Microvesicle-mediated Transfer of MicroRNA-150 from Monocytes to Endothelial Cells Promotes Angiogenesis

**Results:** Secreted miR-150 from monocyte induce endothelial cell tube formation *in vitro* and angiogenesis *in vivo*, and down-regulation of miR-150 inhibits angiogenesis caused by diabetes, cancer, and atherosclerosis.

**Conclusion:** Monocyte-derived miR-150 can induce angiogenesis via targeting endothelial cells.

**Significance:** Our study illustrates the new role of a secreted miRNA in angiogenesis.

Blood vessels deliver oxygen and nutrients to every part of the body, remove waste, and provide gateways for the entry and exit of patrolling immune cells. Angiogenesis, the formation of new blood vessels, is essential for the growth of cells that lack proximity to existing blood vessels (1). Angiogenesis occurs in a number of normal physiological conditions (e.g. embryogenesis, reproduction, and wound healing) (1) as well as in many pathological processes, including various types of cancer (2, 3) and obesity-related disorders such as atherosclerosis and diabetes (4–6).

The angiogenic process involves several cell types and mediators that interact to establish the specific microenvironment suitable for the formation of new capillaries from pre-existing vessels. Monocytes, macrophages, and endothelial cells are active participants in some of the scenarios that can lead to angiogenesis (7–10). Monocytes and macrophages secrete soluble pro-angiogenic factors (e.g. interleukin-1 and tumor necrosis factor-α) and anti-angiogenic factors (e.g. interleukin-12 and interferon-γ) that may affect the proliferation, survival, apoptosis, and migration of endothelial cells (7–10). Therefore, angiogenesis is a result of the net balance between the activities exerted by these positive and negative mediators. For this reason, a full understanding of angiogenesis may depend on our ability to understand these cellular "collaborators" that either promote or mitigate angiogenesis. Despite sustained research efforts, there are still gaps in our understanding of *in vivo* angiogenesis. The molecular mechanism essential for communication between monocytes/macrophages and endothelial cells during angiogenesis remains to be fully elucidated.

The phenomenon of microRNA (miRNA) secretion and its role in intercellular communication has recently gained increased attention. miRNAs are small, endogenous, non-coding RNAs that regulate gene expression by acting on target
mRNAs and promoting either degradation or translational repression (11–13). Intensive studies have provided evidence that microvesicles (MVs) released from many cell types can transfer miRNAs to neighboring or distant cells, where these exogenous miRNAs function similarly to endogenous miRNAs to regulate target gene expression and recipient cell function (14–17). Secreted miRNAs may, therefore, serve as a novel class of signaling molecules for mediating cell-to-cell communication. In the present study we demonstrate for the first time that miR-150 secreted from monocytes enhances angiogenesis in vitro and in vivo. Furthermore, secreted miR-150 may serve as a novel therapeutic target for many angiogenesis-related diseases.

MATERIALS AND METHODS

Cell Culture—The human acute monocytic leukemia cell line THP-1 was purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), and cultured in standard RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Invitrogen). The human microvascular endothelial cell line HMEC-1, which was provided by Dr. E. W. Ades (Centers for Disease Control and Prevention, Atlanta, GA), was cultured in MCDB-131 medium (Invitrogen) supplemented with 10% FBS, 10 ng/ml epidermal growth factor (BD Biosciences), and 10 ng/ml hydrocortisone (Sigma). The human embryonic kidney 293T cells were cultured in DMEM high glucose medium supplemented with 10% FBS.

Patient Characteristics and Clinical Features—We recruited blood samples from 60 normal controls, 4 breast cancer patients, 3 hepatocellular carcinoma patients, 4 non-small cell lung cancer patients, 4 colon cancer patients, 23 type 2 diabetes patients, and 4 atherosclerosis patients from the Ruijin Hospital (Shanghai, China) and the Affiliated Drum Tower Hospital of Nanjing University Medical School (Nanjing, China) between September 2010 to June 2012. These patients were all histologically diagnosed, and all the blood samples were collected before any operation, chemotherapy, and/or radiation treatment. All of the patients or their guardians provided written consent, and the Ethics Committee from Nanjing University approved all aspects of this study. The clinical features of the patients are listed in supplemental Table 1. Five milliliters of venous blood were collected from each participant at his/her first admission to the hospital. Only atherosclerosis patients supplied 50 ml of venous blood for microvesicle isolation. The blood samples were drawn into sterile tubes with 10% anticoagulant to harvest plasma. After leaving these samples standing for 20 min, we centrifuged them at 20 °C, 1500 g for 20 min. Then the supernatant was quickly removed and stored immediately at −80 °C.

Animals—C57BL/6J (male, 8 weeks old) and ob/ob mice (male, 8 weeks old) were purchased from the Model Animal Research Center (MARC) of Nanjing University (Nanjing, China) and were maintained in pathogen-free conditions. All the animal housing and surgical procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition) and were approved by the Institutional Review Board of Nanjing University.

