Original Paper

Exosomes from Adipose-Derived Stem Cells Promotes VEGF-C-Dependent Lymphangiogenesis by Regulating miRNA-132/TGF- β Pathway

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Key Words

Adipose-derived stem cells • Lymphangiogenesis • VEGF-C • miR-132 • Exosome

Abstract

Background/Aims: Lymphangiogenesis plays an important role in the pathogenesis of inflammatory bowel diseases (IBD), and vascular endothelial growth factor-C (VEGF-C) is a powerful lymphangiogenic factor. Adipose-derived stem cells (ADSCs) are a promising therapeutic modality for several diseases because ADSCs secrete growth factors and exosomes, which modulate hostile microenvironments affected by diseases. However, the effect of exosomes on VEGF-C-dependent lymphangiogenesis and its mechanism remain unclear.

Methods: ADSCs were cultured in media with or without recombinant VEGF-C and exosomes were extracted from conditioned medium (CM). Lymphatic endothelial cells (LECs) were treated with ADSCs-derived exosomes, then proliferation, migration and tube formation of LECs were assayed using cell counting Kit-8 (CCK-8), transwell chamber inserts and matrigel-based tube formation assay respectively. **Results:** We identified significantly higher levels of miR-132 in exosomes isolated from VEGF-C-treated ADSCs (ADSCs/VEGF-C) than in those from ADSCs control. miR-132 was directly transferred from ADSCs to the LECs by the mediation of exosomes. The exosomes from ADSCs/VEGF-C promoted LECs proliferation, migration, and tube formation more potently than the exosomes from ADSCs, whereas pretreatment of ADSCs with miR-132 inhibitor attenuates VEGF-C-dependent lymphangiogenic response. Finally we reveal that miR-132 promotes lymphangiogenic response by directly targeting Smad-7 and regulating TGF- β /Smad signaling. **Conclusion:** These data provide new insights into the role of ADSCs-derived exosomes as an important player in VEGF-C-dependent lymphangiogenesis.

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Introduction

Inflammatory bowel disease (IBD) is a complex biologic process, involving genetic, environmental, microbial and immune factors [1, 2]. Growing evidence demonstrated that the microvasculature contributes to chronic inflammation by altering leukocyte recruitment, lymphangiogenesis and angiogenesis leading to tissue remodeling, thus plays a crucial role in the pathophysiology of IBD [3, 4].

Lymphangiogenesis occurs in adult tissues during pathologic conditions such as inflammation, wound healing and tumor metastasis [5], and is a recognized hallmark of inflammatory processes in bowel, skin, heart and airways [6, 7]. Lymphangiogenesis could facilitate the resolution of tissue edema and the mobilization of inflammatory cells in acute and chronic inflammatory settings [8, 9]. On the contrary, lymphangiogenesis could also worsen immune diseases such as the rejection response after transplantation [5]. Thus, lymphangiogenic response can be either a detrimental or a beneficial phenomenon in pathologic conditions, which depends on the pathophysiology of the underlying disease [5, 10]. Emerging studies have identified lymphangiogenesis as hallmark features of chronic gut inflammation, and the expansion of vascular population has been hypothesized to play a pathogenic role in IBD [11, 12].

Vascular endothelial growth factor-C (VEGF-C) is the first powerful lymphangiogenic factor [13, 14]. Lymphangiogenesis is mediated by binding of VEGF-C to VEGFR-3. VEGF-C overexpression markedly induces lymphangiogenesis and reduces the extent of edema in a primary lymphedema mouse model [15]. Additionally, anti-lymphatic treatment in an animal model of IBD has been shown to aggravate inflammation and submucosal edema, and results in an increase of leukocyte infiltration [16].

