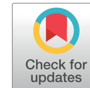

BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 32(4), 432-441 (2024)

Extracellular Vesicles Derived from Adipose Stem Cells Alleviate Systemic Sclerosis by Inhibiting TGF-β Pathway

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Abstract

Systemic sclerosis is an autoimmune disease characterized by inflammatory reactions and fibrosis. Myofibroblasts are considered therapeutic targets for preventing and reversing the pathogenesis of fibrosis in systemic sclerosis. Although the mechanisms that differentiate into myofibroblasts are diverse, transforming growth factor β (TGF-β) is known to be a key mediator of fibrosis in systemic sclerosis. This study investigated the effects of extracellular vesicles derived from human adipose stem cells (ASC-EVs) in an *in vivo* systemic sclerosis model and *in vitro* TGF-β1-induced dermal fibroblasts. The therapeutic effects of ASC-EVs on the *in vivo* systemic sclerosis model were evaluated based on dermal thickness and the number of α-smooth muscle actin (α-SMA)-expressing cells using hematoxylin and eosin staining and immunohistochemistry. Administration of ASC-EVs decreased both the dermal thickness and α-SMA expressing cell number as well as the mRNA levels of fibrotic genes, such as *Acta2*, *Ccn2*, *Col1a1* and *Comp*. Additionally, we discovered that ASC-EVs can decrease the expression of α-SMA and CTGF and suppress the TGF-β pathway by inhibiting the activation of SMAD2 in dermal fibroblasts induced by TGF-β1. Finally, TGF-β1-induced dermal fibroblasts underwent selective death through ASC-EVs treatment. These results indicate that ASC-EVs could provide a therapeutic approach for preventing and reversing systemic sclerosis.

Key Words: Systemic sclerosis, Extracellular vesicle, Exosome, Adipose stem cell, TGF-β

INTRODUCTION

Systemic sclerosis or scleroderma is an autoimmune disease in which fibrosis occurs not only in the skin but also in various internal organs, such as the lungs, heart, and kidneys (Allanore *et al*., 2015). Similar to other fibrotic diseases, fibrosis in systemic sclerosis involves the differentiation of activated fibroblasts into myofibroblasts expressing α-smooth muscle actin (α-SMA), resulting in the excessive accumulation of extracellular matrix (ECM) (Bhattacharyya *et al*., 2011). Fibroblasts transition into myofibroblasts during the wound healing process in response to cytokines, such as TGF-β1 and CTGF (Darby *et al*., 2014). These myofibroblasts play a crucial role in coordinating tissue healing by integrating diverse signals from the surrounding wound microenvironment (Darby *et al*., 2016). Although the mechanism of myofibroblast deactivation after wound healing remains unclear, myofibroblasts can return to homeostatic fibroblasts or undergo apoptosis (Gurtner *et al*., 2008; Kisseleva *et al*., 2012). Myofibroblasts that evade apoptosis or transition to normal fibroblasts can contribute to the excessive production of ECM and drive the progression of the disease towards organ failure and death (Kis *et al*., 2011; Hinz and Lagares, 2020). Therefore, regulation of myofibroblast fate is being investigated as a potential therapeutic approach to prevent and reverse the pathogenesis of fibrosis in systemic sclerosis (Ho *et al*., 2014; Yang *et al*., 2014; Lagares

Open Access **https://doi.org/10.4062/biomolther.2023.191**

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Received Nov 3, 2023 **Revised** Nov 28, 2023 **Accepted** Nov 30, 2023
Published Online Jun 5, 2024

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et al., 2017; He *et al*., 2021).

Mesenchymal stem cells derived from human tissues, such as bone marrow, adipose tissue, and placental tissue, are widely used as therapeutic agents for tissue repair and regeneration (Uccelli *et al*., 2008; Shi *et al*., 2010; Dimarino *et al*., 2013). Adipose-derived stem cells have been proposed as promising candidates for the treatment of tissue defects and degenerative diseases because of their multifaceted functions and demonstrated efficacy in suppressing fibrosis (Rivera-Gonzalez *et al*., 2014; El Agha *et al*., 2017). Despite the promising results of stem cell-based therapies using mesenchymal stem cells and ongoing clinical trials, some challenges remain, including the limitations of expanding cells *in vitro*, low survival rates within the body, and the risk of immunological rejection (Farini *et al*., 2014; Squillaro *et al*., 2016; Zhou *et al*., 2021).

Extracellular vesicles (EVs) are nano-sized (30-200 nm) vesicles that are secreted into the extracellular space (EL Andaloussi *et al*., 2013; Yanez-Mo *et al*., 2015). They are transport to recipient cells and function as messengers communicating with other cells. EVs contain various lipids, proteins, and RNA including mRNA, miRNAs, and non-coding RNA (Thery *et al*., 2002; Ko and Kim, 2023). Stem cell derived EVs have been studied for tissue repair therapy because of their high stability and easy control (Ferreira and Gomes, 2018; Hyvarinen *et al*., 2018). In particular, EVs extracted from adipose stem cells (ASC-EVs) have been shown to exert therapeutic effects on cancer cells (Fatima and Nawaz, 2015). In addition, several groups have demonstrated that ASC-EVs act on fibroblasts and not only affect the proliferation and migration of fibroblasts but also affect the wound-healing process (Hu *et al*., 2016; Wang *et al*., 2017; Choi *et al*., 2019).

This study investigated the effects of ASC-EVs in an *in vivo* systemic sclerosis model induced with bleomycin and *in vitro* in TGF-β1-induced dermal fibroblasts. It was found that ASC-EVs could reduce dermal thickness and fibrotic markers in an *in vivo* model. ASC-EVs also decreased the expression of fibrotic mRNA and protein in TGF-β1-induced dermal fibroblasts. These data indicate that ASC-EVs have therapeutic effects in systemic sclerosis.

