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Secretome from HMGB1 Box A-over-expressing Adipose-derived Stem Cells Shows Potential for Skin Rejuvenation by Senescence Reversal in PM2.5-induced Senescence Cells *via* Stem Cell Induction

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Abstract

Background/Aim: Exposure to particulate matter 2.5 (PM2.5) can lead to cellular senescence by generating reactive oxygen species (ROS). Box A, a DNA-binding domain found in HMGB1, is known for its ability to counteract aging characteristics. This study explored whether BoxA-induced adipose-derived stem cells secretome (BoxA-SC) can reverse senescence in DP and HWPc cells.

Materials and Methods: The stemness characteristics and reversal of senescence by BoxA-SC in PM2.5-induced DP and HWPc cells were assessed at the mRNA level using RT-qPCR and at the protein level using immunofluorescence analysis.

Results: BoxA-SC (1:20) treatment for 48 h induced stemness and reversed PM2.5-induced cell senescence in DP and HWPc cells. BoxA-SC significantly reduced senescence markers, including SA-β-gal staining, and decreased mRNA levels of senescence-associated secretory phenotype factors (IL1α, IL7, CXCL1) in PM2.5-induced senescent cells. DP and HWPc cells exposed to PM2.5 exhibited an increase in p21 and p16 mRNA and protein levels, which was reversed by BoxA-SC. BoxA-SC reduced p21 and p16 in DP senescent cells approximately 3- and 2-fold, respectively, compared to untreated senescent cells.

Conclusion: BoxA-SC can potentially reverse cellular senescence, highlighting the therapeutic potential of stem cells in skin rejuvenation and anti-aging treatments.

Keywords: Stem cell induction, adipose, secretome, PM2.5, senescence reversal.

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Introduction

Aging refers to a biological process characterized by the gradual deterioration of the structures and functions of organisms over time. Numerous studies have shown that particulate matter 2.5 (PM2.5), a component of air pollution, directly contributes to the aging process (1). PM2.5, tiny particles measuring 2.5 micrometers or smaller, can be inhaled into the lungs and enter the bloodstream, posing health risks and affecting the body in various ways (2). Research has demonstrated that exposure to PM2.5 can lead to cell senescence through a mechanism that relies on the generation of reactive oxygen species (ROS) (3).

Senescent cells have previously been suggested to potentially play a role in the decline of the regenerative capacity associated with aging (4). Furthermore, there is growing evidence indicating that senescent cells have a harmful impact on the aging process (5). Cell division and replication are mainly limited by telomere shortening, resulting in an inability to maintain DNA replication and chromosome stability, thereby inducing cell senescence and age-related diseases (6).

Senescence is a condition in which cells halt their cell cycle, leading to impaired division and function, potentially contributing to aging and age-related diseases (7). The senescent cells exhibit specific characteristics, such as larger size compared to non-senescent cells, the presence of senescence-associated beta-galactosidase activity (SA-β-gal), the formation of senescence-associated heterochromatin foci (SAHF), and the secretion of proinflammatory cytokines as part of the senescence-associated secretory phenotype (SASP), which includes increased release of interleukins, matrix metalloproteinases, and growth factors, leading to inflammation and senescence in neighboring cells. The process of cellular senescence is primarily governed by the activation of two pathways: the p16INK4a-pRB pathway and the p53-p21Cip1/WAF1 (p21) pathway (8-10).

While considerable research has explored the effects of PM2.5 on differentiated cells, relatively little is known about its potential to induce reverse senescence in cells. Stem cell therapies hold significant promise in regenerative medicine because of the inherent biological properties of stem cells, including their plasticity, ability to self-renew, and capacity for multi-directional differentiation. Stem cells have the potential to postpone or even reverse the aging process (11).

The stemness refers to the capacity of cells for self-renewal and differentiation into diverse cell types, which is crucial for the formation and maintenance of tissues (12-14). According to previous literature, the transcription factor SRY-box transcription factor 2 (SOX2), which plays a vital role in governing the maintenance of neural progenitor cells, is directly controlled by the cyclin-dependent kinase inhibitor 1A (p21). The expression of SOX2 in neural stem cells is down-regulated by p21, which directly binds to a SOX2 enhancer to govern both the cell cycle and prolonged self-renewal (15).

Box A is a highly conserved DNA-binding domain of high-mobility group box 1 (HMGB1), a protein involved in DNA organization and repair. A plasmid containing Box A was used to rejuvenate aging cells, decreasing both β-galactosidase activity and natural aging in rats (16). Based on previous research, the over-expression of box A within the *HMGB1* gene impacts the expression of stem cell markers OCT4, NANOG, and SOX2 in DP cells and primary human white preadipocytes (HWPc), potentially advancing regenerative strategies (17).

The preadipocyte stem cells found in white adipose tissue are valuable for wound healing and regenerative therapies. Adipose-derived stem cells (ADSCs) are a type of mesenchymal stem cells (MSCs) obtained from adipose tissue (18). The paracrine impact of adipose-derived mesenchymal stem cells (ADMSCs) is notably significant, as they possess the ability to release a range of cell growth factors and chemokines, thereby inducing angiogenesis, triggering the activation of endogenous stem cells, modulating inflammation, and facilitating wound healing (19). Therefore, ADSCs also hold potential for applications in regenerative medicine. Many relevant studies have verified that ADMSCs exhibit low immunogenicity and express histocompatibility complex molecules at a minimal level (11).

