ORIGINAL PAPER

Treatment with adipose-derived regenerative cells enhances ischemia-induced angiogenesis via exosomal microRNA delivery in mice

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ABSTRACT

Adipose-derived regenerative cells (ADRCs), mesenchymal stem/progenitor cells from subcutaneous adipose tissue, have been shown to stimulate angiogenesis in hind limb ischemia, an effect attributed to paracrine action on endothelial cells (ECs) in mice. Despite promising therapeutic effects, the relevant molecules promoting neovascularization in this setting have not been fully elucidated. Extracellular vesicles, crucial mediators of intercellular communication, are recognized as a new therapeutic modality for regenerative medicine. Here, we found that GW4869, an exosome biogenesis inhibitor targeting neutral sphingomyelinase, impaired ADRCs-mediated angiogenesis and improvement of blood perfusion in a murine hind limb ischemia model. In addition, while the supernatant of ADRCs induced murine EC migration, this effect was attenuated by pre-treatment with GW4869. RNA analysis revealed that treatment of ADRCs with GW4869 reduced the expression of microRNA-21 (miR-21), miR-27b, miR-322, and let-7i in ADRCs-derived exosomes. Furthermore, the exosomes derived from GW4869-treated ADRCs induced the expression of the miR-21 targets *Smad7* and *Pten*, and the miR-322 target *Cul2*, in ECs. These findings suggest that several miRNAs in ADRCs-derived exosomes contribute to angiogenesis and improvement of blood perfusion in a murine hind limb ischemia model.

Keywords: adipose-derived regenerative cells (ADRCs), therapeutic angiogenesis, hind limb ischemia, circulating microRNA

Abbreviations:
ADRCs: adipose-derived regenerative cells
ECs: endothelial cells
CLI: critical limb ischemia
HLI: hind limb ischemia
EVs: extracellular vesicles
DMSO: dimethyl Sulfoxide
miRNA: microRNA
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF: polyvinylidene difluoride

INTRODUCTION

Patients with critical limb ischemia (CLI) have ischemic pain at rest and refractory ischemic skin ulcers. Even after surgical or endovascular treatment, a significant number of individuals will experience recurrent ischemia, usually resulting in an untreatable disease state.[1] Therapeutic angiogenesis, which promote new vessel growth in ischemic tissues by gene, protein or stem/progenitor cell implantation, is a promising treatment strategy for severe CLI.[2]

Adipose-derived regenerative cells (ADRCs), which were discovered in subcutaneous adipose tissue in 2001, have typical mesenchymal stem/progenitor cell characteristics.[3] Implantation of ADRCs improves blood perfusion recovery in CLI states of animals and humans.[4,5] Currently, the paracrine effect of ADRCs, including secretion of several growth factors, is considered to be the primary mechanism of these pro-angiogenic outcomes.[6] Circulating microRNAs (miRNAs) are emerging sources of paracrine signaling in cell therapy.[7] However, their contribution to the ADRCs-medicated angiogenesis in an ischemic limb is not fully understood.

Here, we show that pharmacological inhibition of exosomes released from ADRCs impairs the pro-angiogenic faculties of ADRCs in a murine model of hind limb ischemia (HLI). Furthermore, our findings suggest that the delivery of specific miRNAs by exosomes into endothelial cells (ECs) is responsible for enhancing their angiogenic behavior.

MATERIALS AND METHODS

Animal experiments

Male 8- to 10-week old C57Bl/6J mice were obtained from Charles River Laboratories, Japan. Mice were subjected to unilateral hindlimb ischemia surgery as described previously.[8] Briefly, the left femoral artery and vein in C57Bl/6J mice were ligated and excised gently from the proximal portion of the femoral artery to the distal portion of the saphenous artery. The remaining arterial branches, including the perforator arteries, were also ligated. ADRCs (1×10^6 cells per animal) isolated from separate C57Bl/6J mice were pretreated for 24 h with either Dimethyl Sulfoxide (DMSO) (vehicle, n=5 mice), 10 μM GW4869 (Merck, Darmstadt, Germany)(n=7 mice), or 2.5 μM manumycin A (Cayman Chemical, MI)(n=5 mice), and then phosphate-buffered saline (PBS)(n=6 mice) or pretreated-ADRCs were injected into the ischemic adductor muscle area at three different positions with 5 mm spacing starting from one day after hindlimb surgery. Hindlimb blood flow recovery was monitored by Moor LDI (Moor Instruments) before and days 0, 7, 14 after surgery, and was expressed as the ratio of blood flow in left (ischemic) to right (non-ischemic) hindlimb.

