSC CM may increase our understanding of the advantages of using T-MSC CM in the regulation of metabolic diseases.

Oxidative stress is one of the key contributors of aging pathologies. In obesity, systemic oxidative stress is closely associated with the incidence of metabolic syndrome. One potential mechanism of action is that high levels of ROS production may lead to suppressed adiponectin secretion (27,33). In

addition, a recent report demonstrated a positive correlation between plasma adiponectin levels and antioxidant capacity in the elderly (34). The present study elucidated effects of T-MSC CM on the promotion of adiponectin secretion in the setting of both obesity and aging. Furthermore, the results demonstrated that T-MSC CM led to an increase in the HMW adiponectin formation with the amelioration of oxidative stress. Further studies are required to identify potential antioxidant molecules secreted by T-MSCs and their mechanisms of actions.

In conclusion, we demonstrated that T-MSC CM injection reduces mouse body weight and eAT mass. Reduction in body weight increased adiponectin in circulation by upregulating adiponectin expression and multimerization. Amelioration of oxidative stress may be the mechanism by which T-MSC CM leads to increased adiponectin secretion. Furthermore, these results, which, to the best of our knowledge, provide the first description of the role of T-MSC CM on adiponectin synthesis and secretion, may provide the framework for future development of cell therapy to combat obesity or obesity-associated metabolic diseases.

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