Adipose-derived stem cells (ADSCs) are an abundant and readily available population of multipotent progenitor cells that reside in adipose tissue. Their therapeutic use in pre-clinical studies and experimental clinical trials for several diseases has been well documented because of the potential of secreting growth factors and exosomes [17-21]. Exosome, a 30-150nm sized extracellular vesicle, containing nucleic acids and proteins, that act as messengers, delivering factors and signaling molecules to close and distant cells [22-24]. Liang et al. reported that exosomes secreted by mesenchymal stem cells (MSCs) promote endothelial cell angiogenesis by transferring miR-125a [25]. Exosomes from ADSCs ameliorate phenotype of Huntington's disease *in vitro* model [22]. ADSCs also possess the potential of promoting proliferation, migration, and tube formation of lymphatic endothelial cells *in vitro* [13, 26]. However, the role of exosomes in mediating the interaction of VEGF-C with ADSCs and subsequent lymphangiogenesis remains unclear.

Based on above findings, we investigated whether exosomes from ADSCs regulates VEGF-C-dependent lymphangiogenic response. We demonstrated that ADSCs-secreted miR-132 following VEGF-C treatment can be transferred via exosomes to LECs, and this uptake results in enhanced proliferation, migration, and tube formation. miR-132 regulates VEGF-C-dependent lymphangiogenic response by directly targeting Smad7 and activating TGF- β /Smad signaling.

Materials and Methods

Cell isolation and culture

All donors gave informed consent for harvesting of their adipose tissue. Donors with malignancies, infectious or systemic diseases were not included in the present study. ADSCs were isolated as previously described [13, 27]. Briefly, subcutaneous adipose tissue was harvested during surgical operations, as approved by the Hospital's Protection of Human Subjects Committee. Adipose tissues were washed extensively with 2 volumes of PBS and digested at 37°C for 60 minutes with equal volumes of collagenase (final concentration 66.7 U/ml, Sigma Aldrich, USA) and dispase (final concentration 10 U/ml, BD, USA).

The top lipid layer and remaining liquid layer were removed after centrifugation at 200g for 10 minutes. The pellet was re-suspended in DMEM supplemented with 10% FBS and filtered through 40 µm nylon cell strainer (BD Falcon, NJ, USA) after lysing the red blood cells. The isolated cells were then plated and expanded in MesenCult-XF culture medium (Stem Cell Technologies, Vancouver, Canada) supplemented with 1% (v/v) penicillin/streptomycin (Gibco, CA, USA) and 2 mM L-glutamine (Gibco). ADSCs culture was maintained at sub-confluent levels (< 80% confluency) at 37°C in 5% humidified CO₂ and passaged using HyQtase (HyClone, USA).

Human dermal lymphatic endothelial cells (LECs) were purchased from Lonza (Basel, Switzerland) and cultured in endothelial growthmedium-2-MV (EGM-2-MV; Lonza) that consisted of endothelial basal medium-2 (EBM-2; Lonza) plus SingleQuots kit (Lonza), according to the manufacturer's instructions.

Preparation of conditioned medium and exosomes

At approximate 80% confluence, ADSCs were washed with PBS thrice and the culture media were replaced with EBM-2 containing 0.1% fatty acid-free bovine serum albumin (Sigma-Aldrich). After 48 h incubation, the medium was collected, filtered through a 0.22 µm filter (EdLab, NY, USA), and used for assays as ADSCs-cm. Exosomes were extracted using the ExoQuick-TCExosome Precipitation Solution (System Biosciences, Mountain View, CA, USA). Briefly, the appropriate volume of ExoQuick-TCExosome Precipitation Solution was added to the CM and refrigerated overnight. The sample was centrifuged at 1500 r.p.m. for 30 min and then at 3000 r.p.m. for 5 min at 4 °C. The exosome pellet was resuspended in approximate 300 µl of cell medium and incubated 72 h with recipient cells. Exosome-deprived medium was obtained upon 25000 r.p.m. ultracentrifugation for 90 min.

Western blot analysis

After quantification of the protein samples using Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA), equivalent amounts of protein (150 µg) were electrophoresed through a 10% SDS-polyacrylamide gel, and then electro-transferred to PVDF membranes (Millipore, Billerica, MA, USA). The blots were incubated for 2h at room temperature with the indicated antibodies (CD63: ab134045, 1:1000, Abcam, CA, USA; HSP70: ab787, 1:1000, Abcam; CD81: ab109201, 1:1000, Abcam; Smad7: H00004092-M02, Novus Biologicals, 1:1000; Smad2/3: ab63672, 1:1000, Abcam; p-Smad2/3: ab63399, 1:1000, Abcam), and then incubated with goat-anti-rabbit/mouse HRP-linked secondary antibody (Santa cruz, CA, UA). Protein signals were visualized using a chemiluminescence substrate (Pierce Chemical, Rockford, IL, USA). The intensity of the selected bands was quantified using Image J software.