MV Isolation—MVs were isolated from human plasma or cell culture medium by differential centrifugation using a previously published technique (15). Briefly, after cells and other debris were removed by centrifugation at 300 × g, 1200 × g, and 10000 × g, the supernatant was centrifuged at 110,000 × g for 2 h (all steps were performed at 4 °C). The MVs were collected from the pellet and resuspended in FBS-free medium. The BCA method was employed to quantify the total protein content in the MVs. The levels of MVs were determined by measuring the total protein content, which was presented as micrograms of total protein in the MVs.

RNA Isolation and Quantitative RT-PCR of Mature miRNAs—Total RNA was extracted from cells or MVs using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. Quantitative RT-PCR (qRT-PCR) was performed using TaqMan miRNA probes (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Briefly, total RNA was reverse-transcribed to cDNA using avian myeloblastosis virus reverse transcriptase (Takara, Dalian, China) and a stem-loop reverse transcription primer (Applied Biosystems). Real-time PCR was performed using a TaqMan PCR kit and an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems). All reactions, including no-template controls, were performed in triplicate. After the reaction, the CT values were determined using fixed threshold settings. A series of synthetic miRNA oligonucleotides at known concentrations were also reverse-transcribed and amplified to generate the standard curve. By referring to the standard curve, the absolute expression levels of each target miRNA were calculated. The expression levels of miRNAs in cells were normalized to U6 snRNA, whereas the expression levels of target miRNAs in the MVs were directly normalized to the total protein content of the MVs.

Transfection—Synthetic pre-miR-150, anti-miR-150, and scrambled negative control RNAs (pre-ncRNA and anti-ncRNA) were purchased from Ambion (Austin, TX). THP-1 and 293T cells were transfected with equal amounts of anti-miR-150, pre-miR-150, or scrambled negative control RNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Plasmid Construction and siRNA Interference Assay—An siRNA sequence targeting human c-Myb cDNA was designed and synthesized by Invitrogen. A scrambled siRNA was included as a negative control. A mammalian expression plasmid encoding the human c-Myb open reading frame (EX-H3756-M29) was purchased from GeneCopoeia (Germantown, MD). An empty plasmid served as a negative control. The c-Myb expression vector and c-Myb siRNA were transfected into HMEC-1 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Cell Migration Assay—HMEC-1 cells were suspended in serum-free MCDB-131 culture medium at a concentration of 4 × 10⁵ cells/ml and incubated either with or without THP-1 or 293T MVs for 2 h. Next, a 100-μl cell suspension was added to the upper compartment of a Transwell Boyden Chamber (6.5 mm, Costar) (4 × 10⁶ cells/well), whereas 500 μl of MCDB-131 with 10% FBS was added to the lower compartment. After a 4-h plate incubation, non-migrant cells on the upper surface of the
filter membrane were removed using a cotton swab. Cells that had passed through the polycarbonate membrane (8-μm pore size, coated with 0.1% gelatin matrix) were fixed with 90% ethanol for 15 min at room temperature. The membrane was then washed 3 times with distilled water and stained for 15 min with 0.1% crystal violet in 0.1 M borate and 2% ethanol. Images of migrating cells were captured by a photomicroscope (BX51, Olympus, Japan). Cell migration was quantified by blind counting the migrating cells on the lower surface of the membrane; five fields per chamber were counted.

Angiogenesis in Vitro (Capillary Tube Formation Assay)—
The in vitro endothelial tube formation assay was performed as previously described (18, 19). Briefly, 100 μl of Matrigel (BD Bioscience) was added to each well of a 24-well plate and allowed to polymerize at 37 °C for 30 min. HMEC-1 cells were suspended in FBS-free MCDB-131 medium and seeded in each well at a concentration of 1 × 10⁵ cells/well. The cells were incubated at 37 °C in the presence of saline solution (control), THP-1, or 293T MV (20 μg of total protein per well). Each treatment was repeated in three wells. After 6 h, the cells were examined under a light microscope to assess the formation of capillary-like structures. The branch points of the formed tubes, which represent the degree of angiogenesis in vitro, were scanned and quantified in five low-power fields (200×).

Angiogenesis in Vivo (Matrigel Plug Assay)—Matrigel is a soluble basement membrane extract from the Engelbreth-Holm-Swarm tumor. Previous work has shown that when Matrigel is injected subcutaneously, endothelial cells migrate into the Matrigel plug and form vessels. Adding known angiogenic growth factors, such as basic fibroblast growth factor (bFGF), to the Matrigel can accelerate this angiogenic process. The newly formed vessels can contain erythrocytes, which indicates that they are functional capillaries (18, 19).