MATERIALS AND METHODS

Extracellular vesicles preparation and characterization

Extracellular vesicles were isolated from human adipose-derived stem cells (ASCs). Primary human ASCs were purchased from Cefobio Inc (Seoul, Korea). ASCs were cultured in high-glucose Dulbecco's Eagle Medium (DMEM; Capricorn Scientific, Ebsdorfergrund, Germany) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and incubated at 37°C in a 5% CO2 incubator. To isolate extracellular vesicles from the media, the cells were first washed with phosphate-buffered saline (PBS), and the medium was changed to serum-free DMEM for 24 h. The conditioned medium was collected and centrifuged at 300×g for 10 min to remove any remaining cellular debris. A 0.22 μm bottle top filter was used to eliminate extra cell debris and macrovesicles in the conditioned medium. Finally, extracellular vesicles were isolated from the filtered conditioned medium using a tangential flow filtration system with a 300-kDa MWCO ultrafiltration membrane filter (OA300C12, Pall Corporation, Port Washington, NY, USA), as described previously (Jung *et al*., 2020; Kim *et al*., 2022). The morphologies of ASC-EVs were examined using cryogenic transmission electron microscopy (cryo-TEM). The protein concentration of ASC-EVs was measured using a micro-BCA protein assay kit (Thermo Fisher Scientific, MA, USA). The quantity and size distribution of ASC-EVs were analyzed by nanoparticle tracking analysis (NTA; Malvern Panalytical, Malvern, UK).

Flow cytometry

Extracellular vesicles were labeled and analyzed as previously described (Lee *et al*., 2021). Briefly, extracellular vesicles were incubated with microbeads coated with human CD63 antibody (ExoStep, Immunostep, Salamanca, Spain) overnight at room temperature with gentle mixing. PBS was used as a negative control. The extracellular vesicles that were bound to the beads were then washed with buffer and placed on a magnet for 3 min to remove the supernatant. Next, the bead-bound extracellular vesicles were resuspended in buffer and incubated with mouse anti-human CD81 (81Exo-25, Immunostep) for 1 h at 4°C with gentle mixing. The labelled extracellular vesicles were washed with buffer and analyzed using BD FACS AriaIII (BD Biosciences, San Jose, CA, USA). FlowJo software was used to analyze and interpret the data. Beads were observed using confocal laser scanning microscopy (Leica, Solms, Germany).

Mouse model

All animal experiments were performed in accordance with the related laws and guidelines of the Institutional Animal Care and Use Committee (IACUC) of Sungkyunkwan University (SKKUIACUC2019-11-03-1). The institutional committees approved the experiments. Male, aged 6 weeks, DBA/2 mice were purchased from Orient Bio (Seongnam, Korea) and used in this study. Dermal fibrosis was induced by injecting bleomycin (9041-93-4, Cayman, Ann Arbor, MI, USA). Subcutaneous injection of bleomycin (100 μL from a stock of 0.5 mg/ml) was continued every alternate day for 6 weeks (Yamamoto *et al*., 1999). NaCl was used as the control. Five weeks after the first injection of bleomycin, ASC-EVs were administered subcutaneously three times at weeks 5 and 6.

Histological evaluation and immunohistochemistry

Skin tissues were harvested at the last ASC-EVs administration, fixed in formalin, and embedded in paraffin. Histological sections (5 μm) were cut from paraffin blocks and stained with H&E and Masson's trichrome, according to a common protocol (Dees *et al*., 2011). For immunohistochemistry, paraffin sections were deparaffinized and treated with a mouse-specific HRP/DAB (ABC) detection IHC Kit (ab64259, Abcam, Boston, MA, USA), in accordance with the manufacturer's protocol. An α-SMA antibody (A2547, Sigma-Aldrich, Burlington, MA, USA) was used as the primary antibody. Immunopositive cells were counted using ImageJ software (ver 1.54, National Institutes of Health, Bethesda, MD, USA).

Cell culture

Primary human dermal fibroblasts (HDFs) (C0135C, Thermo Fisher Scientific) were used within passages three to seven. HDFs were incubated at 37°C in a 5%CO2 incubator and cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM, DMEM-HPA; Capricorn Scientific) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

For the experiment, cells were seeded in culture dishes and incubated for 24 h. The cells were then treated with TGF-β1 (100-21, Peprotech, Cranbury, NJ, USA) or ASC-EVs for 24 h. BSA (0.1%) and PBS were used as controls for TGF-β1 and ASC-EVs, respectively.

MTT assay

Human dermal fibroblasts were treated with TGF-β1 and incubated at 37°C for 24 h. ASC-EVs were then added to the cells for 24 h, and 50 μL MTT (M6494, Invitrogen, Waltham, MA, USA) was then added to each well. After three hours, the MTT solution and culture media were removed and the cells were incubated with dimethyl sulfoxide for 15 min at 37°C. A plate reader measured the absorbance at a 550 nm wavelength, with a reference wavelength of 690 nm.

Nuclei isolation

HDFs were washed with and collected in 1 mL of PBS. After centrifuging at 4,000 rpm for 1 min at 4°C and removal of the supernatant, the cells were resuspended in 200 μL of buffer A (40 mM Tris-Cl, 10 mM NaCl, 1 mM EDTA, 1 mM DTT, protease inhibitor, and phosphatase inhibitor) and incubated on ice for 15 min to disrupt the cell membrane. The cells were vortexed with 10% NP-40 for 10 sec and centrifuged at 12,000 rpm for 10 min at 4°C. Supernatants were separated as cytoplasmic proteins, and the nuclear protein-containing pellets were washed twice with buffer A. After washing, the pellets were resuspended in 40 μL buffer B (40 mM Tris-Cl, 420 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, protease inhibitor, and phosphatase inhibitor) and incubated on ice for 20 min. Nuclear proteins were extracted by centrifuging at 12,000 rpm for 10 min at 4°C. The supernatants were collected as nuclear proteins.