The secretome, or conditioned medium, refers to the bioactive molecules released by MSCs. It comprises numerous growth factors, cytokines, macromolecules, and extracellular vesicles, such as microvesicles and exosomes, which play a crucial role in stimulating various biological responses, particularly in modulating new tissue formation, making them essential in various therapies, including skin rejuvenation (20, 21). The absence of conventional treatments renders secretome a promising alternative therapy (22).

Therefore, there is significant interest across various research fields regarding the potential of the ADSC secretome to effectively reverse senescence induced by PM2.5 in DP and HWPc cells. Consequently, the secretome represents a significant therapeutic approach aimed at skin rejuvenation through senescence reversal and stem cell induction.

Materials and Methods

PM2.5 sampling. The PM2.5 samples were obtained in a manner similar to that described in previous studies (23).

Construction of hHMGB1-boxA plasmids. The construction of plasmids containing the HMGB1-boxA gene was carried out in accordance with prior research (17).

Plasmid transfection. DP and HWPc cells were transfected with HMGB1-boxA plasmids using Lipofectamine™ 3000 (Thermo Fisher Scientific, Carlsbad, CA, USA), following the manufacturer's instructions.

Preparation of Box A secretome (BoxA-SC). The HWPc cells were transfected with hHMGB1-boxA plasmids for 48 h, followed by refeeding with fresh complete medium for an additional 24 h, after which the conditioned medium was collected as BoxA-SC. The resulting BoxA-SC was then lyophilized at a ratio of 1:20 using a Labconco lyophilizer (Labconco Corporation, Kansas City, MO, USA) (Figure 1).

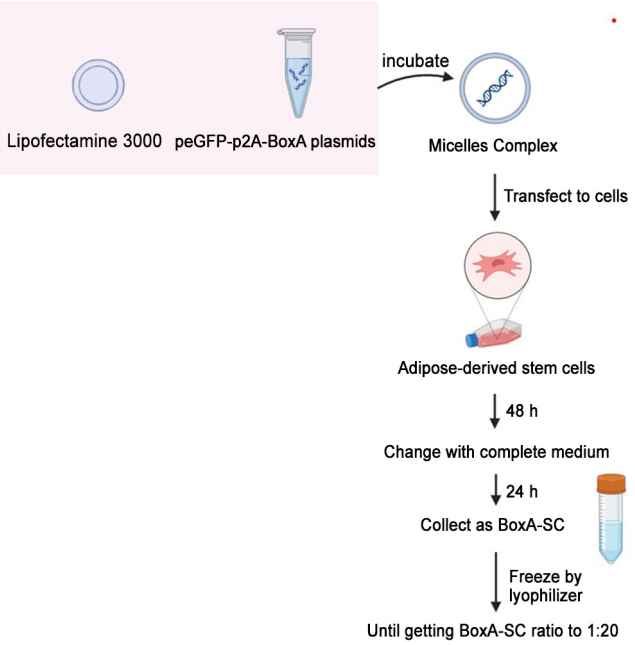

Figure 1. *Preparation of BoxA-SC. The HWPc cells were treated with hHMGB1-boxA plasmids for 48 h, after which fresh complete medium was substituted for another 24 h to yield BoxA-SC. The resulting solution was then freeze-dried to a 1:20 ratio using a Labconco lyophilizer. Created with BioRender.com.*

Cells and reagents. Human primary dermal papilla cells (DP) were acquired from Applied Biological Materials Inc. (Richmond, BC, Canada). These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA,), 2 mM of glutamine, and 1% Anti-Anti (Gibco). DP cells were cultured at 37°C with 5% CO_2.

HWPc were sourced from PromoCell GmbH (Heidelberg, Germany) and cultured in a pre-adipocyte culture medium supplemented with a mix provided by PromoCell GmbH. The cells were cultured without antibiotics or antimycotics at 37°C with 5% CO_2 until they reached a maximum of 10 population doublings, after which they were used for experiments.

The secondary antibody, Alexa Fluor™ 594 goat anti-rabbit IgG (H+L) conjugated, was sourced from Invitrogen through Thermo Fisher Scientific. Hoechst 33342 was obtained from Molecular Probes Inc. (Eugene, OR, USA).

Eurofins Genomics provided all the OCT4, NANOG, SOX2, and GAPDH primers (DNA Sequencing Lab, Louisville, KY, USA). The primers for p21, p16, IL1α, CXCL1, and IL7 were acquired from Marcogen (Seoul, Republic of Korea).

Abcam (Waltham, MA, USA) provided the rabbit monoclonal antibodies OCT4 (cat no: ab19857), NANOG (cat no: ab80892), and SOX2 (cat no: ab97959). The rabbit monoclonal antibodies against p21 Waf1/Cip1 (cat no: 2947) and p16 INK4A (cat no: 92803) were acquired from Cell Signaling (Beverly, MA, USA). Cell Signaling supplied the rabbit secondary antibody anti-rabbit (cat no: 7074).

Treatment of cells with BoxA-SC to measure the level of stemness induction. DP or HWPc cells were seeded at a concentration of 0.8×10^4 cells/well in 96-well plates (for immunofluorescence) and 3×10^5 cells/well in 6-well plates (for RT-qPCR) and cultured for 24 h. The cells were then exposed to BoxA-SC at a ratio of 1:20 for 48 h.

