

BMSC-Derived Exosomes Intervened the Pathogenic Changes of Scleroderma in Mouse Through Its microRNAs

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Abstract

Background

Systemic sclerosis (SSc) is a disease with severe fibrosis of the skin without effective therapy. While bone marrow mesenchymal stem cell (BMSC) derived exosome was a potential stem cell-based candidate in treatment of SSc.

Methods

BMSCs were isolated from the bone marrow of mouse and identified with the surface markers and multi-lineage differentiation. The exosomes were isolated from the BMSCs culture medium with ultracentrifugation and identified with NTA, TEM and western blot. The miRNAs of the BMSC-derived exosomes (BMSC-EXOs) were studied via miRNA sequencing and bioinformatic analysis. The SSc model was established in mice by bleomycin (BLM) subcutaneous injection and the mice were treated with BMSCs or BMSC-derived exosomes. The skin tissues were dissociated and analyzed with H&E staining, RNA sequencing and immunohistochemical staining.

Results

Evident pathological changes like fibrosis and inflammation induced in the skin of BLM-treated mice. Both BMSCs and BMSC-EXOs effectively intervened such pathological manifestations and disease process, in a very similar way. The effects of the BMSC-EXOs were tracked to their microRNAs, which were proved to be involved in regulating the proliferation and differentiation of multiple cell types and in multiple biological processes when the EXOs functioned. Furthermore, the TGF- β 1 positive cells and α -SMA positive myofibroblasts were significantly increased in the scleroderma skin of BLM-treated mice, but evidently reduced in the EXO-treated SSc group. Meanwhile, the number of mast cells as well as the infiltrated macrophages and lymphocytes were evidently increased in the skins of the BLM-treated mice, but significantly reduced by the EXO treatment. Such observations were confirmed by the data of the detected inflammatory cytokines that significantly higher mRNA levels of Il6, Il10 and Tnf- α were found in SSc mice, but reduced following the EXO treatment. Through bioinformatics analysis, TGF β and WNT signaling pathways were revealed to be closely involved in the pathogenic changes in mouse SSc and could be the main targets for treating the disease.

Conclusions

BMSC-derived exosomes could be developed as a potential therapy for treating the dysfunction of the skin in SSc, especially for its similar efficacy with BMSCs but less regulated as compared to cell therapy. Its mechanisms are involved in its microRNAs which alleviate the SSc pathogenic changes through regulating WNT and TGF β signaling pathways.

Introduction

Scleroderma (Systemic sclerosis, SSc) is an autoimmune connective tissue disease with unknown etiology and characterized by a triad of hallmarks: vasculopathy, immune dysfunction, and fibroblast dysfunction which results in excessive accumulation of collagen and fibrosis in the skin and visceral organs [1, 2]. Since there is no effective therapy, scleroderma often causes severe disability and even death [3, 4]. Therefore, in-depth studies on the etiology and effective treatments for scleroderma are necessary to improve quality of life and life expectancy of patients.

Inflammation and vascular injury are reported to drive the autoimmune response and precede fibrosis in the initial stages of SSc [5, 6]. Such fibrosis process is regulated by a combination of profibrotic mediators from autocrine and paracrine, such as transforming growth factor- β 1 (TGF- β 1), interleukin 4 (IL-4), interleukin 13 (IL-13) and interleukin 10 (IL-10). These mediators secreted by macrophages and monocytes promote the tissue-resident fibroblasts to differentiate to myofibroblasts and enhance the production of collagen and other extracellular matrix (ECM) components from local fibroblasts and α -smooth muscle actin (α -SMA)-positive myofibroblasts in the affected organs [6–8]. Although SSc has been considered as an autoimmunity disease, toxin exposure and viral infection could induce its occurrence and development. Subcutaneous injection of bleomycin could induce mouse skin fibrosis similar to human SSc, including dermal fibrosis and abnormalities in ECM deposition, and is the most widely used preclinical animal model in the antifibrotic research [9, 10].

Many regenerative medical therapies such as mesenchymal stem cells (MSCs) transplantation have been explored for relieving or curing such a helpless disease [11]. Alexandre *et al* reported that transplanted allogeneic or xenogeneic bone marrow MSCs (BMSCs) demonstrated similar anti-fibrosis therapeutic effects on SSc [12]. Our previous report also showed that transplanted BMSCs or gene engineered BMSCs attenuated skin fibrosis and reactive oxygen species (ROS) induced apoptosis in bleomycin-induced murine SSc model [10]. However, the colonization number and differentiation efficiency of the MSCs in the injury site of SSc were relatively fewer. MSCs may exert their effects not through their differentiation potential but through their paracrine mechanism such as exosome which contains cytokines, signaling lipids, and regulatory miRNAs involved in cellular communication [13–15]. Recently, exosomes have been identified to contain major paracrine factors released by the outward budding of various types of cells, including MSCs, and could be appealing candidates as vectors of cell efficacy [13, 14]. Intracellular delivery of exosomes has been demonstrated for a number of different cell types and make functional use of the delivered miRNAs [16]. Involvement of exosomes has been implicated in regenerative effects of MSCs in a wide variety of tissues including skin, muscle, lung and vascular tissue [17–20]. MSCs-derived exosomes also showed therapeutic potential in fibrosis diseases, such as renal fibrosis, corneal fibrosis, myocardial fibrosis and hepatic fibrosis [21–23]. However, it is still unclear whether BMSC-derived exosomes can mediate skin fibrosis in SSc.

In the present study, as a continuous effort, we investigate the effects and mechanism of subcutaneous injection of BMSC-derived exosomes in treating bleomycin-induced SSc of mice. The BMSC-derived exosomes significantly relieved the fibrosis and inflammation in the skin, similar to the effects of the BMSCs transplantation. We also identified a group of specific miRNAs in the BMSC-derived exosomes.

Multi-dimensional bioinformatics analysis suggested that these microRNAs contributed to the inhibition of α -smooth muscle actin (α -SMA) expression and collagen deposition, as well as the fibroblast/myofibroblast transition induced fibrosis and inflammation. Therefore, BMSC-derived exosomes could be a potential therapeutic strategy in alleviating inflammation and skin fibrosis in the patients with SSc.

Methods

Experimental animals

All procedures using animal subjects were performed in accordance with the guide for the care and use of laboratory animals, and the experiments were approved and performed following the guidelines of the Institute of Laboratory Animal Resources, the Tongji University. Female C57BL/6 mice were obtained from Shanghai SLAC Laboratory Animal Co., Ltd (China) and used in this study. Skin samples for molecular and histologic analyses were obtained at the time of euthanasia.