Assessment of cell proliferation

Cell proliferation was assessed using Cell Counting Kit-8 (Dojindo, Japan) as our previously described [28]. LECs were plated in 96-well plates in triplicate at approximately 2-5×10⁴ cells per well and cultured in 100 µL of EGM-2-MV overnight at 37°C in 5% humidified CO₂. After the medium was removed LECs were washed with PBS thrice, treated with 100 µL of EBM-2 as a negative control or indicated reagents (exosomes, miR-132 or LY2109761). The numbers of cells per well were measured by the absorbance (450 nm) of reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4 -isulfophenyl)-2H-tetrazolium, monosodium salt) at the indicated time points.

Cell migration assay

LECs migration assay was performed using Transwell chambers with inserts of 8-µm pore size (Corning Costar) as described previously [29]. Briefly, 2 × 10⁵ LECs suspended in serum-free media were plated into the upper chamber for migration assay following treatment with indicated reagents, and media supplemented with 10% FBS was placed into the lower chamber. The cells that had migrated through the membrane to the lower surface were fixed, stained and counted after 48 h.

Tube formation assay

Matrigel tube formation assays were carried out as previously described [13]. Briefly, matrigel (BD Biosciences, Bedford, MA, USA) was placed in 24-well plates (100µL/well) and allowed to gel at 37°C for 30 minutes. 2× 10⁵ LECs were then seeded onto the coated wells and cultured with 500 µL of EBM-2, EBM-2 containing indicated reagents at 37°C for 24 h under 5% CO₂, and then LEC tube formation was assessed

by microscopy, and each well was photographed. The number of tube branches and total tube length were calculated using the MacBiophotonics Image J software.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using Trizol reagent according to the manufacturer's instruction. Reverse transcription (RT) reactions for mRNA were carried out using the Oligo dT primer and an M-MLV Reverse Transcriptase kit. The specific stem-loop RT primers for miR-132 were designed as previously described [30]. qPCR was performed using a standard SYBR Green PCR kit (Toyobo, Osaka, Japan) protocol on Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). U6 and β -actin were used as references for miRNAs and mRNAs, respectively.

miR-132 mimics and inhibitor

Human miR-132 (UAACAGUCUACAGCCAUGGUCG), FAM-labeled miR-132 or 2'-O-methyl modified miR-132 inhibitor (CGACCAUGGCUGUAGAC UGUUA) were purchased from Genepharma (Shanghai, China) and transfected to cells by using Lipofectamine™ 3000 (Invitrogen) according to the manufacturer.

Luciferase reporter assay

pGL3 plasmid encoding a luciferase reporter gene was purchased from Promega (Madison, WI). Recombinant plasmid of pGL3-smad7-3'-UTR (wildtype) or pGL3-smad7-3'-UTR-Mutation was constructed in our laboratory. HepG2 cells (2×10^5 cells/well) were plated in a 24-well plate and cotransfected with 40nM of either miR-132 or miRNA control, 20 ng of either pGL3-smad7-3'-UTR or pGL3-smad7-3'-UTR-Mutation, and 2 ng of pRL-TK (Promega, Madison, WI) by using Lipofectamine™ 3000 (Invitrogen). HepG2 cells were collected 48 h after transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega).

Transmission electron microscopy

For electron microscopy analysis, exosomes were mixed with an equal volume of 4% paraformaldehyde. Sample was then deposited onto Formvar-carbon-coated electron microscopy grids, washed with PBS thrice. Sample was first contrasted in a solution of uranyl oxalate for 5min and then contrasted and embedded in a mixture of 4% uranyl acetate and 2% methyl cellulose for 10 min on ice. The samples were observed under a Hitachi-8100IV transmission electron microscope (Hitachi, Tokyo, Japan) at 100 kV.

