

Therapeutic Potential of Small Extracellular Vesicles Derived from Lipoma Tissue in Adipose Tissue Regeneration – An in Vitro and in Vivo Study

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Keywords: Adipose tissue, Lipoma tissue, Small extracellular vesicles, Adipose tissue regeneration

Posted Date: January 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-144257/v1>

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Version of Record: A version of this preprint was published at Stem Cell Research & Therapy on March 31st, 2021. See the published version at <https://doi.org/10.1186/s13287-021-02291-z>.

Abstract

Objective

To explore the adipogenic effects of the small extracellular vesicles derived from the lipoma tissues (sEV-LT), and to find a new cell-free therapeutic approach for adipose tissue regeneration.

Methods

Adipose tissue-derived stem cells (ADSCs) and small extracellular vesicles derived from the adipose tissues (sEV-AT) were isolated from human adipose tissue, while sEV-LT were isolated from human lipomatous tissue. ADSCs were characterized by using flow cytometric analysis, adipogenic and osteogenic differentiation assays. sEV was identified by electron microscopy, nanoparticle tracking and western blotting. ADSCs were treated with sEV-LT and sEV-AT, respectively. Fluorescence confocal microscopy were used to investigate whether sEV-LT and sEV-AT could be taken by ADSCs. The proliferation, migration and adipogenic differentiation of ADSCs were compared by CCK-8 assays, scratch test and oil red O staining test, and the expression levels of adipogenic-related genes C/EBP- δ , PPAR γ 2 and Adiponectin in ADSCs were compared by real-time quantitative PCR (RT-PCR). The sEV-LT and sEV-AT transplantation tubes were implanted subcutaneously in SD rats, and the neotissues were qualitatively and histologically evaluated in 2, 4, 8 and 12 weeks after transplantation. Hematoxylin and eosin (H&E) staining was used to observe and compare the adipogenesis and angiogenesis in neotissues, while immunohistochemistry was used to examine the expression and distribution of C/EBP- α , PPAR γ , Adiponectin and CD31 at the 4th week.

Results

Both sEV-LT and sEV-AT could be taken up by ADSCs via endocytosis in vitro experiments. The scratch experiment and CCK-8 experiment showed that the migration area and proliferation number of ADSCs in sEV-LT group and sEV-AT group were significantly higher than those in the non-sEVs group ($p < 0.05$). Compared with sEV-AT group, sEV-LT group had larger migration area and proliferation number of ADSCs ($p < 0.05$). Oil red O staining and RT-PCR experiments showed that, compared with the group without sEVs, the lipid droplets and the mRNA expression levels of adipogenesis-related genes PPAR γ 2 and Adiponectin of ADSCs in sEV-LT group and sEV-AT group were significantly up-regulated ($p < 0.05$), while the expression level of C/EBP- δ was not statistically significant compared to the group without sEVs ($p > 0.05$); Compared with sEV-AT groups, ADSCs in sEV-LT groups showed no statistically significant difference in the amount of lipid droplets and adipogenesis-related genes ($p > 0.05$). At 2, 4, 8 and 12 weeks, the adipocyte area and the number of capillaries in neotissues in the sEV-LT groups and sEV-AT groups were significantly increased compared with the Matrigel group ($p < 0.05$); Compared with sEV-AT groups, sEV-LT groups showed no significant difference in adipocyte area and the number of capillaries in neotissues ($p > 0.05$). At the 4th week, neotissues in the sEV-LT groups and sEV-AT groups all

showed positive expression of C/EBP- α , PPAR γ , Adiponectin and CD31 protein, while neotissues in the Matrigel group only showed positive expression of CD31 protein.

Conclusions

This study demonstrated that sEV-LT exerted promotion effects on adipose tissue regeneration by accelerating the proliferation and migration and adipogenic differentiation of ADSCs *in vitro*, recruiting adipocytes and promoting angiogenesis *in vivo*. sEV-LT could serve as an alternative cell-free therapeutic strategy for generating adipose tissue, thus providing a promising application prospect in tissue engineering.

1. Introduction

Soft tissue defects resulting from resection of tumors, as well as from trauma and congenital abnormalities not only lead to disfigurement, but also impair functions, making adipose tissue restoration an urgent clinical need^[1]. In 1893, Neuber first reported the use of autologous fat transplantation to successfully repair tissue defects, but following researches have always shown controversial results in the lasting therapeutic effect of this strategy, due to fat reabsorption, necrotic, liquefaction and possible scar contracture in the donor site^[2-3]. In more recent years, studies have shown that transplantation of adipose tissue-derived stem cells (ADSCs) can enhance adipose tissue regeneration via the paracrine actions of various cytokines and growth factors^[4-6]. For example, our previous studies have demonstrated that fat grafts consisting of platelet-rich plasma and ADSCs constitute an ideal transplant strategy, which may result in decreased absorption and accelerated fat regeneration^[7]. Although cell-based therapy has demonstrated the beneficial effects on adipose tissue regeneration, there are still lots of problems in the application of those mesenchymal stem cells (MSCs), such as immunogenicity, low viability, and potential tumorigenic feature^[8-9]. Importantly, recent works have demonstrated that paracrine factors significantly contribute to the therapeutic effect of stem cells on tissue repair^[10]. In particular, extracellular vesicles (EVs) may play an important role in paracrine mechanisms and have attracted attention in basic research and clinical applications^[11].

EVs are nano-sized membrane vesicles involved in intercellular communication, which have gained the most attraction as a potential and promising cell-free molecule used in clinical therapeutic applications^[11-13]. According to the guideline of MISEV2018 (Minimal information for studies of extracellular vesicles 2018), extracellular vesicles with size less than 200 nm were termed as sEVs (small extracellular vesicles)^[11]. Growing evidence has proved that sEVs are secreted by a variety of cells and mediate local and systemic intercellular communication by transfer their contents into target cells^[14]. In general, sEVs contain messages from the original cell sources including bioactive proteins such as cytokines and growth factors, as well as lipids and nucleic acids (RNA and DNA), which modulate biological behaviors of the target cells^[15-16]. Adipose tissue is an active endocrine organ that can secrete

various factors to regulate adipogenesis via paracrine signals^[17]. Previous studies have proved that sEVs derived from the adipose tissues (sEV-AT) are able to take part in a wide range of biological processes, especially for inducing adipogenic differentiation of ADSCs *in vitro* and promoting adipose tissue regeneration *in vivo*^[17–18].

Lipomas are common benign tumors of adipose tissues that origin in mesenchymal progenitors. While lipomas are usually treated when small, they sometimes can grow larger than 10 cm and can weigh over 1 kg^[19]. Generally, most of the lipoma tissues after surgical resection are considered useless and used to be discarded. In clinical practice, intact lipoma tissues seem to be easier to obtain than normal adipose tissues, which are often fragmented after liposuction. Studies have shown that a large number of adipocytes derived from lipoma tissues were strongly surrounded by Ki67+/CD34 + cells, indicating several altered biological activities such as proliferation, apoptosis and stemness of those adipocytes^[20–21]. Although several researches have indicated that lipoma tissue may have a faster rate of adipogenesis than normal adipose tissue, there is no study about the effect of sEVs derived from the lipoma tissue (sEV-LT) on adipose tissue regeneration. Therefore, in this study, we examined and analyzed sEV-LT. We observed the effects of sEV-LT on the proliferation, migration and adipogenic differentiation of ADSCs. Furthermore, we explored the inductive effect of sEV-LT on adipose tissue regeneration in subcutaneous chamber models of SD rats. Collectively, our study provides a novel theoretical basis and a potential cell-free therapeutic strategy for adipose tissue regeneration.

