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RESEARCH ARTICLE

Exosomes secreted by mesenchymal stem cells promote endothelial cell angiogenesis by transferring miR-125a

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ABSTRACT

Angiogenesis plays crucial roles in various physiological processes including wound healing and tissue repair. It requires a tight interaction between endothelial cells and their surrounding environment. Mesenchymal stem cells (MSCs), one of the non-endothelial cell types present in the perivascular environment, have been shown to secret exosomes to modulate intercellular communications between MSCs and their target cells. In this study, we initially isolated exosomes secreted by human adipose-derived MSCs (adMSC-Exo) and examined their roles in angiogenesis. We found that adMSC-Exo could be taken up by endothelial cells and significantly promote angiogenesis *in vitro* and *in vivo*. Further study showed that miR-125a was enriched in adMSC-Exo, and repressed the expression of the angiogenic inhibitor delta-like 4 (DLL4) by targeting its 3′ untranslated region. Additionally, adMSC-Exo and its exosomal transferred miR-125a could repress DLL4 expression and modulate endothelial cell angiogenesis through promoting formation of endothelial tip cells. In conclusion, our study indicates that adMSC-Exo can transfer miR-125a to endothelial cells and promote angiogenesis by repressing DLL4. adMSC-Exo, as a pro-angiogenic factor, might be a promising candidate for therapeutical tissue repair.

KEY WORDS: Exosome, miR-125a, DLL4, Angiogenesis

INTRODUCTION

Angiogenesis, the formation of new vascular sprouts from pre-existing blood vessels, is a complex multistep progress involving endothelial cell activation, proliferation, migration, invasion, and sprouting of endothelial cells, as well as maturation and stabilization of newly formed sprouts (Adams and Alitalo, 2007; Carmeliet and Jain, 2011). Each of the steps involves a complex interaction between endothelial cells and their corresponding extracellular environment (Patel-Hett and D'Amore, 2011). Several types of cells, such as mesenchymal stem cells (MSCs), have been identified as creating specific microenvironments to control blood vessel density and function (Bronckaers et al., 2014; Watt et al., 2013). Understanding how blood vessels communicate with MSCs in their surrounding microenvironment is a major challenge for diseases involving angiogenesis.

MSCs are multipotent cells that reside in various tissues (da Silva Meirelles et al., 2006). A variety of studies have demonstrated that they can be recruited to sites of inflammation and injury (Gnecchi et al., 2008), and these effects might result from exosomes, an important paracrine mediator between MSCs and target cells (Ranganath et al., 2012; Katsuda et al., 2013). Exosomes are small membraned vesicles (30–100 nm), originating from the inward budding of the endosomal membrane, which can carry complex biological information, including mRNAs, microRNAs (miRNAs) and soluble proteins into target cells (Valadi et al., 2007; Raposo and Stoorvogel, 2013). Accumulated evidence has proved that exosomes secreted by MSCs show promise in different tissue repair processes including reducing injury caused by myocardial ischemia–reperfusion (Lai et al., 2010), promoting neurological recovery from stroke (Xin et al., 2013), and promoting wound healing through improving skin cells survival (Zhang et al., 2014). A few studies have also demonstrated that the underlying mechanism of these effects is most likely acting through miRNAs (Hulsmans and Holvoet, 2013; Ouyang et al., 2014).

miRNAs are highly conserved noncoding RNAs (18–24 nucleotides) that regulate target gene expression by mediating mRNA degradation or translational inhibition (Carthew and Sontheimer, 2009). Increasing evidence indicates that miRNAs are important regulators of angiogenesis (Kane et al., 2014). Here, to better understand the exosome-mediated intercellular communication between endothelial cells and MSCs in angiogenesis, we investigated the roles of exosomes secreted by human adipose-derived MSCs (adMSC-Exo) in angiogenesis. We observed that adMSC-Exo enhanced endothelial cell tube formation both *in vitro* and *in vivo*. Furthermore, advanced studies showed that miR-125a in adMSCs could be transferred into endothelial cells through exosomes and promote tip cell specification through direct suppression of its target delta-like 4 (DLL4). To the best of our knowledge, this is the first report to show that exosomal transferred miR-125a has the ability to promote angiogenesis. Because adMSC-Exo is a pro-angiogenic factor, it might be a potential candidate for tissue repair and other diseases involving angiogenesis.

RESULTS

Isolation and characterization of adMSC-Exo

To study the roles of exosomes in endothelial cell angiogenesis, adMSC-Exo were first isolated and characterized as previously described (Lin et al., 2013) (Fig. S1). Transmission electron microscopy analysis showed that exosomes purified from the adMSCs culture supernatants were round membrane-bound vesicles with a size ranging from 30–100 nm in diameter (Fig. 1A). Western blotting demonstrated that the exosomal marker proteins CD63, TSG101 and HSP70 family proteins, were present in these exosomes as expected (Fig. 1B). To further investigate whether the adMSC-Exo could be transferred into endothelial cells, exosomes were labeled with Dil dye and incubated with human