Western blot analysis

HDFs were lysed using T-PERTM (78510, Thermo Fisher Scientific). Then, 5 μg of protein were electrophoresed on 10% and 12% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% skim milk in TBS-T (10 mM Tris, 150 mM NaCl, and 0.1% Tween 20) for 1 h at RT and incubated overnight at 4°C with primary antibodies against α-SMA, p-SMAD2 (3108S, Cell Signaling Technology, Danvers, MA, USA), SMAD2/3 (07-408, Merck Millipore, Burlington, MA, USA), CTGF (sc-752, Santa Cruz Biotechnology, Dallas, TX, USA), lamin b (ab16048, Abcam), α-tubulin (2144S, Cell Signaling Technology), p-AKT (9271S, Cell Signaling Technology), AKT (2920S, Cell Signaling Technology), BCL2 (PC68, Merck Millipore) and GAPDH (NB300-221, Novus Biologicals, Littleton, CO, USA). After washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at RT. The membranes were treated with ECL solution and the labeled proteins were visualized on X-ray film (AgfaPhoto, Bayern, Germany) using a developer and fixer. ASC-EVs were lysed using 10X RIPA buffer to disrupt the lipid bilayer. ASC and ASC-EVs were detected using the same protocol as for the HDFs and incubated with primary antibodies against CD9 (ab223052, Abcam, Cambridge, UK), CD63 (ab68418, Abcam), and Alix (109201, Abcam). GM130 (12480, Cell Signaling Technology) and Calnexin (AB2301, Merck Millipore) antibodies were used as negative markers for the EVs.

Quantitative real time-PCR

Total RNA from mouse skin tissue and HDFs was extracted using RNAiso plus (9108, Takara Bio, Kusatsu, Japan) according to the manufacturer's protocol. Total RNA was reverse-transcribed into complementary DNA using a kit (RR037A, Takara Bio). Quantitative real-time PCR was performed using TB Green (RR820A, Takara Bio). Primer sequences used for the real-time PCR are listed in Table 1 and 2. GAPDH was used as the housekeeping gene.

Statistical analysis

Statistical analysis was conducted using Prism9 software (GraphPad Software Inc., Boston, MA, USA). One-way ANOVA with Tukey's multiple comparisons test was used to compare more than three samples, while the unpaired Student's t-test was used to assess the significance between two samples. Data are presented as mean ± standard deviation (SD). Statistical significance was determined at $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$), indicating significantly different groups.

Table 1. Human primer list for RT-qPCR

Human gene	Sequence
ACTA2	F: ACC CAC AAT GTC CCC ATC TA
	R: GAA GGA ATA GCC ACG CTC AG
CCN2	F: AGC TG ACCT GGA AGA GAA CAT T
	R: GCT CGG TAT GTC TTC ATG CTG
GAPDH	F: AGC CAC ATC GCT CAG ACA C
	R: GCC CAA TAC GAC CAA ATC C

Table 2. Mouse primer list for RT-qPCR

Mouse gene	Sequence
Acta2	F: ATG GCT CTG GCT CTG TAA G
	R: CCC ATT CCA ACC ATT ACT CC
Ccn2	F: CTG CAG ACT GGA GAA GCA GA
	R: GCT TGG CGA TTT TAG GTG TC
Col1a1	F: CAT GTT CAG CTT TGT GGA CCT
	R: GCA GCT GAC TTC AGG GAT GT
Tgfb1	F: GCA ACA TGT GGA ACT CTA CCA G
	R: CAG CCA CTC AGG CGT ATC A
Timp1	F: CCA GCG TT ATAA GAT CAA GAT GAC
	R: CTG GAC TTG TGG GCA TAT C
Pf4	F: CAT CTC CTC TGG GAT CCA TCT
	R: CCA TTC TTC AGG GTG GCT AT
Ccl2	F: CAG GTG AGT GGG GCG TTA
	R: GCC TGC TGT TCA CAG TTG C
Il6	F: GCT ACC AAA CTG GAT ATA ATC AGG
	R: CCA GGT AGC TAT GGT ACT CCA GAA
Gapdh	F: TTG ATG GCA ACA ATC TCC AC
	R: CGT CCC GTA GAC AAA ATG GT