In vivo Matrigel plug assays were performed as previously described (18, 19). Briefly, 500 μl of growth factor-reduced Matrigel (BD Bioscience) was mixed with 30 units of heparin (Sigma) and 150 ng of bFGF (Sigma). The mixture was subcutaneously injected into the mid-abdominal area of C57BL/6J mice, tumor-implanted mice, or ob/ob mice. Eight mice were used for each group. The injected Matrigel rapidly formed a solid gel that persisted for at least 10 days in mice. Starting on the day the Matrigel was injected, saline solution, THP-1 MVs, or 293T MVs (20 μg of total protein per mouse) were injected intravenously into the mice every other day for a total of 4 injections. The mice were sacrificed after 8 days, and the Matrigel plugs were removed and photographed. The plugs were further fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin. To perform a quantitative analysis, the number of erythrocyte-filled blood vessels in the Matrigel (capillary density) was counted in five independent fields from three sections.

For the subcutaneous xenograft tumor model, mouse sarcoma S-180 cells (1 × 10⁶) were suspended in 100 μl of PBS and then injected subcutaneously into the left armpits of C57BL/6J mice. Fourteen days after tumor implantation, the mice were injected subcutaneously with Matrigel as described above. MVs were injected intravenously at a dose of 20 μg per mouse every other day for a total of four injections per mouse. Control mice were treated with saline. After 8 days, the mice were sacrificed, and the Matrigel plugs were analyzed. The size and the weight of tumors were measured simultaneously.

Western Blotting—The levels of c-Myb protein were quantified by Western blot analysis of whole cell extracts using antibodies against c-Myb (C19, Santa Cruz). Normalization was performed by blotting the same samples with an antibody against GAPDH (6C5, Santa Cruz). Photos of the Western blot assay were analyzed using Bandscan software (Glyko, Novato, CA).

Statistical Analysis—The images of the Western blot and Matrigel plug assay are representative of at least three independent experiments. The values for the qRT-PCR, cell migration, and capillary tube formation assays were from three independent experiments performed in duplicate. In vivo Matrigel plug assays were conducted with eight mice in each group. The data are presented as the means ± S.E. of the mean. Differences were considered statistically significant at p < 0.05 using Student’s t test.

RESULTS

Higher Plasma miR-150 Levels Are Observed in Cancer Patients or Patients with Obesity-related Disorders—The expression levels of a panel of miRNAs from the plasma of patients with various type of cancers (e.g. breast cancer, hepatocellular carcinoma, non-small cell lung cancer, and colon cancer) and patients with type 2 diabetes or atherosclerosis were assayed by TaqMan probe-based qRT-PCR. As shown in supplemental Fig. 1A, miR-150 displayed elevated levels in the plasma of patients with all types of diseases when compared with the control counterparts. Further analysis revealed that the serum levels of miR-150 in the atherosclerosis patients were markedly enriched in MVs (supplemental Fig. 1B), which is one way that miRNAs can be delivered into recipient cells. These results suggest that miR-150 is actively secreted into the circulation under disease conditions, which leads to elevated plasma miR-150 levels in various patients.

THP-1 MVs Enhance Angiogenesis by Facilitating Migration and Capillary Tube Formation of Endothelial Cells—Because miR-150 is an immune-related miRNA abundantly expressed in human monocytes (19), we hypothesized that under inflammatory conditions, monocytes would secrete miR-150 into circulation and that the secreted miR-150 might serve as a signaling molecule that modulates the function of endothelial cells. To test this hypothesis, we assessed the effects of MVs secreted from THP-1, a human monocytic cell line, on the motility capacity of endothelial cells in a Transwell chamber. In this experiment a human microvascular endothelial cell line (HMEC-1) was used as the recipient cell for MVs released from THP-1 cells. The MVs release by 293T cells served as a control. As expected, THP-1 cells contained a significantly higher level of miR-150 than HMEC-1 or 293T cells (supplemental Fig. 2A). Consistent with this observation, the levels of miR-150 in THP-1 MVs were also higher than those observed in 293T MVs (supplemental Fig. 2B). Incubation of HMEC-1 cells with THP-1 MVs dramatically increased the miR-150 levels in HMEC-1 cells (∼12-fold). In contrast, treatment with MVs derived from control 293T cells had no effect on the level of
miR-150 expression in HMEC-1 cells (supplemental Fig. 2C). The protein level of c-Myb, a known target of miR-150 (20, 21), was significantly reduced in HMEC-1 cells after incubation with THP-1 MVs but not with 293T MVs (supplemental Fig. 2D). These results suggest that the secreted miR-150 from monocytes can effectively enter into recipient endothelial cells, where it reduces c-Myb expression levels. Furthermore, the migration of HMEC-1 cells through Transwell filters was significantly enhanced by THP-1-derived MVs, whereas 293T MVs only slightly increased the number of migratory cells (supplemental Fig. 2, E and F), suggesting that MVs containing high levels of miR-150 can enhance the transmigration of endothelial cells across Transwell filters. These results are consistent with our earlier study which also showed that secreted miR-150 in THP-1 MVs can enter into HMEC-1 cells and effectively reduce c-Myb expression and enhance migration in HMEC-1 cells (15).