2. Methods

2.1 Animals and patients

Before surgery, all patients were informed of the purpose and procedures of this study and agreed to offer their excised tissues. Written consent was obtained from all participants involved in this study. All human tissues, including adipose tissues and lipoma tissues, were obtained from male patients (mean age: 36.7 ± 8.3 years, age range: 25–50 years, n = 10) who underwent surgical excision at Xiangya Stomatological Hospital (Changsha, China). SPF Sprague-Dawley (SD) rats were purchased from the Department of Laboratory Animals of Central South University (Changsha, China).

2.2 Isolation and culture of ADSCs

ADSCs were obtained from human subcutaneous adipose tissues. The fresh adipose tissues were washed three times with sterile phosphate-buffered saline (PBS) containing 1% penicillin and streptomycin, chopped by sterile operation scissors into small pieces (1–2mm³) and digested with 3 mg/ml type I collagenase (Sigma, Germany) for 40 min at 37 °C with shaking and then centrifuged at 1400 rpm for 7 min. The sediments were resuspended and expanded in the culture medium consisting of DMEM/F12 medium (BI, Israel), 10% fetal bovine serum (FBS, BI, Israel), 1% penicillin/streptomycin and 2 mM L-glutamine at 37 °C with 5% CO₂.

2.3 Characterization of ADSCs

ADSCs at passage 3 were harvested and counted. Approximately 1×10^5 cells were washed and labeled with fluorescence-conjugated antibodies (Biolegend, USA) (CD105-PE, CD90-PE, CD73-PE, CD34-PE, CD14-PE) at room temperature for 30 min. Isotype control IgG1 and IgG2a were used to stain the cells as a control. After two washes with PBS, the fluorescence of ADSCs were observed. For adipogenic or osteogenic differentiation, the cells were seeded in standard 6-well tissue culture plates (1.5×10^5 cells per well) and incubated with adipogenic differentiation medium (Cyagen, China) for 1 week or with osteogenic differentiation medium (Cyagen, China) for 2 weeks, respectively. Then, the induced cells were stained separately with Oil Red O for 30 min to assess adipogenic differentiation or with Alizarin Red S (Cyagen, China) for 5 min at room temperature to visualize osteogenic differentiation.

2.4. Isolation of small extracellular vesicles

Patients' adipose tissues and lipoma tissues were first minced into small pieces and transferred into a Celstir spinner flask (Wheaton) supplemented with DMEM/F12 and 1% penicillin/streptomycin, respectively. The tissues were cultured at 37 °C with speed at 100 rpm for 2 days. The debris of tissues and cells were removed by centrifugation (2000 *g*, 30 min). An additional centrifugation in Amicon® Ultra-50 Centrifugal Filter Units with Ultracel-3 membrane (3000Mw cutoff membrane, Millipore) at 5000 *g* for 30 min was applied to concentrate lipoma tissue extract (LTE) and adipose tissue extract (ATE). Then, the ATE and LTE were mixed with the Total Exosome Isolation™ reagent (Life Technologies) at 4 °C overnight and a final ultracentrifugation step was performed at 10000 *g* for 1 h at 4 °C. The obtained pellet was resuspended in 400 µl of PBS and stored at -80 °C with known concentration determined by using the Pierce BCA protein assay kit (KeyGEN, China).

2.5 Characterization of small extracellular vesicles

The ultrastructure and size distribution of the sEV-LT and sEV-AT were analyzed by a transmission electron microscopy (TEM) (FEI Tecnai G2 Spirit, USA) and the ZetaView® system (Particle Metrix, Germany), respectively. 20 µg vesicles was dissolved in RIPA Lysis Buffer (KeyGEN, China), and separated on polyacrylamide gels, blotted onto a nitrocellulose membrane. Then, the samples were incubated with a primary antibody CD9 (1:1000, Abcam, ab92726), CD63(1:1000, Abclonal, a5271) and TSG101 (1:1000, Proteintech, 28283-1-ap) at 4 °C overnight, and followed by horseradish peroxidase-coupled secondary antibody for 1 h at room temperature. The labeled protein markers were visualized using ImageQuant LAS 4000 mini (GE Healthcare).

2.6 Small extracellular vesicles labeling and cellular uptake

sEVs were labeled with a membrane-labeling dye DiO (Invitrogen, USA) and were then washed and resuspended in serum free DMEM/F12. Next, ADSCs were co-cultured with DiO-labeled vesicles for 6 h, washed with PBS three times, fixed in 4% paraformaldehyde, stained with phallotoxins (Invitrogen, USA), washed with PBS three times, counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, Germany), washed with PBS three times and imaged by confocal microscopy (Olympus FV1000, Japan).

2.7 Cell migration assay

The effects of sEV-LT and sEV-AT on ADSCs migration were evaluated in a scratch assay. ADSCs were seeded and cultured in 6-well plates at 2×10^5 cells/well. When the cell confluence reached 90%, the medium was replaced with DMEM/F12 after two washes with PBS, the confluent cell monolayer was scratched using a sterile 1000 μ l pipette tip, the cells were washed with PBS. sEV-LT (40 μ g/ml), sEV-AT (40 μ g/ml) and an equal volume of PBS were added to the wells, respectively. Images were recorded 0, 12 and 24 h after the monolayers were scratched. The migration area was measured by using ImageJ software and assessed as follows: migration area (%) = $(A_0 - A_n)/A_0 \times 100$ (A_0 represents the initial wound area ($t = 0$ h) and A_n represents the residual area of the wound at the time of measurement ($t = n$ h)).

2.8 Cell proliferation assay

ADSCs growth was determined by a Cell Counting Kit-8 (CCK-8, KeyGEN, China) assay. ADSCs were seeded at 1500 cells/well in 96-well plates and the medium was replaced with PBS. The cells were cocultured with sEV-LT (40 μ g/ml), sEV-AT (40 μ g/ml) or an equal volume of PBS. At 0, 1, 2, 3, 4, 5 days, 10 mL cell counting solution was added into each well and incubated at 37 °C for 1 h. The optical density (OD) was measured at 450 nm using a microplate reader (BioTek, USA).

2.9 Adipogenic differentiation of ADSCs

ADSCs at passage 3 were seeded at 2×10^5 cells per well into six-well plates, cultured for 24 h, then rinsed with PBS and incubated with 2 ml of one of four different culture media for up to 14 days. The media used were: (1) basal medium (DMEM/F12 supplemented with 10% FBS) as a negative control; (2) basal medium supplemented with sEV-LT (40 μ g/ml); (3) basal medium supplemented with sEV-AT (40 μ g/ml); (4) adipogenic medium (DMEM/F12 supplemented with 10% FBS, 2 mM insulin + 0.5 mM isobutylmethylxanthine + 0.1 μ M dexamethasone + 5 μ M rosiglitazone) as a positive control. The medium was changed every 3 days. The expression of adipogenic genes were analyzed by real-time quantitative PCR (RT-PCR) after 7 days of induction. After 14 days in culture, adipogenic differentiation was determined by Oil Red O (Cyagen, China) staining. Then, Oil Red O in cells was extracted with 100% isopropanol for 15 min. The absorbance was measured at 520 nm with a microplate reader (BioTek, USA).