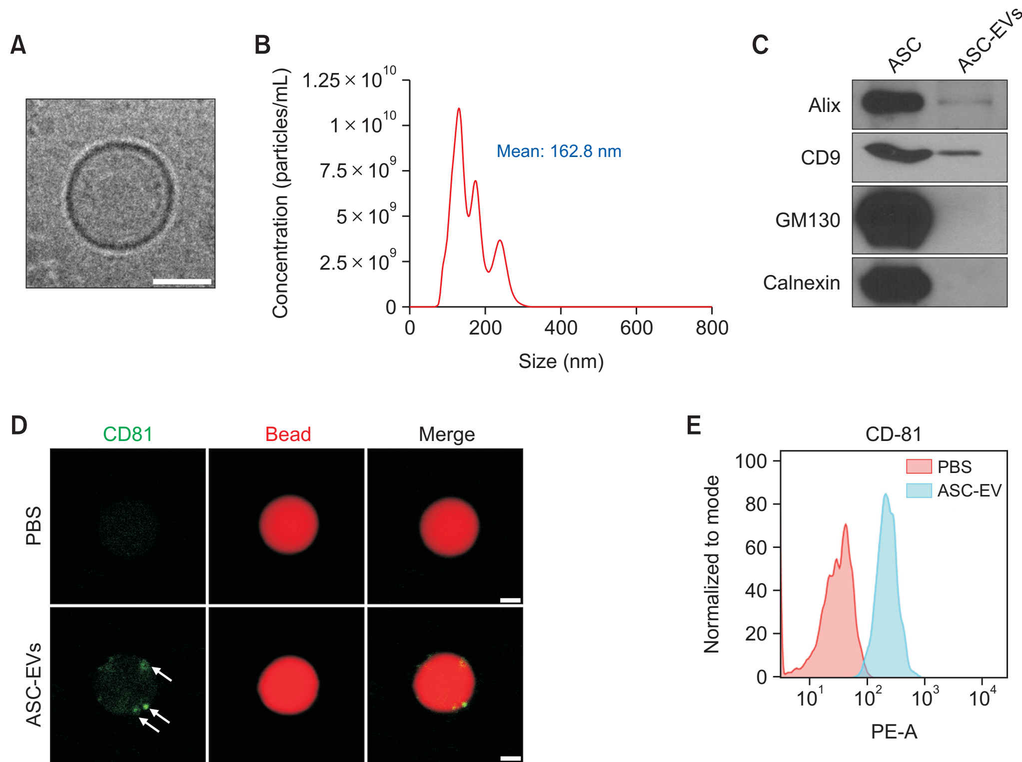

Fig. 1. Characterization of EVs derived from human ASC. (A) Cryo-TEM image of EVs. Scale bar, 50 nm. (B) Particle number (%) of EVs contributed with size analyzed by nanoparticle tracking assay (NTA). (C) Representative western blot image showing the presence of positive EV markers Alix and CD9, as well as the absence of negative markers Calnexin and GM130. (D) CD81 staining of ASC-EVs and fluorescence images of magnetic beads. Scale bar, 2 μm. (E) Flow cytometry of CD81 expression on ASC-EVs.

RESULTS

Characterization of ASC-EVs

To produce ASC-EVs, human adipose-derived stem cells were cultured for 3 weeks, and ASC-EVs were isolated from 1 L of conditioned medium collected during proliferation. The ASC-EVs were characterized by their shape, size, concentration, and protein markers. Cryo-TEM analysis revealed the presence of EVs with a round, spherical shape and a double-layer membrane structure (Fig. 1A). Through NTA, it was found that the mean diameter was 162 nm, which is within the typical size range of EVs (Fig. 1B). Furthermore, the concentration of EVs was determined to be 0.160 μg/μL based on microBCA protein quantification, and the particle number concentration was confirmed to be 2.17×10^7 particles/μL using NTA. As shown in Fig. 1C, ASC-EVs expressed EV markers, such as CD9 and Alix, whereas the negative markers GM130 and Calnexin were not observed. To investigate the surface marker proteins of ASC-EVs, anti-CD63 antibody-coated magnetic beads were incubated with ASC-EVs, which were then detected using an anti-CD81 antibody tagged with PE. The presence of CD81 in ASC-EVs was confirmed by measuring the fluorescence using confocal microscopy and FACS analysis (Fig. 1D, 1E).

Attenuation of HDF fibrogenic activation *in vitro* by ASC-EVs

An *in vitro* fibrosis model for the therapeutic effect of ASC-EVs was constructed by treating human dermal fibroblasts with TGF-β1. After treatment with various concentrations of TGF-β1 for 24 h, α-SMA mRNA and protein levels increased at all concentrations (Supplementary Fig. 1). To validate the therapeutic potential of ASC-EVs, human fibroblasts were treated with TGF-β1 at 1 ng/mL, along with ASC-EVs at a concentration of either 10^7 or 10^8 particles/mL for 24 h. The results showed that the quantity of α-SMA protein significantly decreased in a dose-dependent manner in cells treated with ASC-EVs compared to those treated with PBS when subjected to TGF-β1 for 24 h (Fig. 2A). ASC-EVs were able to significantly decrease the mRNA levels of the fibrotic markers *ACTA2* and *CCN2* in a concentration-dependent manner when the mRNA levels of these markers were increased in fibroblasts treated with TGF- β1 (Fig. 2B).

Therapeutic effect of ASC-EVs in bleomycin-induced *in vivo* scleroderma model

The study investigated the effectiveness of ASC-EVs in skin fibrosis using a mouse model of bleomycin-induced fibrosis. In this model, 50 μg of bleomycin was injected subcutaneously every other day for 6 weeks, and 10^8 particles of ASC-EVs were subcutaneously administered three times in total at weeks 5 and 6 (Fig. 3A). After bleomycin injection, the skin thickness increased, but the administration of ASC-EVs significantly reduced this increase (Fig. 3B). Additionally, through the use of DAB staining, it was observed that the number of myofibroblasts expressing α-SMA was lower in the ASC-EVs

