Blockade of LINC01605-enriched exosome generation in M2 macrophages impairs M2 macrophage-induced proliferation, migration, and invasion of human dermal fibroblasts

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Abstract
Activated M2 macrophages are involved in hypertrophic scar (HS) formation via manipulating the differentiation of fibroblasts to myofibroblasts having the proliferative capacity and biological function. However, the function of exosomes derived from M2 macrophages in HS formation is unclear. Thus, this study aims to investigate the role of exosomes derived by M2 in the formation of HS. To understand the effect of exosomes derived from M2 macrophages on formation of HS, M2 macrophages were co-cultured with human dermal fibroblast (HDF) cells. Cell Counting Kit-8 assay was performed to evaluate HDF proliferation. To evaluate the migration and invasion of HDFs, wound-healing and transwell invasion assays were performed, respectively. To investigate the interaction between LINC01605 and miR-493-3p, a dual-luciferase reporter gene assay was adopted; consequently, an interaction between miR-493-3p and AKT1 was detected. Our results demonstrated that exosomes derived from M2 macrophages promoted the proliferation, migration, and invasion of HDFs. Additionally, we found that long noncoding RNA LINC01605, enriched in exosomes derived from M2 macrophages, promoted fibrosis of HDFs and that GW4869, an inhibitor of exosomes, could revert this effect. Mechanistically, LINC01605 promoted fibrosis of HDFs by directly inhibiting the secretion of miR-493-3p, and miR-493-3p down-regulated the expression of AKT1. Exosomes derived from M2 macrophages promote the proliferation and migration of HDFs by transmitting LINC01605, which may activate the AKT signaling pathway by sponging miR-493-3p. Our results provide a novel approach and basis for further investigation of the function of M2 macrophages in HS formation.

Keywords
hypertrophic scar, exosomes derived from M2 macrophages, LINC01605, miR-493-3p, AKT1

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Introduction
A hypertrophic scar (HS) is dermal fibrosis caused by skin trauma, especially burn injuries. The quality of life of patients with HS is adversely affected by functional limitations and esthetic defects. Currently, HS is challenging to deal with because there are no effective therapeutic methods. Additionally, scar
revision surgery, a treatment most frequently used to treat HS, always leads to the formation of a renewed scar. Therefore, it is important to investigate the pathogenesis of HS and to verify novel strategies for the prevention and treatment of HS.

HS is a type of excessive skin fibroproliferative disease that manifests upon injury, inflammation, or burn wounds. In some cases, HS even develops spontaneously. HS formation is a complex process, usually accompanied by an excessive inflammatory response and abnormal fibroblast transformation and proliferation. Inflammatory reactions promote scar formation. Macrophages play critical roles in the transformation of the inflammatory phase to the proliferative phase, in which they maintain the wound-healing process and ultimately control the degree of scar formation. Macrophages are roughly divided into two types: classically activated macrophages (M1) and alternatively activated macrophages (M2). M1 macrophages are primarily present in early wounds, while M2 macrophages are mainly present in the hyperplasia phase of late wounds and HSs. Moreover, in wounds, the number of M1 macrophages peaks at days 7–14, while the number of M2 macrophages is significantly enhanced by 14–28 days after injury. M2 macrophages promote tissue recovery and healing and are closely associated with excessive fibrosis. It has been demonstrated that reducing the polarization of M2 macrophages can effectively ameliorate HS formation. The fibrosis of HDFs can lead to HS formation, and the mechanisms by which M2 macrophages promote proliferation, migration, and invasion of HDFs remain to be elucidated.

Long noncoding RNA (lncRNA)-derived exosomes play a crucial role in HS formation. Previous studies have shown that lncRNA-ASLNC5088-enriched exosomes derived from M2 macrophages promote HS formation. Furthermore, many microRNAs (miRNAs) are involved in HS development, and regulating these miRNAs’ expression may solve problems associated with HS treatment. The literature suggests that miR-29 and miR-206 are involved in the activation of the TGF-β1 pathway. They can increase fibroblast proliferation and collagen synthesis and induce the formation of HS. LINC01605 was found to play a critical role in promoting the proliferation of bladder cancer cells, while its function in the proliferation of HDFs is still unclear.