BMSCs isolation and culture

BMSCs were isolated from bone marrow of femur and tibia of mouse (4-week-old). The BMSCs were cultured in α -MEM medium (Hyclone, USA) supplemented with 15% fetal bovine serum (FBS, Thermo Fisher Scientific, USA) and 100U/mL Penicillin-Streptomycin solution (Thermo Fisher Scientific, USA). The BMSCs were passaged when reached 80% confluence.

Flow cytometry analysis

Flow cytometry analysis of BMSCs surface markers was performed as the follows. BMSCs cells were suspended in phosphate buffered saline (PBS, Sangon Biotech, China) at a final concentration of 1×10^6 /mL. Then, monoclonal antibodies and the Isotype Control were added to 100 μ L cell suspensions respectively and incubated for 60 minutes at 4°C. The cell suspensions were centrifuged at 2,000 rpm for 3 min to remove the antibodies and washed with PBS for 3 times. Finally, 500 μ L PBS were used to resuspend the cell pellet and analyzed with the CytoFLEX LX system (Beckman Coulter, USA).

Osteogenic and adipogenic differentiation of BMSCs

The mouse BMSCs in passage 3 were cultured in differentiation conditions to identify the capacity for multilineage differentiation. Adipocyte induction of BMSCs: when the cells reached 70–80% confluency, complete medium was replaced with adipogenic induction medium (DMEM medium supplemented with 10% FBS, 10^{-7} M dexamethasone (Sigma Aldrich, USA), 10mM β -glycerol phosphate (Sigma Aldrich, USA), 50 μ M ascorbate-2-phosphate (Sigma Aldrich, USA), 10 μ g/mL insulin (Sigma Aldrich, USA) to culture for 3 weeks. Then, Oil-Red-O staining was performed.

Osteoblast induction of BMSCs: when the cell reached 60–70% confluency, complete medium was replaced with osteogenic induction medium (DMEM medium (Hyclone, USA) supplemented with 10% FBS, 10 mM β -glycerol phosphate (Sigma Aldrich, USA), 50 μ M ascorbate-2-phosphate (Sigma Aldrich,

USA), 100ng/mL recombinant human bone morphogenic protein-2 (peprotech, USA), 10^{-7} M dexamethasone (Sigma Aldrich, USA) in the next 21 days. Then, Alizarin red staining was performed.

Isolation and identification of BMSC-derived exosome

The BMSCs culture medium supplied with exosome (EXO) depleted FBS was collected every 48 hours. The collected culture medium was centrifugated at 300g for 10 minutes at 4°C to eliminate the cell pellets. The supernatant was centrifuged at 2,000g for 20 minutes at 4°C to further remove cell debris. The supernatant was again centrifuged at 10,000g for 30 minutes at 4°C. The supernatant was then filtered through a 0.22- μ m filter (Merck, Germany) and the flow was transferred to new tubes and then ultracentrifuged at 150,000g for 2 hours at 4°C in a SW70Ti rotor (Beckman Coulter, USA) to pellet the exosomes. The supernatant was immediately aspirated upon completion of the first ultracentrifugation and then ultracentrifuged again as described previously. For maximal exosomes retrieval, the exosomes-enriched pellet was resuspended in 200 μ L of cold PBS. The concentration of exosomes was measured according to the protein content using the BCA protein assay kit (Thermo Fisher Scientific, USA). The presence of the exosomes was confirmed with NanoSight NS300 (Malvern Instruments, U.K.). Transmission electron microscopy (Tecnai 12, FEI, USA) and Western blot were applied to detect morphology and exosomal surface markers.

Establishment of SSc model and treatments

Bleomycin (BLM, Thermo Fisher Scientific, USA) was diluted in PBS at a concentration of 1 mg/mL and sterilized by filtration. To establish the murine fibrosis model, 100 μ L BLM solution were subcutaneously injected into the shaved backs (1 cm²) of mice for 28 days using a 27-gauge needle.

For the BMSC or BMSC-EXO treatments, BLM-induced SSc mice were randomly divided into 3 groups and treated with PBS (100 μ L), BMSCs (1×10^6 /100 μ L) or BMSC-derived exosomes (15 μ g/100 μ L). The mice were killed at 14 days after the treatments, and skin tissue samples were collected from the 1 cm² shaved area.

Histochemical analysis

Skin tissue samples from different groups were fixed in 4% paraformaldehyde (PFA) solution for 24 hours. Then, the tissues were embedded in paraffin and cut into 10 μ m sections. The sections of the paraffin-embedded skin tissue were deparaffinized using xylene and rehydrated using decreasing concentrations of ethanol (100, 95, 85 and 75%). Briefly, the sections were stained in hematoxylin for 5 min and further washed with cold running water. After incubated with 1% hydrochloric acid alcohol, the sections were washed and stained with 0.5% eosin dye solution. To analyze the extent of skin fibrosis, randomly selected fields of sections were captured. The sections were stained by Masson trichrome staining (SenBeiJia Biological Technology Co., Ltd, China) according to manufacturer's instructions and the collagen fibers was evaluated under a light microscope. For detection of macrophages, the sections were stained by toluidine blue. Sections were examined and photographed using a microscope (Nikon, Japan).

Immunohistochemical analysis

The sections of the paraffin-embedded skin tissue were deparaffinized as previously description. The section was incubated in 3% H₂O₂ for 5min at room temperature. Then, 5% goat serum (Sangon biotech, China) was used to block the sections for 60 min at room temperature. The primary antibodies which are listed in supplemental table 1 diluted in 5% goat serum solution were added to the sections and incubated overnight at 4°C. The horseradish peroxidase (HRP) labeled secondary antibodies were applied and incubated for 60 min at room temperature. Diaminobezidin (DAB) solution was applied to show the positive signaling.

Hydroxyproline measurement

Collagen content of skin samples was quantified with the hydroxyproline test kit (Nanjing JianCheng Bioengineering Institute, China) according to the recommendations of the manufacturer. Hydroxyproline content was measured as follows: (tested OD value - blank OD value) / (standard OD value - blank OD value) * standard sample concentration (5µg/mL) * total hydrolysate volume (10 mL) / tissue wet weight (mg).

Western blotting analysis

Samples (20 µg total protein) were separated by SDS-PAGE (10%) and transferred to PVDF membranes (Merck, Germany). After blocking in 5% nonfat milk for 1 hour, the membranes were incubated with primary antibodies against CD9, CD63 and GAPDH at 4°C overnight. After washing, the membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. Signals were detected using a Tanon chemiluminescence image detection system (Tanon, China).

Quantitative real-time PCR

Total RNA was extracted and purified using Trizol reagent (Takara, Japan) following the manufacturer's instructions, and cDNA was synthesized using the primeScript™ RT Master Mix (Takara, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR Green Real-time PCR Master Mix (Tiangen, China). The oligonucleotide primers used are listed in supplemental table 2. Gene expression was normalized to GAPDH mRNA levels in each sample. The fold change in expression was calculated using the $2^{\Delta\Delta Ct}$ method.

