Photomodulation Alleviates the Senescence of Adipose-Derived Stem Cells Aging in Vitro

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Short Report

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Abstract

Adipose-derived stem cells (ASCs) therapies are emerging as a promising approach to therapeutic regeneration. Therapeutic persistence and reduced functional stem cell following cell delivery remain a critical hurdle for the clinical investigation due to the senescence of freshly isolated ASCs, extensive in-vitro passage. Cultured ASCs were derived from subcutaneous white adipose tissue isolated from mice fed a normal diet (NFD) or high-fat fed (HFD). ASCs were passaged in vitro and prepared for subculture up to passage 9, after light and non-light-treated ASCs. We performed senescence-associated-β-galactosidase (SA-β-gal) staining and real-time PCR to evaluate the time-dependent changes in expression levels of mRNA related to cell cycle, cellular senescence markers, and senescence-associated secretory phenotype (SASP). The mRNA expression levels of senescence and SASP markers were significantly increased at passages 7 and 9 of ASCs-NFD. At the same passage number, ASCs-HFD showed a markedly higher mRNA level of senescence and SASP than that in ASCs-NFD. At different passages (Passages 3, 5, 7, and 9), these cells were performed by light treatment. Photomodulation reduced the expression of senescence, SASP, and SA-β-Gal in all cells at passage. Moreover, the light-treated ASCs-derived exosomes decrease the expression of senescence, SASP, and SA-β-Gal in the later passage cells. Furthermore, Light treatment significantly increased the Ca 2 + influx compared to the non-light-treated ASCs. Collectively, ASCs can undergo cellular senescence in-vitro passage. Photomodulation may be better preserved over senescence in aged ASCs. Light-treated ASCs-derived exosomes could be served as e a new protective paradigm for the cellular senescence in-vitro passage.

Introduction

Accumulating evidence has indicated that the therapeutic value of mesenchymal stem cells (MSCs) in regenerative medicine. For in-vitro culture, MSCs must be expanded over replicative, multiple population doublings to obtain an available and sufficient number of cells for administration [1, 2]. MSCs isolated from aged individuals or MSCs that had been cultured for many passages *in vitro*. However, in either case, cultured MSCs gradually lose their replicative potential with a concomitant weaken in cellular lifespan and quality. Previous studies have primarily shown the different conditions of population doublings from the initial MSCs passage to senescence, which is linked to loss of the replication and differentiation capability [3–7].

Cellular senescence occurs due to multiple factors such as in vitro cell aging, telomere attrition, irradiation, oncogene activation, oxidative damage, and mitochondrial dysfunction. The senescent cell cycle arrest is mainly related to upregulation of p16, p21, p53, and the age-dependent senescence of MSCs has been linked to mitochondrial dysfunction and abnormal change in levels of reactive oxygen species (ROS) [8, 9].

Senescent cells and the senescence-associated secretory phenotype (SASP), as well as the senescence-associated induction of beta-galactosidase (SA-β-Gal), are believed to excel in the occurrence of aging, such as degenerative, hyperproliferative, and inflammatory [10, 11]. Several aging-related experimental

therapeutic approaches, such as senolytics, antioxidant, and exercise, have been shown to delay the onset of cellular senescence or inhibit the SASP [12–14]. Understanding the molecular mechanism involved in aging-associated deterioration of stem cell function is crucial in developing effective new therapeutic methods. Despite the observations that senolytics have the potential to improve outcomes [15, 16], senolytics can also target and kill non-senescent cells, which are major drivers of fibrosis [17].

Photomodulation involves transmitting specific packets of light that can be absorbed by photoreceptors or molecules associated with the mitochondria of cells. Numerous studies have demonstrated the benefits of photomodulation as a promising physical approach in various pathologies, especially for promoting cell and tissue regeneration. In vitro ASCs culture, our recent study has shown that light treatment significantly increased cell proliferation compared with non-light treatment [18]. Light application to ‘healthy’ cells and tissue induces cell proliferation while inducing apoptosis signaling pathways in inflamed tissue [19]. Hence, photomodulation could be a potential approach as a preventive way in cellular senescence for ASCs-based in-vitro passage.

Methods

Animals

6–8 weeks old C57BL/6J mice were obtained from the Chongqing Medical University Animal Center, Chongqing, China. All protocols for animal use were reviewed and approved by the Animal Care Committee of Southwest Medical University in accordance with Institutional Animal Care and Use Committee guidelines.