In this study, we found that LINC01605-enriched M2 macrophage-derived exosomes promoted fibrosis of HDFs by directly inhibiting the secretion of miR-493-3p and increased the expression of AKT1, p-AKT1, mTOR, and p-mTOR. Blockade of LINC01605-enriched exosomes derived from M2 macrophages impaired M2 macrophage-induced HS formation. Our results provide a novel approach and basis for understanding the effect of M2 macrophage-derived exosomes on HS formation.

Materials and methods

Cell culture

Human dermal fibroblasts (HDFs) were purchased from the American Type Culture Collection (PCS-201-012, ATCC, VA, USA). The cells were cultured in DMEM (Grand Island, NY, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS, Grand Island, NY, USA), 1% (v/v) nonessential amino acid solution (NEAA, Grand Island, NY, USA), and 100 μg/mL penicillin and streptomycin (Grand Island, NY, USA) at 37°C in an atmosphere of 5% CO2 and 90% relative humidity.

THP-1 cell culture and treatment

THP-1 cells were purchased from the American Type Culture Collection (TIB-202, ATCC, VA, USA) and cultured in RPMI 1640 medium (Gibco, CA, USA) supplemented with 10% (vol/vol) FBS (Gibco, CA, USA), 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, 10 mM HEPES, 4500 mg/L glucose, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO2 atmosphere having 90% relative humidity.

THP-1 cells were induced with PMA (10 ng/mL, 72 h) to differentiate into M0 macrophages. M0 macrophages were then treated with 1 μg/mL LPS and 10 ng/mL IFN-γ for 48 h to induce M1 macrophage polarization or treated with 2 ng/mL IL-4 and IL-13 for 48 h to induce M2 macrophage polarization.

Cell co-culture

Transwells were used to co-culture M1/M2 macrophages and HDFs. M1/M2 macrophages were plated in upper chambers, and HDFs were plated at the bottom of the chambers. To inhibit exosome generation, macrophages were pre-treated with 10 mM GW4689 (Sigma Aldrich, St. Louis, MO, USA) for 2 h.
Transfection

The LINC01605 sequence was obtained from NCBI (no. NR 121620.2). The full-length sequence of LINC01605 was cloned into the pcDNA3.1(+) vector, and the empty pcDNA3.1(+) vector was used as a control. Plasmid transfections were performed with Lipofectamine 3000 according to the manufacturer’s protocols (Thermo Fisher Scientific, former Savant, MA, USA).

Quantitative polymerase chain reaction (qPCR) and western blotting

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA). Reverse transcription was performed using QuantiTect Reverse Transcription Kit (Qiagen, NY, USA). qPCR was performed using the SuperRT One-Step RT-PCR Kit (CWBIO, Beijing, China), following the manufacturer’s guidelines. qPCR analysis was performed in quadruplicate for each sample with specific primers (the primers are shown in Table 1).

Western blotting analysis

Cultured cells were collected and incubated in RIPA lysis buffer. The cell suspension was incubated on ice for 30 min, after which the suspension was centrifuged at 14,000 rpm for 3–5 min at 4°C. Proteins were separated via 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, MA, USA). All primary antibody incubations were performed at 4°C overnight. The primary antibodies used in the experiment were as follows: collagen I (ab34710, Abcam, Cambridge, MA, USA), collagen III (ab7778, Abcam, Cambridge, MA, USA), AKT1 (No. 2967, Cell Signaling Technology, CA, USA), phospho-Akt1 (Ser473) (#9018, Cell Signaling Technology, CA, USA), mTOR (#2972, Cell Signaling Technology, CA, USA), phospho-mTOR (Ser2448) (#2971, Cell Signaling Technology, CA, USA), CD63 (sc-15363, Santa Cruz Biotechnology, TX, USA), TSG101 (sc-7964, Santa Cruz Biotechnology, TX, USA), and β-actin (sc-8432, Santa Cruz Biotechnology, TX, USA). The expression level of proteins was determined using an ECL reagent (Millipore, MA, USA) after incubation with the secondary antibody (Santa Cruz Biotechnology, TX, USA).