RNA-seq and bioinformatic analysis

For miRNA-Seq of exosomes, preparation of tagged miRNA sequencing libraries, sequencing and NGS data analysis were performed by LC Sciences (USA). The library was sequenced with Illumina Hiseq2500 SE50. Raw reads were subjected to an in-house program, ACGT101-miR (LC Sciences, USA) to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, unique sequences with length in 18 ~ 26 nucleotide were mapped to specific species precursors in miRBase 22.0 by BLAST search to identify known miRNAs and novel 3p- and 5p- derived miRNAs. The criteria that the number of reads is higher than the average copy of the data set was used to

filter the high-level miRNAs. The R package (multiMiR, version 3.12) was used for the miRNA target scan and prediction [24], while the cluster analysis of target genes was using the R package (clusterProfiler).

For RNA-Seq of tissue sample, the skin tissue was dissected under the microscope and placed to the TRIzol reagent immediately. The total RNA was isolated with TRIzol reagent (Takara, Japan). The library was sequenced with Illumina Novaseq 6000 PE150. The criteria $|\logFC| > 1$ and $P < 0.05$ was applied to filter the differential expressed genes. The gene ontology and KEGG analysis with R package (clusterProfiler). The results were shown with GOplot.

Statistical analysis

All data are expressed as the standard error of the mean. Data analysis was performed using GraphPad Prism software (USA). ANOVA was used for comparisons among multiple groups, and unpaired Student's t tests were used for comparisons between two groups. A value of $P < 0.05$ was considered statistically significant.

Results

1. Identification of mouse BMSCs and BMSC-derived exosomes

Following isolation and culture as described above, the mouse BMSCs were identified according to the criteria of International Society of Cellular Therapy [25]. As shown in Fig. 1a, the isolated mouse BMSCs were adhered to the culture dish with fibroblast-like and spindle-shaped morphology. After adipogenic induction, the small cytoplasmic lipid droplets of BMSCs were observed upon oil-red O staining (Fig. 1b). After osteoblast induction, positive alizarin red staining could be seen (Fig. 1c). The surface markers of the BMSCs were analyzed with flow cytometry and the results were shown in Fig. 1d; BMSCs high expressed CD73 (100%), CD105 (100%), CD44 (97%), and CD90 (95%). Thus, the mouse BMSCs exhibit typical mesenchymal stem cell characteristics.

The exosomes (EXOs) isolated from the mouse BMSCs cultured media were analyzed with nanoparticle tracking analysis (NTA). As shown in Fig. 1e, most of these exosome vesicles ranged from 90 to 230 nm in size, and their morphology was revealed as round or elliptical vesicles with uneven size and intact capsule under transmission electron microscopy (TEM) (Fig. 1f). Moreover, compared to BMSCs, exosome-specific markers of CD9 and CD63 were significantly enriched in these EXOs (Fig. 1g). Taken together, the mouse BMSCs and BMSCs-derived EXOs were isolated successfully.

2. BMSCs and BMSC-exosomes treatments reduce bleomycin-induced dermal thickening and fibrosis

In order to confirm the effects of BMSCs on SSc as we previously reported, and examine if mouse BMSC-EXOs have any effects on bleomycin (BLM)-induced SSc pathology, the mouse model was established

with BLM, daily subcutaneously injected for 4 weeks, followed by subcutaneous injections of the mouse BMSCs or the BMSC-EXOs (Fig. 2a). All the BLM, BMSCs and BMSC-EXOs were subcutaneously injected into the centers of the depilated skins for even distribution of the liquid and effects (Fig. 2b).

Two weeks after the BMSC and EXO treatments, in comparison with normal mouse skin, the dermis layer of PBS treated model mice was thickened ($P < 0.05$), the subcutaneous adipose layer was lost, and the dermal architecture was disrupted (Fig. 2c, 2d). Both the BMSC and EXO treated skin samples showed normal thickness of subcutaneous adipose layer, and in both cases, the hypodermic adipose thickness was significantly thicker than that in the PBS-treat mice ($P < 0.05$) (Fig. 2c, 2e). Furthermore, when inflammatory cell infiltration was examined as another parameter to evaluate the effects of the treatments on SSc, as shown in Fig. 2f, the inflammatory cell counts in skin tissue sections in both the BMSC and EXO groups were significantly lower than that in SSc mice ($P < 0.05$), indicating the attenuated leucocytic infiltration. These results demonstrated that subcutaneously injected BMSC-EXOs could significantly improve the dermal damage and abnormalities in the SSc model, and its effects were as strong as those of the mouse BMSCs.

3. BMSCs and BMSC-exosomes treatments reduce abnormal deposition of collagen in mouse SSc model

Considering that abnormal ECM deposition in dermis is atypical character of SSc, Masson's trichrome staining was used to evaluate the effects of the BMSCs and the BMSC-EXOs in ameliorating the deposition of excess ECM including collagen. As shown in Fig. 3a, BLM-induced scleroderma skin exhibited an abundance of collagen formation and dense dermis ECM structure, but the ECM deposition was significantly reduced by the treatments of BMSCs or BMSC-EXOs. Consistently, as shown in Fig. 3b, the BMSC and BMSC-EXO treatments significantly decreased the hydroxyproline content, which was increased in the BLM-treated mice ($P < 0.05$), and there was no significant difference between the BMSC group and BMSC-EXO group ($P > 0.05$). Furthermore, mRNA expressions of Col1 and Fn1 were found significantly elevated in the PBS-treated SSc mice, but were significantly reduced in the mice treated with either BMSCs or BMSC-EXOs (Fig. 3c, 3d). However, in these two cases, the effects of BMSC-EXOs were not as strong as BMSCs (Fig. 3c, 3d). These data indicate that both BMSCs and BMSC-EXOs could significantly reduce the BLM-induced abnormal deposition of ECM and collagen density.

4. Analysis of BMSC-EXOs and their specific microRNAs

Since both mouse BMSCs and their EXOs attenuated the accumulation of disordered dermis structure and ECM deposition in the skin of BLM-induced SSc mouse to a similar degree, we focused on BMSC-EXO and its functional microRNAs in the experiments below in order to understand the related mechanism and develop a more convenient and efficient therapy. First, the global expression of microRNAs in the BMSC-EXO was analyzed via high throughput microRNA sequencing approaches. As listed in supplemental table 3, BMSC-EXO highly expressed a cluster of specific abundance miRNAs, such as mir-21a, mir-143, mir-27b, mir-29a and let-7. These highly expressed miRNAs were then studied for

their target genes by miRNA target scan analysis and GO function cluster analysis to predict their potential functional processes. As shown in Fig. 4a, the results showed that these highly expressed miRNAs were involved in regulating the proliferation and differentiation of multiple cell types including muscle, T cell and fat cell, and in multiple processes such as ECM origination including collagen, and cell adhesion and junction. In addition, WNT, TGF β , Notch and T cell receptor signaling pathways were also predicted to play certain roles in regulating the homeostasis of skin (Fig. 4b, 4c, 4d).