Statistical analysis

The data presented are mean $\pm$ standard deviation of at least three independent experiments. Averaged data were compared using unpaired Student's t test or one-way ANOVA, followed by the Scheffé test. A two-tailed P value < 0.05 was deemed statistically significant. All statistical analyses were done using SPSS statistical software version 22 for Windows (IBM Corp., USA).

Results

Exosomes from VEGF-C-treated ADSCs (ADSCs/VEGF-C) have higher miR-132 expression

Cell-to-cell communication is an important mechanism for information exchange promoting cell survival, proliferation and migration, and exosomes play a vital role in cell-to-cell communication [31, 32]. In the study we investigated the role of exosomes in the interaction of ADSCs with LECs. Exosomes were first isolated from the conditioned media collected from ADSCs/VEGF-C and were characterized by electron microscopy, western blot and qNano analysis. Electron micrographs showed that the collected products had a distinctive cup shape (Fig. 1A). qNano analysis revealed that the collected products had diameters ranging from 80 to 120 nm (Fig. 1B). Western blot analysis further showed the expression of the exosomal markers CD63, HSP70 and CD81 (Fig. 1C) but the absence of the cis-Golgi compartment-specific marker GM130 (data not shown).

miRNAs that transfer between living cells that are involved in cell-to-cell communication are frequently encapsulated in exosomes [32]. To identify miRNAs that are transferred by exosomes secreted from ADSCs to LECs, and involved in lymphangiogenesis, we searched

Fig. 1. Exosomes from ADSCs/VEGF-C have higher miR-132 expression. (A) Electron micrograph analysis of whole-mount exosomes isolated from ADSCs/VEGF-C conditioned medium. Scale bar = 100nm. (B) qNano analysis of the exosomes diameter (nm) isolated from ADSCs/VEGF-C conditioned medium. (C) Western blot analysis showing exosome marker CD63, HSP70 and CD81 in the exosome. (D) Heat map representation of qPCR data about the expression levels of 15 miRNAs involved in lymphangiogenesis in exosomes isolated from ADSCs and ADSCs/VEGF-C.

Fig. 2. miR-132 markedly promotes LECs proliferation. 2×10^5 cells LECs were plated in 96-well plates and cultured in 100 μ L of EGM-2-MV overnight. LECs were treated with control, miR-132, miR-181a, miR-27a or miR-486-5p, and cell proliferation was assayed by using CCK-8 and at the indicated time points. * $p < 0.05$.

PubMed and found 15 functional miRNAs involved in lymphangiogenesis (Table 1). The expression of these miRNAs was assayed using qPCR array in exosomes isolated from ADSCs with or without VEGF-C treatment. The qPCR analysis identified 4 upregulated miRNAs and 3 downregulated miRNAs in exosomes from VEGF-C-treated ADSCs compared to exosomes from ADSCs control (Fig. 1D, Table 2). Of the 4 upregulated miRNAs, miR-132 was selected to further study because miR-132 possessed the most potential of promoting LECs proliferation (Fig. 2).

Direct transfer of miR-132 from ADSCs to LECs

To determine whether miR-132 can be directly transferred from ADSCs to LECs, ADSCs were transiently transfected with miR-132 or control, then the conditioned medium, changed 8 h post-transfection, was collected from transfected cells at 48 h and processed for exosomal purification. The expression level of miR-132 was assayed by qPCR in transfected cells and in the isolated exosomes. As shown in Fig. 3A and B, miR-132 level was significantly upregulated in miR-132-treated ADSCs and its exosomes compared