Extraction of exosomes

The extraction of exosomes was performed according to the previous study. Briefly, macrophages were cultured for 4 days, and the supernatant was collected. To remove cell debris, the supernatant was centrifuged at 2000×g for 45 min at 4°C. After that, 0.22-micron syringe filters (Merck Millipore, Billerica, MA, USA) were used to remove vesicles and other impurities larger than 220 nm in the supernatant. Finally, 15 mL supernatant was added into 100 KD ultrafiltration tubes (Merck Millipore, Billerica, MA, USA) and centrifuged at 2000×g for 35 min at 4°C to concentrate the exosomes. The concentrated exosomes were stored at −20°C.

Flow cytometry analysis

The cells were digested and separated using trypsin (Invitrogen, Grand Island, NY, USA), collected in a tube (1 × 10^5 cells/tube), and centrifuged at 1200 rpm for 10 min; the supernatant was then discarded. After washing with phosphate-buffered saline, cells were re-suspended in 500 μL annexin V binding buffer. After incubation at room temperature for 15 min, 10 μL propidium iodide was added to each tube, and the tubes were incubated in the dark for 5 min, followed by flow cytometry (Becton Dickinson, Immunocytometry Systems, Mountview, CA).

Cell counting kit-8 (CCK8) assay

The viability of differently treated cells was evaluated using the CCK8 assay. CCK8 solution (10 μL; Beyotime Biotechnology, Shanghai, China) was added to 100 μL of the medium, followed by incubation of cells at 5% CO₂ in a humidified incubator at 37°C for 1–4 h. The absorbance was measured at
450 nm using a microplate reader (Former Savant, MA, USA).

**Wound-healing assay**

Cell migration capacity was evaluated using a wound-healing assay. Cells were cultured in serum-free medium in 24-well culture plates for 24 h. A sterile pipette tip (200 μL) was used to create a “scratch-wound” on the monolayer of cells. According to the experimental design, the cells were divided into different groups and photographed under the microscope at 0 and 24 h. Cell migration ability was evaluated by determining the cell-free area around the wound.

**Transwell invasion assay**

To evaluate the invasion capacity of cells, a transwell assay was performed. Briefly, $2 \times 10^5$ cells were seeded in the upper Matrigel-coated chamber. The bottom of the chamber was filled with a medium containing 10% FBS. After 24 h, the cells were fixed and stained with 1.0% crystal violet for 10 min. Finally, the assays were performed, and the number of cells was obtained under a microscope.

**Luciferase reporter assay**

Cells were cultured in 24-well plates and mixed with 100 ng pGL3-AKT1-wild, pGL3-AKT1-mut, pGL3-LINC01605-wild, or pGL3-LINC01605-mut. Lipofectamine 2000 Reagent (Invitrogen, USA) was used to transfect the plasmids (5 ng pRL-SV40, 50 nM pre-miR-493-3p, or pre-neg) into cells, in accordance with the manufacturer’s instructions. After these cells were digested by pancreatin for 48 h, they were analyzed via the luciferase assay using the Dual-Luciferase Reporter Assay kit (Promega, USA) according to the manufacturer’s instructions. Each experiment was repeated three times.