In order to confirm the functional predictions of the BMSC-EXO derived miRNAs in vivo, high throughput RNA sequencing was performed to analyze the skin tissues of the BLM-treated mice with or without a 7-day BMSC-EXO treatment. As shown in Fig. S1a, in comparison with the normal control, the skin tissue of the SSc mice highly expressed genes involved in hedgehog signaling pathway, WNT pathway, cell cycle and cellular senescence signaling, but the skin of BMSC-EXO treated SSc mice highly expressed the genes related to Focal adhesion, Hippo signaling pathway and Fatty acid biosynthesis. Reasonably, following the BMSC-EXO treatment, the genes which were hyperactivated by BLM stimulation were significantly lowered, especially those in Wnt pathway, or related to ECM-receptor interaction and cell cycle (Fig. 4e-4g), except those of fatty acid synthesis which was up-regulated. There were only few genes which were differently expressed in BMSC-EXO-treated group and normal control (Fig. S1b).

These results suggest that the BMSC-EXO may function through regulating WNT signaling pathway, TGF β signaling and inflammatory response in treating the BLM-induced SSc.

5. BMSC-EXO treatment decreases myoblast cell differentiate to fibroblast

Considering the important roles of TGF- β signaling pathway in inducing fibroblast activation and myofibroblast differentiation, both are important characteristic during the progression of BLM-induced animal models of fibrosis, TGF- β 1 positive cells and TGF- β 1 mRNA expression were examined in the skin samples of the mice. As shown in Fig. 5a, 5c, the TGF- β 1 positive cells were significantly accumulated in the dermis layer in comparison than those in the normal mice, but evidently reduced in the EXO-treated group ($P < 0.05$), similar to those in normal group. Such changes were also consistent with those in the comparison of mRNA level ($P < 0.05$) (Fig. 5d).

Another typical character of SSc, increased α -SMA positive myofibroblasts in fibrotic skin [26, 27] was also examined in the same model. As shown in Fig. 5b, 5e, the number of α -SMA⁺ myofibroblasts was evidently increased in the scleroderma skin of BLM-treated mice, but reduced back to normal level in the EXO treatment mice. Consistently, the mRNA level of α -SMA in SSc model was significantly higher and was reduced to normal level after the BMSC-EXO treatment (Fig. 5f). These results confirmed that BMSC-EXO could inhibit fibroblast activation through down-regulating the expression of TGF- β 1 in the dermis.

6. BMSC-EXO treatment reduces inflammatory infiltration in dermis in Scleroderma

To find more evidence to support our suggestions above, more parameters related to SSc, like degranulation of mast cell and accumulation of macrophage and lymphocyte were examined [28]. As shown in Fig. 6a, 6b, the number of mast cells in the dermis of the BLM-treated mice was significantly increased as showed by Toluidine blue staining, as compared to the normal control ($P < 0.05$), and the BMSC-EXO treatment significantly reduced the mast cell number back to the level comparable to the normal group ($P < 0.05$). Moreover, as shown in Fig. 6c-6h, immunohistochemical examination demonstrated that BMSC-EXO significantly reduced the infiltration of F4/80⁺ labeled macrophages and CD4⁺/CD8⁺ labeled lymphocytes, both were significantly increased in BLM-treated SSc mice ($P < 0.05$).

To investigate the effects of BMSC-EXO on BLM-induced inflammation, the mRNA expressions of inflammatory cytokines, including Il-10, Il-6 and Tnf- α in the skin samples were detected. The results showed that BLM-treated mice had significantly higher mRNA levels of Il-6, Il-10 and Tnf- α as compared to the normal control ($P < 0.05$), while the levels of these inflammatory cytokines in the EXO-treated group were significantly reduced ($P < 0.05$) (Fig. 6i, 6j, 6k). Thus, BMSC-EXO treatment can significantly reduce the infiltration of inflammatory cells and inhibit the release of inflammatory factors in the skin of the BLM-induced SSc mice.

Discussion

Curing scleroderma (SSc) is now a realistic clinical challenge [3, 4], since its etiology and pathogenesis are unclear. The key clues about its possible mechanisms are limited to vasculopathy, immune dysfunction and fibroblast dysfunction [1, 2]. On the other hand, BMSCs-based therapies, including our previous work[10], showed some therapeutic effects like attenuating skin fibrosis and apoptosis in BLM-induced SSc model. One of the observations drew our interests that the colonization number and differentiation efficiency of the BMSCs were fewer or lower in the injected sites [12]. It may indicate that the therapeutic mechanism of these BMSCs in treating SSc involves paracrine rather than differentiation. Therefore, in this study, we used BMSCs as a tool to explore the pathogenesis and to develop a potentially more practical treatment for scleroderma, through exploring BMSC-EXOs and their components. Again, BLM-induced SSc in mice was used to mimic the diseases since the pathological changes like increased dermal thickness and collagen accumulation were paralleled with those in the patients with SSc [28].

The MSCs have been reported to play an anti-fibrotic role in fibrotic diseases such as liver fibrosis [29], kidney fibrosis [30], lung fibrosis [31, 32], and skin fibrosis [10]. However, there are still unresolved and unavoidable risks of MSC clinical application such as iatrogenic tumor formation, cellular rejection and infusion toxicity [33]. Also, cells as drugs are still in a very beginning stage and their evaluations and approvals for clinical use has a long way to go. On the other hand, exosomes derived from MSCs have been shown to be the key factors in MSC-to-surrounding cells communication [34], and are considered as stem cell-based, cell-free drugs and as carrier of siRNA [35]. As compared to BMSC, the BMSC-EXO has several advantages over the cells, like simpler production and storage procedures, and easier to control the quality. The safety of intravenously and intraperitoneally injection of exosome has been verified in

animal experiments [36, 37]. Furthermore, the safety and efficiency of MSC-derived exosomes have been evaluated in several clinical trials for various diseases, including inhalation in severe acute respiratory syndrome coronavirus 2 (NCT04276987, ChiCTR2000030261) and intravenous injection in diabetes mellitus (NCT02138331). Therefore, we designed the present study to verify if BMSC-EXO could mediate the effects as BMSCs in treating SSc so that could be developed into a potential therapy without the risks of MSCs. As we expected, subcutaneous BMSC-EXO treatment significantly improved the BLM-induced dermal damage and abnormalities, and reduced the ECM deposition and collagen density. The therapeutic effects of such BMSC-EXOs were as strong as those of the BMSCs. So, BMSC-EXO has great potential to be developed as a new therapy for SSc.