Effect of BoxA-SC on PM2.5-induced senescence. DP or HWPc cells were treated with 50 μg/ml concentration of PM2.5 for four days to establish cellular models of senescence. Afterward, the PM2.5-induced senescent cells were treated with BoxA-SC (1:20) ratio for 48 h.

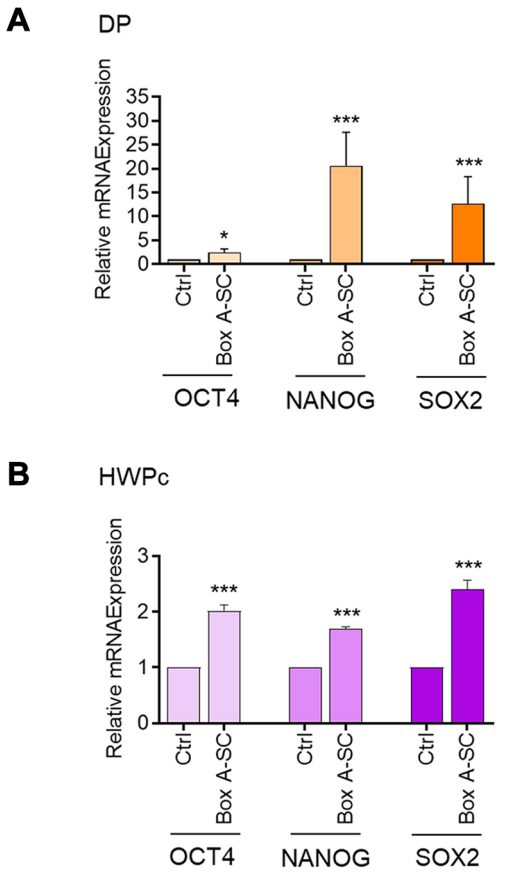

Figure 2. *Continued*

Senescence-associated β-galactosidase (SA-β-gal) activity. The β-galactosidase staining kit (cat no: #9860) was obtained from Cell Signaling. The cells were fixed in a fixative solution (1×) for 15 min at room temperature. The cells were then exposed to the β-galactosidase staining solution and incubated overnight at 37˚C. Senescence cells exhibited a blue color, which was captured under a 20× magnification.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from treated cells (3×10^5 cells per well in 6-well plates) using GENEzol reagent. Subsequently, cDNA was generated from total RNA using SuperScript III reverse transcriptase (Invitrogen). Following cDNA synthesis, 100 ng of cDNA was utilized for RT-qPCR with Luna Universal qPCR Master Mix (New England Biolabs, NEB Headquarters, Ipswich, MA, USA) in a final volume of 20 μl. The PCR reaction was performed in a CFX 96 Real-time PCR system (Bio-Rad, Hercules, CA, USA). The RT-qPCR conditions involved an initial denaturation step at 95˚C for 1 min, followed by 45 cycles consisting of denaturation at 95˚C for 15 s, and primer annealing and extension at 60˚C for 30 s. Melting curve analysis was employed to ascertain primer specificity. Gene expression levels were normalized using GAPDH as an internal control. The relative mRNA expression levels of each gene were determined from comparative Cq values.

Immunofluorescence. Following treatment, the cells were fixed with 4% paraformaldehyde for 15 min. Subsequently,

