Effect of adipose-derived stem cells-conditioned medium extracellular vesicles on senescent fibroblast and E2F1 expression

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ABSTRACT

BACKGROUND Adipose-derived stem cells (ADSCs) are well-known for their regenerative properties, especially towards senescent cells. Extracellular vesicles derived from ADSCs are believed to influence the expression level of the E2 promoter binding factor (E2F1) protein, one of the key proteins regulating the cell cycle. This study aimed to investigate the impact of extracellular vesicles from ADSC-conditioned medium (ADSC-CM) on E2F1 levels and their potential to improve aging cells.

METHODS Extracellular vesicles from ADSC-CM were introduced into senescent fibroblasts through transfection. Then, the E2F1 protein levels were measured and compared between transfected and untransfected cells. A total of 18 samples were calculated based on Federer's formulas. E2F1 protein levels were counted using a cell-based enzyme-linked immunosorbent assay. Senescence-associated beta-galactosidase staining was used to quantify the number of senescent cells in each group, and the microculture tetrazolium technique assay was used to assess cellular metabolic activity.

RESULTS The number of senescent cells was lower in the transfected group compared to the untransfected group. ADSC-CM extracellular vesicles-transfected fibroblasts exhibited higher levels of E2F1 protein (0.19 [0.17] ng/ml) compared to untransfected fibroblast (0.06 [0.049] ng/ml; $p = 0.048$). Higher E2F1 protein levels were associated with reduced senescent fibroblasts and increased metabolic viable fibroblasts in the transfected group.

CONCLUSIONS ADSC-CM extracellular vesicles positively affected senescent cells by enhancing the level of E2F1.

KEYWORDS adipose-derived stem cells, extracellular vesicles, E2F1, senescence

Cellular senescence is the main pathogenesis of skin aging.[1,2] Senescent fibroblasts lose their ability to produce collagen and elastin, contributing to various aging phenotypes.[3] Modern medical research has increasingly focused on the potential of stem cells and their conditioned media to support new cell development and maintain cellular homeostasis.[4–6] Mesenchymal stem cells (MSCs), specifically adipose-derived stem cells (ADSCs), are believed to have regenerative abilities by releasing cytokines, growth factors, and microRNAs, which can subsequently affect target cells.[2,7,8]

To exert their influence, ADSCs require an effective method of transporting genetic material to target cells, such as extracellular vesicles. They are known to serve as carriers for genetic material and other molecular components between cells, reflecting the capabilities of the parent cell.[2,7,8] Therefore, extracellular vesicles derived from ADSCs are expected to exert regenerative effects on senescent fibroblasts.

E2 promoter binding factor 1 (E2F1) is known to inhibit cellular senescence.[9] As the key regulator of the cell cycle, the retinoblastoma protein (pRb) is bound to E2F1. Usually, mitogenic signals will activate cyclin-dependent kinase 4 and 6 (CDK4/6) and cyclin D, leading to the phosphorylation of pRb and the subsequent release of E2F1. The released E2F1 will bind to the promoters of pro-proliferative genes, facilitating the transition from the G1 phase to the S phase of the cell cycle.[10]

Although the impact of ADSCs on the cell cycle has been extensively studied, no study has specifically addressed the influence of ADSCs on E2F1 protein levels. Therefore, this study aimed to investigate the effect of extracellular vesicles from ADSC-conditioned medium (ADSC-CM) on E2F1 protein expression levels in senescent fibroblasts. Using senescence-associated beta-galactosidase (SA-β-gal) staining, we identified and quantified the population of senescent cells, and the microculture tetrazolium technique (MTT) assay was used to evaluate changes in cellular metabolic viability.

METHODS

In total, 18 samples were used in this study, with nine replicates each for the ADSC-CM extracellular vesicles-transfected and untransfected groups, based on Federer's formula. This study has received ethical clearance from the Medical Research Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, Malang (No. 240/EC/KEPK-PPDS/09/2019).

Fibroblast subculture

Cryopreserved human dermal fibroblasts (HDFs) and ADSCs collected from previous research[11] were preserved in the Biomedical Laboratory of the Faculty of Medicine, Universitas Brawijaya. A 70–90% confluent flask of HDFs was used for multiple subculture sequences. Cells were removed from the bottom of the flask using 0.25% trypsin-EDTA before replacing the medium with 1 ml of high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) and 1.25% antibiotic-antimycotic solution. Half of the total volume was transferred to a second flask. The flasks were then filled with a complete medium containing 20% FBS to a total 5–6 ml volume and incubated at 37°C and 5% CO_2. HDFs were passaged multiple times to induce senescence. The medium was replaced every 3 days and repeated until passage 16–17; senescence typically occurs after passage 11–14.[12]