2.10 Real-time PCR analysis

The TRIzol® reagent (Invitrogen, USA) was used to extract total RNA, which was reverse transcribed into cDNAs using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The synthesized cDNAs were amplified with SYBR Premix ExTaq (TaKaRa Biotechnology, Japan) using a RT-PCR System (Biometra Tone, Germany). The PCR cycling parameters were 95 °C for 2 min, 44 cycles of 95 °C for 5 s, and 60 °C for 30 s (The primers used for RT-PCR were shown in Table 1).

Table 1
Primers used for RT-PCR

| Gene | Primers |
|-----------------|---|
| PPAR γ 2 | 5'-GCCCTTTGGTGACTTTATGGAG-3' |
| | GCAGCAGGTTGTCTTGGATGT |
| C/EBP- δ | CTGCCATGTATGACGACGAGAG CGCTTTGTGATTGCTGTTGAAG |
| ADIPOGENCTIN | CGTTCTCTTCACCTACGACCAGT |
| | ATTGTTGTCCCCTTCCCATAAC |
| GAPDH | TCAACGGCACAGTCAAGG |
| | ACCAGTGGATGCAGGGAT |

2.11 Adipose tissue regeneration experiments in an animal model

All operations were performed on 8-week-old SD rats (200 ± 20 g, $n = 16$) under general anesthesia (1% pentobarbital sodium, 10 mL/kg, intraperitoneal injection). A silicone tube with an internal diameter of 5.0 mm and a height of 5.0 mm was subcutaneously implanted into the longitudinal incision (about 4 cm) on the back of rats. The incision was closed with 4/0 nylon suture. Each rat was implanted with 3 tubes: (1) 100 mL Matrigel (Corning, USA) alone; (2) 100 mL Matrigel containing 100 mg sEV-LT by injection; (3) 100 mL Matrigel containing 100 mg sEV-AT by injection. Rats were sacrificed at 2, 4, 8, and 12 weeks, respectively ($n = 4$ per time point), and their implanted tubes and contents were harvested and analyzed for further investigation.

2.12 Histology

The excised neo-tissue samples from the silicone tubes were fixed in 10% formalin, dehydrated with a graded alcohol series, embedded in paraffin. Sections with a thickness of 4 μ m were stained with hematoxylin and eosin (H&E). Images of the histologic sections were examined microscopically (Leica, Germany) at magnification of 100 \times . To quantitatively analyze the area of intact adipocytes and the number of mature capillaries, images in 8 random fields per section from each group (100 \times magnification) were examined by using ImageJ software. Immunochemical staining for C/EBP- α (Bioss, China, cat.bs1630R), PPAR γ (Bioss, China, cat.bs4888R), Adiponectin (Bioss, China, cat.bs0471R) and CD31 (Sino Biological, China, cat.50408-T16) was performed to determine the extent of adipose tissue and blood vessel formation in new growth tissues on the 4th week.

2.13. Statistical analysis

Each experiment was repeated at least three times. Data are expressed as mean \pm standard (SD). Statistical analysis was performed with a paired Student's t-test. A probability (p) value < 0.05 was considered statistically significant.

3. Results

3.1. Characterization of ADSCs, sEV-LT and sEV-AT

The morphology of ADSCs at passage 3 exhibited spindle-like in shape which is typical for mesenchymal stem cells under the inverted microscope (Fig. 1A). ADSCs also displayed adipogenic differentiation and osteogenic differentiation abilities, which were demonstrated by Oil red O staining for adipocytes (Fig. 1B) and Alizarin Red S staining for osteoblasts (Fig. 1C), respectively. Flow cytometry analysis (Fig. 1D) showed that these cells were highly positive for CD105, CD90 and CD73, while negative for CD34 and CD31. These results were consistent with previous studies in the characterizations of ADSCs^[7]. Therefore, all these data unequivocally confirmed that ADSCs were successfully isolated from human normal adipose tissues.

We then isolated sEV-LT and sEV-AT from the tissue extracts. The cupshaped morphology of the extracellular vesicles was observed by TEM (Fig. 1E). The size of ADSC Exos was directly tracked by using a Particle Metrix Analyzer named the ZetaView system. The average size of sEV-LT and sEV-AT was 121.3 nm and 121.1 nm, respectively (Fig. 1F). Western blots also showed that the exosomal markers CD9, CD63 and TSG101 (Fig. 1G) were all expressed in sEV-LT and sEV-AT. These results indicated that sEV-LT and sEV-AT were successfully isolated as they were consistent with the defining characteristics of sEV^[14].

3.2 The biological responses of ADSCs to sEV-LT and sEV-AT

To explore whether sEV-LT and sEV-AT could be internalized by ADSCs, ADSCs were co-cultured with the DiO-labeled extracellular vesicles for 6 h and then observed by fluorescence confocal microscopy. The DiO-labeled (green) sEV-LT and sEV-AT were seen to localize predominantly at the perinuclei region after entering cells, which indicated that endocytosis might be the main mechanism through which ADSCs internalized extracellular vesicles (Fig. 2A).

In order to preferably examine the effects of sEV-LT on the migration and proliferation of ADSCs, we used sEV-AT for comparison. sEV-LT and sEV-AT promoted the migration of ADSCs at different time points. The results from the scratch closure test demonstrated that the migration of ADSCs increased after 12 h and 24 h in the presence of sEV-LT and sEV-AT compared to the migration in the control group. At 12 h, cell migration of sEV-AT group was higher than that of sEV-LT group. However, cell migration of sEV-LT group was higher than that of sEV-AT group at 24 h (Fig. 2B–C). Likewise, CCK-8 analysis showed that compared to the control group, sEV-LT and sEV-AT at a concentration of 40 µg/mL both increased the proliferation of ADSCs, while sEV-LT had a more stronger promotion effect than sEV-AT (Fig. 2D).

To examine the effects of sEV-LT on adipogenesis, ADSCs were continuously co-cultured with sEV-LT (40 µg/ml) for 14 days. Lipid droplets were showed when ADSCs were treated with both sEV-LT and sEV-AT after 14 days induction (Fig. 2E). Adipogenesis was further determined by Oil Red O staining. The

results showed that the OD value of extracted Oil Red O was much higher in both sEV-LT group and sEV-AT group than in the negative control group. However, there was no significant difference in OD value between sEV-LT group and sEV-AT group (Fig. 2F). Similarly, RT-PCR revealed that the mRNA levels in sEV-LT group and sEV-AT group for the adipogenic genes encoding PPAR γ 2 and ADIPONECTIN, were remarkably elevated on day 7 compared to that in the negative control group, while there was no significance in mRNA expression between sEV-LT group and sEV-AT group (Fig. 2G). Moreover, the expression of mRNA encoding C/EBP δ remained at a similarly low level (Fig. 2G).