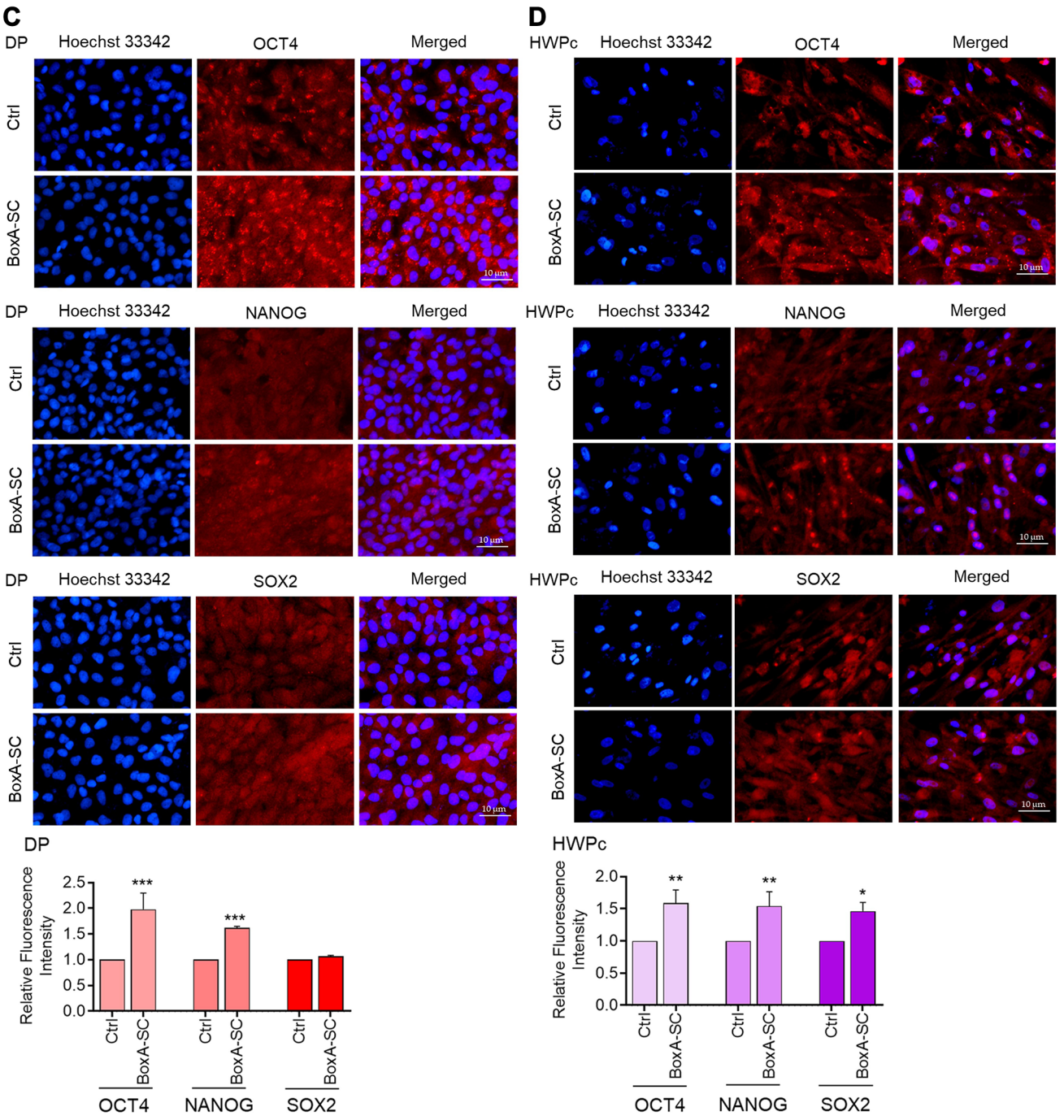

Figure 2. *The BoxA-SC-induced expression of stemness transcription factors in DP and HWPc cells. (A) DP cells were treated with BoxA-SC (1:20) for 48 h, and the mRNA expression levels of the stemness transcription factors OCT4, NANOG and SOX2 were determined using the RT-qPCR method. The mRNA level was normalized to the housekeeping mRNA of GAPDH. The relative mRNA expression was calculated by using comparative Ct cycles. (C) Expression of the stemness transcription factors OCT4, NANOG and SOX2 was examined using immunofluorescence and the fluorescence intensity was measured using Image J software. (B, D) HWPc cells were assessed for the mRNA levels and protein expression of stemness transcription factor by RT-qPCR and immunofluorescence analysis, respectively. Data are presented as mean±SD (n=3). Significant compared to the control group, *p<0.05, **p<0.01, ***p<0.001 versus untreated control cells.*

they were permeabilized with 0.5% Triton-X for 5 min, followed by blocking of nonspecific proteins with 10% FBS in 0.1% Triton-X PBS for 1 h at room temperature. The cells were exposed to a 1:400 dilution of primary antibodies (OCT4, NANOG, SOX2, p21, p16) at 4˚C overnight. On the subsequent day, the cells were exposed to a 1:500 dilution of the secondary antibody Alexa Fluor 594 conjugated goat anti-rabbit IgG (H+L) (Invitrogen) and stained with Hoechst 33342 for 1 h at room temperature to visualize the nuclei. The cells were then rinsed with PBS and covered with 50% glycerol. Images were acquired using a fluorescence microscope (Olympus IX 51 with DP70, Olympus America Inc., Center Valley, PA, USA), and the fluorescence intensity was quantified using Image J software (Image J 1.52a, National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. The findings are expressed as the mean±standard deviation (SD) from a minimum of three independent experiments. Multiple comparisons were conducted using one-way ANOVA analysis with post hoc testing in GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Statistical significance was considered at a p-value <0.05.

Results

BoxA-SC induced high expression of stemness transcription factors in DP and HWPc cells. Stem cells exhibit self-renewal properties through increased expression of stemness transcription factors such as OCT4, NANOG, and SOX2 (12). To investigate the effect of BoxA-SC on the stemness properties of DP and HWPc cells, the cells were treated with BoxA-SC (1:20) for 48 h. Following this, the mRNA expression levels of stemness transcription factors were quantified using the RT-qPCR technique, while protein levels were assessed through immunofluorescence analysis.

The mRNA expression levels of OCT4 in DP cells increased 2-fold following treatment with BoxA-SC. The mRNA level of another transcription factor, NANOG, showed a substantial 20-fold increase following treatment of DP cells with BoxA-SC. The mRNA expression of SOX2 increased approximately 12-fold in DP cells treated with BoxA-SC (Figure 2A).

Based on the immunofluorescence analysis, the protein expression of OCT4 was also increased 2-fold in DP cells treated with BoxA-SC. However, the fluorescence intensity of NANOG protein expression increased 1.5-fold in DP cells treated with BoxA-SC (Figure 2C).

The mRNA and protein levels of the transcription factor OCT4 were also significantly elevated in HWPc cells treated with BoxA-SC. The mRNA and protein levels of NANOG showed a 1.5-fold increase in HWPc cells treated with BoxA-SC. Furthermore, the mRNA level of SOX2 showed a 2-fold increase, while the protein levels increased 1.5-fold in HWPc cells treated with BoxA-SC (Figure 2B and D).