An important step in angiogenesis is the stimuli-specific migration of endothelial cells out of existing blood vessels to organize new capillaries (1). To investigate the effect of secreted miR-150 on angiogenic morphogenesis, we used an in vitro assay that allowed the visualization of the capillary-like tube structures, which resemble microvascular networks on Matrigel (18, 19). We first assessed the effects of EGF and THP-1 MVs on the capacity of HMEC-1 cells to form tubes (Fig. 1A). As expected, HMEC-1 cells developed capillary-like structures when treated with EGF (supplemental Fig. 3, A and B). When a gradual series of THP-1 MVs containing an increased level of miR-150 (Fig. 1B) were incubated with HMEC-1 cells, miR-150 levels were consistently increased in the recipient HMEC-1 cells (supplemental Fig. 3C). Likewise, the branch points of capillary-like structures increased dramatically to levels comparable with those observed for EGF treatment when HMEC-1 cells were solely incubated with THP-1 MVs, and this effect was dose-dependent (Fig. 1, C and D). Compared with THP-1 MVs, 293T MVs, which contained low levels of miR-150, did not significantly affect miR-150 levels in the recipient HMEC-1 cells (supplemental Fig. 3D) and displayed a reduced capacity for promoting tube formation in HMEC-1 cells (Fig. 1, E and F).

We next conducted Matrigel plug assays using a mouse model to investigate whether THP-1 MVs are capable of stimulating in vivo angiogenesis (18, 19). The design of the experiment is shown in Fig. 1G. The THP-1 MV-treated mice displayed significantly elevated plasma levels of miR-150 compared with their control counterparts (Fig. 1H). The plugs from the mice treated with saline solution or 293T MVs were pale and pink in color, whereas the plugs in mice treated with THP-1 MVs were a dark red color (Fig. 1I), indicating that a large number of new blood vessels had been formed in these Matrigel plugs. The histochemical examination and quantitative evaluation of results indicated that the Matrigel plugs from the THP-1 MV-injected mice contained a large number of erythrocyte-filled blood vessels (Fig. 1J).

The Role of Monocyte-secreted miR-150 in Promoting Angiogenesis—Although we showed that the THP-1 MVs that contain high levels of miR-150 enhance the tube formation capability of HMEC-1 cells, it could also be possible that MV components other than miR-150 are responsible for the angiogenic characteristics of endothelial cells. To confirm the pro-angiogenic effect of secreted miR-150 in monocyteic MVs, we performed several experiments. First, we knocked down miR-150 expression in THP-1 cells by transfecting these cells with antisense oligonucleotides against miR-150 (anti-miR-150). Efficient knockdown of miR-150 in THP-1 cells is shown in supplemental Fig. 4A. The knockdown of miR-150 in the THP-1 cells resulted in the production of THP-1 MVs that contained a low level of miR-150 (supplemental Fig. 4B). Next, original THP-1 MVs and the “modified” THP-1 MVs that were miR-150-deficient (THP-1 MV/miR-150 Δ) were used to treat HMEC-1 cells. Although THP-1 MVs increased miR-150 levels in HMEC-1 cells, miR-150-deficient THP-1 MVs had no effect on miR-150 levels in HMEC-1 cells (supplemental Fig. 4C). In agreement with this, the THP-1 MV-induced increase in tube formation in HMEC-1 cells was largely reversed after miR-150 was specifically depleted in the THP-1 MVs (Fig. 2, A and B). However, when we knocked down miR-150 in THP-1 cells to obtain miR-150-defective MVs, it is quite likely that multiple targets regulated by miR-150 are affected in the producer cells, and these target proteins might be the ones to regulate angiogenesis in the MVs. To ensure that the phenotype of the target cells is only modulated by miR-150 in the MVs but not by other molecules that are themselves directly or indirectly modulated by miR-150 in the MV-producing cells, we treated the endothelial cells with original THP-1 MVs and then knocked down miR-150 in the endothelial cells with anti-miR-150 to analyze the tube formation. Although THP-1 MVs enhanced tube formation in HMEC-1 cells, anti-miR-150 significantly attenuated the effect of THP-1 MVs on tube formation (supplemental Fig. 5, A and B). The results suggest that it was miR-150 in the THP-1 MVs that accounted for the pro-angiogenic effect. Second, although 293T cells and 293T MVs contain low levels of miR-150, the overexpression of miR-150 in the 293T cells with pre-miR-16 (a synthetic RNA oligonucleotide duplex mimicking mir-16 precursor) (supplemental Fig. 4D) resulted in the production of 293T MVs that contained high levels of miR-150 (293T MV/miR-150) (supplemental Fig. 4E). Although 293T MVs did not affect the levels of miR-150 in HMEC-1 cells, miR-150-overexpressed 293T MVs significantly up-regulated miR-150 levels in HMEC-1 cells (supplemental Fig. 4F). Consistent with this, the normal 293T MVs did not affect tube formation in HMEC-1 cells, whereas the modified MVs, 293T MV/miR-150, promoted tube formation (Fig. 2, C and D). Additionally, HMEC-1 cells were directly treated with pre-miR-150 followed by assessment of tube formation. Efficient overexpression of miR-150 after transfection with pre-miR-150 is shown in supplemental Fig. 4G. Elevated exogenous miR-150 significantly enhanced tube formation in the HMEC-1 cells (Fig. 2, E and F). Finally, HMEC-1 cells were treated with siRNA against c-Myb and assessed for tube formation. Efficient interference of c-Myb expression was shown in supplemental Fig. 4H. As expected, the direct siRNA-induced knockdown of c-Myb significantly increased tube formation in the HMEC-1 cells (Fig. 2, G and H). To determine whether the effect of miR-150-containing THP-1 MVs on tube formation in HMEC-1 cells was dependent on c-Myb silencing, a recombinant plasmid carrying the miR-150-resistant form of c-Myb (c-Myb ORF) was constructed to trans-
efect THP-1 MV-treated HMEC-1 cells. Efficient overexpression of c-Myb in HMEC-1 cells is shown in supplemental Fig. 5C. Although THP-1 MVs significantly promoted tube formation in HMEC-1 cells, overexpression of c-Myb reversed the function of miR-150-containing THP-1 MVs (supplemental Fig. 5, D and E), suggesting that c-Myb as the direct target of miR-150 may be responsible for the pro-angiogenic effect of secreted miR-150 in THP-1 MVs. Taken together, these results demonstrate that the miR-150 in the MVs accounts for the enhancement of HMEC-1 tube formation, probably via the inhibition of c-Myb.