Hfd-fed Mouse Model

Eight-week-old male C57BL/6J mice were fed a high fat diet (HFD) (TP2330055A; Research Diet, Trophic Animal Feed High-tech Co. Ltd, China) for 14 weeks as described previously [18]. Age-matched male mice that were fed a normal diet were used as controls. Blood samples were obtained from the tail vein and blood glucose levels were measured using an automatic glucometer (Accu-Chek; Roche Diagnostics, Mannheim, Germany). Body weight was monitored every seven days. Blood was collected and centrifuged at 1500 $\times g$ for 10 min to measure fasting serum glucose.

Culture Of Ascs

Mouse adipose-derived stem cells (ASCs) were isolated from inguinal subcutaneous fat of C57BL/6 mice that were fed normal chow (NFD) or high fat chow (HFD). The cells were cultured as described previously [18]. Briefly, subcutaneous adipose tissues were digested with collagenase type 1 (Sigma-Aldrich, St. Louis, MO, USA) in PBS (Phosphate-buffered saline) by incubation in a shaker at 37°C for 30 min. Cells were suspended in complete medium made of DMEM/F12 (Dulbecco modified Eagle medium) medium

supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Cells were cultured in 37°C at 5% CO2 incubator. Flow cytometry is used to identify the ASCs with surface markers as described previously by us [18, 20]. ASCs at passage 3–10 were used in all the experiments.

Photomodulation

Cultured ASCs were transferred into a sterile syringe and subjected to light treatment for 30 min using a CellRegena Device (HarmonyRegena CO., Ltd, China) (18,20). The syringe was then placed into the device and rotationally activated by light-emitting diode (LED) light. This device integrates monochromatic lights of three different wavelengths, including 575–595 nm (5–20 mW), 630–635 nm or 660–670 nm (10–100 mW) and/or 510–540 nm (10–60 mW) of monochromatic light.

After light-treated for 30 min, ASCs were prepared for subculture every 10 days up to passage 9 and culture media were completely renewed every 3 days. A second set of cells was subjected to non-light treatment for 30 min as control group, which cultured ASCs were transferred into a sterile syringe without light treatment.

Quantitative Real-time Pcr

ASCs were collected and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA samples were pre-treated with deoxyribonuclease I (Invitrogen Life Technologies, Carlsbad, CA, USA), and a SuperScript kit (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to synthesize cDNA according to the manufacturer's recommendations. qRT-PCR was analyzed using miScript SYBR Green PCR Kits (Qiagen). Levels of macrophage polarization and oxidative stress markers mRNAs were determined by ABI PRISM 7700 cycler (Applied Biosystems, Foster City, CA). Each sample was analysed in duplicate with ribosomal 18S RNA as an internal control. All fold changes in gene expression were determined using the 2 − ΔΔCT method. The values are presented as the mean ± SEM. All primers are listed in Supplemental Table 1.

Ascs-derived Exosomes

Collection of supernatant

Under the condition of non-light treatment or light treatment for 30 minutes, the cell density reached 60%-70% and then replaced with DMEM medium containing 10% exosome-free serum. After culturing for 24h-48h, collect the supernatant and perform a preliminary centrifugation: 300g, centrifugation for 10min, carefully aspirate the supernatant, 3000g, centrifugation for 15min.

The supernatant was then passed through a 0.22 μm filter sieve, and transferred to an ultrafiltration concentration tube Vivaspi Turbo 15 for concentration. Use the PS affinity exosome extraction kit

MagCaptureTM Exosome Isolation Kit PS (Wako, 293-77601) to collect exosomes.

Dilute 10 μl of the above exosomes suspension with calcium and magnesium ion-free PBS buffer to 1 ml, and the particle size and concentration of exosomes were evaluated by Nano Sight NS3000.

Westernblotting

Take the PBS suspension containing exosomes collected above (the number of particles is $1x10^8$-10^9), directly add SDS loading buffer, and place it in a 95°C metal bath for denaturation. The equal amounts of protein were subjected to SDS-polyacrylamide electrophoresis and transferred to polyvinylidene difluoride membranes by electroblotting. After blocking, the membranes were incubated with antibodies directed against CD9 (#98327; Cell Signaling Technology), CD63 (ab217345; Abcam), CD81 (#10037; Cell Signaling Technology), TSG101 (#ab125011; Abcam). Secondary antibody was horseradish-peroxidase (HRP)-conjugated goat IgG raised against IgG (Santa Cruz Biotechnology). Blots were developed with ECL substrate (Pierce).