3.3 sEV-LT promotes adipose tissue regeneration *in vivo*

3.3.1 Gross observations and weights

In order to explore the effects of sEV-LT on adipose tissue regeneration *in vivo*, Matrigel mixed with sEV-LT and sEV-AT was injected into the silicone tube and then transplanted into the back of SD rats, respectively, while Matrigel alone served as the control (Fig. 3A). The gross appearance of tubes was shown and their appearance at 2, 4, 8, 12 weeks revealed active angiogenesis and adipogenesis in the sEV-LT group and sEV-AT groups, since there were a few growths of capillaries and adipose-like tissue from host into the tubes, while in control group, tubes filled with Matrigel were only encapsulated by integral fibrous fascia (Fig. 3B-C). The neotissues in the tubes were harvested and the macroscopic images at all time points were taken (Fig. 3D). The weights of neotissues were systematically calculated at each time-point and found to be gradually increased in all groups. Furthermore, the weights of neotissues were significantly increased in the sEV-LT group and sEV-AT group compared to control groups, but there was no significant difference in that between sEV-LT group and sEV-AT group (Fig. 3E).

3.3.2 Histological observations and newly formed capillaries

Neotissues from all groups were stained with hematoxylin and eosin (H&E), and quantitative comparisons of the newly formed areas of adipocytes and capillary numbers have been carried out. In sEV-LT group and sEV-AT group, light microscopy revealed that immature adipocytes with irregular morphologies and small diameters showed up after 2 weeks of transplantation, and neovascularization was seen beside the adipocytes, but no obvious adipocytes grew in the Matrigel group (Fig. 4A). In the 4th week, the light microscopy showed that most of the Matrigel contained in each group had been absorbed completely, and an increased large number of regular adipocytes and capillaries were seen in the sEV-LT group and sEV-AT group (Fig. 4A-C). At the 8th and 12th week, the adipocyte areas and the numbers of capillaries observed in the sEV-LT group and sEV-AT group decreased, while the granulation and fibrous tissue areas gradually increased (Fig. 4A-C). Although some of the adipocytes were replaced by fibroblasts and granulosa cells, the remaining were maintained for 12 weeks and there still had significant increase in adipose tissue in the sEV-LT group and sEV-AT group than Matrigel groups (Fig. 4B-C).

3.3.3 Immunohistochemistry

Furthermore, we used immunohistochemistry to ascertain the adipogenesis and angiogenesis effects of sEV-LT. Neotissue sections at the 4th week were stained with antibodies against C/EBP- α , PPAR γ , Adiponectin and CD31, which were all positive expressed in cells of sEV-LT group and sEV-AT group. However, in Matrigel group, only positive expression of CD31 protein was found. In addition, we found that many adipocytes and endothelial cells were positively stained near the blood vessels, while those cells located at a distance from the blood vessels were negative (Fig. 5).

4. Discussion

Previous studies in cell-free therapeutic approaches for adipose tissue regeneration have demonstrated certain effects to contribute to new adipose tissue development, including using synthetic or natural biomaterial scaffolds loaded with adipogenic growth factors, or providing a microenvironment suitable for originally existing cells in the body to migrate, proliferate, and differentiate to form adipose tissues^[22–23]. However, problems such as immune rejection responses or potential side effects of high growth factor concentrations may make it difficult to apply in clinic extensively. In recent studies, researchers have found and confirmed that cell-free adipose tissue extract (ATE) could effectively induce adipogenesis and angiogenesis^[5,24]. Furthermore, sEV-AT, as an indispensable component of ATE, has also been found that they can modulate proliferation, migration, and differentiation of the target cells by transferring functional proteins, mRNAs, and miRNAs^[16,25]. For example, sEV-AT under hypoxic conditions might promote lipid stimulation in 3T3-L1 adipocytes by increasing the levels of lipogenic enzymes, including fatty acid synthase (FASN), glucose-6-phosphate dehydrogenase (G6PD) and acetyl-CoA carboxylase (ACC), which may contribute to adipose tissue homeostasis or dysfunction^[26]. In addition, Zhang *et al*^[17] found that sEV-AT could induce adipogenesis differentiation through a mechanism involving transfer of proadipogenic miR-450a-5p. And miR-450a-5p could promote adipogenesis through repressing expression of WISP2 by targeting its 3' untranslated region. To conclude, from a perspective of paracrine effect, sEV-AT can be delivered into target cells and give rise to adipogenesis ultimately. Lipoma tissues, as adipose tissues of benign tumors, were always defined as useless tissues in the application of regenerative medicine and tissue engineering^[27]. Previous studies basically focused on lipoma-derived stem cells. However, the exact biological functions of sEV-LT are still needed to be found and determined.

Our study was among the first giving details in the characteristics and adipogenic analysis of sEV-LT and comparison with sEV-AT from human. In our studies, we showed evidence that lipoma tissue can release sEV, and ADSCs could uptake both sEV-LT and sEV-AT in a form of endocytosis. Furthermore, we found that sEV-LT and sEV-AT were able to effectively promote the proliferation, migration and adipogenic differentiation of ADSCs. Compared with sEV-AT, sEV-LT had a stronger ability to promote the proliferation and migration of ADSCs, while there was no significant difference in inducing adipogenic differentiation of ADSCs. These results indicated that the local progressive growth of the lipoma tissue may be related to the increasing migration and proliferation of the surrounding ADSCs induced by sEV-LT. In fact, previous studies have also confirmed that EVs secreted by terminally differentiated cells can influence biological functions of stem cells. For instance, EVs from osteoblasts, vascular endothelial cells

and skeletal muscle cells were found to be involved in the regulation of osteogenic differentiation, angiogenic differentiation, and myogenic differentiation of stem cells, respectively^[28-30]. Once the stem cells differentiate into mature cells, they, in turn, regulate stem cells to differentiate through EVs, thus a positive-feedback loop mechanism is formed during differentiation. Therefore, both sEV-LT and sEV-AT presented similar effects on ADSCs. However, we observed that sEV-LT showed stronger effects in promoting proliferation and migration of ADSCs than sEV-AT, these findings should be related to the differences in the functional proteins and miRNA and other genetic materials contained in the two kinds of sEV, which may be associated with the core reason for the tumorigenicity of lipoma tissue.

In order to further investigate the potential functions of sEV-LT in adipose tissue regeneration, we transplanted sEV-LT into subcutaneous chamber models of SD rats and observed the effects over a long-term period of 12 weeks *in vivo*, which was sufficient for complete adipogenesis and neotissue stabilization. We found that capillaries were first visible at 2 weeks on the surface of the silicone tubes in the sEV-LT group and sEV-AT groups, while none of that were seen on the surface of the cannula in the Matrigel group. At 2, 4, 8 and 12 weeks, the adipocytes were all detected under the microscope in the sEV-LT and sEV-AT groups, and mainly appeared around the newly formed blood vessels. Compared with the Matrigel group, the adipocyte areas, and the number of capillaries in the sEV-LT and sEV-AT groups were increased and statistically significant at all time points. Collectively, these results showed that the sEV-LT were able to promote adipogenesis and angiogenesis *in vivo* and the positive regulation relationship between adipogenic differentiation and angiogenesis, which was consistent with past studies. It has been shown that the increased resorption of a fat graft is often related to debilitated and weakening neovascularization^[31]. Vascular-derived factors regulate the metabolism and accumulation of adipose tissue by affecting the formation and reconstruction of adipose tissue vascular network^[32]. Due to the close relationship between adipogenesis and angiogenesis, we used immunohistochemistry to analyze the expression of adipogenesis-related proteins PPAR γ , C/EBP- α and Adiponectin and angiogenesis-related protein CD31 in each group at 4 weeks. And we found that all these proteins were positively expressed in the sEV-LT and sEV-AT groups, and adipogenesis-related proteins were mostly aggregated and expressed in adipocytes around capillaries. In contrast, they are relatively less expressed in adipocytes far away from microvessels. These results also confirmed the positive regulatory relationship between adipogenesis and angiogenesis.