To further assess the pro-angiogenic properties of secreted miR-150 in vivo, we evaluated the infiltration of blood vessels into Matrigel plugs when THP-1 MV, THP-1 MV/miR-150$^{\text{def}}$,
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293T MV, and 293T MV/miR-150 treatment were administered to mice. Injections of THP-1 MVs after the subcutaneous implantation of Matrigel increased the plasma levels of miR-150 and the number of perfused vessels that invaded the Matrigel plugs in vivo, but this induction was largely blocked by the removal of miR-150 from the THP-1 MVs (Fig. 2, J–K). Moreover, although normal 293T MVs showed no effect on in vivo angiogenesis, 293T MV/miR-150 significantly increased the plasma levels of miR-150 and the number of erythrocyte-filled blood vessels in Matrigel plugs (Fig. 2, L–N). Furthermore, we mixed the 293T MV, THP-1 MV, and THP-1 MV/miR-150 into the Matrigel before implantation and then evaluated the infiltration of blood vessels into Matrigel plugs. The plugs from the mice with different treatments were pale in color (supplemental Fig. 6), indicating that none of new blood vessels had been formed in these Matrigel plugs. Thus, in the absence of pro-angiogenic stimuli from miR-150-containing MVs, endothelial cells that were secreted miR-150 has specific pro-angiogenic properties in vivo and may be important in the switch from quiescence to activation of endothelial cells.

MV Delivery of Anti-miR-150 Abolishes the Effects of Secreted miR-150 on Angiogenesis—We next tested whether “neutralization” of the secreted miR-150 could suppress angiogenesis. Taking advantage of the low expression of miR-150 in 293T cells, we transfected the 293T cells with anti-miR-150 to load the MVs with an anti-miR-150 inhibitor (293T MV/anti-miR-150). We used these MVs to treat mice bearing subcutaneous S-180 sarcomas, as depicted in Fig. 5A. The plasma miR-150 levels were significantly lower in the mice treated with 293T MV/anti-miR-150 than in the control counterparts when both groups were implanted with S-180 sarcomas (Fig. 5B), suggesting significant suppression of the sarcoma-induced secretion of miR-150 into circulation. Consequent development of erythrocyte-filled blood vessels in the Matrigel plugs after 293T MV treatment was strongly inhibited by the addition of anti-miR-150 into the 293T MVs (Fig. 5, C and D). Likewise, ob/ob mice treated with 293T MV/anti-miR-150 (Fig. 5E) displayed reduced plasma miR-150 levels (Fig. 5F), and the number of newly formed blood vessels in their Matrigel plugs was significantly decreased (Fig. 5, G and H).

DISCUSSION

Angiogenesis is a complex process that controls new blood vessel formation and is essential for various physiological and pathological events (1). For example, in addition to being the most effective mechanism to overcome the lack of nutrients and oxygen and to remove waste products from a growing tumor, angiogenesis provides the principal route by which tumor cells exit the primary tumor site and enter the circulation (2, 3). Without new blood vessels, tumors cannot grow beyond a critical size or metastasize to new organs (2, 3). Furthermore, angiogenesis plays a crucial role in the modulation of adipogenesis and obesity (4–6). Adipose tissue is a highly vascularized body tissue, as capillaries encircle each adipocyte. To supply growing adipose tissue with nutrients and oxygen, the vasculature increases the number and/or size of blood vessels (4–6). Thus, adipose tissue expansion requires a parallel growth of its capillary network. Although the importance of angiogenesis is well understood, the molecular mechanisms underlying pathophysiological angiogenesis are largely unknown. Recent studies have unveiled important roles for intercellular communication.