Table 1. miRNAs involved in lymphangiogenesis

miRNAs	References
miR-126	Br J Cancer, 2012, 107(4): p. 700-6.
miR-132	Arterioscler Thromb Vasc Biol, 2016, 36(12): p. 2381-2393.
miR-9	Nat Med, 2010, 16(8): p. 909-14.
miR-1236	Am J Physiol Cell Physiol, 2015, 309(10): p. C680-92.
miR-181a	Arterioscler Thromb Vasc Biol, 2012, 32(3): p. 633-42.
miR-31	Arterioscler Thromb Vasc Biol, 2014, 34(3): p. 603-15.
miR-466	Blood, 2010, 116(13): p. 2395-401.
miR-146a	Blood, 2010, 116(13): p. 2395-401.
miR-146b	J Biomed Sci, 2015, 22: p. 3.
miR-155	Cancer Med, 2017, 6(5): p. 1036-1048.
miR-221	Cancer Med, 2017, 6(5): p. 1036-1048.
miR-222	Atherosclerosis, 2011, 215(2): p. 286-93.
miR-27a/b	Blood, 2011, 118(10): p. 2896-905.
miR-486-5p	Blood, 2011, 118(10): p. 2896-905.
miR-93	PLoS One, 2017, 12(10): p. e0186718.
	Clin Sci (Lond), 2016, 130(17): p. 1523-33.
	Onco Targets Ther, 2016, 9: p. 2865-71.
	Cancer Med, 2017, 6(5): p. 1036-1048.

to control. Fig. 3C showed that the incubation with ADSCs-secreted exosomes results in increase of miR-132 level in recipient LECs compared to control, indicating that the recipient LECs could uptake the exosomes and their cargo. To further determine whether miR-132 can be directly transferred from ADSCs to LECs, ADSCs were transiently transfected with fluorescein amidite (FAM)-tagged miR-132, and were co-cultured with LECs. Confocal microscopy detected FAM-labeled miR-132 in the LECs co-cultured with miR-132-transfected ADSCs but not in those co-cultured with mock-transfected ADSCs, suggesting that miR-132 was transferred from ADSCs to LECs (Fig. 3D). Exosome was then isolated from conditioned media collected from ADSCs that had been transfected with FAM-tagged miR-132 or control and was added to LECs. Confocal microscopy revealed fluorescently labelled signals in the LECs incubated with exosomes obtained from FAM-tagged miR-132-transfected cells compared to control (Fig. 3E), confirming that miR-132 was transferred from ADSCs to LECs via exosomes.

Exosomes from-ADSCs/VEGF-C (exosome-ADSCs/VEGF-C) promote LECs proliferation, migration and tube formation

We then investigated whether exosomes-ADSCs/VEGF-C promotes lymphangiogenesis by regulating LECs proliferation, migration and tube formation. LECs were treated with exosome-ADSCs or exosome-ADSCs/VEGF-C and cell proliferation was assayed using CCK-8. Fig. 4A showed that exosome-ADSCs/VEGF-C promotes LECs proliferation compared to exosome-ADSCs or control. We also assessed the role of exosome-ADSCs/VEGF-C in the regulation

Table 2. Differently expressed miRNA between Exo/ADSC control and Exo/ADSC+VEGF-C

	Exo/ADSC control	Exo/ADSC+VEGF-C	Up or Down
miR-126	153.9508	101.71	DOWN
	124.2514	111.3253	
	101.4858	118.9297	
	6.020987	17.15981	
miR-132	5.038304	16.6152	UP
	5.528374	15.32034	
	5.528374	5.320338	
	8.987999	10.61512	
miR-9	9.872763	12.17262	DOWN
	60.5865	38.34589	
	57.14203	35.47851	
	56.6141	34.5948	
miR-1236	37.66102	41.51627	UP
	39.47288	54.10103	
	40.27287	45.8273	
	112.674	131.2645	
miR-181a	114.281	134.393	UP
	115.8297	125.5152	
	1.641713	2.091984	
	2.748301	2.802094	
miR-466	1.857362	3.110209	DOWN
	1.902637	1.128296	
	1.702726	1.030202	
	1.826356	1.100204	
miR-146a	4.902637	3.128296	UP
	5.702726	4.030202	
	4.826356	5.100204	
	91.51627	37.66102	
miR-146b	94.10103	39.47288	DOWN
	95.8273	40.27287	
	0.632529	1.016558	
	0.741817	1.229374	
miR-221	0.600063	0.637263	UP
	0.832529	1.023514	
	0.784652	1.115246	
	0.686587	0.965874	
miR-27a	112.674	171.2645	UP
	114.281	174.393	
	115.8297	175.5152	
	3.021657	13.05356	
miR-486-5p	3.602155	15.33022	UP
	3.852031	17.03127	
	28.60254	18.81746	
	28.03936	27.81728	
miR-93	26.83836	17.01827	DOWN