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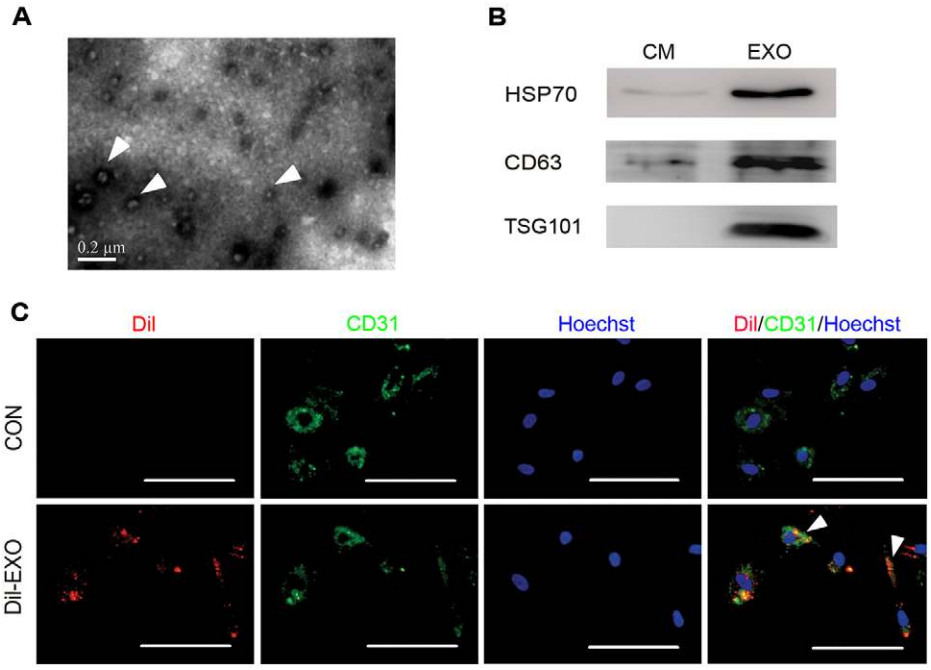

Fig. 1. Characterization of adMSC-Exo. (A) Electron micrograph of exosomes isolated from MSC-conditioned medium. Arrowheads indicate exosomes. Scale bar: 200 nm. (B) Western blotting was performed with adMSC-Exo (EXO) or MSC-conditioned medium (CM). CD63, TSG101 and HSP70 expression in exosomes was detected. (C) HUVECs were incubated with Dil-labeled exosomes (Dil-EXO; Dil is shown in red) or carrier control (CON, control) and uptake of exosomes was observed in HUVECs. Endothelial cells were co-stained with antibodies against CD31 (green) and nuclei were stained with Hoechst 33342 (blue). Arrowheads indicate Dil-labeled exosomes within CD31-positive endothelial cells. Scale bars: 50 μm.

umbilical vein endothelial cells (HUVECs) *in vitro*. The uptake was confirmed by fluorescence microscope. After 6 h, over 90% of HUVECs were Dil positive, showing that the Dil-labeled exosomes had been taken up and transferred to cytoplasm compartments (Fig. 1C). The results shown in Fig. S2 further confirmed that the red label in Fig. 1C was caused by real exosome internalization, not by free Dil dye. Taken together, these data suggest that adMSC-Exo can be successfully isolated and could be transferred to HUVECs efficiently.

adMSC-Exo promotes endothelial cell angiogenesis *in vitro* and *in vivo*

To examine the effect of adMSC-Exo on angiogenesis, primary HUVECs were treated with various concentrations of exosomes (25 μg/ml, 50 μg/ml, 100 μg/ml and 200 μg/ml) for 48 h. We found that the expression levels of the pro-angiogenic genes Ang1 (also known as ANGPT1) and Flk1 (also known as KDR) in HUVECs were upregulated when the concentration of exosomes is between 0–100 μg/ml (Fig. 2A,B) and that expression of the anti-angiogenic genes Vash1 and TSP1 (also known as THBS1) were substantially downregulated (Fig. 2C,D). Moreover, we found that Flk1 and Ang1 expression levels were highest in the cells treated with 100 μg/ml adMSC-Exo. Thus, we treated the HUVECs with 100 μg/ml exosomes in the following experiments. We further evaluated the effects of adMSC-Exo on angiogenesis *in vitro* by performing a tube formation assay. As expected, tube formation of HUVECs was significantly enhanced in the exosome-treated group as determined by the increase of the tube length (Fig. 2E,F) and the number of branches (Fig. 2E,G). To assess the role of adMSC-Exo in endothelial cell angiogenesis *in vivo*, we performed Matrigel plug assays in immunodeficient mice by subcutaneously injecting HUVECs alone or HUVECs mixed with adMSC-Exo. Consistent with the *in vitro* data, immunohistochemical analysis showed that the number of vascular structures was much higher in the exosome-treated group than in the untreated group *in vivo* (Fig. 2H). Taken together, our observations suggest that adMSC-Exo promotes angiogenesis of endothelial cells both *in vitro* and *in vivo*.

Exosomal transferred miR-125a mediates pro-angiogenic activity of adMSC-Exo

Previous studies have demonstrated MSCs release a substantial amount of microvesicles containing coding and noncoding RNAs, including miRNAs with multiple functional properties (Valadi et al., 2007). Thus, we hypothesized that adMSC-Exo might enhance angiogenesis of HUVECs by transferring specific miRNA. Hence, we performed microarray-based miRNA expression profiling (data not shown). According to this data, we found that miR-125a was enriched in adMSC-Exo, and we validated this result by miRNA-specific quantitative real-time PCR (qRT-PCR) analyses (Fig. 3A). To investigate whether the miR-125a was transferred into HUVECs, we first transfected adMSCs with FAM-miR-125a and then labeled the secreted exosomes containing FAM-miR-125a with Dil. As expected, both the red and green signals were detected in the cytoplasm of HUVECs exposed to these Dil-labeled exosomes under fluorescence microscopy (Fig. 3C). Moreover, the miR-125a level was significantly increased in HUVECs treated with exosomes compared to in the untreated group (Fig. 3B). To further evaluate the effect of exosomal transferred miR-125a on angiogenesis, we performed miR-125a knockdown in adMSCs using a miR-125a inhibitor (miR-125aI). Data showed that the miR-125a level was significantly decreased in exosomes secreted by adMSCs transfected with miR-125a inhibitor (125aI-Exo) compared to those from cells transfected with inhibitor control (NCI-Exo) (Fig. 3D). For HUVECs treated with 125aI-Exo, qRT-PCR analysis showed that the expression levels of pro-angiogenic genes (Ang1 and Flk1) were significantly downregulated, which was accompanied with upregulation of anti-angiogenic genes (Vash1 and TSP1) (Fig. 3E,F). A tube formation assay also showed that the tube length and number of branches in the 125aI-Exo group was significantly decreased (Fig. 3G–I). Additionally, to reinforce the effects of MSC-secreted exosomal miR-125a on angiogenesis, we co-cultured HUVECs with MSCs transfected with miR-125a or miR-125aI and performed a tube formation assay. As expected, tube formation of HUVECs was significantly enhanced when co-cultured with MSCs