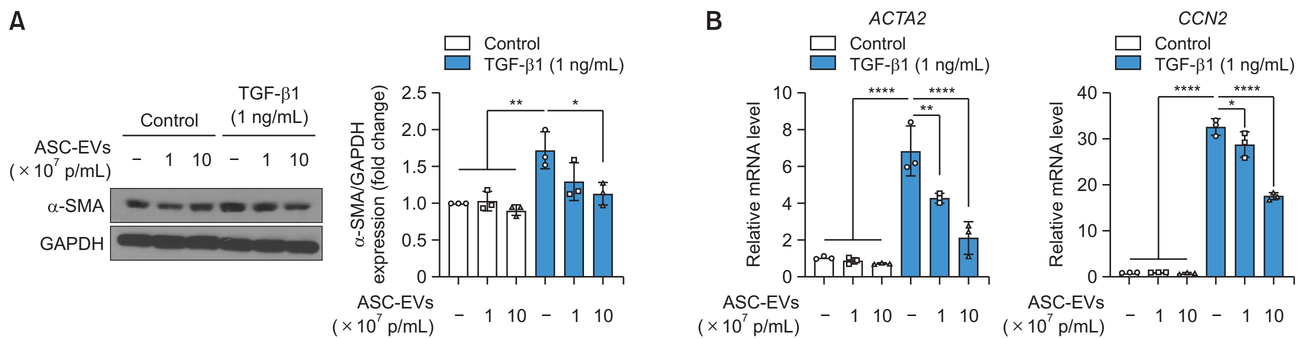

Fig. 2. Activation of dermal fibroblast to myofibroblast was inhibited by ASC-EVs. (A) Representative western blot image of α-SMA expression level in TGF-β1 treated dermal fibroblasts (left). The relative α-SMA expression compared to GAPDH was quantified using ImageJ software (right). (B) The fibrotic gene expression in dermal fibroblasts was determined by qPCR. The relative gene expression was normalized to a housekeeping gene (*GAPDH*) and expressed as the fold change compared to control (0.1% BSA and PBS treated dermal fibroblasts). At least three independent experiments were conducted. Statistical analysis was processed using a one-way ANOVA with Tukey's multiple comparison test (**p*<0.05, ***p*<0.01, *****p*<0.0001).

treated group than in the group treated with NaCl for skin fibrosis (Fig. 3C). Furthermore, mRNA analysis revealed that several fibrosis markers, including *Acta2*, *Ccn2*, *Col1a1*, *Tgfb1*, and *Timp1*, which are expressed by fibroblasts, as well as *Pf4* and *Ccl2* expressed by immune cells, exhibited significant decreases following injection of ASC-EVs (Fig. 4).

Effects of ASC-EVs on the TGF-β1 pathway *in vitro*

The TGF-β/SMAD signaling pathway is overactive in fibrosis and plays a major role in systemic sclerosis (Wynn, 2008). Therefore, inhibition of the TGF-β/SMAD signaling pathway is a major target for systemic sclerosis treatment (Walton *et al*., 2017). When TGF-β binds to receptors on the fibroblast surface, the activated receptor phosphorylated the SMAD complex (Liu *et al*., 2016). The phosphorylated SMAD complex enters the nucleus and acts as a transcription factor that induces the transcription of genes related to fibrosis and ECM production (Heldin *et al*., 1997; Joan Massague, 2000). Thus, changes were observed in canonical SMAD signaling to determine whether ASC-EVs inhibited the TGF-β /SMAD signaling pathway and showed therapeutic effects. First, phosphorylation of SMAD2 increased after treatment with TGF-β1 for 24 h at various concentrations (Supplementary Fig. 1). When 1 ng/mL or 2.5 ng/mL of TGF-β1 and ASC-EVs were co-treated for 24 h, the phosphorylation of SMAD2 was inhibited when compared with TGF-β1/PBS treatment (Fig. 5A, 5B). Next, to identify the effects of ASC-EVs on the nuclear localization of the SMAD complex, changes were observed in the cytoplasmic and nuclear level of phosphorylated SMAD2 in the nuclear fraction. Nuclear localization of total SMAD2/3 did not change significantly, but localization of phosphorylated SMAD2 decreased when ASC-EVs were co-treated with 1 ng/mL of TGF-β1 (Fig. 5C, 5D).

ASC-EVs induce cell death in myofibroblasts

Next, the study aimed to determine the effect of ASC-EVs on the fate of myofibroblast. Myofibroblasts have the potential to either revert to fibroblasts or undergo cell death, which is typically suppressed (Hinz and Lagares, 2020). Furthermore, the AKT pathway plays a crucial role in the transformation of differentiation of cells comprising the skin tissue (Ko and Kim, 2023). Initially, it was confirmed that treatment of dermal fibroblasts with TGF- β1 at a concentration of 2.5 ng/mL for 24 h increased the levels of phosphorylated AKT, which contributes to cell proliferation, as well as BCL-2, an anti-apoptotic marker (Supplementary Fig. 2A). To confirm the effect on activated myofibroblasts, fibroblasts were stimulated with TGF- β1 for 24 h and subsequently exposed to ASC-EVs at a concentration of 10^8 particles/mL for 24 h. ASC-EVs treatment significantly decreased the expression levels of not only α-SMA, but also phosphorylated AKT and BCL-2 (Fig. 6A, 6B). Prior to examining the effects of ASC-EVs on cell viability, we confirmed a significant increase in cell proliferation after treatment with 2.5 ng/mL of TGF- β1 for 24 h (Supplementary Fig. 2B). To investigate whether ASC-EVs could affect cell viability, dermal fibroblasts were treated with ASC-EVs for 24 h following the activation of cell proliferation with a 24 h treatment with 2.5 ng/mL of TGF- β1. ASC-EVs treatment did not affect cell viability under normal conditions. However, under conditions where cell proliferation was enhanced by TGF- β1, ASC-EVs treatment significantly reduced cell viability (Fig. 6C). These results indicate that ASC-EVs induce cell death in a myofibroblast-specific manner.