Senescence-Associated-β-Galactosidase (SA-β-gal) Assay

The SA-*β*-gal activity was measured using Senescence Assay Kit (ST429, Beyotime) based on the manufacturer's instructions. Briefly, cells were incubated in ONPG at room temperature for 12 hours then stained with the Staining Mixture at 37°C without CO_2 overnight. Subsequently, cells were observed and visualized under a light microscope (Zeiss HAL 100). The values were normalized to total protein of cell lysates assessed with a bicinchoninic acid (BCA) protein assay (Pierce).

Intracellular Ca^{2+} concentration

To evaluate the changes in intracellular calcium concentrations, a calcium-sensitive fluorescence indicator, Fluo-8, AM (AAT Bioquest, Sunnyvale, CA, USA), was used to stain the cells according to the manufacturer's instructions. Briefly, Cells were washed in serum- and phenol red-free DMEM containing 4 μM Fluo-8 AM plus 0.08% Pluronic F127 (AAT Bioquest, Sunnyvale, CA, USA) for 20 min at 37°C, 5% CO_2 to load the dye into the cells. 25 mM Probenecid (AAT Bioquest, Sunnyvale, CA, USA) solution was used to reduce leakage of intracellular dye. The fluorescence of Fluo-8 AM was excited at the wavelength of 490 nm and measured using a fluorescence microplate reader (Cytation 5, USA). Three independent experiments were averaged.

Statistical analysis

Data are presented as the mean ± SEM of triplicate experiments. Experimental groups were compared by the two-tailed Student's t-test or

one-way analysis of variance (ANOVA). All analyses were performed with SPSS software (version 24.0 for Windows; Armonk, NY, USA), and a level of $P < 0.05$ was defined as indicative of statistical significance.

Results

Light treatment prevents the onset of senescence in ASCs

We first evaluate the cellular senescence in-vitro passage of cultured ASCs derived from the normal-fat diet (NFD) C57BL/6J mice. Following the schematic timeline in the experimental model (Fig. 1A), we observed that the in vitro increased passage number of ASCs led to a significantly greater expression in the cell cycle gene p16 and p21 as well p53 at passage 5, 7, and 9 (P5, P7, and P9) (Fig. 1B-D). Conversely, the levels of senescence genes were the same regardless of P3-P9 when ASCs were activated with light at P3. Based on these changes, we further examined the relative senescent phenotypes of the HFD-fed mice. We found that the expression levels of p16, p21, and p53 were significantly higher in HFD-P5 compared with HFD-P3, and light-treated HFD-P5 was much less than non-light-treated HFD-P5 (Fig. 1E-G). Moreover, at P3, compared with NFD-ASCs, HFD-ASCs presented a marked increase in the expression levels of p16, p21, and p53. Light-treated ASCs also exhibited a decrease in SA-β-galactosidase activity in both NFD- and HFD-ASCs (Fig. 1H-I).

A senescent phenotype is related to a secretory phenotype, which would also affect the SASP. We confirmed that light treatment decreased the mRNA levels of SASP specific markers, including PAI-1, MMP3, CD68, IL-6, MCP-1, and IGF-1 in HFD-ASCs by qPCR (Fig. 1J-N). These findings demonstrated that photomodulation effectively reduced the expression of the critical cellular senescence markers and SA–β-gal for the cellular senescence in-vitro passage.

Light- Treated-ascs-exos Presented A Marked Efficacy In The Prevention Of Cellular Senescence

We explore the effects of photomodulation on exosome secretion. ASCs at P3 were treated with light for 30 min. Then, exosomes were obtained, and Transmission electron microscopy (TEM) analysis confirmed that light treatment did not change the morphology of the exosomes (Fig. 2A). All the exosomes isolated were within the range of 50–150 nm (Fig. 2B), as evaluated by nanoparticle tracking analysis (NTA). The concentration of light-treated ASCs-Exos was approximately a fivefold increase in the number of exosomes secretion compared with non-light-treated ASCs-Exos (Fig. 2C). Moreover, Westernblotting analysis of the exosomal inclusive markers CD9, CD63, CD81, and TSG101 confirmed the identity of the exosomes (Fig. 2D).

Aging ASCs were stimulated with ASCs-derived exosomes for 24 hours. P9 treated with ASCs-Exos showed a significant decrease in the mRNA levels of p16, p21, and p53. Notably, the light-treated ASCs-Exos group presented marked lower levels than the non-light-treated ASCs-Exos group (Fig. 2E). It is important to note that photomodulation exhibits a dominant efficacy in preventing cellular senescence.