All procedures of animal care and animal use were reviewed and approved by the Animal Ethics Review Board of Nagoya University Graduate School of Medicine.

Cell culture

Isolation and culture of ADRCs was performed as previously described with slight modification.[4] Subcutaneous inguinal adipose tissue from C57Bl/6J mice was isolated, minced and digested with 2 mg/mL type I collagenase (Wako, Japan) at 37°C for 1 h, followed by filtration through a 100-μm filter (BD Falcon, Bedford, MA) and centrifugation at 200 × g for 5 minutes. The precipitated cells were used as ADRCs, maintained in DMEM (D6046, Sigma) supplemented with 10% fetal bovine serum (FBS) and used until passage 5. The supernatant from ADRCs incubated in serum-free DMEM with DMSO or with 10 μM GW4869 for 48 h was used as conditioned medium (CdM). MS1 cells, murine immortalized ECs isolated from pancreas, were obtained from

American Type Culture Collection (ATCC). MS1 cells were cultured in DMEM supplemented with 5% FBS. Cultured cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Western blot analysis

ADRCs were treated with DMSO or with 10 μM GW4869 for 24 h, washed with PBS, lysed with Cell Lysis Buffer (#9803, Cell Signaling), and quantified with BCA protein assay reagent (Thermo Fisher Scientific). Equal amounts of protein per condition were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with the indicated antibodies, followed by incubation with secondary antibodies conjugated with horseradish peroxidase. Protein signals were detected with SignalFire ECL Reagent (#6883, Cell Signaling) using the ChemiDoc imaging system (BIO-RAD). The blots were quantified with the gel analysis function in Fiji software.[9,10] The antibodies are shown in Table 1.

Table 1 List of primers and antibodies used in the present study

mRNA	Forward	Reverse	Amplicon Length
Cul2	TGAGTGTCAGCAGCGGATGGTA	CACTGGACACAGCACGGAGTAA	128 bp
Gapdh	AAAGGGTCATCATCTCCGCC	GCCCTTCCACAATGCCAAAG	171 bp
Pten	TGAGTTCCCTCAGCCATTGCCT	GAGGTTTCCTCTGGTCCTGGTA	138 bp
Smad7	GTCCAGATGCTGTACCTTCCTC	GCGAGTCTTCTCCTCCCAGTAT	143 bp

miRNA	Product information	Catalog number
miR-21	Mm_miR-21_2 miScript Primer Assay	MS00011487
miR-27b	Mm_miR-27b_1 miScript Primer Assay	MS00001358
miR-30b	Mm_miR-30b_1 miScript Primer Assay	MS00001386
miR-126	Mm_miR-126-5p_1 miScript Primer Assay	MS00006006
miR-130a	Mm_miR-130a_1 miScript Primer Assay	MS00001547
RNU6B	Hs_RNU6-2_1 miScript Primer Assay	in the miScript PCR Starter Kit
miR-210	Mm_miR-210_2 miScript Primer Assay	MS00032564
miR-296	Mm_miR-296-5p_1 miScript Primer Assay	MS00016436
miR-322	Mm_miR-322_2 miScript Primer Assay	MS00029218
let-7f	Mm_let-7f_1 miScript Primer Assay	MS00005866
let-7i	Mm_let-7i_1 miScript Primer Assay	MS00001253
miR-39	Ce_miR-39_1 miScript Primer Assay	MS00019789

Name	Company (Catalog number)	Dilution
anti-Alix (E69PB) Rabbit mAb	Cell Signaling (#92880)	1:1000
anti-beta-Actin (C4) Mouse mAb	Santa Cruz (#sc-47778)	1:2000
anti-rabbit IgG HRP-linked	Cell Signaling (#7074)	1:2000
anti-mouse IgG H&L (HRP)	Abcam (#ab6728)	1:2000

Cell Migration assay

MS1 cells were seeded at 2.1×10^4 cells per well into silicon culture inserts with two individual wells (#ib80209, Ibidi, Munchen, Germany) on cell culture slides (Ibidi) for 24 h. The culture inserts were removed, and cells were incubated with DMSO- or GW4869-treated CdM. Images were acquired using a BZ-X710 microscope (Keyence, Osaka, Japan) at 0, 24 and 36 h after cell migration, and were analyzed with Fiji software.