The effect of PM2.5 exposure on cellular senescence and the effect of BoxA-SC on senescent cells. The cells were exposed for four days to 50 μg/ml of PM2.5, and subsequently, they were stained for SA-β-gal. As depicted in Figure 3, there was an increase in the proportion of SA-β-gal positive cells after four days of treatment.

To explore if BoxA-SC could reverse senescence in PM2.5-treated cells, the cells were initially exposed to PM2.5 for 4 days. Subsequently, the senescent cells were treated with BoxA-SC (1:20) for an additional 48 h. The data depicted in Figure 3 illustrate that treatment with BoxA-SC significantly decreased the number of SA-β-gal-positive cells compared to the control group of PM2.5-treated senescent cells. The BoxA-SC has the potential to reverse PM2.5-induced senescence in DP and HWPc cells.

Effect of BoxA-SC on PM2.5-induced cell cycle arrest. Cellular senescence is associated with the halting of the cell cycle (24). The mRNA levels of senescence-related markers p21 and p16 were assessed using RT-qPCR.

The findings indicated that exposure of DP cells to PM2.5 led to an increase in the mRNA levels of p21 and p16 (Figure 4A). To assess BoxA-SC's potential to reverse senescence in

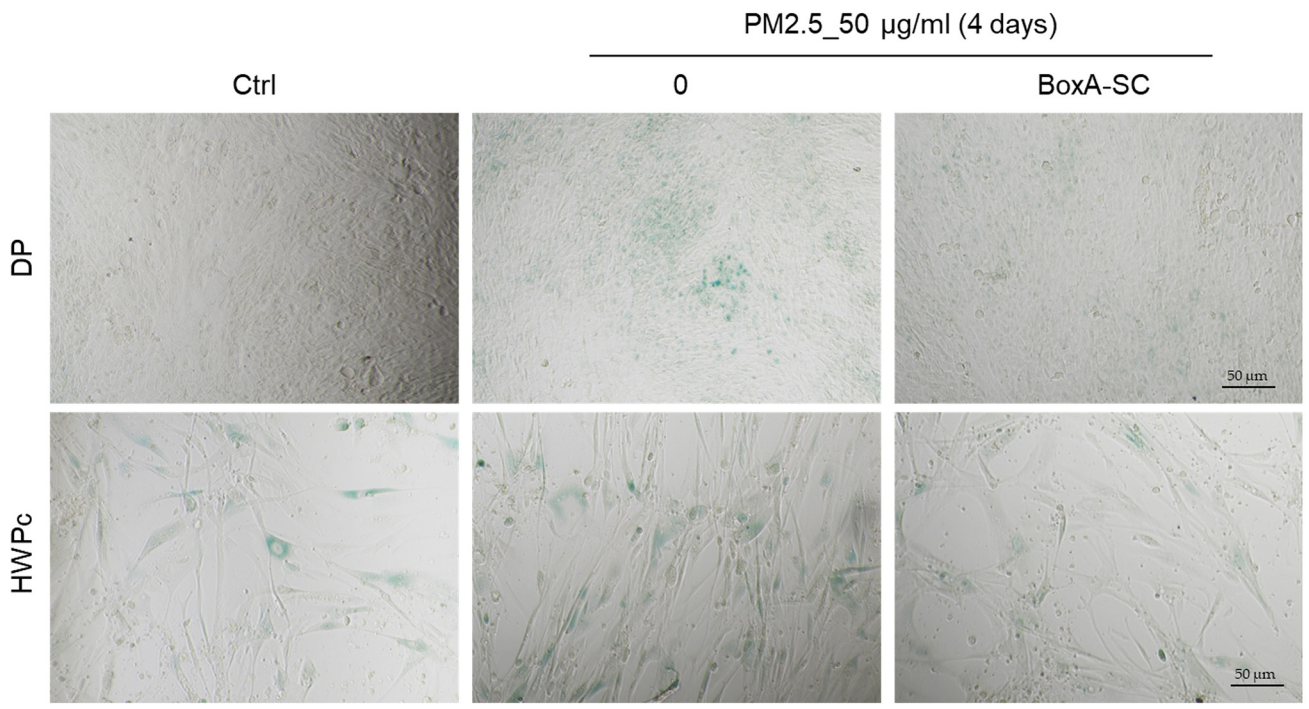

Figure 3. *The BoxA-SC reverses senescence in DP and HWPc cells induced by PM2.5 as assayed using staining for senescence-associated-β-galactosidase (SA-β-gal). The blue color indicates senescent cells. DP and HWPc cells were treated with 50 µg/ml PM2.5 for four days.*

PM2.5-induced DP senescent cells, we employed RT-qPCR to quantify the mRNA levels of p21 and p16.

PM2.5-induced senescent DP cells were treated with BoxA-SC (1:20) for 48 h, followed by measurement of p21 and p16 mRNA levels. The results suggested that treatment with BoxA-SC led to a 5-fold decrease in the mRNA level of p21 compared to the senescent DP cell population. BoxA-SC successfully reversed the senescence induced by PM2.5 in DP cells, resulting in a significantly reduction in the levels of p16 (Figure 4A).

Immunofluorescence analysis was employed to evaluate the protein expression levels of p21 and p16 in senescent cells treated with PM2.5. Based on our findings, the population of DP senescent cells induced by PM2.5 exhibited a 3-fold increase in the protein expression level of p21 and a 1.5-fold increase in the protein expression level of p16 (Figure 4C).