Fig. 3. Direct transfer of miR-132 from ADSCs to LECs. (A) qPCR analysis of miR-132 expression in ADSCs after miR-132 mimics treatment * p<0.05. (B) qPCR analysis of miR-132 expression in exosomes isolated from ADSCs with or without miR-132 treatment * p<0.05. (C) qPCR analysis of miR-132 expression in LECs co-cultured with ADSCs with or without miR-132 treatment * p<0.05. (D) Fluorescence microscopy analysis was performed to assay the green fluorescent signals in LECs co-cultured with ADSCs following FAM-labeled miR-132 treatment. Scale bar = 2 µm. (E) Fluorescence microscopy analysis was performed to assay the green fluorescent signals in LECs co-cultured with exosomes isolated from ADSCs with or without FAM-labeled miR-132 treatment. Scale bar = 2 µm.

of LECs migration. Fig. 4B and C showed that exosome-ADSCs/VEGF-C significantly promoted LECs migration compared with exosome-ADSCs. We further assessed the role of exosome-ADSCs/VEGF-C in the regulation of LECs tube formation. As shown in Fig. 5A and B, exosome-ADSCs/VEGF-C promotes tube formation of LECs compared with exosome-ADSCs. Importantly, pretreatment with miR-132 inhibitor attenuated the effect of these exosomes on LECs proliferation, migration and tube formation (Fig. 4 and Fig. 5). These data demonstrated that exosomal transfer of miR-132 from ADSCs to LECs promoted LECs proliferation, migration and tube formation.

miR-132 promotes lymphangiogenic response by regulating TGF- β /Smad signaling

Transforming growth factor- β (TGF- β) is known to play an important role in lymphangiogenesis [33, 34], but the precise relationship between TGF- β and lymphangiogenesis is not well known. Additionally, the correlation of miR-132 with TGF- β /Smad signaling was revealed in several cancer, including glioma [35], prostate cancer [36] and non-small cell lung cancer [37]. Therefore, we next investigated whether miR-132 promotes lymphangiogenic response by regulating TGF- β /Smad signaling. As shown in Fig. 6A, miR-132 overexpression promoted LECs proliferation, whereas Ly2109761 (a specific inhibitor of TGF- β /Smad) treatment attenuated the effect of miR-132 on LECs proliferation. Similarly, Ly2109761 attenuated miR-132-induced migration and tube formation of LECs (Fig. 6B-D).

We then investigated whether miR-132 directly regulates TGF- β /Smad signaling. Fig. 7A showed that overexpression of miR-132 in LECs increased the level of p-Smad2/3, whereas Ly2109761 treatment inhibited miR-132-induced increase of p-Smad2/3. To identify the molecular mechanism of miR-132 involved TGF- β /Smad pathway, we assayed the expression level of main components (TGF β RI, TGF β RII, smad1, smad2, smad3, smad4, smad5 and smad7) in TGF- β /Smad pathway. As shown in Fig. 7B, smad7 expression was significantly inhibited following miR-132 overexpression among these components of TGF- β /Smad pathway. To further verify that miR-132 directly targets smad7 and inhibits its expression, we constructed luciferase reporter vector containing 3'-UTR of smad7. The reporter assay showed that miR-132 was able to markedly inhibit luciferase expression, whereas mutation