FIGURE 1. THP-1 MVs promote angiogenesis in vitro and in vivo. A, shown is a flow chart depicting the experimental design. Matrigel was placed on a 24-well plate at 37 °C for 30 min to allow solidification. HMEC-1 cells were seeded on Matrigel and allowed to form capillary-like structures when incubated with MVs at 37 °C for 6 h. Quantitative analysis of the formed tubes was obtained by counting the number of branch points from five fields per well. B, the levels of miR-150 in different amounts of THP-1 MVs are shown. C, representative images of HMEC-1 cells on Matrigel that were treated with different amounts of THP-1 MVs are shown. D, quantitative analysis of the experiments in panel C (n = 3) is shown. E, shown is representative images of HMEC-1 cells on Matrigel that were treated with saline (control) or with THP-1 or 293T MVs. F, shown is quantitative analysis of the experiments shown in panel E (n = 3). G, shown is a flow chart depicting the experimental design. Matrigel containing heparin and bFGF was injected into the abdominal area of mice. Immediately after the implantation, mice were given an equal dose of THP-1 or 293T MVs via a tail vein injection once every 2 days. Animals that received saline served as controls. Eight days later plugs were excised and fixed for subsequent H&E staining of microvessels formed within the Matrigel. H, shown is the qRT-PCR analysis of the plasma miR-150 levels in mice that were injected with saline (control) or with THP-1 or 293T MVs (n = 3). J, shown is morphology of the Matrigel plugs excited from mice that were injected with saline (control) or with THP-1 or 293T MVs. J, left panel, shown are H&E-stained sections from the Matrigel plugs shown in panel I, with arrows pointing to the luminal structures containing red blood cells. Right panel, shown is a quantitative analysis of the numbers of newly formed vessels in Matrigel sections (n = 3). *, p < 0.05; **, p < 0.01.
between monocytes/macrophages and endothelial cells in the angiogenic process (7–10). In normal conditions, intercellular signaling can trigger signal transduction mechanisms within target cells and modulate cell behavior, thus coordinating cellular responses and maintaining tissue homeostasis (7–10). Additionally, in pathological situations such as cancers and...
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FIGURE 2. Secreted miR-150 promotes angiogenesis in vitro and in vivo. A, shown is representative images of HMEC-1 cells on Matrigel that were treated with saline (control) or with normal or modified THP-1 MVs (named THP-1MV/miR-150\textsuperscript{def}), which were miR-150-deficient and were harvested by transfecting the cells with anti-miR-150. B, shown is quantitative analysis of the experiments in panel A (n = 3). ND, not determined. C, representative images of HMEC-1 cells on Matrigel that were treated with saline (control) or with normal or modified 293T MVs (named 293T MV/miR-150), which were miR-150-sufficient and were harvested by transfecting the cells with pre-miR-150. D, shown is quantitative analysis of the experiments in panel A (n = 3). E, shown are representative images of HMEC-1 cells on Matrigel treated with saline (control) or with scrambled negative control RNAs or pre-miR-150. F, shown is quantitative analysis of the experiments in panel E (n = 3). G, shown are representative images of HMEC-1 cells on Matrigel treated with saline (control) or with scrambled control siRNA or c-Myb siRNA. H, shown is quantitative analysis of the experiments in panel C (n = 3). I, shown is the qRT-PCR analysis of the plasma miR-150 levels in mice that were injected with saline (control) or with normal THP-1 MV or THP-1MV/miR-150\textsuperscript{def} (n = 3). J, shown is morphology of the Matrigel plugs excised from mice that were injected with saline (control) or with normal 293T MV or 293T MV/miR-150 (n = 3). M, shown is morphology of the Matrigel plugs excised from mice that were injected with saline (control) or with normal 293T MV or 293T MV/miR-150. N, left panel, shown are H&E-stained sections from the Matrigel plugs in panel M, with arrows pointing at the luminal structures containing red blood cells. Right panel, shown is quantitative analysis of the numbers of newly formed vessels in Matrigel sections (n = 3). *, p < 0.05; **, p < 0.01.

FIGURE 3. Secreted miR-150 from plasma of atherosclerosis patients enhances angiogenesis. A, shown is a flow chart depicting experimental design. The C57BL/6J mice were injected subcutaneously with Matrigel containing bFGF. Simultaneously, they were injected via tail vein with saline (control) or with MVs from normal donors or atherosclerosis patients once every 2 days. Matrigel plugs were analyzed 8 days later. B, qRT-PCR analysis of the plasma miR-150 levels in mice injected with saline (control) or with MVs from normal donors or atherosclerosis patients (n = 3). C, shown is morphology of the Matrigel plugs excised from mice injected with saline (control) or with MVs from normal donors or atherosclerosis patients. D, left panel, shown are H&E-stained sections from the Matrigel plugs in panel C, with arrows pointing at the luminal structures containing red blood cells. Right panel, shown is quantitative analysis of the numbers of newly formed vessels in Matrigel sections (n = 3). *, p < 0.05; **, p < 0.01.

obesity-related disorders, angiogenesis may serve a maladaptive role that promotes disease progression (7–10). The mechanism by which this signaling crosstalk contributes to angiogenesis remains to be elucidated.