Exosomes serve as carriers that transport functional proteins, mRNAs and miRNAs to various cells, where these factors act as mediators of intercellular communication and signaling pathway [38, 39]. Studies proved that MSC-EXO microRNAs possess the abilities to promote cell proliferation, accelerate injured tissues' repairment, and inhibit fibrotic diseases [16, 40–42]. In this study, we further demonstrated that there was a series of miRNAs of the BMSC-EXOs, which contributed to the alleviation of SSc through regulating related signaling pathways. Previously, TGF β pathway, Toll-like receptor signaling and WNT pathway were reported to be the main dysfunctional signaling pathways in the skin of the patients with systemic sclerosis [43], and TGF β and Wnt/ β -catenin pathways were found hyperactivated to promote ECM production and induce fibrosis [44, 45]. In this study, we focused more on these signal pathways and our data suggested that BMSC-EXO might function through regulating TGF β and WNT pathways as well as the inflammatory response in treating the BLM-induced SSc. The EXOs inhibited fibroblast activation through down-regulating the expression of TGF- β 1 in the dermis, significantly reduced the infiltration of inflammatory cells and inhibit the release of inflammatory factors in the skin of the BLM-induced SSc mice. All these effects were related to the miRNAs of the BMSC-EXOs.

The microRNA let-7 family contains let-7a, 7b, 7c, 7d, 7e, 7f, 7g, 7i and 7j, mir-29, mir-125 and mir-21. They were all found to highly express in the mouse BMSC-EXOs in this study. let-7 is the first discovered microRNA and functionally conserved in vertebrata [46], and was reported to inhibit production of proinflammatory cytokines such as Il8 and receptors such as Il1r1 and Il23r, to negatively regulate the differentiation of Th17 cell, and to regulate natural killer T cell [47–49]. Both EXOs and microRNAs are closely related to TGF β signaling pathway when they play regulating roles. For example, the down-regulations of Let-7, mir-29 and mir-30 families in idiopathic pulmonary fibrosis were related to the TGF β pathway [50, 51], and mir-29 knockdown significantly upregulated the TGF β signaling in the induction of the pulmonary fibrosis [50]. What's more, let-7 cooperated with mir-99a and mir125b, both high expressed in BMSC-EXO, when targeted receptor subunits and SMAD signaling transducers to block of the TGF β pathway [52]. TGF β signaling and Wnt signaling were found to promote each other to induce the fibrosis in SSc[53, 54]. Mir-21 and mir-29 were reported to target the transducers of Wnt signaling [55, 56]. Thus, addition of microRNAs from BMSC-EXOs could regulate the dysfunctional signals like TGF β and Wnt to ameliorate the SSc condition include ECM deposition and inflammatory infiltration. This study enriched the understanding on the molecular regulations of exosome mediated microRNAs in the SSc pathogenesis and its treatment with the BMSC-EXOs.

Conclusion

BMSC-derived EXOs could effectively treat the dysfunction and fibrosis of skin in murine SSc model, demonstrating its potential in replacing the related stem cell therapies. The microRNAs of the BMSC-EXOs might be the effectors in alleviating the ECM deposition and inflammatory infiltration via regulating the TGF β and WNT signaling pathways. For the first time, BMSC-EXO was proved to be able to intervene SSc in mice and may provide a potential cure for the patients with SSc.

Abbreviations

BLM: Bleomycin

BMSC: Bone marrow mesenchymal stem cell

ECM: Extracellular matrix

EXO: Exosome

FBS: Fetal bovine serum

GO: Gene ontology

HRP: Horseradish peroxidase

IL interleukin

ROS: Reactive oxygen species

MSC: Mesenchymal stem cell

NTA: Nanoparticle tracking analysis

PBS: Phosphate buffered saline

PFA: Paraformaldehyde

SSc: Systemic sclerosis

TEM: Transmission electron microscopy

TGF β : Transforming growth factor β

Declarations

Ethics approval

All animal procedures were performed according to the institutional guidelines and the Guide for the Care and Use of Laboratory Animals issued by the NIH and the guidelines of the animal experimentation ethics committee of Tongji University (Approved NO. TJHBLAC-2019-019).

Consent for publication

Not applicable.

Availability of data and material

RNA-seq data generated in the study can be accessed at the Gene Expression Omnibus under accession code GSE164965 and GSE165117. (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164965> and <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165117>). All other data are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JJ and QO conceived and designed the experiments, performed the experiments, analyzed the data, and drafted the paper. ZW and QO analyzed the bioinformatic data. JZ, G-T X, CJ and LL contributed to overall supervising the project, designing the experiment, financial support, manuscript drafting and revising as well as final approval of manuscript submission. The others contributed to reagents/materials/analysis tools. All authors read and approved the final manuscript.