**Statistical analysis**

All data were expressed as the mean ± standard deviation (SD), and each experiment was performed in triplicate. The one-way analysis of variance (ANOVA) was used to evaluate the difference, followed by Tukey’s HSD post hoc test to compare multiple groups (>2). The two-tailed Student’s $t$-test was used for comparison between two groups. Significance values were set at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

**Results**

**M2 macrophages promoted fibrosis of HDFs**

The expression of M1 macrophage marker genes, including IL-6, IL-8, IL-1β, TNF-α, and iNOS, and the expression of M2 macrophage marker genes, including IL-10, DC-SIGN, SOCS1, MRG-1, Arg, and CD206, were detected via qPCR (Figure 1a and b). To investigate the effect of different macrophages on the proliferation of HDFs, a co-culture of M1/M2 macrophages with HDF was done, and the CCK8 assay was performed. M2 macrophages significantly promoted the proliferation of HDFs, whereas M1 macrophages had no effect on it (Figure 1c). Similarly, M2 macrophages significantly promoted the migration and invasion of HDFs, but M1 macrophages had no effect on them (Figure 1d and e). M2 macrophages also induced collagen I and collagen III expressions, but M1 macrophages did not affect them (Figure 1f), suggesting that M2 macrophages might promote the cell growth of HDF by secreting exosomes.

**GW4869 impaired M2 macrophage-induced HDF fibrosis**

M2 macrophages can secrete exosomes to participate in the activation of fibroblasts. To determine whether exosomes derived from M2 macrophages can promote fibrosis of HDFs, GW4869, an inhibitor of exosomes, was used in our experiments. CCK8 assay results indicated no significant difference in proliferation between the GW4869-treated group (HDF+GW4869) and its control (HDF). The results were the same for the groups of HDF+M1+GW4869 and HDF+M2+GW4869 (Figure 2a). Besides, GW4869 significantly down-regulated collagen I and collagen III (Figure 2b) and impaired M2 macrophage-induced migration and invasion of HDFs (Figure 2c and d), confirming that M2 macrophages could promote the proliferation, migration, and invasion of HDFs by secreting exosomes.

**M2 macrophages induced fibrosis of cells via LINC01605 transfer by secreting exosomes**

To further explore if over-expression of LINC01605 in HDFs caused the reversal of the
M2 macrophage-induced fibrosis of cells, exosomes derived from M1 and M2 macrophages (M1-Exo and M2-Exo, respectively) were collected, and the expression of CD63 and TSG101 was evaluated for the identification of concentrated exosomes (Figure 3a). qPCR results

**Figure 1.** M2 macrophages promoted fibrosis of HDF. (a and b) The expression of M1 and M2 macrophages marker genes was detected during qPCR. Statistical significance was determined using Student’s t-test, ***P < 0.01. (c) CCK8 assay was applied to evaluate HDF proliferation. **P < 0.001 by one-way ANOVA with Tukey’s HSD post hoc test. (d and e) The migration and invasion of HDFs were evaluated using wound-healing and transwell assays, respectively. Statistical significance was determined using Student’s t-test, ****P < 0.0001, HDF + M2 group versus HDF group. (f) The expression levels of collagen I and collagen III in HDFs were determined via western blotting. Statistical significance was determined using Student’s t-test***P < 0.001, HDF + M2 group versus HDF group.
showed that LINC01605 was highly expressed in the exosomes derived from M2 macrophages (Figure 3b). CCK8 analysis suggested that cell proliferation was significantly slower during the inhibition of exosome-secretion by GW4869. However, the promotion of cell proliferation was reversed upon inhibition of exosome-secretion by GW4869 and upon over-expression of LINC01605 in HDFs (Figure 3c). Moreover, we obtained similar results indicating that over-expression of LINC01605 in HDF reversed the migration and invasion of cells and the expression of collagen I and collagen III upon inhibition of exosome-secretion by GW4869 and over-expression of LINC01605 in HDF (Figure 3d–f). Therefore, M2 macrophage-derived exosomes promoted the proliferation, migration, and invasion of HDFs by transferring LINC01605.