Previous studies have shown that exosome release is regulated by a calcium-dependent mechanism [21, 22]. With light treatment, we used Fura Red fluorescence to measure cytoplasmic calcium concentration

alteration in the cultured ASCs. Our data showed that light treatment significantly increased the Ca 2 + concentration compared to the non-light-treated ASCs group. These data indicated that photomodulation stimulates exosomes release of ASCs is associated with increased intracellular calcium concentration. Future studies need to be conducted to look into the effect of photomodulation on ASCs-Exos and miRNA signaling and other proposed mechanisms.

Discussion

Extracellular vesicles (EVs) shedding can be induced by cell activation by chemical or mechanical factors or stress. As shown here, we demonstrated that photomodulation has the potential to improve the cellular lifespan of ASCs in vitro passage. The effects were dependent on the wavelength of the applied light and associated with the reduced markers of senescence, SASP, and SA-β-Gal activity in all cells at passage. Furthermore, the light-treated ASCs-derived exosomes decreased cellular senescence, and light treatment increased the Ca 2 + influx. In this study, we, for the first time, demonstrated that photomodulation could alleviate the senescence of ASCs aging in vitro, possibly via releasing exosome production.

Based on the wavelength of the applied light, the application of photomodulation was reported to have beneficial effects on cellular functions, including proliferation and migration in a variety of cell types [18, 23]. Our previous study has shown that ASCs responded to light-increased secreted proteins related to endothelial functions (proliferation, migration, and chemotaxis), cell differentiation, and ECM. In addition, oxidative stress plays a causal role in the dysfunction of ASCs. Light-treated ASCs secretome might have a more prone antioxidative profile when compared with non-light-treated ASCs [18]. Thus, the response to photomodulation appears to be related to the delay in replicative senescence through cell growth. In the current study, photomodulation targets senescent molecules formed at this stage in a passage number-dependent manner.

The potential mechanism of how light promotes exosome release might rely on the influx of Intracellular calcium. The endosomal sorting complex for transport required (ESCRT) is involved in the formation of microvesicles and vesicles of the multivesicular body. Intracellular calcium plays a crucial role in ESCRT recruitment and is related to the endosomal release. The encoded protein p53 responds to cellular stresses to regulate target genes involved in cell cycle arrest, DNA repair, apoptosis, or senescence. It is noted that the activation of P53 may enhance the expression of genes that regulate endosome production generation during photomodulation, while the detailed mechanism remains unclear. Our study here suggests that promoted exosome secretion and thus the associated Ca 2 + influx might be an alternative mechanism for the function of photomodulation.

Conclusion

In this study, preventing replicative senescence by short-time treatment with photomodulation may benefit the in-vitro passage of ASCs. Although further mechanisms remain to be investigated,

photomodulation could be served as e a new protective paradigm for the replicative cellular senescence in-vitro passage.

Abbreviations

ASCs: Adipose-derived stem cells;EAT: Epididymal adipose tissue; HFD: High-fat diet; MSCs: Mesenchymal stem cells; NFD: Normal diet; SA-β-gal:

Senescence-Associated-β-Galactosidase

Declarations

Acknowledgements

All authors are acknowledged for their contribution to the study.

Authors' contributions

XS, JW designed and performed experiments, analyzed data; XS, JW, TZ, XM, ZD, LW, NC, YZ, and ML performed collection and/or assembly of data, data analysis and interpretation; JW wrote the manuscript.

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Availability of data and materials

The data generated or analyzed during this study are included in this article,

or if absent are available from the corresponding author upon reasonable

request.

Ethics approval and consent to participate

All animal procedures were approved and performed under the supervision

of the Institutional Animal Care and Use Committee (IACUC) at Southwest

Medical University, China.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests. JW is inventor

on a patent application filed on this work.