However, along with the decrease of blood vessels, the adipocytes recruited in the sEV-LT and sEV-AT groups began to decrease and were replaced by fibroblasts and granular cells at 8 and 12 weeks. We hypothesized the main reason could be related to rats' immune responses to those silicone tubes and limited quantities of EVs which gradually exhausted. Nevertheless, from a long-term perspective, the newly formed adipose tissue in sEV-LT could still display a certain vitality at the 12th week, which indicated the therapeutic potential of sEV-LT in fat regeneration and tissue engineering.

There are some limitations to our studies, and several problems must be solved before the results can be applied in the clinic setting. For instance, although we have verified the adipogenesis and angiogenesis effects of sEV-LT, the exact proteins or miRNAs involved in sEV-LT-mediated differentiation are still

needed to be determined in the future. Another limitation is that we need to further evaluate and confirm the tumorigenicity and biosafety of sEV-LT, since they are extracted from tumor tissue. Moreover, the number of SD rats in the study was too small, a larger sample size should be included for further study. Additional studies will be performed in the future; however, our studies have provided a potential new method for adipose tissues regeneration and these findings will direct better clinical treatment.

5. Conclusion

In summary, in this study, we first provided direct evidence that sEV-LT exert promotion effects on adipose tissue regeneration by accelerating the proliferation and migration and adipogenic differentiation of ADSCs *in vitro*, recruiting adipocytes and promoting angiogenesis *in vivo*. Notably, the neotissue induced by sEV-LT could maintain viability lasting for 12 weeks. Results from our group suggested that sEV-LT could serve as an alternative cell-free therapeutic strategy for generating adipose tissue, providing a promising application prospect in tissue engineering.

Abbreviations

ADSCs

Adipose tissue-derived stem cells

ACC

Acetyl-CoA carboxylase

ATE

Adipose tissue extract

H&E

Hematoxylin and eosin

FASN

Fatty acid synthase

G6PD

Glucose-6-phosphate dehydrogenase

LTE

Lipoma tissue extract

MSCs

Mesenchymal stem cells

PBS

Phosphate-buffered saline

sEVs

Small extracellular vesicles

sEV-AT

sEVs derived from the adipose tissues

sEV-LT

sEVs derived from the lipoma tissues

TEM

Transmission electron microscopy

Declarations

Acknowledgements

We thank and appreciate Zhigang Yao, Long Li, Gui Liu, Lin Cui and Xinjia Cai's (Department of Oral Pathology, Xiangya Stomatological Hospital, Central South University, China.) assistance in the interpretation and preparation of the histological section.

Funding

This work has been supported by the National Natural Science

Foundation of China (81800952), National Natural Science

Foundation of China (81671003), the Natural Science Foundation of Hunan Province, China (2018JJ3712), and the Fundamental Research Funds for the Central Universities of Central South University(2018zzts838).

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The study was approved by the Ethical Committees of the Department of Laboratory Animals, Central South University, China (approvals no. 2020sydw0045) and the Ethical Committees of the Xiangya Stomatological Hospital & School of Stomatology, Central South University, China (approvals no. 20190058).

Consent for publication

Not applicable

Authors' contributions

Pengyu Hong, Kun Li and Zhangui Tang designed the experiment; Pengyu Hong performed the experiments, analyzed the data, and wrote the manuscript; Xiaoyang Xu and Xin Hu discussed the research designs and results; Hao Yang, Yue Wu and Juan Chen revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