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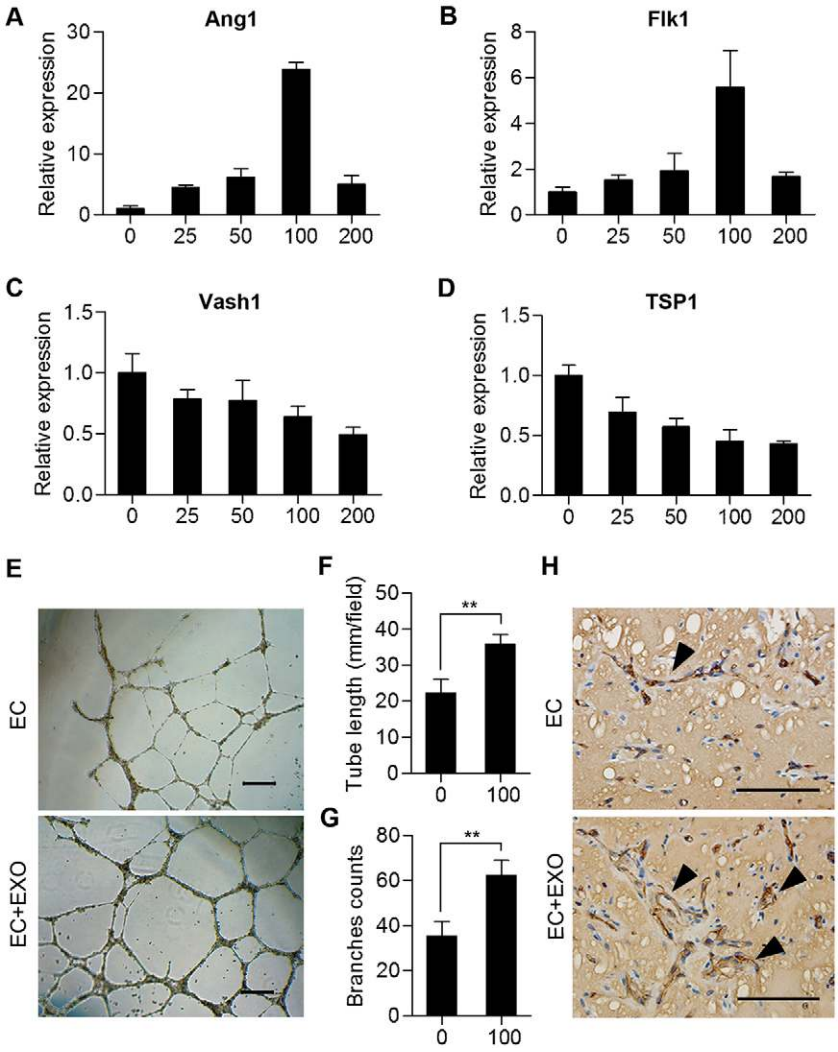

Fig. 2. adMSC-Exo promotes endothelial cell angiogenesis *in vitro* and *in vivo*. (A–D) HUVECs were incubated with various concentrations of adMSC-Exo (25 µg/ml, 50 µg/ml, 100 µg/ml and 200 µg/ml) or carrier control (PBS) for 48 h. The mRNA levels of Ang1, Flk1, Vash1 and TSP1 were evaluated by qRT-PCR. Results are mean±s.d. (n=3 for each group). (E) Screening for enhanced HUVEC (EC) tube formation *in vitro* after treatment with or without adMSC-Exo (100 µg/ml). Scale bar: 200 µm. (F,G) Quantitative evaluation of tube length and number of branches after treating HUVECs with adMSC-Exo; the tube length and number of branches were calculated in three random fields. Results are mean±s.d. $^{**}P$<0.01 (Student's t-test). (H) Microvessel structures were analyzed by a Matrigel plug assay *in vivo*. Arrowheads indicate microvessel structures. Scale bars: 50 µm.

transfected with miR-125a, but was decreased when co-cultured with MSCs transfected with miR-125aI (Fig. S3). Thus, our results indicate that the pro-angiogenic activity of adMSC-Exo is mediated by miR-125a.

Overexpression of miR-125a in HUVECs enhances angiogenesis *in vitro*

To further study the effect of miR-125a on angiogenesis, synthesized miR-125a mimics were transfected directly into cultured HUVECs. qRT-PCR analysis showed that the expression levels of pro-angiogenic genes (Ang1 and Flk1) were substantially upregulated in HUVECs transfected with miR-125a mimic, which was accompanied by downregulation of anti-angiogenic genes (Vash1 and TSP1) compared to the mimic control (NC) group (Fig. 4A,B). Furthermore, a tube formation assay showed that the tube length and number of branches in HUVECs transfected with miR-125a mimics significantly increased (Fig. 4C–E). Combined with results of miR-125a knockdown in adMSC-Exo by miR-125a inhibitor, these results confirm that miR-125a in exosomes plays a crucial role in endothelial cell angiogenesis.