Cell proliferation assay

MS1 cells were seeded at 7.5×10^3 per well in a 96-well plate for 24 h, followed by incubation with DMSO- or GW4869-treated CdM. After 24 h, cells were incubated with Cell Count Reagent SF (#07553-15, NACALAI TESQUE, Kyoto, Japan) for 2.5 h. Cell viability was measured by determining the absorbance at 450 nm (reference: 650nm).

Isolation of exosomes

After 48 h incubation with 6 ml of serum-free DMEM in 100 mm dish, 6 ml (for MS1 cell culture) or 12 ml (for RNA isolation) of the supernatant was collected, filtered through Millex-GP 0.22 μm filter unit (#SLGVM33RS, Merck Millipore), and centrifuged at $10000 \times g$ for 60 min. The supernatant was transferred into Amicon Ultra-15 filter (10kDa MWCO) (Merck) and centrifuged at $5000 \times g$ for 40 min. The concentrated medium was washed twice with PBS and used for MS1 cell culture or RNA isolation.

Quantitative RT-PCR analysis

To analyze mRNA expression, total RNA was extracted from MS1 using miRNeasy Micro Kit (Qiagen). Complementary DNA (cDNA) was generated with a ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). The quantitative PCR (qPCR) was performed on a CFX96 real-time PCR detection system (BIO-RAD) using THUNDERBIRD SYBR qPCR Mix (TOYOBO). Primer pairs are listed in Table 1. Data are presented following normalization with *Gapdh*.

For miRNA expression analysis, total RNA was isolated from ADRCs or exosomes obtained using the miRNeasy Micro Kit. Briefly, 200 μl exosomes solution was lysed by 1 ml QIAzol Lysis Reagent. After 5 minutes incubation for homogeneity, 3.5 μl of Spike-In control (Ce-miR-39, RNA Spike-in Kit for RT, Qiagen) were added, mixed thoroughly and RNA isolation was performed following manufacturer's instructions. cDNA synthesis and qPCR were performed using miScript PCR Starter Kit (Qiagen). All miScript Primer Assays are listed in Table 1. RNU6B was used to normalize gene expression for ADRCs. Ce-miR-39 Spike-In control was used as an internal control for exosomes.

Statistical analysis

Data are presented as the mean ± SEM for given experiments. All statistical analyses for experiments were performed using GraphPad Prism 8 (GraphPad Software Inc, San Diego, USA). Comparisons between two groups were performed using unpaired two tailed student's t-tests. For more than two groups, we performed one-way ANOVA with Tukey's multiple comparison test to assess statistical significance with a 95% confidence interval. Significance was defined as $P<0.05$.