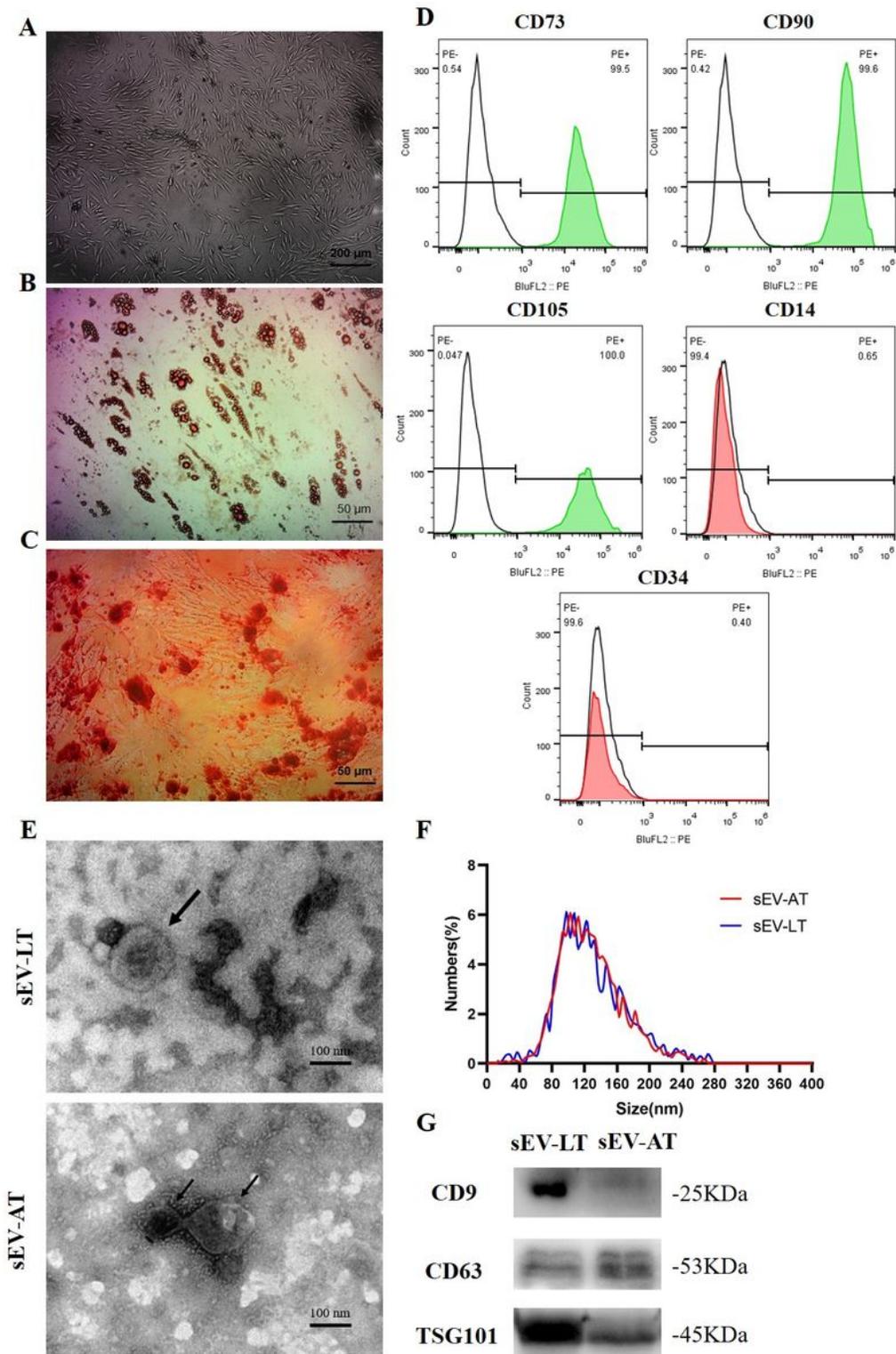


Figure 1

Characterization of ADSCs, sEV-LT and sEV-AT. (A) ADSCs exhibited the typical spindle-shaped morphology, as shown in the picture. Scale bar=200 μ m. (B) Representative photographs of Oil red O staining for adipocytes. Scale bar=50 μ m. (C) Representative photographs of Alizarin Red S staining for osteoblasts. Scale bar=50 μ m. (D) Flow cytometry analysis showed that these cells were highly positive for CD105, CD90, CD73 but negative for CD34 and CD31. Abbreviations: PE, phycoerythrin. (E) The

ultrastructure of sEV-LT and sEV-AT under transmission electron microscopy. Scale bar= 100 nm. (F) The size distribution profile of sEV-LT and sEV-AT by dynamic light scattering. (G) The expression of the sEV markers CD9, CD63 and TSG101 was confirmed by immunoblotting.

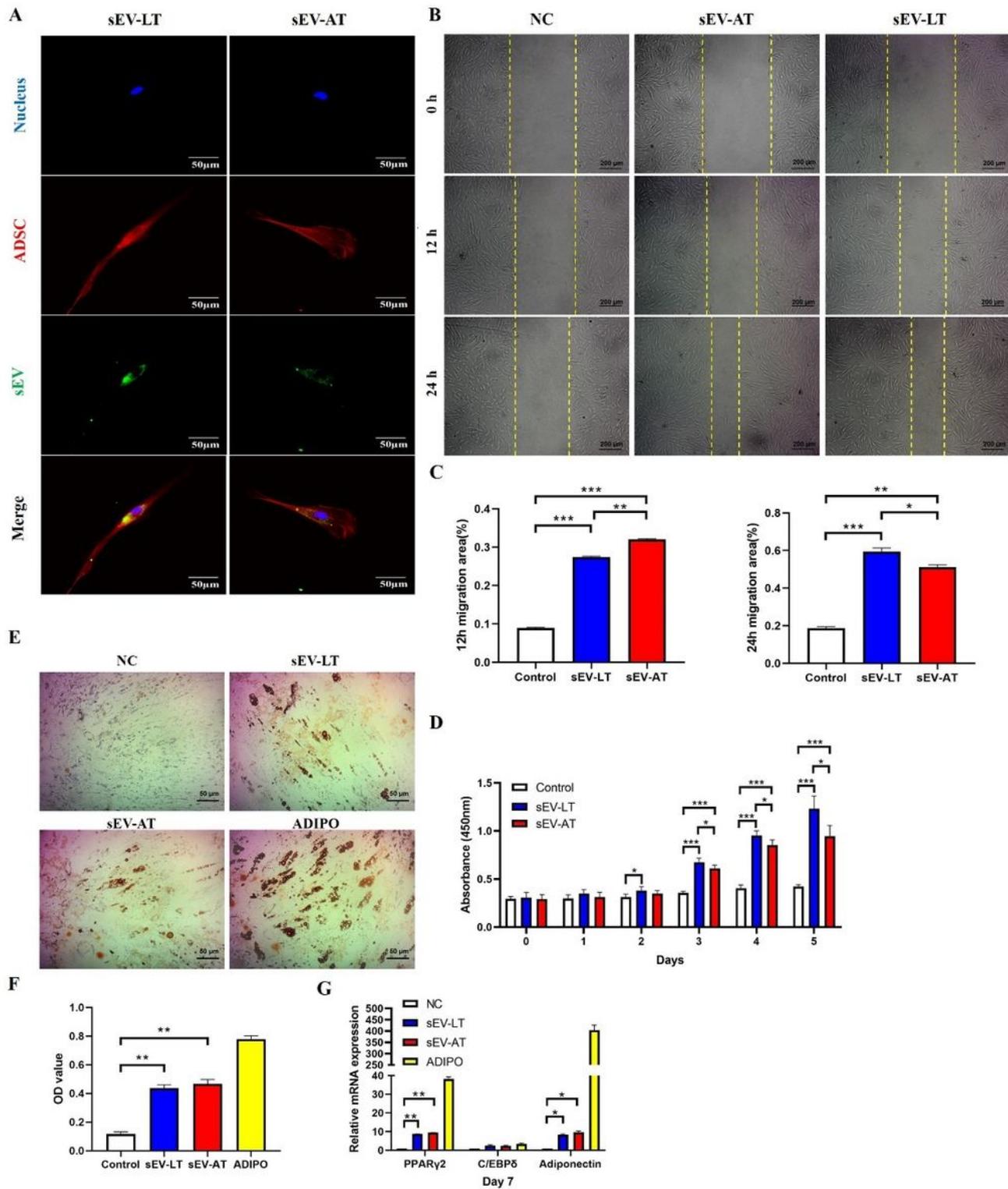


Figure 2

The biological responses of ADSCs to sEV-LT and sEV-AT (A)The uptake of sEV-LT and sEV-AT by ADSCs. ADSCs were incubated with DiO-labeled extracellular vesicles (green) and stained with phalloxins

(red). Nuclei were stained with DAPI (blue). Scale bar=50 μm . (B) Representative images from the scratch wound assay. Scale bar=200 μm (C) Quantitative analysis of cell migration in each group at 12 h and 24 h. (D) CCK-8 assay. (E) ADSCs cultured with sEV-LT and sEV-AT for 14 days, and the lipid droplets were stained with Oil Red O to determine the level of adipogenesis. ADSCs cultured with basal culture medium (NC) or adipogenic medium (ADIPO) were used as a negative and positive controls, respectively. Scale bar=50 μm . (F) Quantification of the amount of Oil Red O. (G) The relative expressions of mRNA encoding C/EBP δ , PPAR γ 2, Adiponectin was measured by RT-PCR on day 7 after induction. Results are present as mean \pm s.d. (n=3). *p < 0.05, **p < 0.01 and ***p < 0.001.