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Figures

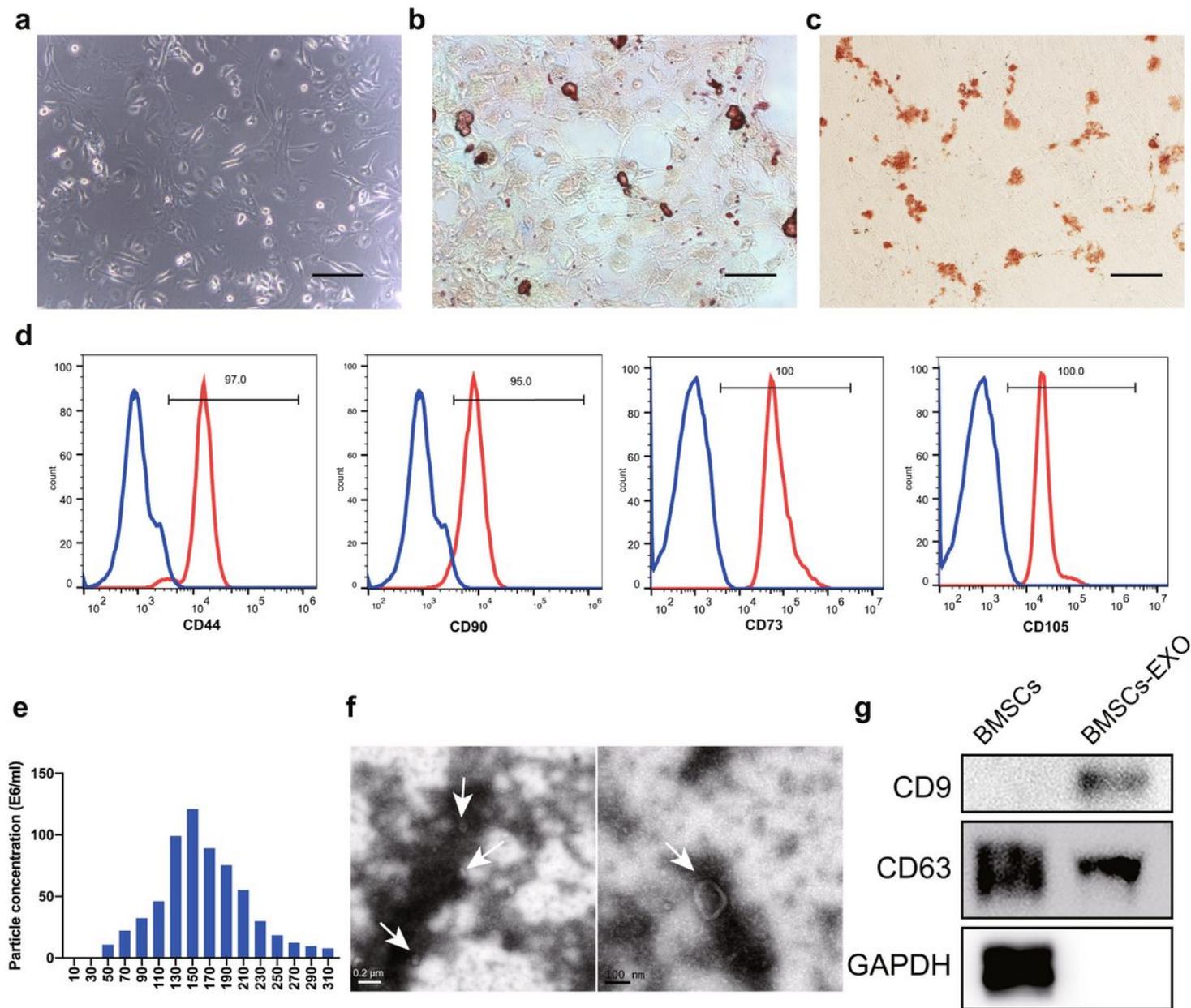


Figure 1

Identification of mouse BMSCs and BMSC-derived exosomes. (a) Morphology of BMSCs cultured in the regular medium at passage 3. Scale bar: 100µm. (b) Oil-Red-O staining of adipogenic induction of BMSCs for 21 day. Scale bar: 200µm. (c) Alizarin red staining of osteogenic induction of BMSCs for 21 day. Scale bar: 200µm. (d) Flow cytometry plots show that the BMSCs were positive for CD44, CD105, CD90 and CD73. (e) Particle size distribution of BMSC derived exosomes measured by nanoparticle tracking analysis (NTA). (f) Morphology of exosome in the transmission electron micrograph (TEM). (g) Western blot analysis of the protein profiles of EXO. BMSCs: Bone marrow mesenchymal stem cells; EXO: exosomes derived from mouse BMSCs.

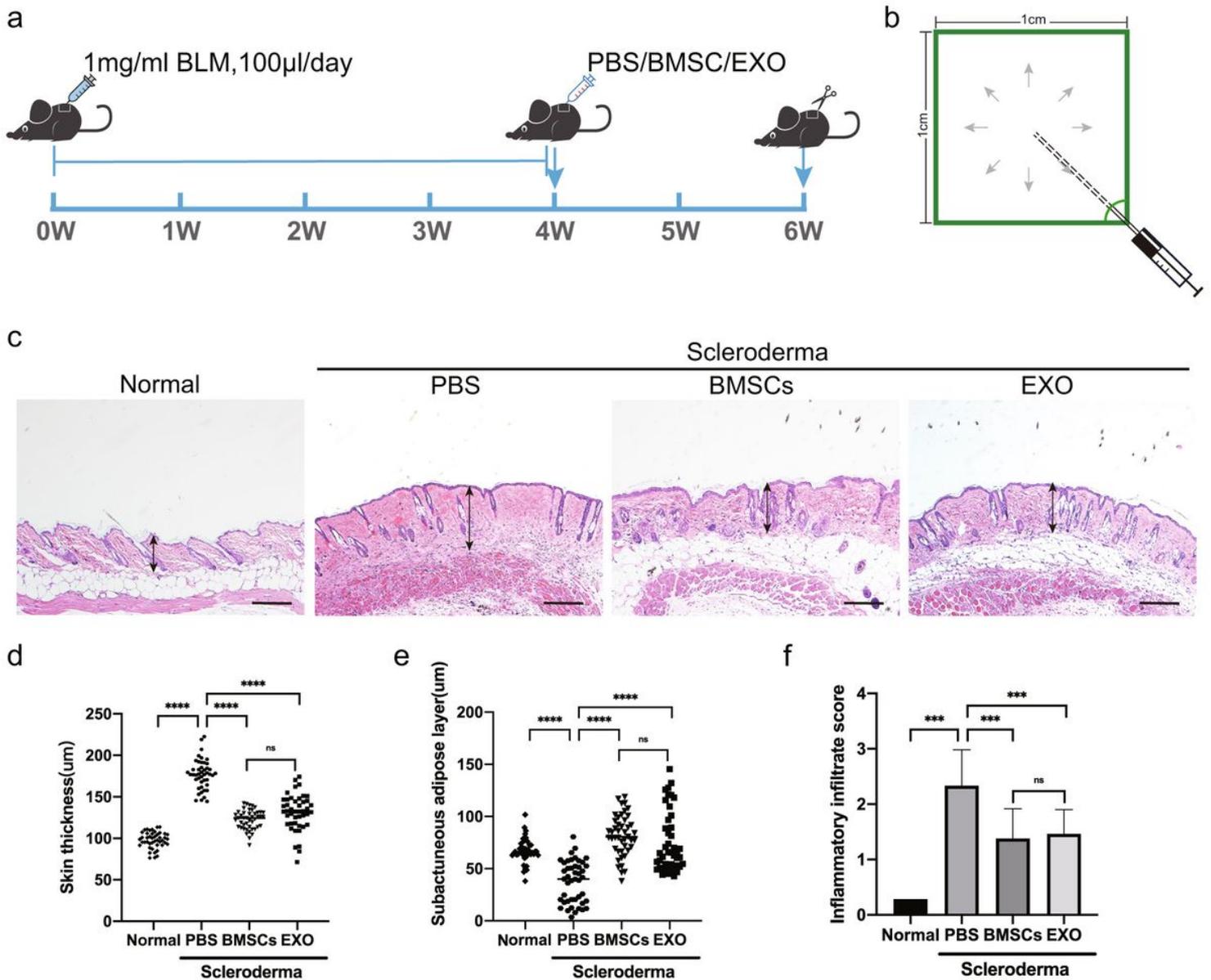


Figure 2

BMSCs and BMSC-EXOs treatments reduce bleomycin-induced dermal thickening and fibrosis. (a) Schematic chart shows the modeling and treatment process of mouse SSc model. (b) Schematic image shows the injection technique and strategy to distribute the bleomycin, BMSCs and BMSC-EXOs as evenly as possible. (c) H&E staining of representative skin section from mouse skin tissue. Arrows shows the dermal thickness. Scale bar: 200µm. (d, e) The thickness of dermis skin and subcutaneous fat layer was measured with image J on HE stained images. (f) Inflammatory cells were counted and determined in the H&E staining. SSc: Systemic sclerosis.