DLL4 is a direct downstream target of miR-125a

To further reveal the molecular mechanism underlying the pro-angiogenic activity of miR-125a, we predicted potential downstream targets of miR-125a by in silico analyses using TargetScan. Among the predicted candidates, we focused on DLL4, a ligand of Notch signaling pathway, which has been proven to repress angiogenesis (Tung et al., 2012). According to the computational analysis, the miR-125a target site in the 3′UTR of DLL4 is highly conserved among vertebrates and partially complementary to miR-125a (Fig. 5A). To validate whether miR-125a repressed the expression of DLL4 through binding to 3′UTR of DLL4, a luciferase assay was performed. Luciferase reporters that contained either a wild-type (pRL-TK-DLL4-WT) or mutated (pRL-TK-DLL4-MUT) DLL4 were transfected into 293T cells, along with either the miR-125a mimic or the mimic control. Overexpression of miR-125a significantly suppressed the luciferase activity of the pRL-TK-DLL4-WT reporter plasmid (~49.6%, compared to mimic control) but not that of pRL-TK-DLL4-MUT reporter plasmid (Fig. 5B). Therefore, these results indicate that DLL4 is a direct target of miR-125a through the specific binding site in its 3′UTR.

Exosomal transferred miR-125a modulates endothelial tip cell specification through DLL4

Previous studies have indicated that DLL4 regulates endothelial cell sprouting during angiogenesis by inhibiting endothelial tip cell formation (Tung et al., 2012). In order to confirm that adMSC-

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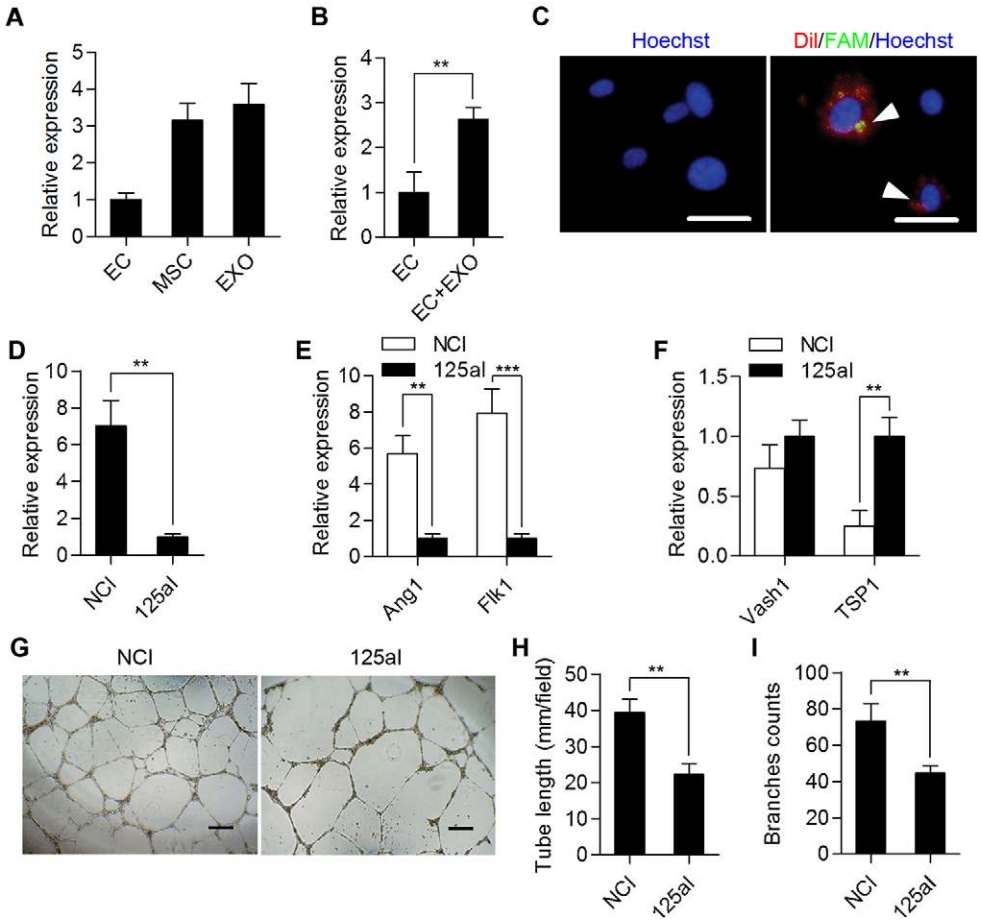

Fig. 3. Exosomal transferred miR-125a mediates the pro-angiogenic activity of adMSC-Exo. (A) The mRNA levels of miR-125a in endothelial cells (EC), adMSCs and adMSC-Exo (EXO) were analyzed by qRT-PCR. (B) The mRNA levels of miR-125a in HUVECs treated with adMSC-Exo were analyzed by qRT-PCR. (C) Exosomes derived from adMSCs transfected with FAM-miR-125a were labeled with DiI and then added to HUVECs. FAM-miR-125a signals are green and DiI is red. Nuclei were stained with Hoechst 33342 (blue). Arrowheads indicate the exosomal transferred miR-125a in cytoplasm of HUVECs. Scale bars: 50 µm. (D) The levels of miR-125a in NCI-Exo and 125aI-Exo were analyzed by qRT-PCR. (E,F) HUVECs were incubated with inhibitor control NCI-Exo or 125aI-Exo (100 µg/ml). The mRNA levels of Ang1, Flk1, Vash1 and TSP1 were evaluated by qRT-PCR. The qRT-PCR values are presented as the mean±s.d. (n=3 for each group). $^{**}P$<0.01; $^{***}P$<0.001 (Student's t-test). (G) Screening for enhanced HUVEC tube formation *in vitro* after treatment with NCI-Exo or 125aI-Exo (100 µg/ml). Scale bars: 200 µm. (H,I) Quantitative evaluation of tube length and branches after treating HUVECs with NCI-Exo or 125I-Exo (100 µg/ml), the tube length and number of branches were calculated in three random fields. Results are mean±s.d. $^{**}P$<0.01 (Student's t-test).