Identification of senescent fibroblasts

Senescent fibroblasts were identified by examining the morphological changes after passages 16–17. Senescent cells exhibit several morphological changes, including a flattened, irregular shape, and inhomogeneity (200× magnification, Light Microscope; Olympus, Japan). Additionally, senescent fibroblasts were SA-β-gal-positive, as indicated by blue staining under the microscope (200× magnification, IX71 Inverted Microscope; Olympus Microscope, Japan). This staining method was performed by fixing fibroblasts with 25% glutaraldehyde for 5 min, followed by overnight staining with a cell-staining solution obtained from a commercial kit (BioVision, Inc., USA).

Isolation of ADSC-CM

ADSC cultures were obtained from previous research,[11] which were isolated from human subcutaneous adipose tissue and preserved in the Biomedical Laboratory of the Faculty of Medicine, Universitas Brawijaya. Informed consent for sampling and research was obtained from all donors. The cells were identified as plastic adherent, able to grow and migrate under standard culture conditions, and expressed the CD34 and CD44 markers.[11]

For the collection of CM, ADSCs at 80–90% confluence were initially washed with phosphate-buffered saline (PBS) 3 times before replacing the medium with a serum-free medium and incubating at 37°C and 5% CO_2 for 30 min. The medium was aspirated and replaced with fresh medium before incubation for an additional 48 hours. Following incubation, the cell homogenate was centrifuged at 165 × *g* for 5 min and filtered through a 0.22 µm syringe filter to obtain the ADSC-CM.

Purification of extracellular vesicles from ADSC-CM

The ADSC-CM was centrifuged at 200 × g (≈1,000 rpm) for 15 min to remove cells and debris. The extracellular vesicles was purified from the ADSC-CM using an Exosomes Purification Kit (Norgen Biotek, Canada). The flow-through supernatant containing the extracellular vesicles was analyzed using a scanning electron microscope (Hitachi Ltd., Japan)

and PKH26 Red Fluorescence Cell Linker Kit (Merck Ltd., Germany).

ADSC-CM extracellular vesicle transfection to fibroblasts

Transfection was performed as described on a previously published method.[13] The fibroblast culture medium was discarded, and the cells were washed with PBS. Extracellular vesicles at a concentration of 1 µg/ml in DMEM were added to each well. This concentration was chosen following the use of 1 µg/ml of MSC extracellular vesicles in previous research, which induced the proliferation and migration of fibroblasts.[14] The plates were incubated at 37°C and 5% CO_2 for 3 days.

Detection of ADSC-CM extracellular vesicle uptake by senescent fibroblast cultures

Following the manufacturer's protocol, pelleted extracellular vesicles-transfected fibroblast and negative control fibroblast were resuspended in 1 ml of Diluent C to produce a 2× cell suspension. A dye solution was prepared by mixing 4 ml of PKH26 ethanol staining solution (PKH26 Red Fluorescent Cell Linker Kit for general membrane labeling; Sigma-Aldrich, USA) with 1 ml of Diluent C. Thus, 1 ml of 2× dye solution was added to 1 ml of 2× cell suspension. Reddish glow spots inside the fibroblasts examined under a fluorescence microscope indicated the successful uptake of extracellular vesicles.

E2F1 detection using a cell-based enzyme-linked immunosorbent assay (ELISA)

Cells from both groups were cultured overnight in 96-well plates at 37°C until 70–80% confluent before being washed 3 times in PBS. E2F1 levels were measured using a cell-based ELISA, following the manufacturer's protocol (LifeSpan BioSciences, Inc., USA). The results were read at an optical density (OD) of 450 nm using a spectrophotometer and converted to a concentration in ng/ml.

SA-β-gal quantification

SA-β-gal-positive cells were quantified following the protocol by Noren Hooten and Evans.[15] The number of blue-stained cells was counted per 100 cells in five randomly chosen fields of view for each treatment condition. The percentage of SA-β-gal-positive cells was compared between the transfected and untransfected fibroblast groups.

MTT assay

Following the manufacturer's protocol, the medium was aspirated, and the wells were washed 3 times with PBS, each for 10 min. Fresh medium (200 µl) and MTT solution (50 µl at 50 mg/ml concentration) were added to each well. The plates were covered with aluminum foil and incubated at 37°C for 6 hours. After incubation, the medium was aspirated, and 200 µl of dimethyl sulfoxide and 25 µl of glycine buffer were added. Absorbance was measured using a spectrometer at an OD of 570 nm.