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Figures

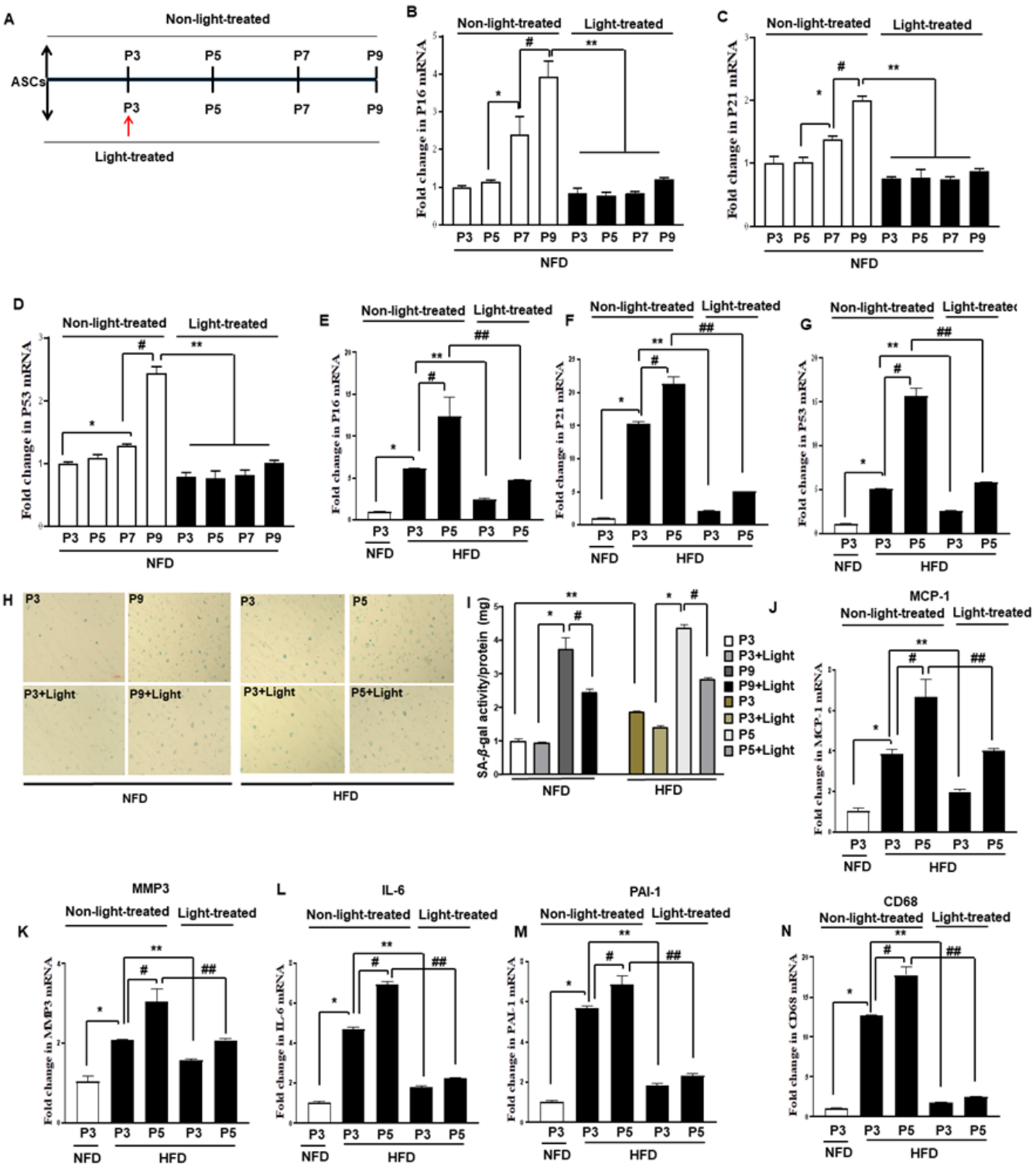

Figure 1

Light treatment prevents the onset of senescence in ASCs. **A**. Schematic timeline of ASCs transplantation model. Light treatment was performed at passage 3 (P3). After light-treated for 30 min, ASCs were prepared for subculture every 10 days up to passage 9 and culture media were completely renewed every 3 days. A second set of cells was subjected to non-light treatment for 30 min as control group. The expression of p16 (**B**), p21 (**C**), and p53 (**D**) was evaluated by qPCR from P3-P9 in ASCs from normal diet

fed (NFD) C57BL/6J mice. **E-G**. The expression of p16 (**E**), p21 (**F**), and p53 (**G**) was assessed by qPCR at P3 and P5 in ASCs from high fat diet (HFD) fed C57BL/6J mice. NFD-P3 as compared a control. **H**. Representative images of SA-β-galactosidase-positive cells.**I**. Senescence was evaluated in terms of SA-β-galactosidase activity and expressed as the ratio of cells protein (mg). Scale bar: 50μm. The mRNA levels of MCP-1(**J**), MMP3 (**K**), IL-6 (**L**), PAI-1 (**M**) and CD68 (**N**) of HFD-ASCs as grown in the absence or presence of light treatment, quantified by qPCR. n=3-5 per group. Data shown as mean±SEM. *P<0.05; **P<0.05; #P<0.05; ##P<0.05.