Exo and miR-125a modulated angiogenesis through DLL4, we examined the DLL4 protein expression level and tip cell formation of HUVECs either exposed to adMSC-Exo or transfected with miR-125a mimic. Western blotting results showed that DLL4 protein expression was significantly decreased in HUVECs exposed to exosomes and transfected with miR-125 compared to the control group (Fig. 5C,D). Tip cell formation was evaluated by staining for CD34, a marker of endothelial tip cells (Siemerink et al., 2012). An immunostaining assay showed that HUVECs treated with adMSC-Exo or transfected with miR-125a mimics had an increased

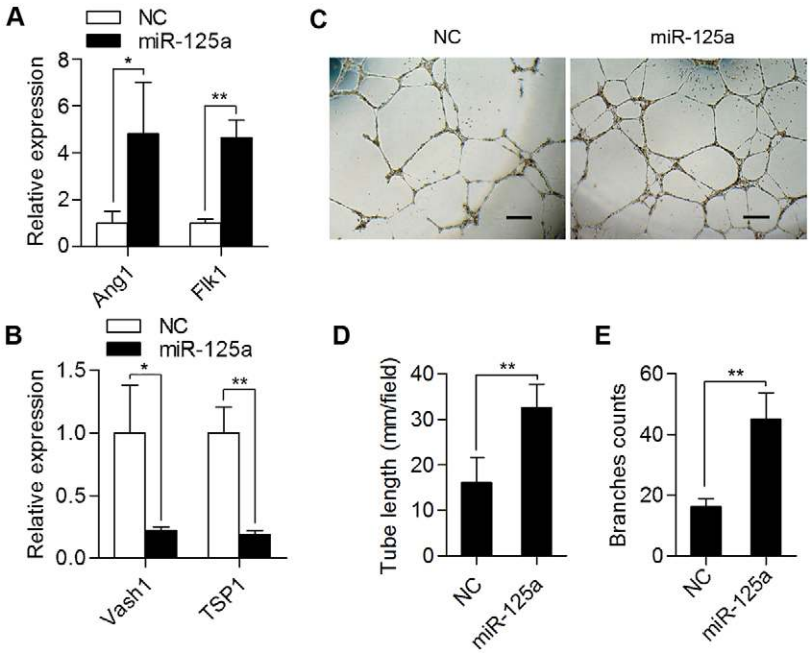

Fig. 4. Overexpression of miR-125a in HUVECs enhances angiogenesis *in vitro*. (A,B) HUVECs were transfected with mimic control (NC) or miR-125a mimics. The mRNA levels of Ang1, Flk1, Vash1 and TSP1 were evaluated by qRT-PCR. The qRT-PCR values are presented as the mean±s.d. (n=3 for each group). $^{*}P$<0.05; $^{**}P$<0.01 (Student's t-test). (C) Screening for tube formation in HUVECs transfected with NC or miR-125a mimics. Scale bars: 200 µm. (D,E) Quantitative evaluation of tube length and number of branches after HUVECs were transfected with NC or miR-125a mimics, the tube length and number of branches were calculated in three random fields. Results are mean±s.d. $^{**}P$<0.01 (Student's t-test).

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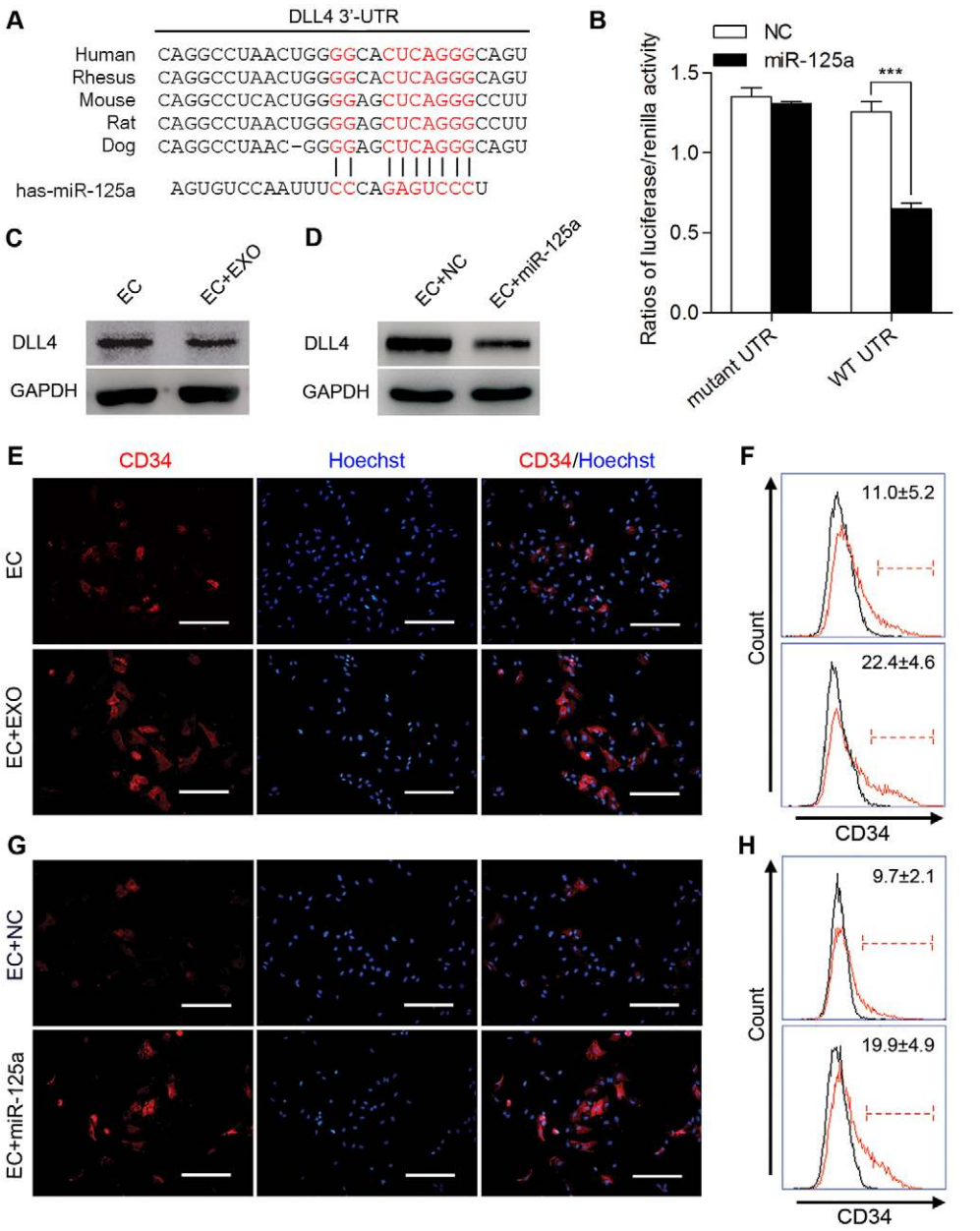