After treating PM2.5-induced senescent DP cells with BoxA-SC for 48 h, we evaluated the protein expression levels of p21 and p16 using immunofluorescence analysis. The results demonstrated that BoxA-SC treatment of PM2.5-induced senescent cells resulted in a significant 3-fold decrease in the protein expression level of p21 and a 1-fold reduction in the protein expression level of p16 (Figure 4C).

The senescent HWPc cells induced by PM2.5 also showed elevated protein expression levels of p21 and p16, with increases of 3.5-fold and 2-fold, respectively. Treatment of senescent HWPc cells induced by PM2.5 with BoxA-SC also resulted in a significant reduction in both mRNA and protein levels of p21 and p16 (Figure 4B and D).

Effect of PM2.5 on senescence-associated secretory phenotype (SASP) production and BoxA-SC reduces the level of SASP in PM2.5-induced cellular senescence in DP and HWPc cells. The release of different cytokines, chemokines, and proteinases by senescent cells is mainly linked to disease development (25). Senescent DP cells treated with

PM2.5 displayed SASP, as evidenced by the analysis of mRNA levels of SASP-related genes. PM2.5-induced DP cells exhibited a notable increase in the expression of genes associated with SASP. Specifically, the mRNA levels of IL1α, CXCL1, and IL7 increased 3-fold, 3-fold, and 5-fold, respectively (Figure 5A).

The senescent cells were generated as outlined above and treated with BoxA-SC in a similar manner. The findings from the SASP analysis in PM2.5-induced senescent DP cells indicated that BoxA-SC led to a 2-fold decrease in the mRNA expression level of IL1α (Figure 5A). In PM2.5-induced senescent DP cells, BoxA-SC treatment resulted in a 2-fold decrease in the mRNA expression level of CXCL1 and a 10-fold decrease in the mRNA expression level of IL7 (Figure 5A).

PM2.5-induced HWPc cells demonstrated a considerable elevation in the expression of genes associated with SASP. Notably, the mRNA levels of IL1α, CXCL1, and IL7 increased 5-fold, 20-fold, and 10-fold, respectively (Figure 5B).

We validated the reversal effect of BoxA-SC on PM2.5-induced senescence in HWPc cells. In PM2.5-induced senescent HWPc cells, BoxA-SC treatment resulted in a 5-fold decrease in the mRNA expression level of IL1α and a 2-fold decrease in the mRNA expression level of CXCL1 (Figure 5B). The results suggested that treatment with BoxA-SC markedly decreased the mRNA expression level of IL7 in senescent cell populations induced by PM2.5 (Figure 5B).

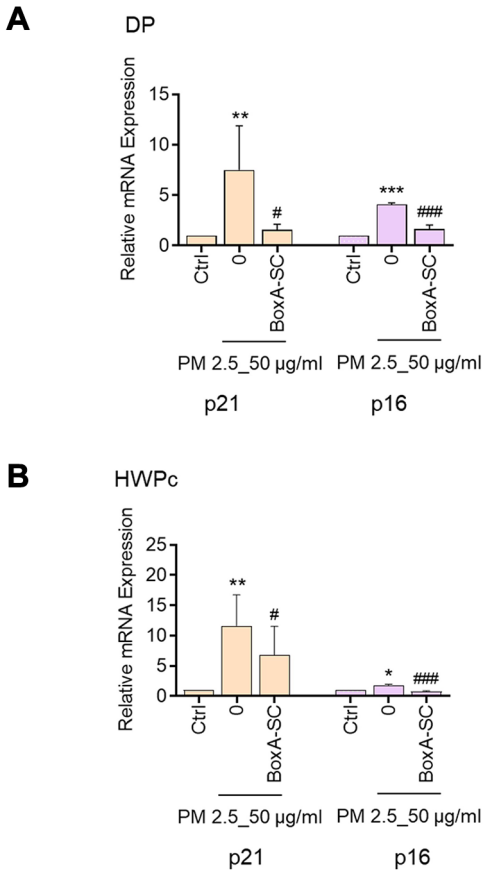

Figure 4. *Continued*

Discussion

Upon exposure of the skin to air pollution, PM2.5 trigger processes of cellular detoxification. Prolonged activation of these mechanisms can lead to senescence associated characteristics such as DNA and protein damage, increased levels of ROS, increased expression of SASP, and lipid peroxidation, leading to skin aging (1).

ADSCs, abundant in adipose tissue, have the capacity to differentiate into keratinocytes, dermal fibroblasts, and other skin cell types. This ability allows them to repair injured and apoptotic cells or promote cell regeneration through paracrine mechanisms. These mechanisms have the potential to enhance a range of skin issues, including wrinkles, skin thickness, skin lightening, and damage caused by ultraviolet exposure (26).