DISCUSSION

Systemic sclerosis is an autoimmune disease characterized by inflammatory reactions and fibrosis. Although the exact cause of systemic sclerosis remains unclear, the pathogenesis of systemic sclerosis, which accumulates ECM by activation of fibroblasts via an inflammatory reaction, has been well studied. Myofibroblasts, which directly produce ECM, are the main therapeutic targets. Several groups have demonstrated that stem cell-derived extracellular vesicles can regulate fibroblast differentiation in wound-healing models (Hu *et al*., 2016; Wang *et al*., 2017). TGF-β is a major factor that activates the differentiation of systemic sclerosis fibroblasts into myofibroblasts. Therefore, studies are underway to inhibit the TGF-β signaling pathway during systemic sclerosis treatment. Stem cell-derived EVs have been shown to include components associated with the TGF-β pathway and can inhibit this pathway in dermal fibroblasts (Fang *et al*., 2016). In particular, ASC-EVs contain miRNAs that target the TGF-β pathway (Choi *et*

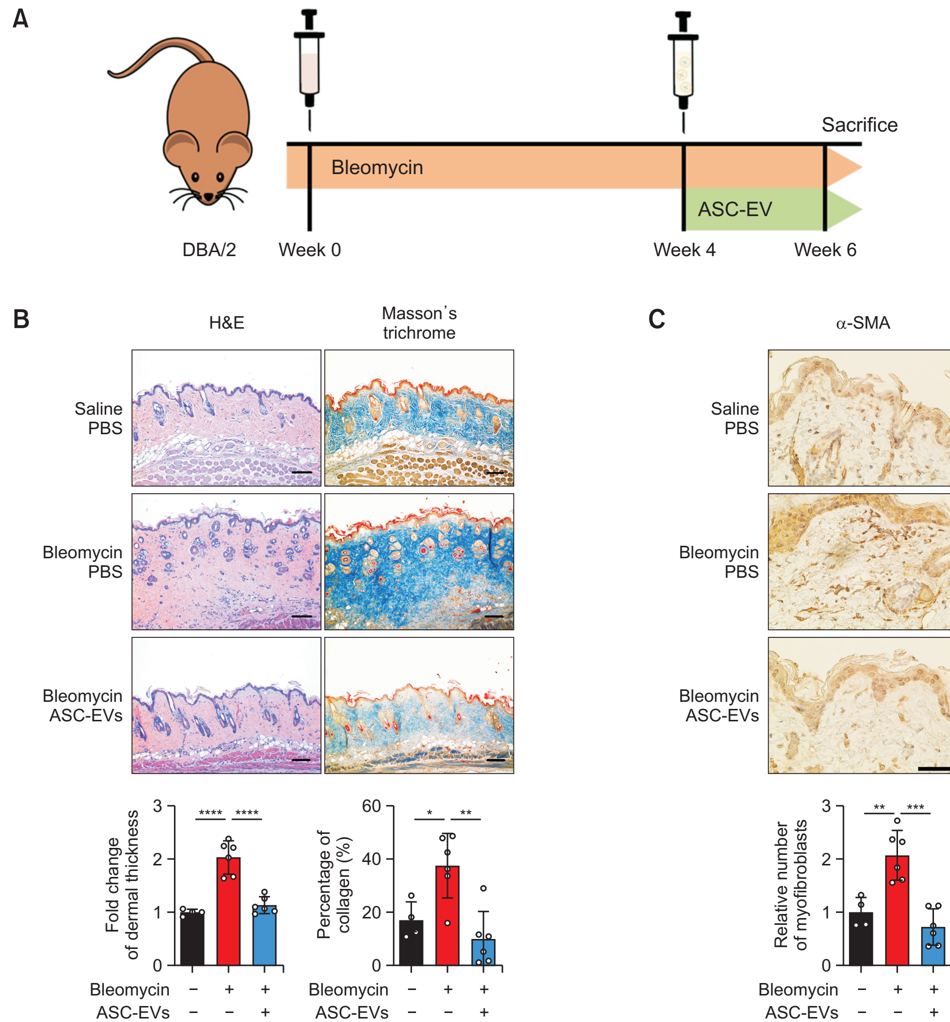

Fig. 3. ASC-EVs alleviate skin fibrosis of bleomycin-induced systemic sclerosis model. (A) Experimental design of confirm the therapeutic effect of ASC-EVs in bleomycin-induced systemic sclerosis model. (B) Representative Hematoxylin & Eosin (H&E) quantification data of dermal thickness (left) and Masson's Trichrome images and area percentage of collagen from 6 weeks saline- versus bleomycin-treated mice (right). Results were expressed as the fold change compared to saline treated group. Scale bar, 100 μm. (C) Immunohistochemistry image of α-SMA in bleomycin-induced systemic sclerosis mice treated with PBS or ASC-EVs and quantification data of α-SMA positive myofibroblast number compared to saline treated group. Scale bar, 50 μm. n=4 for saline treated group, n=6 for each bleomycin treated group. Statistical analysis was processed using a one-way ANOVA with Tukey's multiple comparison test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

al., 2019).

We hypothesized that ASC-EVs could alleviate fibrosis in systemic sclerosis fibrotic models, both *in vivo* and *in vitro*. By activating human dermal fibroblasts with TGF-β1 and treating them with ASC-EVs at two different concentrations, we verified a decrease in the mRNA levels of fibrotic genes and a reduction in the expression of α-SMA. A reduction in dermal thickness and the number of cells expressing α-SMA in the bleomycin-induced systemic sclerosis model upon administration of ASC-EVs. Furthermore, the administration of ASC-EVs to the mouse model decreased the mRNA expression levels of fibrotic genes. ASC-EVs have been shown to inhibit the phosphorylation level and nuclear localization of phosphorylated SMAD2 and reduce the level of BCL-2, thereby inducing cell death in myofibroblasts. As TGF-β signaling is also involved in myofibroblast apoptosis resistance, based on these results, the therapeutic effects of ASC-EVs are mediated through inhibition of the TGF-β signaling pathway (Zhang and Phan, 1999). In addition to fibrotic genes, inflammatory response genes expressed by immune cells also decreased when ASC-EVs were injected into the bleomycin-induced mouse model. The fact that stem cell derived EVs reduce the expression of genes involved in the inflammatory response is known and studied through experiments in various models (Blazquez *et al*., 2014; Cho *et al*., 2018; Hyvarinen *et al*., 2018; Zhao *et al*., 2018). As bleomycin induces an inflammatory response after the initial injection (Yamamoto, 2006), it is thought that the suppressed inflammatory response resulted in a therapeutic effect in this model. However, ASC-EVs were injected after fibrosis, suggesting that this effect is not exerted by suppressing the inflammatory response alone. In accordance with these studies, it could be suggested that the effect of ASC-