Fig. 5. Exosomal transferred miR-125a modulates endothelial tip cell specification by targeting DLL4. (A) Schematic diagram of the putative miR-125a-binding site in the 3′UTR regions of DLL4. The seed sequence of miR-125a matches 3′UTR regions of DLL4 (red). (B) Dual luciferase assay on wild-type (WT) or mutant DLL4 3′UTR in 293T cells transfected with mimic control (NC) or miR-125a, and calculated as the luciferase activity ratio of firefly to *Renilla* luciferase. Results are mean±s.d. ***P<0.001 (Student's t-test). (C,D) HUVECs (EC) were treated with adMSC-Exo (100 µg/ml) or transfected with miR-125a. The expression of DLL4 was determined by a western blot assay. (E,G) Immunofluorescence analysis of CD34$^+$ (red) endothelial tip cells after HUVECs were treated with adMSC-Exo (100 µg/ml) or transfected with miR-125a. Nuclei were stained with Hoechst 33342 (blue). Scale bars: 50 µm. (F,H) HUVECs were treated with adMSC-Exo (100 µg/ml) or transfected with miR-125a. The CD34$^+$ population was analyzed by flow cytometry. Data are means±s.d. from three independent experiments.

formation of endothelial tip cells compared with the control group (Fig. 5E,G). To confirm this finding, we used flow cytometry to quantify CD34 expression. The results showed that the proportion of CD34$^+$ endothelial tip cells in the exosome- (22.4±4.6%) and miR-125a-treated group (19.9±4.9%) was significantly increased compared to the untreated group and mimic control group (11.0±5.2% and 9.7±2.1%) (mean±s.d.; n=3 for each group) (Fig. 5F,H). Thus, we conclude that adMSC-Exo and its exosomal transferred miR-125a can repress DLL4 expression and modulate endothelial cell angiogenesis through promoting endothelial tip cell formation.

DISCUSSION

Intercellular communication is required for various physiological and pathological processes. Accumulated evidence suggests that, apart from releasing soluble factors, cells can communicate through exosomes (Bang and Thum, 2012; Schorey et al., 2015), a small membraned vesicle, which can directly transfer various bioactive molecules including mRNAs, microRNAs and proteins from donor cells to recipient cells. Exosomes also have an important effect in a wide range of diseases (EL Andaloussi et al., 2013; Danielson and Das, 2014; Hwang, 2013). They have been shown to promote cell migration and metastasis (Luga et al., 2012), reduce myocardial ischemia–reperfusion injury (Arslan et al., 2013), regulate the cytotoxic activity of natural killer cells (Reiners et al., 2014), and facilitate tumor cell survival and expansion of the tumor (Pap et al., 2011). In the present study, we successfully isolated and purified exosomes from human adipose-derived MSCs. Besides demonstrating that Dil-labeled exosomes could be transferred to the cytoplasm of HUVECs, we further showed that adMSC-Exo increased endothelial cell tube formation *in vitro* and vascularization of implanted Matrigel plugs *in vivo*. These results predict that adMSC-Exo is a positive regulator of angiogenesis.

Recently, many miRNAs have been identified to be selectively packaged into exosomes and several studies have confirmed that exosomal transferred miRNA can modulate the activities of target cells (Xin et al., 2012; Umezu et al., 2013, 2014). However, the

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effects of miRNAs secreted by adMSC-Exo on endothelial cell angiogenesis are poorly understood. Our previous study has found that a group of miRNAs are highly expressed in human adipose-derived MSCs. In this study, we predicted that miRNAs might modulate angiogenesis of HUVECs by in silico analyses. Indeed, we found that knockdown of miR-125a in adMSC-Exo partially diminished their pro-angiogenic activity. To confirm the role of miR-125a in this process, we overexpressed miR-125a by directly transfecting miRNA into HUVECs. The results were consistent with those from knocking down miR-125a in adMSC-Exo, and this suggests that miR-125a plays crucial roles in the pro-angiogenic activity of adMSC-Exo. To our knowledge, this is the first study demonstrating the modulation of endothelial cell angiogenesis by miRNA transferred from adMSC-Exo.