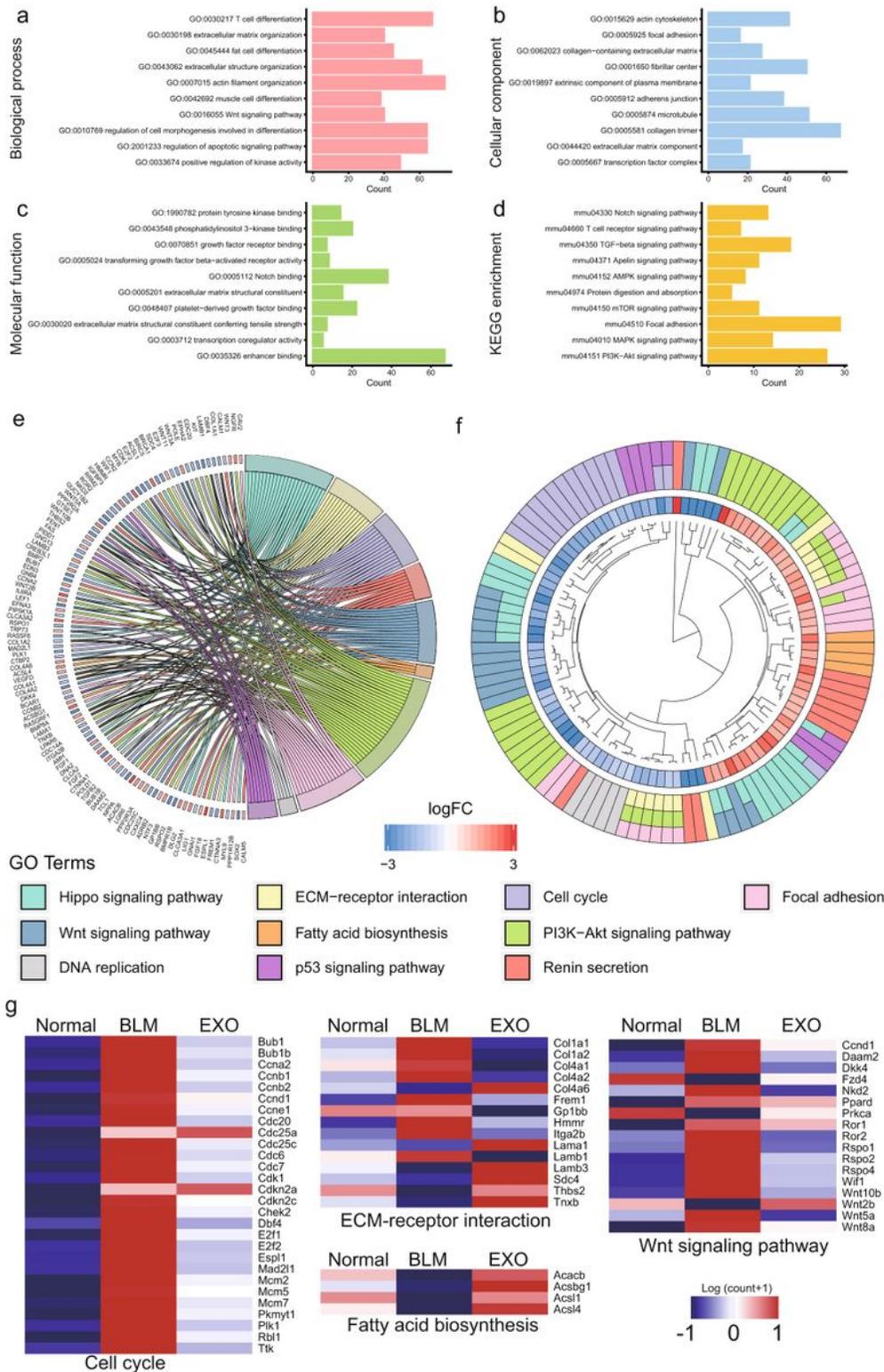


Figure 4

High-throughput sequencing analyze microRNAs of BMSC-EXO and transcriptome of the skin tissues (a-d) Gene ontology analysis of the targeted genes of high expression microRNA in the BMSC-EXO. (e-f) Gene ontology and GOchord show the differential expressed genes of BMSC-EXO treated skin tissues which compare to the PBS treated group. (g) Heatmap shows the relative mRNA expression of related signaling pathway.