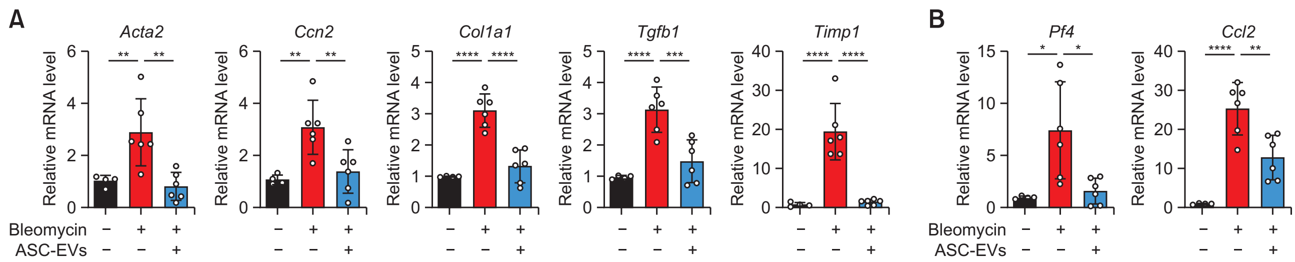

Fig. 4. ASC-EVs reduce expression of systemic sclerosis markers in bleomycin-induced systemic sclerosis model. (A) Fibrotic markers were significantly downregulated by treating ASC-EVs. (B) ASC-EVs administration could reduce inflammatory markers in systemic sclerosis mouse model. Relative gene expression was normalized to a housekeeping gene (*Gapdh*) and expressed as a fold change compared to saline treated group. n=4 for saline treated group, n=6 for each bleomycin treated group. Statistical analysis was processed using a one-way ANOVA with Tukey's multiple comparison test (**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

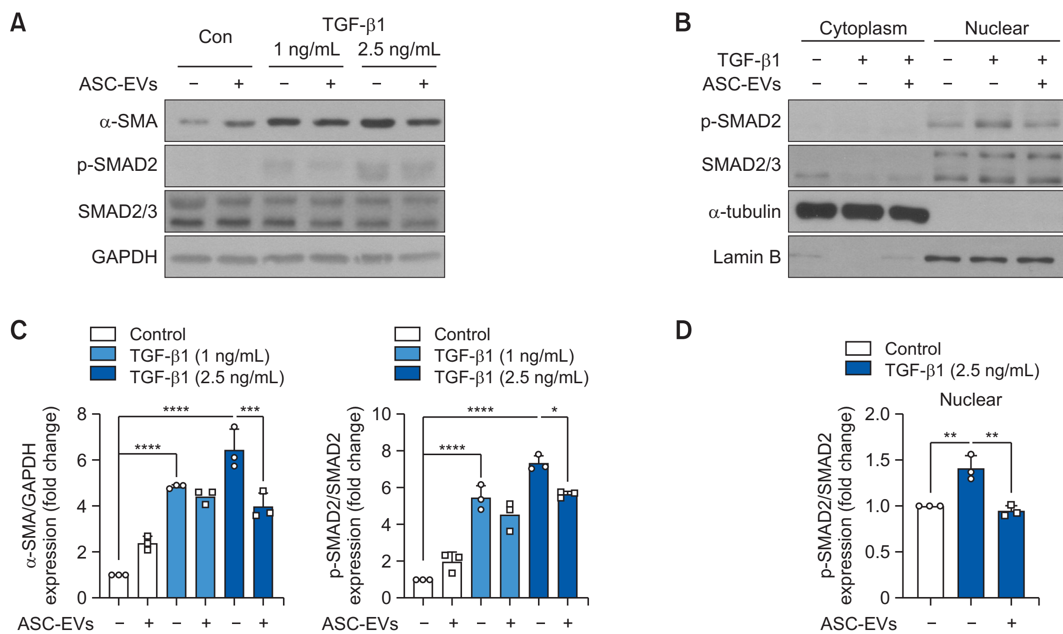

Fig. 5. ASC-EVs have a suppressive effect on TGF-β signaling pathway. (A) Representative western blot image showing the effect of ASC-EVs on phosphorylated SMAD2 levels. (B) Quantification of α-SMA and p-SMAD2 immunoblotting in activated dermal fibroblasts treated for 24 h with PBS or ASC-EVs. Three independent experiments were conducted. (C) Western blot analysis of cytoplasmic and nuclear fractions from dermal fibroblasts demonstrating reduced nuclear localization of phosphorylated SMAD2 following ASC-EVs treatment. (D) Quantification of α-SMA and p-SMAD2 immunoblotting in activated dermal fibroblasts treated for 24 h with PBS or ASC-EVs. Three independent experiments were conducted. Statistically significant differences were assessed as compared to the controls. Statistical analysis was processed using a one-way ANOVA with Tukey's multiple comparison test (**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

EVs on myofibroblasts is more significant than the suppression of inflammation.