In the present study, we found that the expression levels of the pro-angiogenic genes Ang1 and Flk1 in HUVECs were upregulated when treated with adMSC-Exo. As the pro-angiogenic genes Ang1 and Flk1 were not detectable within adMSC-Exo, this suggests that the exosome is not simply transferring the mRNA to target cell HUVECs but is actually causing induction of these mRNA. Various studies have shown that many miRNAs are involved in angiogenesis (Urbich et al., 2012; Schober et al., 2014). Here, to determine the role and molecular mechanism of exosomal transferred miR-125a during angiogenesis, we used computational bioinformatics to predict that DLL4 is a potential target of miR-125a. Western blot and luciferase assays confirmed that miR-125a bound directly to the 3′UTR of DLL4 mRNA and inhibited its translation. DLL4, a transmembrane protein and Notch ligand, is required to regulate the balance between endothelial tip and stalk cell specification. There is a high expression of DLL4 in endothelial tip cells; this protein then interacts with Notch1 receptors in neighboring endothelial cells, and thereby prevents the those endothelial cells from being specified as tip cells and, hence, suppresses angiogenesis (Sainson et al., 2005). Recent studies also show that inhibition of DLL4–Notch signaling increases the number of the endothelial cells committed to the tip cell fate and augments tube branch density (Leslie et al., 2007; Niessen et al., 2011; Napp et al., 2012). Hence, we considered that adMSC-Exo and exosomal transferred miR-125a might promote angiogenesis through induction of endothelial tip cell formation. And as expected, HUVECs treated with adMSC-Exo or overexpressing miR-125a effectively increased the proportion of $CD34^+$ tip cells, in agreement with observations of inhibition of DLL4 in endothelial cells (Hellstrom et al., 2007). These findings indicate a new role of exosomal transferred miR-125a in regulating endothelial tip cell specification by modulating its target DLL4.

The study of angiogenesis will lead to better understanding of various physiological and pathological processes, such as vascular disease, wound healing and tumorigenesis (Carmeliet, 2005). Although our study demonstrates a potential role of exosomal miR-125a, further study is required in order to determine the overall importance of exosomal miR125a compared to the wider secretome, as well as the mechanisms behind exosome-induced mRNA expression. In summary, our findings provide novel insights into the intercellular communications between endothelial cells and MSCs in the surrounding microenvironment. The further investigation of exosomal transferred miRNAs might offer opportunities for the therapeutic modulation of diseases involving angiogenesis.

MATERIALS AND METHODS

Cell culture

Human adipose tissues and human umbilical cords were obtained with informed consent and all experiments were approved by the Ethics Committee at the Chinese Academy of Medical Sciences and Peking Union Medical College, and all clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki.

MSCs were isolated from human adipose tissues obtained from patients undergoing tumescent liposuctions as previously described. After isolation, cells were plated in a 75-cm^2 culture flask at a density of 2×10^6 in 12 ml of regular growth medium (Cao et al., 2005) and incubated at 37°C and 5% CO_2. adMSCs from the third passage were used in the experiments.

HUVECs were prepared and cultured as routinely described (Baudin et al., 2007). In brief, veins were isolated and washed twice with PBS, and endothelial cells were flushed out after digestion with 0.1% collagen P (Roche) for 15 min at room temperature. HUVECs were seeded onto gelatin-coated plastic dishes in Medium 200 containing Large Vessel Endothelial Supplement (Life Technologies).

Exosome purification

Exosome purification was performed as previously described (Ohshima et al., 2010). Briefly, adMSC culture medium was collected and centrifuged at 800 ***g*** for 5 min and then for an additional 10 min at 2000 ***g*** to remove lifted cells. Then the supernatant was subjected to filtration on a 0.2-µm pore membrane filter, followed by concentration using a 100,000-kDa molecular mass cut-off membrane (Millipore) to remove large membrane vesicles. The supernatant was ultracentrifuged at 100,000 ***g*** for 1 h at 4°C using a 70Ti rotor (Beckman Coulter). The resulting pellets were resuspended in 10 ml PBS and ultracentrifuged at 100,000 ***g*** for 1 h at 4°C.

Exosomes uptake

Purified adMSC-Exo were labeled with 1 µM Dil (Invitrogen) as previously described. Briefly, adMSC-Exo were mixed with 1 µM Dil, and the exosome–dye suspension was incubated for 5 min with regular mixing. Excess dye from the labeled exosomes was removed by ultracentrifugation at 100,000 ***g*** for 1 h at 4°C using a 70Ti rotor (Beckman Coulter), and the exosome pellets were washed three times by resuspending them in PBS. The final pellets were resuspended in PBS. Dil-labeled exosomes were co-cultured with HUVECs for 6 h, then HUVECs were washed with PBS, and fixed in 4% paraformaldehyde (PFA). The uptake was observed by fluorescence microscopy.

miRNA mimics and miRNA inhibitor transfection

HUVECs at 80% confluency were transfected with 50 nM miRNAs using Lipofectamine 2000 in Opti-MEM (Invitrogen) according to the manufacturer's procedures. The synthetic miR-125a mimic, miR-125a inhibitor (miR-125aI), mimic control (NC) and inhibitor control (NCI) were purchased from Invitrogen. After transfection for 5 h, the culture medium was replaced with Medium 200 containing Large Vessel Endothelial Supplement (Life Technologies).

Tube formation assay in Matrigel

In vitro capillary network formation was determined by performing a tube formation assay in Matrigel (BD Biosciences). HUVECs were resuspended in culture medium with the indicated concentration of adMSC-Exo for 48 h, then HUVECs (10^5) were seeded onto Matrigel-coated 48-well plates and cultured in high-glucose Dulbecco's modified Eagle's medium (H-DMEM) supplemented with 10% fetal bovine serum (FBS) for 16 h at 37°C. Triple wells were set for each concentration. Tube formation was examined by microscopy (Olympus, Tokyo, Japan), and the branch density and tube length was quantified by randomly selecting three fields per well.

Matrigel plug assay

Male BALB/c nude mice (6–8 weeks old) were purchased from Vital River Laboratories (VRL, Beijing, China), and the experimental setup was approved by the Ethical Committee for Animal Research at PUMC (Beijing, China). Eight mice were randomly divided into two groups. HUVECs treated with or without adMSC-Exo (100 µg/ml) were harvested and resuspended in PBS. Aliquots of the cells (3×10^6 cells in 200 ml) were mixed with 200 ml of Matrigel. Nude mice were inoculated by subcutaneous injection on the dosal region with the mixture. On day 7 after the implantation, the Matrigel plugs were trimmed out and collected.