**LINC01605 may regulate AKT1 expression by sponging miR-493-3p**

To investigate the mechanism by which LINC01605 promotes the proliferation and migration of HDFs, Starbase 2.0 was used to predict the target of LINC01605 and its downstream signaling pathway (Figure 4a). Luciferase reporter assay was then carried out, and the result verified our assumption (Figure 4b). Luciferase reporter assay results indicated that miR-493-3p could directly interact with AKT1 (Figure 4c), suggesting that LINC01605 might regulate the activation of the AKT signaling pathway.
Figure 3. M2 macrophage-derived exosomes induced fibrosis of cells by transferring LINC01605. (a) To identify the concentrated exosomes, the expression of CD63 and TSG101 was detected via western blotting. (b) Relative expression analysis of LINC01605 in M1/M2 macrophage-derived exosomes using qPCR. Statistical significance determined using Student’s t-test, **P < 0.01, ****P < 0.0001, M2 group versus group; ##P < 0.01, M2 + GW4689 group versus M2 group. (c) CCK8 assay was performed to analyze cell proliferation in different cell experimental groups. **P < 0.001 by one-way ANOVA with Tukey’s HSD post hoc test. (d and e) Analysis of migration and invasion of different cell experimental groups by wound-healing and transwell assays, respectively. Statistical significance was determined using Student’s t-test, ***P < 0.001, ****P < 0.0001, OE-NC + M2 + GW4689 group versus OE-NC + M2 group; ####P < 0.0001, OE-LINC01605 + M2 + GW4689 versus OE-NC + M2 + GW4689 group. (f) Analysis of expression of collagen I and collagen III in different cell experimental groups using western blotting. Statistical significance was determined using Student’s t-test, **P < 0.01, ****P < 0.0001, OE-NC + M2 + GW4689 group versus OE-NC + M2 group; ####P < 0.0001, OE-LINC01605 + M2 + GW4689 versus OE-NC + M2 + GW4689 group.
pathway via a mechanism involving interaction with miR-493-3p.

**Over-expression of LINC01605 in HDFs promoted fibrosis of cells by regulating AKT1 via sponging miR-493-3p**

While investigating whether LINC01605 secreted from M2 macrophages affects the fibrosis of HDFs by regulating miR-493-3p, we found that over-expression of LINC01605 in HDFs significantly promoted cell proliferation compared to the control, while over-expression of miR-493-3p abolished the promotion of proliferation (Figure 5a). Additionally, over-expression of LINC01605 markedly promoted the migration and invasion of HDFs, but up-regulation of miR-493-3p in HDF abrogated the promotion of migration and invasion (Figure 5b and c). In addition, over-expression of LINC01605 up-regulated collagen I, collagen III, AKT1, p-AKT1, mTOR, and p-mTOR in HDFs, while miR-493-3p abolished the up-regulation of fibrosis markers and activation of the AKT/mTOR signaling pathway (Figure 5d and e), indicating that LINC01605 might promote the fibrosis of cells by activating the AKT signaling pathway by sponging miR-493-3p.

**Discussion**

HS is a pathological scar characterized by excessive tissue proliferation during the process of scar repair and could significantly affect the appearance and function of an individual. A variety of cell
types are involved in the HS formation, but the underlying molecular mechanisms are not fully understood.

Macrophages are a major component in the process of HS formation, promoting the initiation and formation of scars. This study found that M2 macrophages significantly promoted proliferation, migration, and invasion of HDFs. Additionally, we found that M2 macrophages significantly enhanced the levels of collagen I and collagen III. These results were similar to those of Chen et al., who found that myofibroblasts highly expressed α-smooth muscle actin (α-SMA) could enhance the production of collagen I and collagen III, thereby promoting HS formation.

The present study aimed to explore a novel mechanism by which M2 macrophages contribute to HS formation. In recent years, the role of miRNAs in HS has attracted significant attention. MiRNAs are involved in a variety of physiological and pathological processes. They can participate in the process of HS formation by regulating the TGF-β signal.