Both *in vivo* and *in vitro* studies have shown the efficacy of ASC-EVs; however, further studies are needed. Most importantly, the specific components of ASC-EVs responsible for inhibiting the TGF-β signaling pathway, inducing myofibroblast death, and exhibiting a therapeutic effect in the systemic sclerosis model, remain unclear. We suggest that miRNAs play a role because miRNAs that target the TGF-β pathway are present among the miRNAs contained in stem cell-derived EVs that can inhibit the TGF-β/SMAD signaling pathway (Fang *et al*., 2016). In addition, TGF-β pathway is involved in the top 12 pathways targeted by miRNAs in ASC-EVs (Choi *et al*., 2019). However, ASC-EVs contain various elements besides miRNAs (Ferguson and Nguyen, 2016), and other factors can affect vital pathways in addition to the TGF-β pathway and thus have a therapeutic effect. Therefore, future studies are required to confirm whether miRNAs target the TGF-β signaling pathway and can exhibit a therapeutic effect.

In conclusion, we demonstrated that ASC-EVs have therapeutic effects in a bleomycin-induced mouse model and TGF-β-induced human dermal fibroblasts *in vitro*. ASC-EVs downregulate the expression of fibrotic genes and decrease the accumulation of extracellular matrix proteins in both mouse and human dermal fibroblast (Fig. 7). Furthermore, we observed that ASC-EVs inhibit the TGF-β signaling pathway by decreasing the phosphorylation and nuclear localization of SMAD2. Moreover, ASC-EVs affected the survival and viability

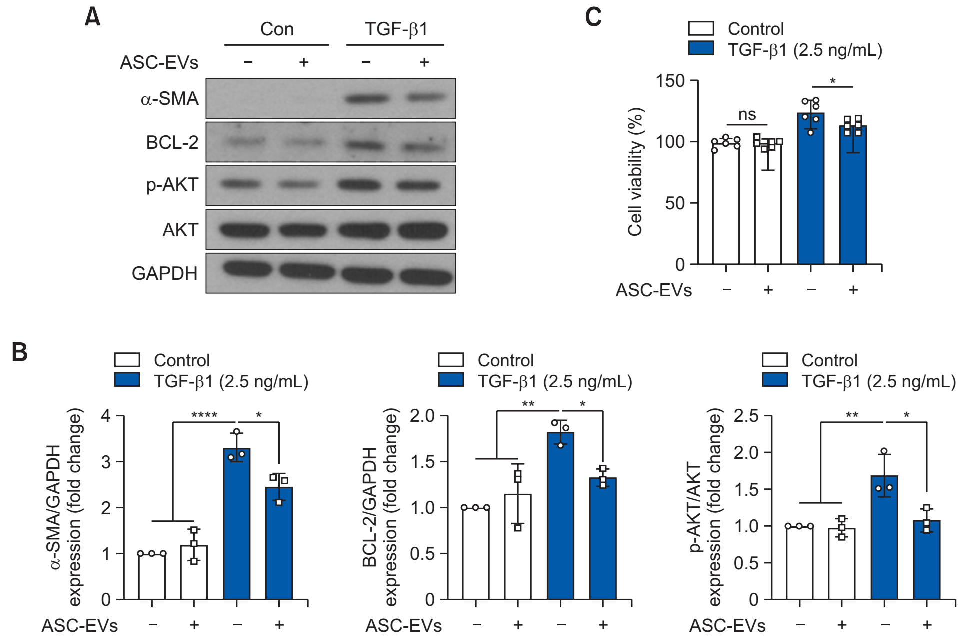

Fig. 6. ASC-EVs induce the cell death in antiapoptotic myofibroblasts. (A) Representative images and (B) quantification of α-SMA, p-AKT and BCL-2 immunoblotting in activated dermal fibroblasts treated for 24 h with PBS or ASC-EVs. Three independent experiments were conducted. (C) Cell viability (MTT assay) of activated dermal fibroblasts treated with PBS or ASC-EVs for 24 h. Statistically significant differences were assessed as compared to the controls. Statistical analysis was processed using a one-way ANOVA with Tukey's multiple comparison test (*p<0.05, **p<0.01, ****p<0.0001).

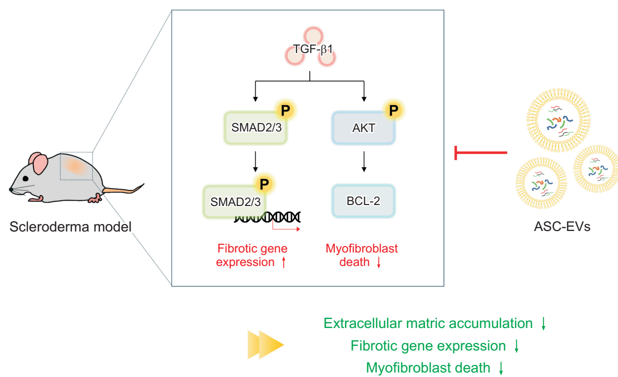

Fig. 7. Graphical summary of ASC-EVs' therapeutic effects on systemic sclerosis model. ASC-EVs inhibit the TGF-β signaling pathway by decreasing the phosphorylation and nuclear localization of SMAD2. Moreover, ASC-EVs affect to survival and viability of myofibroblasts through the modulation of phosphorylated AKT and BCL-2 protein levels. Consequently, ASC-EVs downregulate the expression of fibrotic genes and reduce the accumulation of extracellular matrix proteins in both mouse and human dermal fibroblasts.

of myofibroblasts through the modulation of phosphorylated AKT and BCL-2 protein levels. These results indicate that ASC-EVs could provide a therapeutic approach for preventing and reversing systemic sclerosis.

CONFLICT OF INTEREST

DGJ, JHP, and YWC are stockholders of ExoStemTech, Inc.

ACKNOWLEDGMENTS

This study was supported by the National Research Foundation of Korea (NRF-2019R1A2C3011422, NRF-2019R1A5A2027340). This work was also supported by the Korea Drug Development Fund (KDDF-HN21C1266) and the Ministry of Oceans and Fisheries' R&D project, Korea (1525011845).

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