RESULTS

GW4869 inhibits ADRCs-mediated blood perfusion recovery in hind limb ischemia

To evaluate the effect of exosomes derived from ADRCs on angiogenesis in hind limb
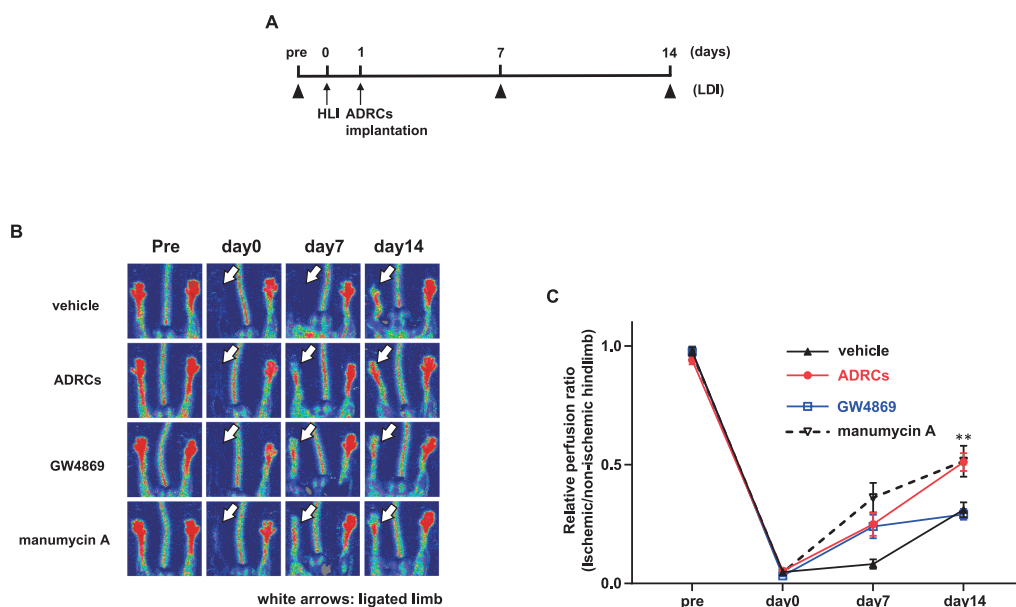
Fig. 1 Treatment of an exosome inhibitor, GW4869, attenuates pro-angiogenic effects of adipose-derived regenerative cells (ADRCs) in a murine hind limb ischemia model

Fig. 1A: Scheme showing the time points of surgical femoral artery ligation, ADRCs implantation, and laser Doppler imaging (LDI) for the blood perfusion recovery analysis.

Fig. 1B: Representative laser Doppler images of mice in the supine position. White arrows indicate the ligated limb.

Fig. 1C: Quantification of blood perfusion recovery in the ischemic limbs showed significant decreased blood perfusion in mice injected with GW4869-treated ADRCs, but not with another exosome inhibitor, manumycin A-treated ADRCs, compared with DMSO-treated ADRCs at day 14 post-injection. PBS was used as vehicle.

(At day 14 post-injection, **$P<0.01$ ADRCs vs. vehicle or GW4869-treated ADRCs, manumycin A-treated ADRCs vs. vehicle or GW4869-treated ADRCs, n=5–7, one-way ANOVA with Tukey's multiple comparison post hoc test.) Data presents mean±SEM.

ischemia, we utilized a surgical ligation of the femoral artery approach, the most commonly used mouse model of HLI as described above. Before implantation into HLI mice, ADRCs were exposed to either of two pharmacological exosome inhibitors, GW4869 and manumycin A. GW4869 inhibits neutral sphingomyelinase to regulate ceramide synthesis,[11] while manumycin A targets farnesyltransferases resulting in the inhibition of Ras activity.[12] ADRCs were injected into the ischemic muscles one day after femoral artery ligation, followed by the assessment of blood perfusion seven and fourteen days after injection with laser Doppler imaging (Fig. 1A). Mice treated with ADRCs showed robust improvement in tissue perfusion compared with controls (Fig. 1B, C). Interestingly, the ischemic limbs injected with GW4869-pretreated ADRCs, but not with manumycin A-pretreated ADRCs, showed impaired limb perfusion (Fig. 1B, C), suggesting that a subpopulation of ADRCs-derived exosomes plays an essential role in pro-angiogenic effects *in vivo*.

ADRCs-derived exosomes facilitate EC migration

Angiogenesis is a highly dynamic and coordinated process of endothelial cell behaviors including migration, proliferation, sprouting and adhesion.[13] To investigate the paracrine effects

of ADRCs on EC behavior, a wound healing assay was performed to monitor proliferation and coordinated migration. Mouse vascular MS1 endothelial cells were exposed to supernatant from ADRCs treated with vehicle or GW4869 (Fig. 2A). Treatment with supernatant from GW4869-treated ADRCs significantly impaired collective EC migration compared with that from DMSO-treated ADRCs (Fig. 2B, C). In contrast, no significant differences of MS1 cell proliferation were seen between the supernatant from ADRCs treated with vehicle and GW4869 (Fig. 2D), suggesting that exosomes from ADRCs are important for EC migration *in vitro*.