Figure 5. Over-expression of LINC01605 in HDF promoted fibrosis of cells by regulating AKT1 via miR-493-3p sponging. (a) Analysis of cell proliferation, via CCK8 assay, in different cell experimental groups. **P < 0.001 by one-way ANOVA with Tukey’s HSD post hoc test. (b) and (c) Analysis of migration and invasion of different cell experimental groups by wound-healing assay and transwell assay, respectively. Statistical significance was determined using Student’s t-test, *P < 0.05, **P < 0.01, ***P < 0.001, OE-LINC01605 group versus OE-NC group; #P < 0.05, ##P < 0.01, ###P < 0.001, OE-LINC01605 + miR-493-3p-mimic group versus OE-LINC01605 group. (d) and (e) Analysis of expression of collagen I, collagen III, AKT1, p-AKT1, mTOR, and p-mTOR in different cell experimental groups using western blotting. Statistical significance was determined using Student’s t-test, **P < 0.01, ***P < 0.001, OE-LINC01605 group versus OE-NC group; #P < 0.05, ##P < 0.01, ###P < 0.001, OE-LINC01605 + miR-493-3p-mimic group versus OE-LINC01605 group.
pathway, extracellular matrix deposition, and fibroblast proliferation and differentiation. In the present study, we found that GW4689 inhibited the secretion of exosomes in M2 macrophages and significantly decreased the expression of LINC01605. Furthermore, over-expression of LINC01605 in HDFs promoted the fibrosis of cells, suggesting that exosomes derived from M2 macrophages could promote fibrosis of cells by transferring LINC01605.

Mechanistically, our luciferase reporter assay verified that M2 macrophage-derived exosomes inhibited miR-493-3p in HDF by transferring LINC01605, up-regulating AKT1 levels, and activating the Akt/mTOR signaling pathway. Xu et al. reported that LSKL peptide inhibited the ability of HS fibroblasts by suppressing the phosphorylation of PI3K, AKT, and Mtor. In the present study, we found that LINC01605 up-regulated collagen I and collagen III and activated the AKT/mTOR signaling pathway, thereby promoting the fibrosis of HDFs. It has been reported that miR-4269, miR-382, miR-6836, and miR-495 also regulate collagen synthesis and participate in HS formation. Previous studies have shown that miR-4269 and miR-382 are up-regulated, while miR-203 and miR-205 are down-regulated, in keloids; however, they can be potential therapeutic targets against HS. Given the important role of the Akt/mTOR signaling pathway and miRNAs in HS formation, this suggests that these may be potential therapeutic targets against HS. However, the present study only explored the effect of exosomes derived from M2 macrophages on the proliferation and migration of HDFs in vitro. Further studies are needed to determine the promotion of HSs by exosomes derived from M2 macrophages in vivo. And in this study, the normal human dermal fibroblasts were used, and hypertrophic scar-derived fibroblasts were not incorporated. Therefore, further investigation of the regulatory effect of M2 macrophage exosomes on the proliferation of hypertrophic scar-derived fibroblasts is needed. In addition, while GW4869 inhibits the exosome secretion of M2 macrophages, it may also inhibit the exosome secretion of other cells such as keratinocytes, melanocytes, or endothelial cells, which may affect wound-healing. Therefore, our current findings can only prove that GW4689 can inhibit the specific mechanism of HDFs proliferation by inhibiting the secretion of exosomes from M2 macrophages. To explore whether GW4689 can have potential for clinical transformation, we need to explore whether GW4689 can inhibit the formation of HSs without affecting wound-healing in vivo.

Conclusion

In conclusion, our study elucidated that M2 macrophage-derived exosomes might be involved in HS formation via a mechanism involving the transfer of LINC01605, which could inhibit miR-493-3p in HDFs and activate the Akt/mTOR signaling pathway.

Declaration of conflicting interests

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