Numerous studies have revealed important roles for miRNAs in regulating angiogenesis. The term "angiomiR" has been adopted for miRNAs that regulate angiogenesis; pro-angiomiRs promote angiogenesis by targeting negative regulators of angiogenic signaling pathways, and anti-angiomiRs inhibit angiogenesis by targeting positive regulators of angiogenesis (22). Presently, it seems that the group of angiomiRs classically recognized as intracellular pro-angiomiRs and anti-angiomiRs should be expanded to include the newly discovered intercellular miRNAs. In this study we showed that MVs shed from the monocytic cell line THP-1 were enriched in miR-150 and could be taken up by cultured endothelial cells, upon which these vesicles exerted a pro-angiogenic effect. By depleting the miR-150 levels from THP-1 MVs and enhancing the miR-150 levels in control 293T MVs, we demonstrated that the miR-150 in the MVs accounted for the enhanced angiogenesis of endothelial cells. Under pathological conditions, such as in the tumor-implanted mouse model and diabetic ob/ob mouse model, the secretion of miR-150 into the circulation was increased, which significantly promoted angiogenesis.

The discovery of mRNA secretion opens new frontiers in the study of miRNAs but leaves many unanswered questions. For example, the physiological relevance of most secreted miRNAs is currently unclear. The work described herein extends our understanding by illustrating the potential for secreted miRNAs, as extracellular effectors of cell-to-cell communication, to exert paracrine effects on recipient cells. The ability of miRNAs to modulate multiple target genes makes them well suited for such a role. However, it is unclear which mechanism accounts for the increased secretion of miR-150. In addition, the direct target of miR-150 in recipient endothelial cells is not fully known. Further studies are necessary to unveil how miR-150 is
sorted into MVs and whether or how this process is regulated. It will also be essential to uncover the molecular machinery through which exogenous miR-150 exerts its effect in endothelial cells and to establish the roles of this mechanism in the development and progression of pathological angiogenesis. Furthermore, angiogenesis is a complicated process that requires multiple functions of endothelial cells. Besides migration and tube formation, proliferation of endothelial cells is also an important process. In the present study we have demonstrated that secreted miR-150 in THP-1 MVs can enter into HMEC-1 cells and enhance migration tube formation of HMEC-1 cells. To fully validate the pro-angiogenic activity of secreted miR-150 in MVs, the role of secreted miR-150 in modulating proliferation of HMEC-1 cells should also be investigated in the future.

In this study we also found that miR-150-containing MVs collected from the plasma of atherosclerosis patients enhance angiogenesis in vivo. However, angiogenesis is not a direct reason of atherosclerosis and debate surrounds the pathogenic role of angiogenesis in atherosclerosis (22). Although microves- sels are a feature of advanced human atherosclerotic plaques, it remains unclear whether angiogenesis either plays a central role in the development of atherosclerosis or is responsible for plaque instability (22). Furthermore, current evidence from clinical trials of both pro-angiogenic and anti-angiogenic therapies does not suggest that inhibition of angiogenesis is likely to be a viable therapeutic strategy for atherosclerosis (22). Although our results only indicate that secreted miR-150 plays a role in angiogenesis in atherosclerosis but does not provide evidence that secreted miR-150 contributes to the pathogenesis of atherosclerosis, an understanding of the function of secreted miRNAs in atherosclerosis is timely and will help to illuminate some of the major unresolved issues about the role of angiogen- esis in atherosclerosis. Definitely, more experimental work is needed to address these key outstanding questions.

Because angiogenesis contributes to a number of diseases, including cancers, atherosclerosis, and diabetes, the search for novel anti-angiogenesis targets will have significant clinical