The regenerative effects of ADSCs mainly come from the release of bioactive substances, including proteins, nucleic acids, lipids, and extracellular vesicles, with paracrine properties. This collection of substances is termed the secretome or conditioned medium. ADMSCs release different cytokines that have the potential to activate hair follicles and facilitate the regeneration of hair. The researchers treated patients with alopecia using protein injections from adipose stem cells, which led to increased hair growth (27). An animal study investigated that impact of transplanting adipose-derived mesenchymal stem cells

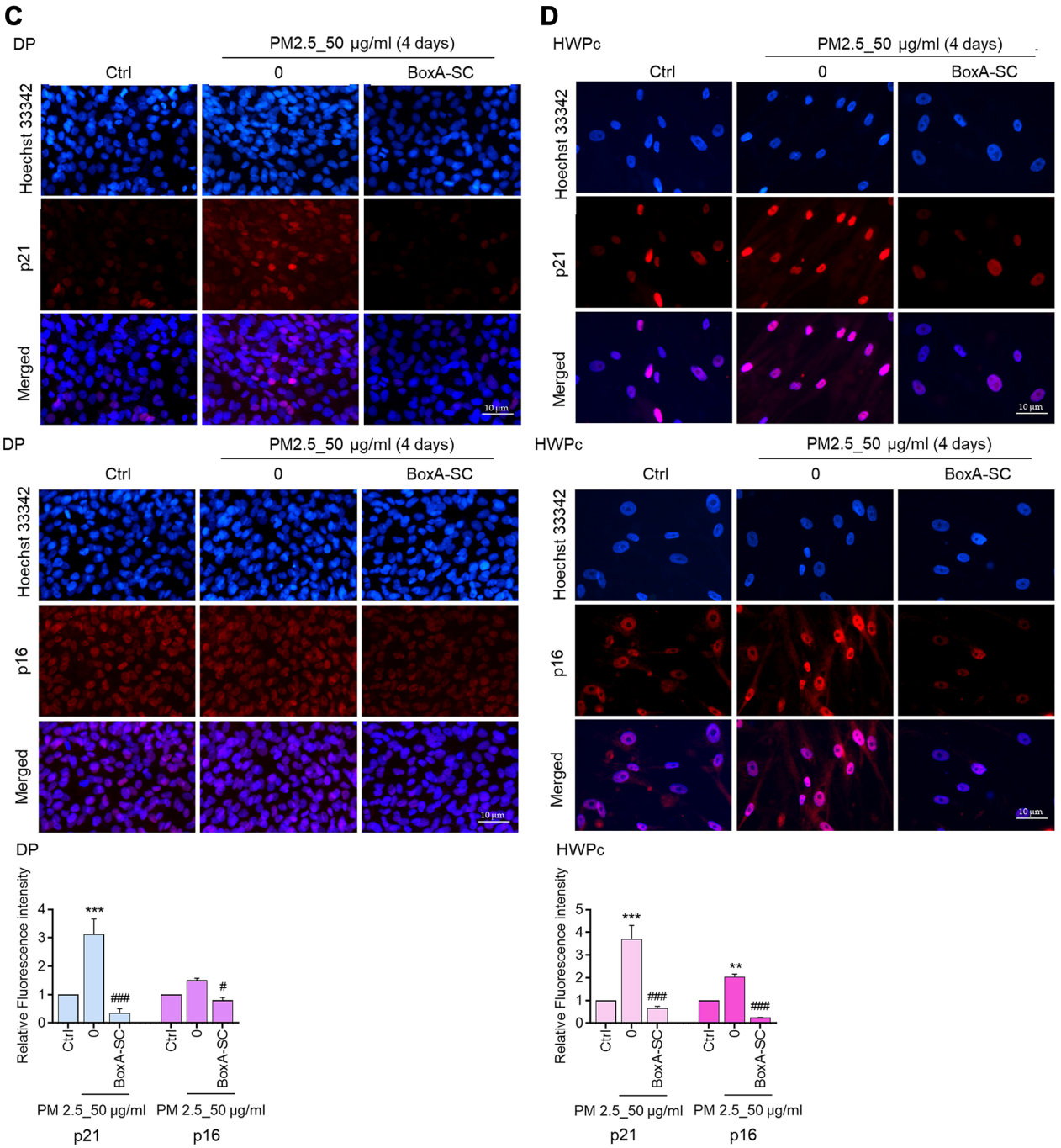

Figure 4. *Effect of PM2.5 on cellular senescence evaluated based on p21/p16 level and reversal of senescence by BoxA-SC. DP and HWPc cells were treated with 50 µg/ml PM2.5 treatment for four days. Then, PM2.5-treated senescent cells were treated with BoxA-SC (1:20) for 48 h. (A) The mRNA expression levels of p21 and p16 were assayed using RT-qPCR in DP cells. The mRNA level was normalized to the mRNA of the housekeeping GAPDH gene. The relative mRNA expression was calculated by using comparative CT cycles. (C) The expression of p21 and p16 was assayed using immunofluorescence staining and the fluorescence intensity was determined using Image J software. (B, D) HWPc cells were assessed for the mRNA levels and protein expression level of p21 and p16 by RT-qPCR and immunofluorescence analysis, respectively. Data are presented as mean±SD (n=3). Significant compared to the control group, *p<0.05, **p<0.01, ***p<0.001 versus untreated control DP or HWPc cells and, #p<0.05, ###p<0.001 versus PM2.5-induced senescence in DP or HWPc cells.*