ADRCs-derived exosomes impact gene expression in donor ECs

Recent studies have shown that exosomes transfer miRNAs to modulate the function of target cells.[14] To gain more insight into the molecular mechanisms of ADRCs-derived exosomes in angiogenesis, we performed quantitative reverse transcription PCR (RT-qPCR) analysis to quantify the expression levels of 10 established pro-angiogenic miRNAs. The expression of miR-21, miR-27b, miR-322, and let-7i was reduced in exosomes isolated from GW4869-treated ADRCs in comparison with that from controls (Fig. 3A), but GW4869 treatment led to a slight, but not statistically significant, reduction of miR-21, miR-27b, miR-322, and let-7i expression in ADRCs (Fig. 3B). Immunoblotting of Alix, an exosome biogenesis marker, showed no obvious changes between control and GW4869-treated ADRCs (Fig. 3C). To further clarify the mechanisms by which miRNAs, delivered by exosomes, could act in MS1 cells, the target genes of miR-21 and miR-322 were investigated. RT-qPCR analysis revealed that the expression levels of *Smad7* and *Pten*, miR-21 targets,[15] and the miR-322 target *Cul2*,[16] were increased in MS1 cells upon exposure to exosomes derived from GW4869-treated ADRCs, when compared to exosomes from controls (Fig. 3D).

Taken together, these results suggest that paracrine effects of ADRCs on therapeutic angiogenesis can be partly attributed to a subpopulation of exosome-mediated miRNAs.

DISUCUSSION

The present studies show that a subpopulation of exosomes derived from ADRCs play a pivotal role in improving angiogenesis and blood perfusion in hind limb ischemia, and that this function stems in part from exosome-mediated delivery of select miRNAs which can have pro-angiogenic effects on ECs.

Cell therapy is a promising approach for cardiovascular regenerative medicine for ischemic diseases.[17] A recent meta-analysis of 19 randomized controlled trials reveals that cell therapy for CLI reduces the risk of amputation and improves amputation-free survival and wound healing.[18] Paracrine signaling including soluble growth factors, cytokines and chemokines released from implanted cells has been shown to play an important role in angiogenesis. Extracellular vesicles (EVs) are known to play pivotal roles in the maintenance of physiological functions, homeostasis and regeneration, provide a novel intercellular signaling mechanism, and are emerging as potential therapeutic modalities.[19] It has been reported that exosomes derived from human CD34+ stem cells promote angiogenesis and improve limb perfusion in a rodent hind limb ischemia model.[20] Though a promising therapeutic approach, a key requirement for the safe and effective use of EVs in clinical settings would be unambiguous identification and quality control of the desired kind and cargo of such agents. EVs, which include not only exosomes but also microvesicles, consist of heterogeneous populations with distinct composition and function.[21] Several different cellular mechanisms for the biogenesis of exosomes exist, and the resulting heterogeneity may be a function of which pathway was used to produce a particular exosome. Exosomes are generated