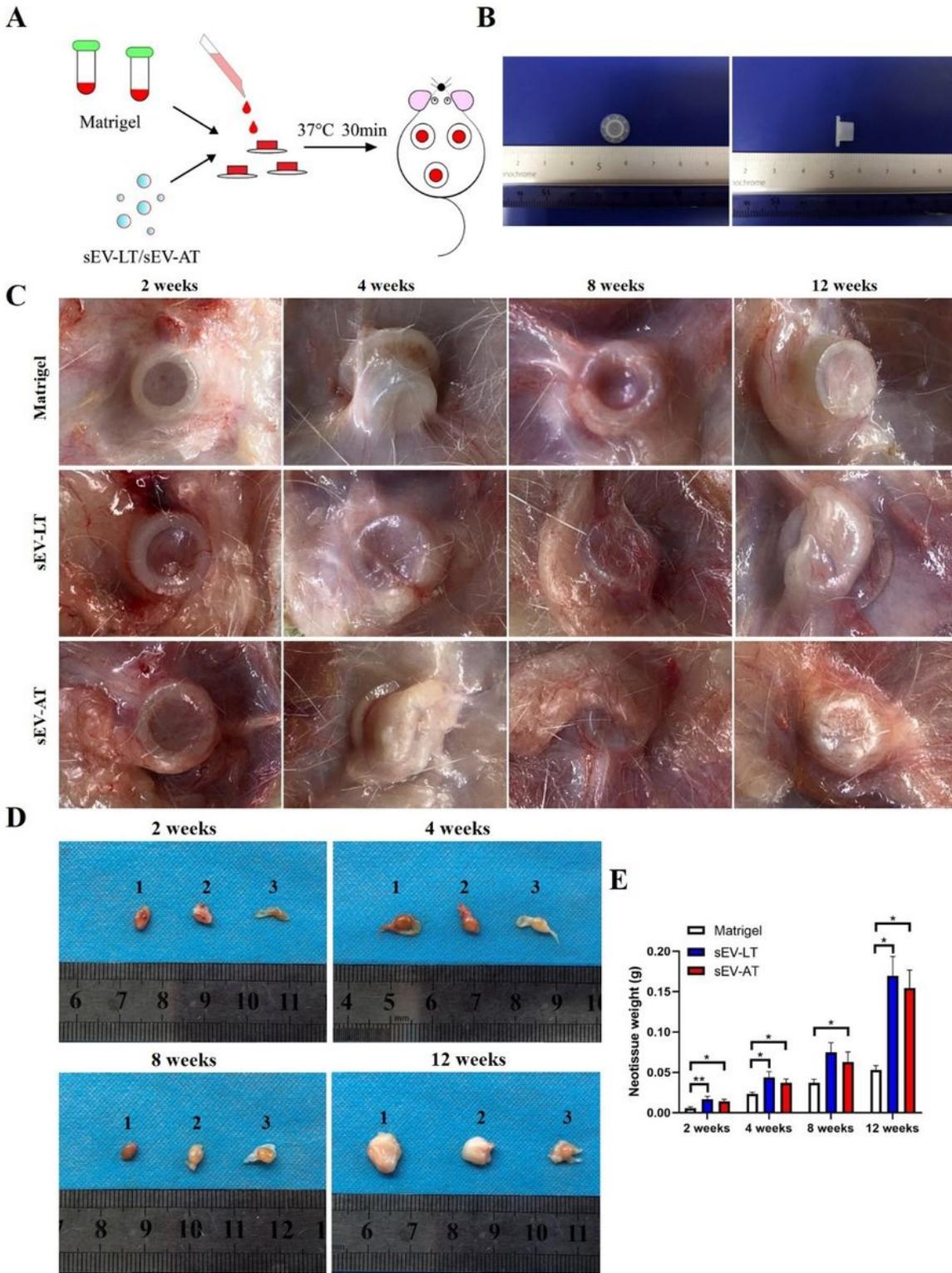


Figure 3

sEV-LT promotes neotissue formation in vivo (A) The schematic view of the experimental operation process. (B) The implanted silicone tubes, with an internal diameter of 5.0mm and a height of 5.0mm. (C) The gross observation of the implanted Matrigel mixed with sEV-LT and sEV-AT within the tube at week 2,4,8 and 12 after transplantation. (D) Dimensions of neotissues at week 2,4,8 and 12 after

transplantation. 1: sEV-LT. 2: sEV-AT. 3: Matrigel group. (E) Weight comparisons of neotissues at week 2, 4, 8 and 12 after transplantation. * $p < 0.05$, ** $p < 0.01$.

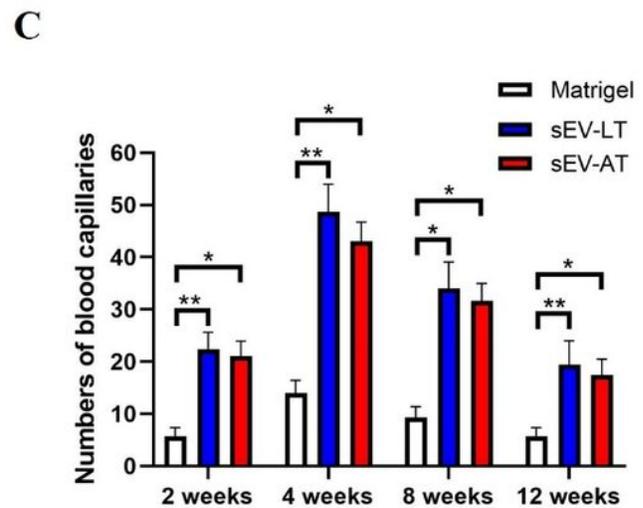
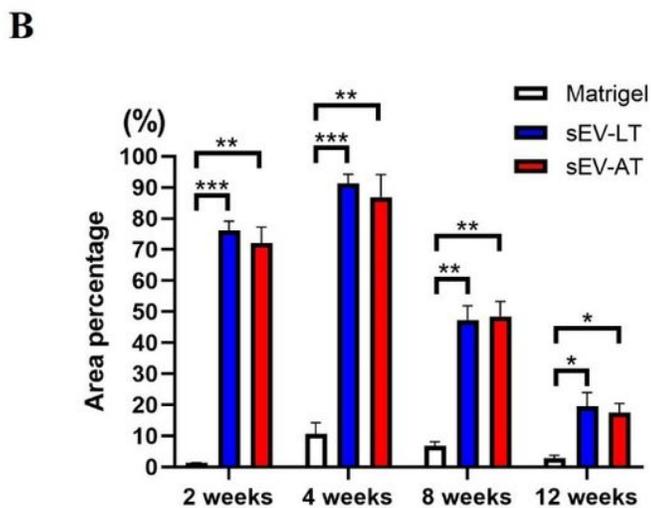
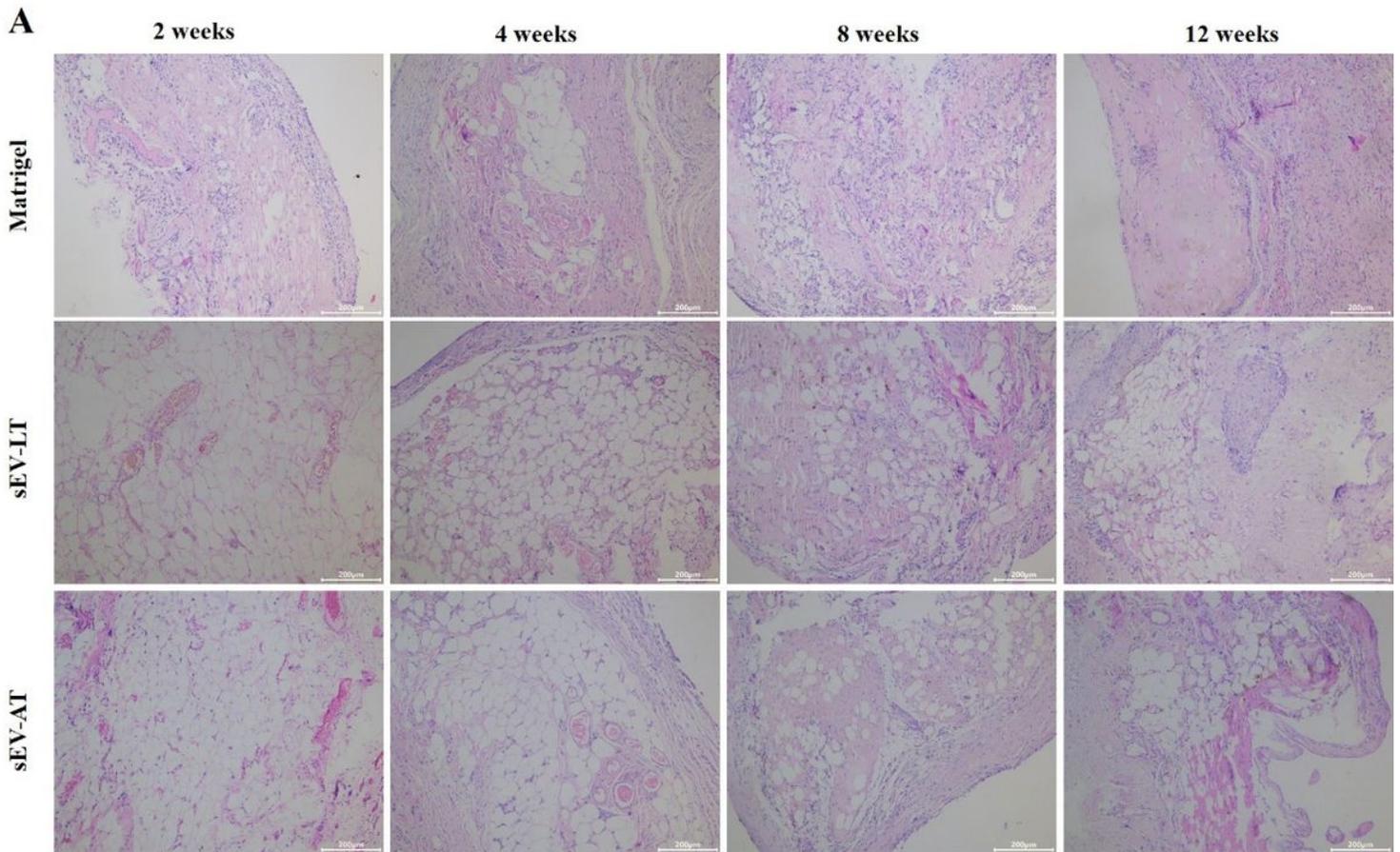