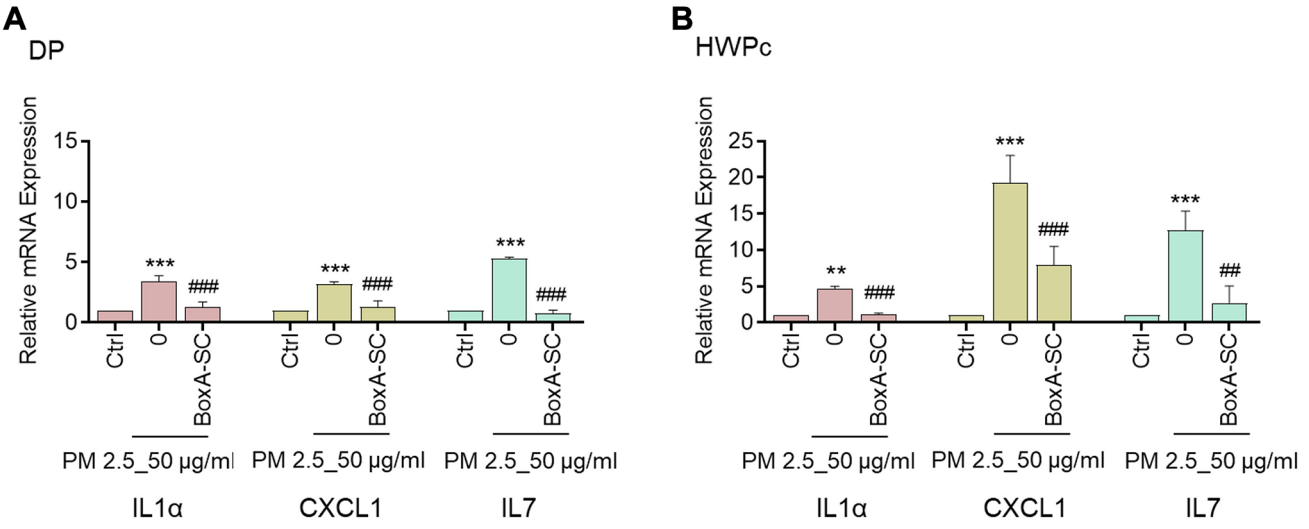

Figure 5. *PM2.5 induces senescence-associated secretory phenotype (SASP), which was reduced following treatment with BoxA-SC. DP and HWPc cells were treated with 50 μg/ml PM2.5 for four days. Then, PM2.5-treated senescent cells were treated with BoxA-SC (1:20) for 48 h. The mRNA expression level of SASP cytokines was assessed using RT-qPCR; (A) DP, (B) HWPc. The mRNA level was normalized to the mRNA of the housekeeping GAPDH gene. The relative mRNA expression was calculated using comparative CT cycles. Data are presented as mean±SD (n=3). Significant compared to the control group, *p<0.05, **p<0.01, ***p<0.001 versus untreated control DP or HWPc cells, and $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 versus PM2.5-induced senescence in DP and HWPc cells.*

(ADMSCs) during autologous ovarian transplantation in mice. It was found that this transplantation improved both the structure and function of the transplanted ovaries (28). Another study revealed that transplanting ADSCs effectively reduces lung injury and cardiac dysfunction induced by PM2.5 (3).

In our research, we utilized the secretome produced by adipose stem cells induced by Box A of HMGB1 to reverse cellular senescence. Box A of HMGB1 has a genome-stabilizing function. This could facilitate cell regeneration and provide potential for epigenetic therapies aimed at age-related diseases (16, 29). In our previous study, activating Box A within the HMGB1 gene was found to affect the expression of stem cell markers like OCT4, NANOG, and SOX2 in DP cells and HWPc. This finding could potentially advance regenerative strategies (17).

Interestingly, our findings demonstrated that BoxA-SC possess the capability to revert senescence in both DP and HWPc cells through the induction of stemness markers OCT4, NANOG, and SOX2 (Figure 2). Furthermore, the administration of BoxA-SC reduced the expression of the β-galactosidase enzyme in PM2.5-induced senescent DP and HWPc cells compared to untreated cells (Figure 3).

In our current study, exposure to PM2.5 resulted in a higher percentage of SA-β-gal blue stained cells and elevated expression levels of p21 and p16 in DP and HWPc cells (Figure 3, Figure 4). In addition, we revealed that treatment of PM2.5-induced senescent DP and HWPc cells with BoxA-SC decreased the expression of factors associated with senescence, such as p21Cip1/WAF1 and p16INK4a, compared to senescent cells that were not treated with BoxA-SC (Figure 4).

Moreover, this was accompanied by increased secretion of SASP cytokines such as IL1α, IL7, and CXCL1 in PM2.5 treated DP and HWPc cells. Our results suggested that PM2.5 contributes to the induction of senescence in DP and HWPc stem cells. Additionally, treatment PM2.5-induced senescent DP and HWPc cells with BoxA-SC resulted in a reduction in the secretion of SASP, including IL1α, IL7, and CXCL1 (Figure 5).

The findings of this study offer clear evidence that BoxA-SC can reverse senescence in PM2.5-treated DP and HWPc

cells by regulating senescence markers through the induction of stemness. Hence, BoxA-SC can serve as an important therapeutic approach for reversing senescence, and contributing to skin rejuvenation during the aging process.

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Conflicts of Interest

All Authors declare that there are no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization: PC. Data curation: ZZE, AM, PC. Formal analysis: ZZE, PC. Funding acquisition; PC. Methodology: ZZE, AM, PC. Project administration: ZZE, AM, PC. Supervision: PC. Validation: PC. Visualization: ZZE, PC. Writing-original draft preparation: ZZE. Writing—review and editing: PC. All Authors have read and agreed to the published version of the manuscript.

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