Data analysis

An independent sample *t*-test was used to compare E2F1 levels between untransfected and ADSC-CM extracellular vesicles-transfected senescent fibroblasts. Normality assumptions were tested using the Shapiro–Wilk test prior to comparison. A *p*-value of <0.05 was considered to be statistically significant. All data analyses were performed using SPSS software version 21 (IBM Corp., USA).

RESULTS

Senescence in fibroblasts was confirmed by a distinct flat morphology, irregular edges, and non-uniform shapes, as observed under an electron microscope (Figure 1a). These senescent cells were further identified by their characteristic blue coloration following SA-β-gal staining (Figure 1b). Additionally, extracellular vesicles were identified using an electron microscope and appeared as round structures ranging from 30 to 100 nm in size (Figure 1c).

In order to pinpoint the location of extracellular vesicles within fibroblasts, the vesicle membranes were labeled with the PKH26 fluorescent dye, which emitted a red glow under a fluorescence microscope. The cell nuclei were stained with 4',6-diamidino-2-phenylindole dilactate (DAPI), a compound emitting blue light, under a fluorescence microscope. PKH26-labeled extracellular vesicles were spread around the DAPI-stained nuclei within the cell membrane, indicating effective uptake by fibroblasts (Figure 2).

The cell profiles were evaluated using the SA-β-gal marker and MTT assay. The SA-β-gal marker helps determine the proportion of senescent cells in a culture, as senescent cells accumulate hazardous by-products, such as hydrolase enzymes, indicated by their blue staining. The untransfected group exhibited a higher

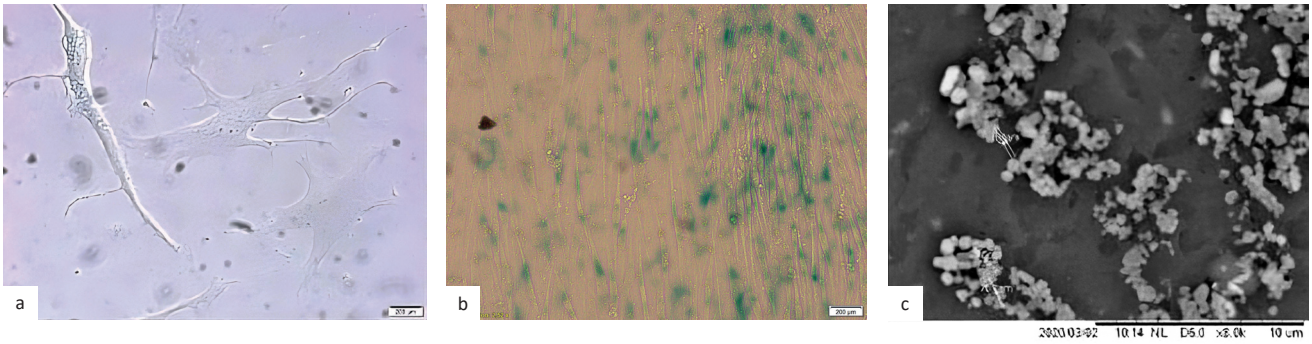

Figure 1. Senescent fibroblast characteristic and identification of extracellular vesicles. Senescent fibroblast demonstrated the morphology of flattened, irregular, and non-uniform cells (a) and exhibit the blue color as SA-β-gal-stained cells (b). TEM was used to identify the presence of extracellular vesicles, shown as clusters of round-shaped structures with sizes of 30–100 nm (c). SA-β-gal=senescence-associated beta-galactosidase; TEM=transmission electron microscope