A


B

C

D
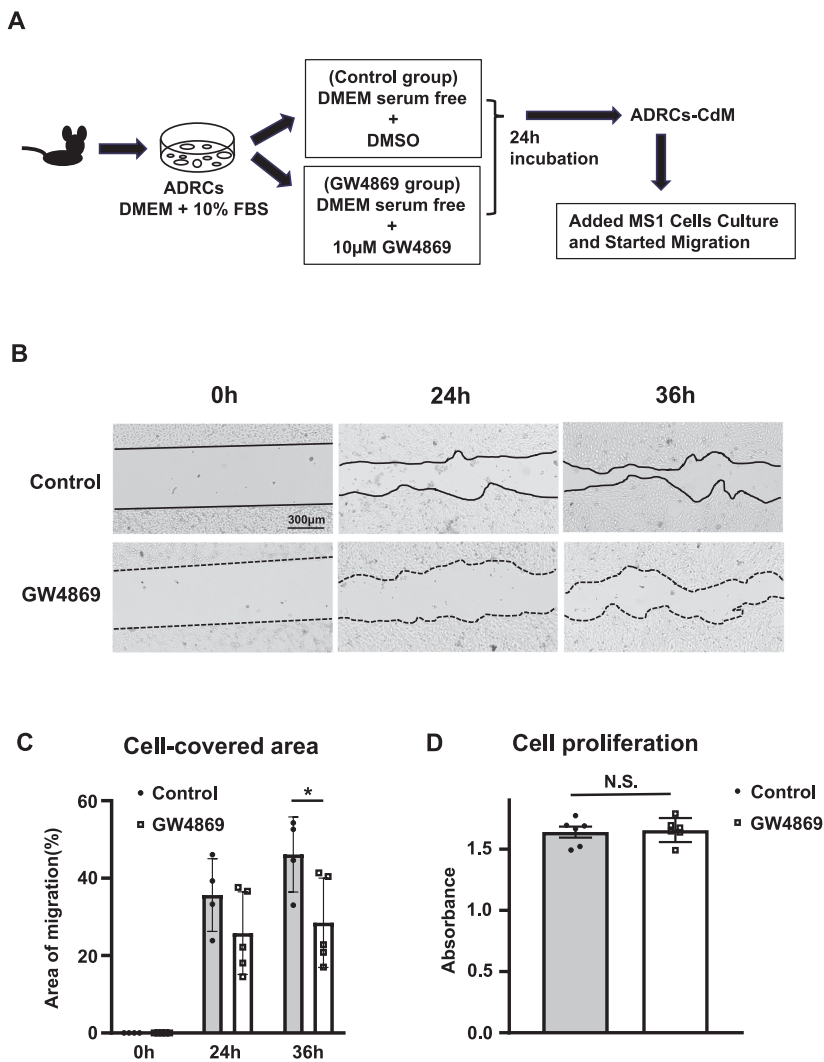
Fig. 2 Supernatant derived from ADRCs promotes endothelial cell (EC) migration

Fig. 2A: Scheme showing the experimental procedure of ADRCs-conditioned medium (CdM) preparation and MS1 cell (murine EC) migration assay.

Fig. 2B: Representative images of cell migration assay. Scale bar, 300 μm.

Fig. 2C: Quantification of the cell-covered area showed impaired cell motility in GW4869 group compared with that in ADRCs group. (*P<0.05, n=4–5, unpaired t-test)

Fig. 2D: Cell proliferation assay using WST-8 colorimetric reagent of MS1 with ADRCs-CdM with or without 10 μM GW4869 for 48 h revealed that there was no significant difference between the two groups. MS1 cells were exposed to ADRCs-CdM for 24 h. (n=6, unpaired t-test) Data presents mean±SEM.

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A miRNA expression in exosomes from ADRCs-CdM
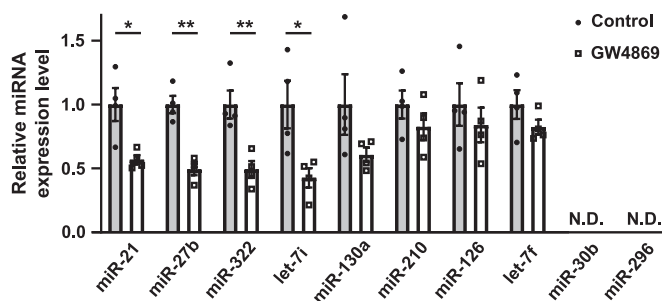
B miRNA expression in ADRCs
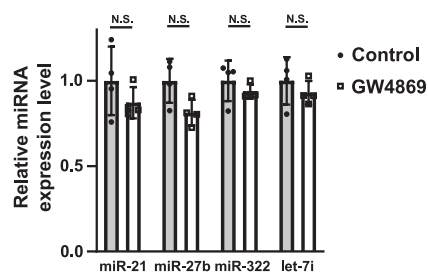
C
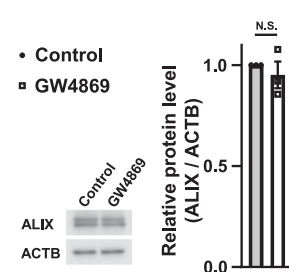
D miRNA expression in MS1 Cells
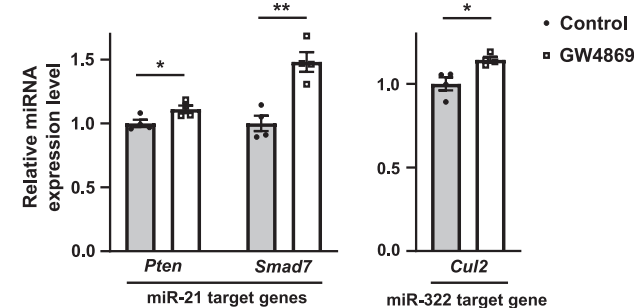
Fig. 3 GW4869 attenuates pro-angiogenic effects of ADRCs by inhibiting miRNA transfer