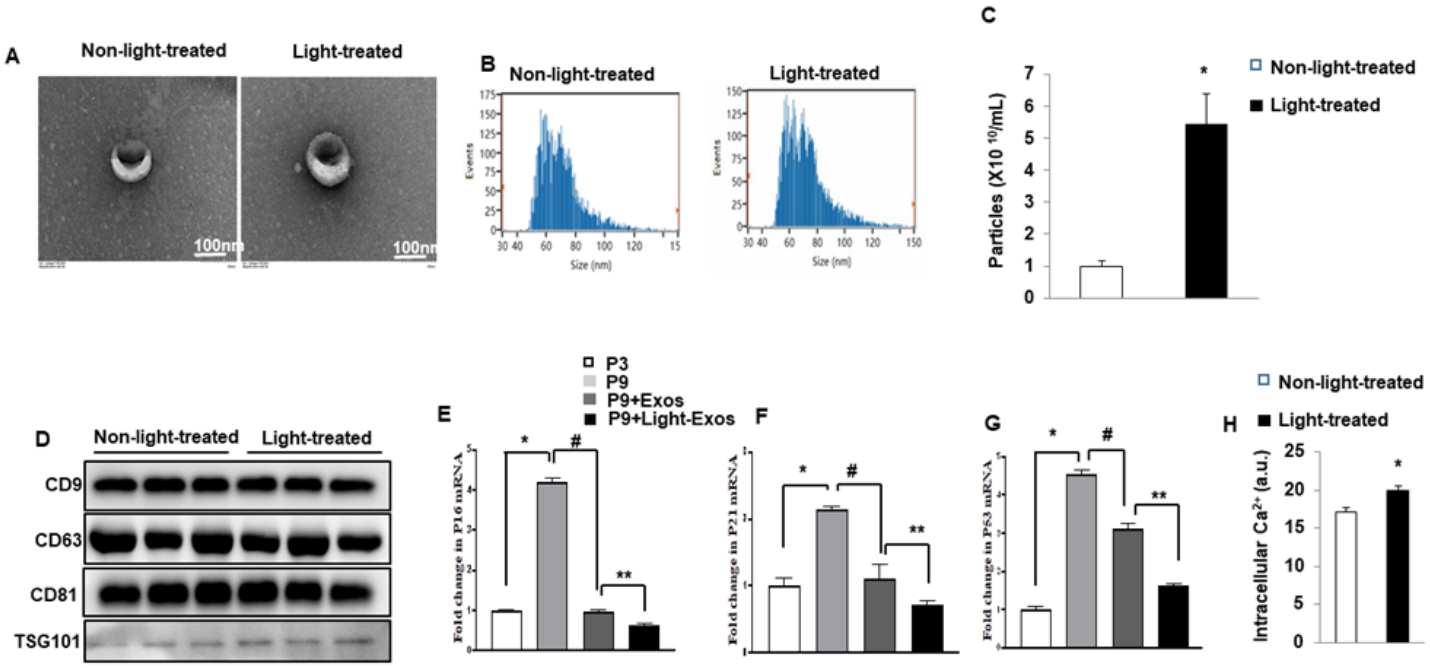

Figure 2

Light- treated-ASCs-Exos presented a marked efficacy in the prevention of cellular senescence.A. TEM analysis of the morphology of the exosomes from ASCs with indicated treatments. Scale bar = 100 nm. **B**. Size distribution of the exosomes isolated from ASCs with indicated treatments as analyzed by Nano Sight. **C**. The relative concentration of exosomes from from ASCs with indicated treatments.**D**. The expression of exosome markers, CD9, CD63, CD81, and TSG101, were detected by Westernblotting. **E-G.** $1x10^8$/mL of exosomes were added in the cultured NFD-ASCs for 24 hours as indicated treatment. The expression of p16 (**E**), p21 (**F**), and p53 (**G**) was assessed by qPCR. **H.**Intracellualr calcium concentration was measured by using Fura Red fluorescence in NFD-ASCs as indicated treatment. Light treatment was performed at passage 5 (P5). n=3-5 per group. Data shown as mean±SEM. *P<0.05; **P<0.05; #P<0.05; ##P<0.05.

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