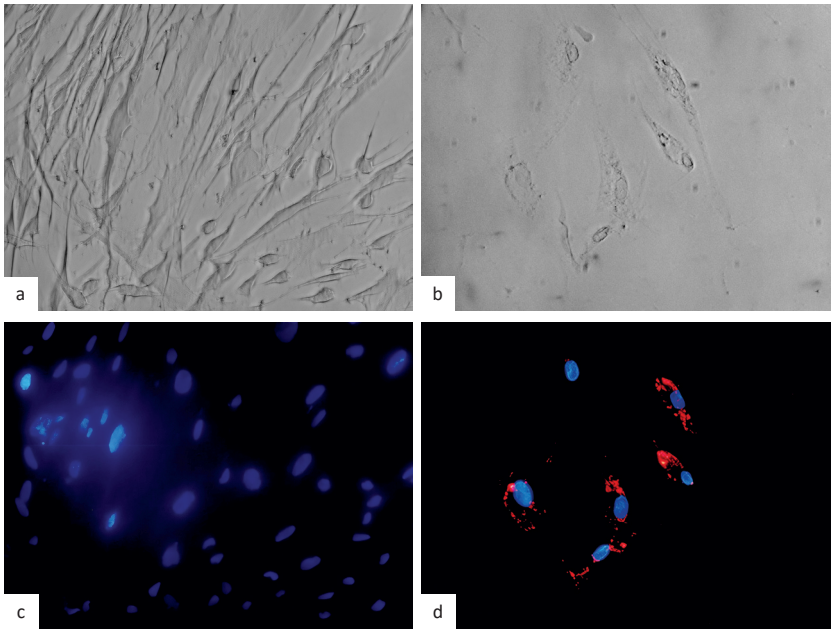

Figure 2. Extracellular vesicle uptake detection. Negative control fibroblast (a) and extracellular vesicles transfected fibroblast (b), both stained with PKH-26 fluorescent dye, showed under inverted microscope without fluorescence. In the same field, the cells with fluorescence showed no red glow spots in the negative control (c) and multiple red glow spots in the transfected cells (d). Red dots indicated extracellular vesicles stained with PKH-26. Blue oval bodies indicated nucleus stained with 4',6-diamidino-2-phenylindole, dilactate (DAPI)

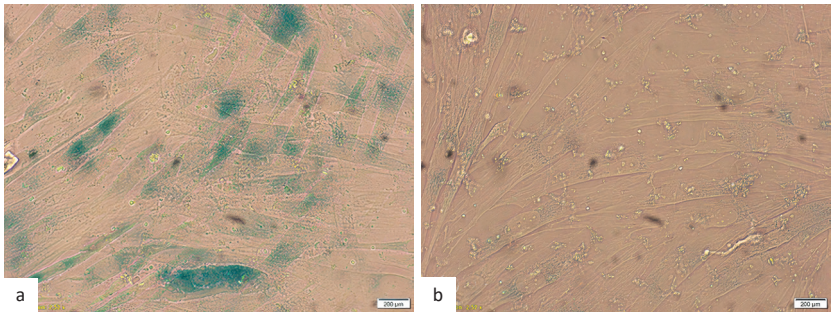

Figure 3. Senescence-associated beta-galactosidase (SA-β-gal) marker examination after extracellular vesicle treatment on fibroblast. The population of SA-β-gal positive fibroblast cells (indicated as tosca-blue color) in untransfected group (a) and 1 µg/ml extracellular vesicles transfected group (b)

percentage of blue-stained cells (66%, Figure 3a) than the transfected group (27%, Figure 3b), suggesting that the extracellular vesicles-transfected group had fewer senescent cells.

Fibroblast viability was determined using the MTT assay, which measures mitochondrial dehydrogenase activity by quantifying the reduction of MTT to purple formazan crystals at 570 nm. The absorbance of the formazan crystals correlates with the number of viable cells. Fibroblasts transfected with extracellular vesicles at 1 µg/ml had a slightly higher average absorbance (0.505 absorbance unit [AU]) than the untransfected

Table 1. Concentration level of E2F1 between untransfected and 1 µg/ml ADSC-CM vesicles transfected fibroblast groups

Samples	Total OD	Total concentration (ng/ml)	GAPDH OD (ng/ml)	GAPDH concentration (ng/ml)	E2F1 concentration (ng/ml)
Untransfected	0.09	0.952	0.101	1.122	0
	0.043	0.227	0.05	0.335	0
	0.064	0.551	0.063	0.535	0.015
	0.09	0.952	0.086	0.890	0.062
	0.088	0.921	0.083	0.844	0.077
	0.047	0.289	0.042	0.211	0.077
	0.049	0.319	0.043	0.227	0.093
	0.094	1.014	0.087	0.906	0.108
	0.099	1.091	0.09	0.952	0.139
1 µg/ml ADSC-CM vesicles transfected	0.095	1.029	0.096	1.045	0
	0.071	0.659	0.067	0.597	0.062
	0.099	1.091	0.095	1.029	0.062
	0.039	0.165	0.032	0.057	0.108
	0.056	0.427	0.044	0.242	0.185
	0.06	0.489	0.048	0.304	0.185
	0.104	1.168	0.085	0.875	0.293
	0.101	1.122	0.079	0.782	0.34
	0.124	1.477	0.089	0.937	0.54

ADSC-CM=adipose-derived stem cell-conditioned medium; E2F1=E2 promoter binding factor 1; GAPDH=glyceraldehyde-3-phosphate dehydrogenase; OD=optical density
GAPDH is an internal control

group (0.498 AU). This result was then converted into the number of viable cells, which indicated a higher number of viable cells in the transfected group than in the untransfected group (98.8% *versus* 96.4%, respectively).