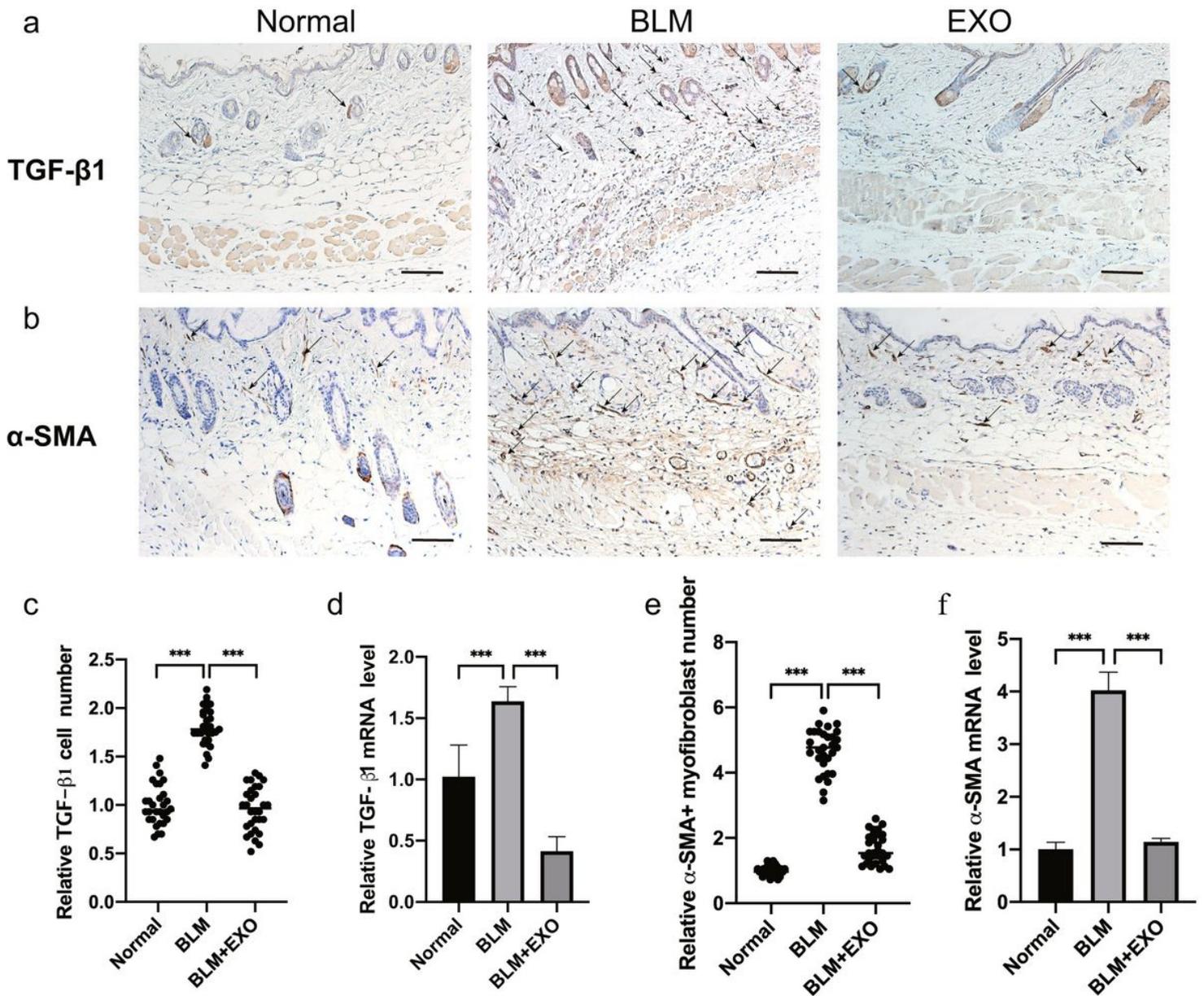


Figure 5

BMSC-EXO treatment decreases myoblast cell differentiate to fibroblast. (a, b) Representative images of TGF-β1 and α-SMA positive cells detected by immunohistochemical staining. Scale bar: 100μm. (c, e) Quantitative analysis of TGF-β1 and α-SMA positive cell numbers. (d, f) Expression levels of α-SMA and TGF-β1 were detected with real time PCR. BLM: bleomycin; EXO: BMSC derived exosomes; BLM: bleomycin; α-SMA: α-smooth muscle actin; TGF-β1: transforming growth factor-β1.

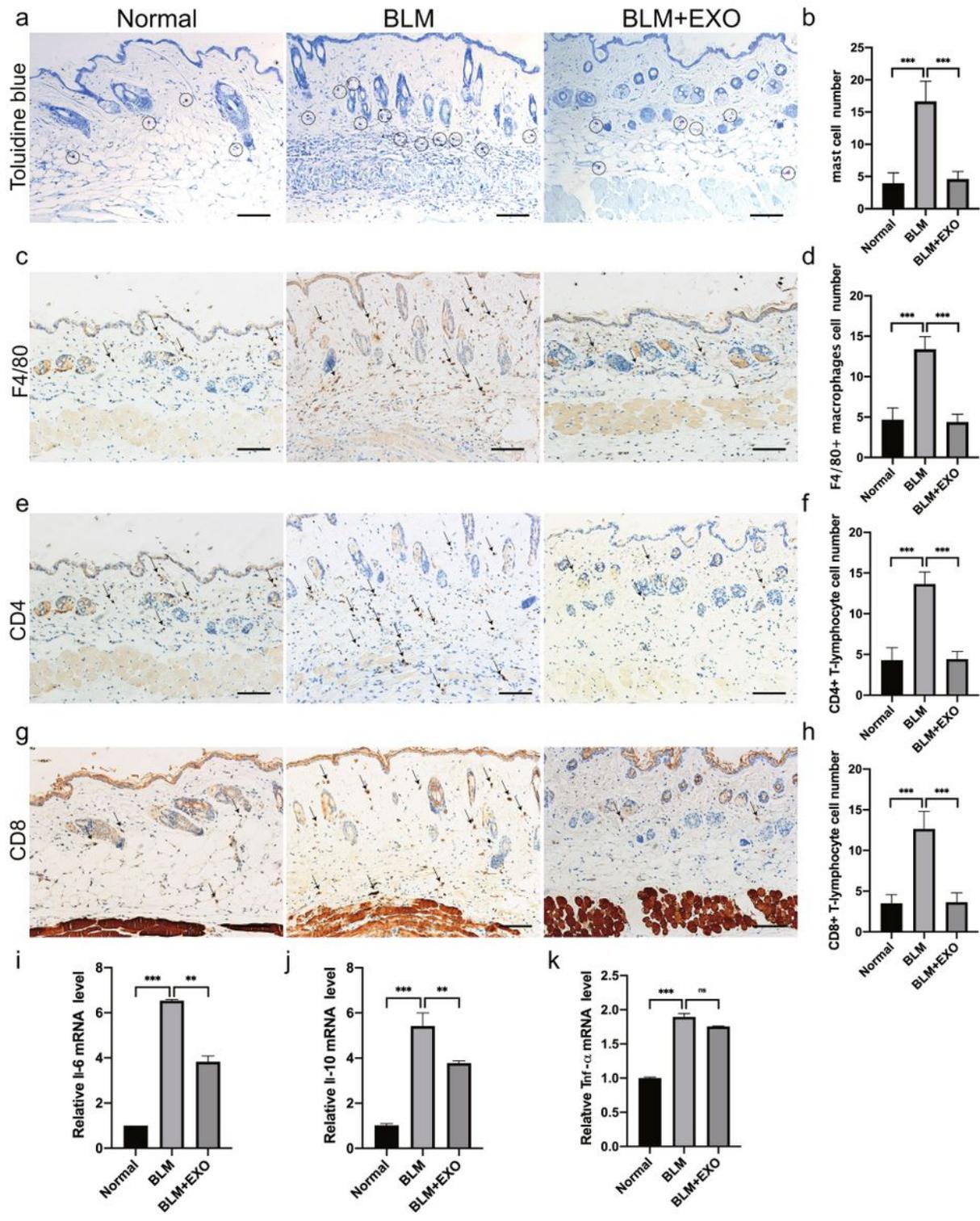


Figure 6

BMSC-EXO treatment reduces inflammatory infiltration in dermis in Scleroderma. (a-h) Representative images of the mast cell degranulation detected by toluidine blue staining (a, b) F4/80+ positive macrophages (c, d), CD8+ and CD4+ positive lymphocyte (e, f, g, h) infiltration in the skin detected by immunostaining and their corresponding quantification. Scale bar \square 100 μ m. (i-k) Quantification of mRNA

of Il6, Il10 and Tnf- α . EXO: BMSC derived exosomes; BLM: bleomycin; Il6: Interleukin-6; Il10: Interleukin-10; Tnf- α : tumor necrosis factor α .

Supplementary Files

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