Fig. 4. Exosomes from ADSCs/VEGF-C promote LECs proliferation and migration. (A) 2×10^5 cells LECs were plated in 96-well plates and cultured in 100 μ L of EGM-2-MV overnight. After the medium was removed LECs were treated with 100 μ L of EBM-2 control, EBM-2 containing exosome-ADSCs, exosome-ADSCs/VEGF-C or exosome-ADSCs/VEGF-C+miR132 inhibitor. Cell proliferation was assayed by using CCK-8 and at the indicated time points. * $p < 0.05$. (B) 2×10^5 LECs suspended in serum-free media were plated into the upper chamber for migration assay after treatment with indicated reagents, including EBM-2 control, exosome-ADSCs, exosome-ADSCs/VEGF-C or exosome-ADSCs/VEGF-C+miR132 inhibitor. Media supplemented with 10% FBS was placed into the lower chamber. The cells that had migrated through the membrane to the lower surface were fixed, stained and counted after 48 h. * $p < 0.05$.

of 4 nucleotides in 3'-UTR of smad7 resulted in complete abrogation the suppressive effect (Fig. 7C). Forced expression of miR-132 significantly inhibited the smad7 protein level, whereas miR-132 inhibition resulted in an increase of smad7 protein level in LECs (Fig. 7D). It is well known that smad7 negatively regulates TGF- β /Smad signaling. Therefore current data suggest that miR-132 enhances the activation of TGF- β signaling, at least in part by inhibiting smad7.

Discussion

In the present study, the role of ADSCs in VEGF-C-dependent lymphangiogenesis and its underlying mechanism were investigated *in vitro*. The current data demonstrate that (i) Exosomes from ADSCs/VEGF-C have higher miR-132 expression, (ii) miR-132 can be directly transfer from ADSCs to LECs, (iii) Exosomes from ADSCs/VEGF-C promote LECs proliferation, migration and tube formation, (iv) miR-132 promotes lymphangiogenic response by regulating TGF- β /Smad signaling. These results reveal the interaction of ADSCs with LECs in VEGF-C-dependent lymphangiogenesis and identify exosome-derived miR-132 play an important role in lymphangiogenesis by regulating TGF- β /Smad signaling.

Fig. 5. Exosomes from ADSCs/VEGF-C promote LECs tube formation. 2×10^5 LECs were seeded onto the coated wells and cultured with 500 μ L of EBM-2, EBM-2 containing exosome-ADSCs, exosome-ADSCs/VEGF-C or exosome-ADSCs/VEGF-C+miR132 inhibitor. LEC tube formation was assessed by microscopy, and each well was photographed. The number of tube branches and total tube length were calculated using the MacBiophotonics Image J software.

Fig. 6. miR-132 promotes lymphangiogenic response by regulating TGF- β /Smad signaling. 2×10^5 LECs were plated in 96-well plates and cultured in 100 μ L of EGM-2-MV overnight. After the medium was removed LECs were treated with 100 μ L of EBM-2 control, EBM-2 containing miR-132, miR-132 plus LY2109761. Cell proliferation (A) and migration (B) were assayed. * $p < 0.05$. (C and D) 2×10^5 LECs were seeded onto the coated wells and cultured with 500 μ L of EBM-2, EBM-2 containing miR-132, miR-132 plus LY2109761. LEC tube formation was assessed by microscopy, and each well was photographed. * $p < 0.05$.

Fig. 7. miR-132 regulates TGF- β /Smad signaling by targeting smad7. (A) Following transfection with miR-132 (50nM), LECs were treated with LY2109761 (5 μ M) and the protein levels of total Smad2/3, p-Smad2/3 were assayed 30 minutes after treatment. (B) Following transfection with miR-132 (50nM), the expression level of main components (TGF β RI, TGF β RII, smad1, smad2, smad3, smad4, smad5 and smad7) in TGF- β /Smad pathway was assayed using quantitative RT-PCR analysis. (C) The 3'UTR reporter assay was carried out in HepG2 cells overexpressed with miR-132. pGL3-smad7-3'-UTR-WT or pGL3-smad7-3'-UTR-Mutation was co-transfected with pRL-TK. Luciferase assays were performed 48 h after transfection. Firefly luciferase activity was standardized to Renilla luciferase control. (D) Western blot analysis for endogenous smad7 protein level after miR-132 overexpression or inhibition in LECs. *p<0.05.