Immunohistochemistry staining

The Matrigel plug samples were fixed in 10% formalin and embedded in paraffin; 5-µm thick sections were cut for immunohistochemistry staining and examined under a microscope. Tissue sections underwent citrate-based antigen retrieval, and blocking with 5% goat serum for 1 h. The sections were incubated in anti-human CD31 antibodies (1:100; Abcam, ab24590) at 4°C overnight. The sections were developed with DAB and counterstained with haematoxylin. Different sections were prepared from three Matrigel plugs in each group.

Immunofluorescence

HUVECs were fixed in 4% PFA for 15 min and extensively washed with PBS to remove fixative. Cells were blocked for 1 h at room temperature using PBS supplemented with 5% BSA, then incubated with primary monoclonal mouse anti-CD34 antibody (1:100; Abcam, ab8536) diluted in blocking solution at 4°C overnight, followed by secondary phycroethrin-conjugated anti-mouse-IgG antibody (1:200; Santa Cruz Biotechnology) for 2 h at room temperature. Cultures were extensively washed with PBS. Nuclei were stained with 1 mg/ml Hoechst 33342 (Sigma-Aldrich). The stained cells were examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Luciferase assays

The putative miR-125a recognition element from the DLL4 gene was cloned in the 3′UTR of the firefly luciferase reporter vector according to the manufacturer's guidelines (catalog number C8021, Promega). The oligonucleotide sequences were designed to carry the *Xba1* and *Not1* sites at their ends to facilitate ligation into corresponding sites of pRL-TK-Report (Promega). The oligonucleotides used in these studies are as follows: DLL4 sense, 5′-TCGAGTCCCACCAGCCAAGGGTGCCAGGCCTAACTGGGGCATTTTTTTCAGTGTGTTGGAAATTCCACTGAGGGGGAAATCAGC-3′ and DLL4 antisense, 5′-GGCCGCTGATTTCCCCTCAGTGGAATTTCCAACACACTGAAAAAAATGCCCCAGTTAGGCCTGGCACCCTTGGCTGGTGGGAC-3′. For the luciferase activity assay, 5.0×10^4 cells in 24-well plates were co-transfected with 0.5 µg of the indicated pRL-TK firefly luciferase construct and 40 ng of a PGL3 *Renilla* luciferase normalization control, together with 200 nM miR-125a mimic or its corresponding control. Luciferase assays were performed 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each transfected well. All experiments were performed in triplicate.

Flow cytometry

Flow cytometric analysis was performed with a flow cytometer BD Accuri™ C6 (BD Biosciences). Cells were stained with the endothelial tip cell marker FITC-conjugated antibodies against CD34 (1:100; ebioscience, 11-0349) using standard procedures. Data were analyzed with CFlow plus 1.0.

RNA isolation and qRT-PCR analysis

Total RNA was isolated from culture cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total exosome RNA was extracted with the Total Exosome RNA & Protein Isolation Kit (Life Technologies) as described by the manufacturer. Relative expression of mRNA or miRNA was evaluated by the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH or U6, respectively. Primers for real-time PCR used were as follows: miR-125a reverse transcription primer, 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAGG-3′; miR-125a PCR primers, sense, 5′-CTGGAGUCCCUGAGACCCUUUA-3′ and anti-sense, 5′-ACGCTTCACGAATTTGCGTGTC-3′; U6 reverse transcription primer, 5′-AAAATATGGAACGCTTCACGAATTTG-3′; U6 PCR primers, sense, 5′-CTCGCTTCGGCAGCACATATACT-3′ and anti-sense, 5′-ACGCTTCACGAATTTGCGTGTC-3′; Ang1, sense, 5′-CTTCAAGGCTTGGTTACTCGTC-3′ and anti-sense, 5′-CTCTTCCTCTCTTTTTCCTCCC-3′. Flk1 sense, 5′-AACGACTGCCTTATGATGCC-3′ and anti-sense, 5′-ACTGTCCTGCAAGTTGCTGTC-3′; Vash1 sense, 5′-CTCAAGATTGGCAAAGGGACG-3′ and anti-sense, 5′-TTTCACTGCGGCTGTTCCTGC-3′; TSP1 sense, 5′-GGTGATGTATGAAGGGAAGAA-3′ and anti-sense, 5′-GGAAGCCAAGGAGAAGTGAT-3′.

Western blot analysis

Cells were harvested in RIPA lysis buffer (Beyotime) with 1 mM phenylmethanesulfonyl fluoride (PMSF), quantified by a BCA Protein Assay Kit (Beyotime), and separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were blocked in 5% non-fat milk in TBST for 1 h, and incubated in primary antibodies overnight at 4°C, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Signals were visualized with an immobilon western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore) and detected by an ImageQuant LAS 4000 mini imaging system (GE Healthcare). β-actin was used as an internal control. Primary antibodies used were as follows: DLL4 (1:100; goat IgG, Santa Cruz Biotchnology, sc-18640), CD63 (1:100; rabbit IgG, SBI, EXOAB-KIT-1), TSG101 (1:100; rabbit IgG, abcam, ab133586), HSP70 (1:100; rabbit IgG, SBI, EXOAB-KIT-1), β-actin (1:1000; mouse IgG, Santa Cruz, sc-8432); and HRP-conjugated anti-rabbit-IgG (NeoBioscience), HRP-conjugated anti-goat-IgG (NeoBioscience), HRP-conjugated anti-mouse-IgG (NeoBioscience).

Statistics and data analysis

Statistical significance ($P<0.05$) was determined using the Student's *t*-test. Data are presented as the mean±s.d., and the data are representative of at least three separate experiments conducted in triplicate.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Xiaolei Liang and Lina Zhang performed the experiments, collected and assembled data, analyzed and interpreted data, and wrote the manuscript. Shihua Wang analyzed and interpreted data, and wrote the manuscript. Qin Han designed the study, provided study material, and approved the final version of the manuscript. Robert Chunhua Zhao conceived and designed the study, provided study material, and approved the final version of the manuscript.

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Supplementary information

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