Fig. 3A: Quantitative reverse transcription PCR (RT-qPCR) analysis of miRNAs in exosomes isolated from ADRCs-conditioned medium (CdM). The expression levels of miR-21, -27b, -322 and let-7i were significantly decreased in GW4869 group, whereas the expression of other angiogenic miRNAs was unchanged. ADRCs were treated with DMSO or 10 μM GW4869 for 48 h. (*P<0.05 in miR-21 and let-7i; **P<0.01 in miR-27b and miR-322, n=4, unpaired t-test) N.D.: not detectable.

Fig. 3B: RT-qPCR analysis of miRNAs in total ADRCs lysates revealed that there were no significant differences of miR-21, -27b, -322 and let-7i expression between control and GW4869 group. ADRCs were exposed to DMSO (control) or 10 μM GW4869 for 48 h. (n=4, unpaired t-test)

Fig. 3C: Western blot analysis of ALIX and ACTB expression in total ADRCs lysates. ADRCs were treated with DMSO (control) or 10 μM GW4869 for 24 h. (n=3) Data presents mean±SEM.

Fig. 3D: RT-qPCR analysis of *Smad7* and *Pten* (miR-21 targets), and *Cul2* (miR-322 target) expression in MS1 cells. MS1 cells were exposed to ADRCs-CdM for 24 h, which was derived from ADRCs treated with DMSO (control) or 10 μM GW4869 for 48 h. (*P<0.05 in *Pten* and *Cul2*; **P<0.01 in *Smad7*, n=4, unpaired t-test)
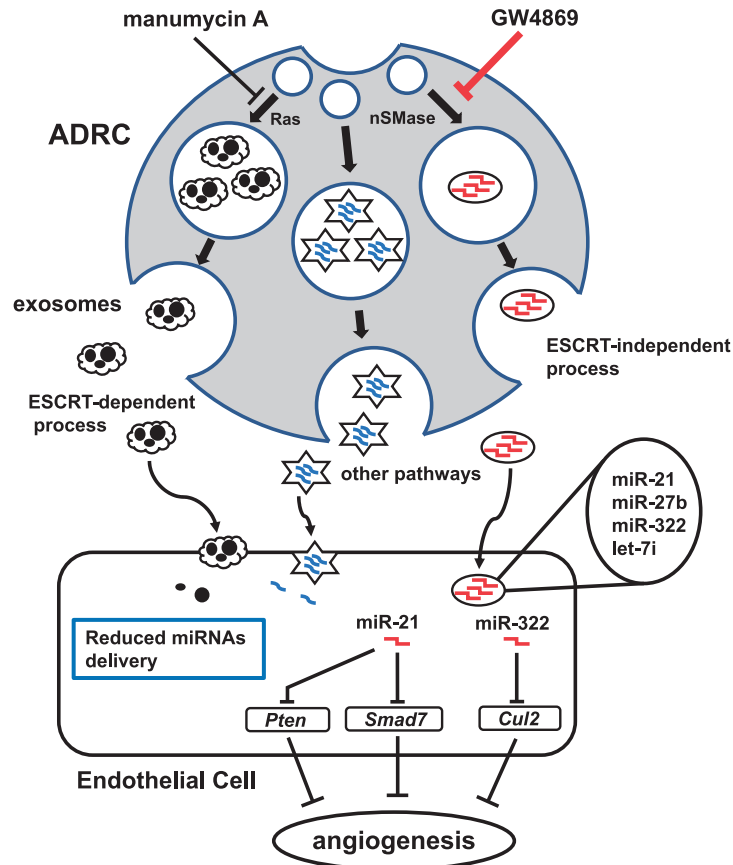
Fig. 4 Model of EC angiogenic responses to ADRCs-derived exosomal miRNAs
GW4869 inhibits exosome biogenesis in the ESCRT-independent process by inhibiting membrane neutral sphingo-myelinase (nSMase), resulting in the reduced expression of miR-21, miR-27b, miR-322, and let-7i in exosomes derived from GW4869-treated ADRCs. Endothelial cells (ECs) receive a reduced amount of those miRNA from exosomes, which leads to up regulation of *Pten*, *Smad7*, and *Cul2* in ECs. These molecules act to suppress the angiogenic ability of ECs.
Black dots, blue lines, and red lines indicate miRNAs.
ESCRT: endosomal sorting complex required for transport