Figure 4

sEV-LT promotes adipose tissue regeneration in vivo. (A) Histology of Matrigel group, sEV-LT group and sEV-AT group sections at week 2, 4, 8 and 12 after transplantation. Sections were stained with hematoxylin and eosin. (B) Comparison of the average adipocyte areas in experimental and control groups at 2, 4, 8 and 12 weeks after transplantation. (C) Comparison of the mean capillary numbers of

neotissue sections in the experimental and control groups at 2, 4, 8 and 12 weeks. Scale bar =100 μ m.
* p <0.05, ** p <0.01, *** P < 0.001.

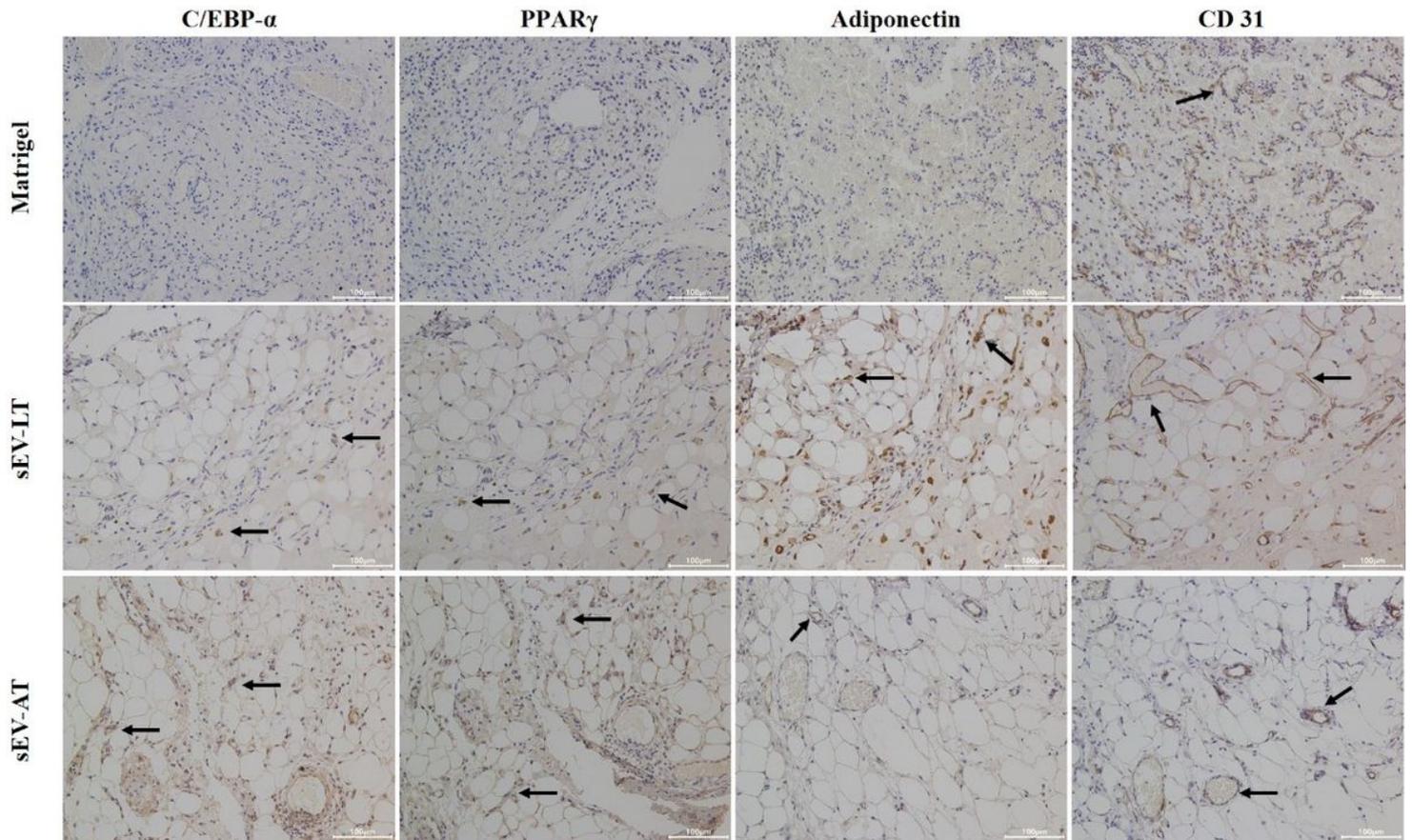


Figure 5

Immunohistochemistry of neotissue sections in the experimental and control groups at week 4 after transplantation. Representative photographs of C/EBP α , PPAR γ , Adiponectin and CD31 immunostaining in Matrigel group, sEV-LT group and sEV-AT group, respectively. The black arrows indicated the positive stained protein. Scale bar=100 μ m.