Modified cell-based angiogenic therapy is a promising novel strategy for several diseases such as ischemic heart, limb diseases and inflammation-related diseases [38]. Currently, major studies focus on myoblast or mesenchymal stem cells (MSCs) transplantation. However emerging studies showed extensive expansion of MSCs may contain the risk for clonal selection and subsequent malignant transformation [39, 40]. Adipose-derived stem cells (in contrast to bone marrow) are abundantly available and can be easily harvested during liposuction or surgery. Due to high paracrine activity and availability ADSCs are thought to be a preferable cell type for the therapy of several diseases. The therapeutic potential of modified ADSC is gradually being revealed [38]. Shevchenko et al. reported that ADSCs modified with VEGF165 present a more efficient angiogenic response in ischemic skeletal muscle [38]. In the study, we investigated the role of ADSCs in VEGF-C-dependent lymphangiogenic response. VEGF-C-treated ADSCs secrete exosomes, and the exosomes from ADSCs/VEGF-C promote LECs proliferation, migration and tube formation.

Emerging studies reported that cell-to-cell communication is an important mechanism for information exchange promoting cell survival via microvesicles, including ectosomes (100-1,000 nm) and exosomes (30-150 nm) [41]. Exosomes are extracellular membrane vesicles secreted from almost all human cell types [42]. Secreted exosomes can be engulfed by tissues locally or carried in biological fluids to distant sites in the body. Target cells can engulf exosomes through endocytosis, resulting in the intracellular release of bioactive molecules. We thus investigated whether exosomes from ADSCs/VEGF-C promote lymphangiogenesis by regulating LECs proliferation, migration and tube formation. Our results showed that cm-ADSCs/VEGF-C or exosome-ADSCs/VEGF-C promotes LECs proliferation, migration and tube formation.

Studies have shown that exosomes contain non-coding RNAs such as miRNAs. miRNAs that transfer between living cells that are involved in cell-to-cell communication are

frequently encapsulated in exosomes [32]. Xu et al. reported that miR-27a overexpression in hLECs promotes lymphangiogenesis by targeting SMAD4 [34]. Here we demonstrated that exosomes from VEGF-C-treated ADSCs had higher miR-132 expression. Furthermore, miR-132 could be directly transferred from ADSCs to LECs by the mediation of exosomes. Functionally, miR-132 promoted lymphangiogenic response, whereas miR-132 inhibition suppressed LECs proliferation, migration and tube formation. TGF- β plays an important role in lymphangiogenesis [33, 34], and the correlation of miR-132 with TGF- β /Smad signaling was revealed in several cancer [35, 37]. We thus investigated whether miR-132 promoted lymphangiogenic response by regulating TGF- β /Smad signaling. Current data demonstrated that miR-132 overexpression promoted lymphangiogenic response, whereas Ly2109761 (a specific inhibitor of TGF- β /Smad) treatment partially inhibited the effect of miR-132 on LECs proliferation, migration and tube formation. Finally, we demonstrated that miR-132 enhances the activation of TGF- β signaling, at least in part by inhibiting smad7.

Conclusion

ADSCs treated with VEGF-C results in a more efficient lymphangiogenic response by secreting exosomes and may provide a new therapeutic modality for boosting the efficacy of therapeutic lymphangiogenesis.

Abbreviations

IBD (Inflammatory bowel diseases); VEGF-C (Vascular endothelial growth factor-C); ADSCs (Adipose-derived stem cells); LECs (Lymphatic endothelial cells); CM (Conditioned media); ADSCs-CM (ADSCs-conditioned medium); Exo (Exosome).

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The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

The article does not contain any individual person's data in any form.

XL-W was responsible for conducting the study, under the supervision of HC-W, and contributed to the experimental design; HC-W, JL-C, and C-Y did the experiments and analyzed the data. XL-W and HC-W did immunohistochemical staining and image analysis; XL-W wrote the paper, and all authors read and approved the final manuscript.

All research and procedures involving human subjects were approved by the ethics committee of Shanghai Tenth People's Hospital. The methods in this study were carried out in accordance with the approved guidelines by Shanghai Tenth People's Hospital. All donors provided written informed consent for participation.

Disclosure Statement

All authors declare that they have no competing interests.

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