in an endosomal sorting complex required for transport (ESCRT)-dependent or -independent mechanism in different cell types.[22] In our study, administration of ADRCs pretreated with manumycin A, that inhibits the ESCRT-dependent pathway, restored the perfusion of ischemic limb, whereas the use of ADRCs pretreated with GW4869, inhibiting the ESCRT-independent pathway, did not. These results suggest that a subpopulation of exosomes might be important for the pro-angiogenic properties of ADRCs.

miRNAs represent a class of small noncoding RNAs that can bind to complementary target sites in specific mRNA molecules and repress translation or cause mRNA degradation. Recent studies have shown that miRNAs are key regulators of cardiovascular functions in physiology and pathology.[23] While we cannot exclude a possibility that growth factors and cytokines in EVs contribute to promoting neovascularization, VEGF and miR-126 levels in exosomes obtained from

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human placenta-derived MSCs are known to be responsible for proangiogenic effects on limb ischemia in a murine model.[24] Administration of EVs enriched with miR-210 and VEGF from mouse bone marrow-derived MSCs promoted vessel formation in the ischemic limb.[25] Treatment with exosomes from rat adipose-derived MSCs overexpressing miR-21, which were abundant in SDF-1, HIF-1α and VEGF, enhanced the angiogenic ability of cultured ECs.[26] Our results indicate that the expression level of miR-126 in exosomes isolated from GW4869-treated ADRCs did not show significant reduction. Instead, we found reduced expression of miR-21, miR-27b, miR-322, let-7i in GW4869-treated ADRCs-derived exosomes. We have shown that the expression of *Smad7*, *Pten* and *Cul2* in MS1 cells was increased by exosomes isolated from GW4869-preconditoned ADRCs. Transforming growth factor β (TGF-β) family signaling is important for the regulation of angiogenesis through a variety of cellular responses, such as proliferation, differentiation and migration. SMAD7 is originally identified as a vascular SMAD and known as an antagonist of TGF-β family signaling by specifically inhibiting the phosphorylation of receptor-regulated SMADs.[27] Smad7 has been described to attenuate peritoneal angiogenesis.[28] Likewise, Smad7-deficient mice exhibit massive growth retardation with reduced viability.[29] A recent study showed that SMAD7 is a key regulator of EC quiescence.[30] Another signaling pathway, the phosphoinositide 3-kinase (PI3K) signaling pathway, is a potent regulator of many aspects of cell function and is essential for angiogenesis downstream of a variety of receptors. The Ser/Thr protein kinase AKT is the major downstream effector of PI3K signaling, while PTEN, a lipid phosphatase, antagonizes PI3K-mediated signaling of cell growth, survival and migration.[31] It has been shown that PTEN and SMAD7 are direct targets of miR-21 in different cell types, resulting in modulation of AKT- and SMAD-dependent signaling.[15] Hypoxia-inducible factors (HIFs) control physiological and pathological angiogenesis.[32] miR-424, and its murine orthologue miR-322, reduced CUL2 expression, a scaffolding protein involved in the assembly of the ubiquitin ligase system, resulting in the stabilization of HIFs.[16] Taken together, our data suggest that miRNAs in ADRCs-derived EVs may coordinate different key signaling pathways in ECs to tune and promote angiogenesis in hind limb ischemia (Fig. 4).

In summary, our study suggests that ADRCs-derived exosomes play an important role in improving angiogenesis and blood perfusion recovery in hind limb ischemia. These findings could pave the way for the application of exosomes from ADRCs as a useful, cell-free therapeutic agent to stimulate angiogenesis in ischemic cardiovascular diseases in the future.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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