We observed that ADSC-CM extracellular vesicle transfection significantly increased E2F1 expression. The average and median E2F1 levels were higher in the 1 µg/ml ADSC-CM extracellular vesicles-transfected group (0.19 [0.17] ng/ml) compared to the untransfected group (0.06 [0.049] ng/ml). The normality test showed $p = 0.435$, indicating a normal data distribution. An independent *t*-test revealed a significant difference in mean E2F1 levels between the two groups ($p = 0.048$). Detailed data on E2F1 concentrations for each fibroblast sample are provided in Table 1.

DISCUSSION

As human skin cells age, they undergo replicative senescence after repeated divisions until they reach the lowest point of clonogenicity. *In vitro*, cell senescence can be induced by culturing cells through several passages,[12] which was used in this study to induce senescence in HDFs. Morphological characteristics and positive staining for SA-β-gal confirmed senescent fibroblasts.

This study reveals several findings regarding the role of ADSC-CM extracellular vesicles in senescent HDFs. After transfecting extracellular vesicles from ADSC-CMs, E2F1 protein levels increased. This finding also implies senescence reversal on the transfected cells, shown by a decrease in SA-β-gal and higher cell metabolic activity in the MTT assay.

Previous studies have shown that CM and exosomes from human ADSCs can accelerate cutaneous wound healing and fibroblast migration.[16,17] Senescent cells can appear at any stage of the cell cycle; however, traditionally, they occur during the G1 phase and are often triggered by the deterioration of telomeres in aging cells. This phenomenon is part of the reaction to DNA damage, leading to the buildup of CDK inhibitors p21 and p16, blocking the deactivation of the tumor suppressor pRb phosphorylation, and

stopping DNA replication.[18] In response to DNA damage, E2F1 promotes DNA repair and supports cell survival.[19]

This study demonstrated a higher E2F1 level in extracellular vesicle-transfected senescent fibroblasts than in untransfected fibroblasts (Figure 3). The specific mechanism by which extracellular vesicles from ADSC-CM affect E2F1 levels remains unclear; however, it has been hypothesized that the genetic components within these extracellular vesicles may influence gene transcription and translation, leading to altered protein synthesis in recipient cells. MicroRNAs (miRNAs) are key genetic elements in extracellular vesicles that modulate gene expression by regulating messenger RNA (mRNA) stability and translation into specific proteins. These interactions typically inhibit translation and suppress protein synthesis.[7,8] Previous studies have explored how miRNAs and mRNAs can diminish CDK inhibitor proteins; however, no studies have linked specific miRNAs and mRNAs to E2F1 protein production.[20] This study did not examine the genetic material within the extracellular vesicles, which hinders a complete understanding of how ADSC-CM extracellular vesicles influence E2F1 levels.

The elevation of E2F1 levels in the transfected group correlated with decreased senescence marker SA-β-gal and increased cell viability. As a senescence indicator, elevated SA-β-gal expression has been observed in senescent fibroblasts affected by ultraviolet B (UVB) radiation exposure[21] and in brain cells affected by contusion trauma.[22] This study also found a reduction in SA-β-gal expression in the treatment group compared to the control group, suggesting that extracellular vesicle transfection from ADSC-CM can reverse cellular senescence.

Senescent cells typically show reduced viability, characterized by decreased mitochondrial activity and an inability to convert tetrazolium salt (MTT) into formazan.[23] Oh et al[22] found that if senescent cells were exposed to UVB radiation and treated with exosomes, they exhibited higher viability based on the MTT assay than untreated cells. Consistent with this study, the extracellular vesicles-transfected group exhibited an absorbance value of 0.505, indicating a viability rate of 98.8%, which was higher than that of the untransfected group, which had an absorbance value of 0.498 and a viability rate of 96.4%. The limitations of this study are the manual cell counting process and the small sample size, which prevented statistical analysis and resulted in minimal differences in numerical values between the treatment and control groups.

In conclusion, extracellular vesicles from ADSC-CM stimulate the reversal of senescence in high-passage fibroblasts, which is mediated by increased E2F1 expression. This study provides valuable insights into the effect of ADSC-CM extracellular vesicles on E2F1 levels. It contributes to our understanding of the anti-aging properties of ADSCs, paving the way for the development of stem cell-based treatments for aging. However, this study was limited by the inability to fully characterize the extracellular vesicles and genetic material transferred to fibroblasts. Further research should focus on elucidating the mechanisms by which extracellular vesicles and their genetic components influence E2F1 and other cell cycle-related proteins, which could lead to the development of novel anti-aging therapies using ADSC-derived exosomes.

Conflict of Interest

The authors affirm no conflict of interest in this study.

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