FIGURE 4. Secreted miR-150 enhances angiogenesis in tumor-implanted and ob/ob mouse model. A, a flow chart depicting the experimental design is shown. The C57BL/6J mice were first implanted with S-180 sarcoma. After 14 days, mice were injected subcutaneously with Matrigel containing bFGF. Eight days later Matrigel plugs were analyzed. B, shown is qRT-PCR analysis of the plasma miR-150 levels in mice with or without sarcoma implantation (n = 3). C, morphology of the Matrigel plugs excised from mice with or without sarcoma implantation is shown. D, left panel, shown are H&E-stained sections from the Matrigel plugs in panel C, with arrows pointing at the luminal structures containing red blood cells. Right panel, shown is quantitative analysis of the numbers of newly formed vessels in Matrigel sections (n = 3). D, shown is a flow chart depicting the experimental design. The C57BL/6J and ob/ob mice were injected subcutaneously with Matrigel containing bFGF. Eight days later Matrigel plugs were analyzed. F, qRT-PCR analysis of the plasma miR-150 levels in C57BL/6J and ob/ob mice (n = 3) is shown. G, morphology of the Matrigel plugs excised from C57BL/6J and ob/ob mice is shown. H, left panel, shown are H&E-stained sections from the Matrigel plugs in panel G, with arrows pointing at the luminal structures containing red blood cells. Right panel, shown is quantitative analysis of the numbers of newly formed vessels in Matrigel sections (n = 3).
FIGURE 5. Inhibition of angiogenesis by 293T MVs that contained anti-miR-150. A, a flow chart depicting the experimental design is shown. The C57BL/6J mice were first implanted with S-180 sarcoma. After 6 days mice were injected subcutaneously with Matrigel containing bFGF. Simultaneously, they were injected via tail vein with saline (control) or with normal or modified 293T MVs (named 293T MV/anti-miR-150) once every 2 days. The modified 293T MVs were harvested by transfecting the cells with anti-miR-150. Nine days later, Matrigel plugs were analyzed, and tumor sizes were measured. B, shown is qRT-PCR analysis of the plasma miR-150 levels in tumor-implanted mice injected with saline (control) or with normal 293T MV or 293T MV/anti-miR-150 (n = 3). C, shown is morphology of the Matrigel plugs excised from tumor-implanted mice injected with saline (control) or with normal 293T MV or 293T MV/anti-miR-150. D, left panel, shown are H&E-stained sections from the Matrigel plugs in panel C, with arrows pointing at the luminal structures containing red blood cells. Right panel, shown is quantitative analysis of the numbers of newly formed vessels in Matrigel sections (n = 3). E, shown is a flow chart depicting the experimental design. The ob/ob mice were injected subcutaneously with Matrigel containing bFGF. Simultaneously, they were injected via tail vein with saline (control) or with normal 293T MV or 293T MV/anti-miR-150 once every 2 days. Three days later, Matrigel plugs were analyzed. F, shown is qRT-PCR analysis of the plasma miR-150 levels in ob/ob mice injected with saline (control) or with normal 293T MV or 293T MV/anti-miR-150 (n = 3). G, shown is morphology of the Matrigel plugs excised from ob/ob mice injected with saline (control) or with normal 293T MV or 293T MV/anti-miR-150. H, left panel, shown are H&E-stained sections from the Matrigel plugs in panel G, with arrows pointing at the luminal structures containing red blood cells. Right panel, shown is quantitative analysis of the numbers of newly formed vessels in Matrigel sections (n = 3). *, p < 0.05; **, p < 0.01.
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applications. In the present study the identification of secreted miR-150 as a key regulator of angiogenesis has opened a new avenue for disease therapies. Indeed, we have successfully blocked the secretion of miR-150 into circulation, thereby decreasing angiogenesis by employing MVs to deliver anti-miR-150 in both a tumor-implanted mouse model and a diabetic ob/ob mouse model. These results indicate that MVs derived from cells engineered to express miRNAs or siRNAs may be capable of delivering these small RNAs to the local cellular environment. In fact, the therapeutic use of MV-delivered miRNAs has long been thought to have unequivocal advantages over traditional treatment. First of all, because MVs are derived from endogenous cells, they have reduced toxicity and would potentially be tolerated by the immune system when serving as therapeutic delivery agents. Second, considering the complexity and diversity of miRNA-mRNA interactions, the impact of MV-mediated transfer of miRNA on the recipient’s translational machinery may be quite extensive. Thus, this method provides a natural means of normalizing the expression of disease genes, potentially avoiding the undesired effects caused by switching a single target gene on or off.

Taken together, the results of the present study suggest that secreted miR-150 may act directly on angiogenesis, which results in the aberrant formation of capillary-like tubes in vitro and the abnormal generation of new vessels in disease states in vivo. Discovery of this new form of intercellular communication may herald a new era in our understanding of signaling and molecule transfer between cells.

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REFERENCES
1. Folkman, J. (2003) Fundamental concepts of the angiogenic process. Curr. Mol. Med. 3, 643–661
2. Carmelet, P., and Jain, R. K. (2000) Angiogenesis in cancer and other diseases. Nature 407, 249–257
3. Carmelet, P., and Jain, R. K. (2011) Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. Nat Rev. Drug Discov. 10, 417–427
4. Cao, Y. (2010) Adipose tissue angiogenesis as a therapeutic target for obesity and metabolic diseases. Nat. Rev. Drug Discov. 9, 107–115
5. Cao, Y. (2007) Angiogenesis modulates adipogenesis and obesity. J. Clin. Invest. 117, 2362–2368
6. Lijnen, H. R. (2008) Angiogenesis and obesity. Cardiovasc. Res. 78, 286–293
7. Naldini, A., and Carraro, F. (2005) Role of inflammatory mediators in angiogenesis. Curr. Drug Targets Inflamm. Allergy 4, 3–8
8. Moldovan, L., and Moldovan, N. I. (2005) Role of monocytes and macrophages in angiogenesis. EXS 94, 127–146
9. Moldovan, N. I. (2002) Role of monocytes and macrophages in adult angiogenesis. A light at the tunnel’s end. J. Hematother. Stem Cell Res. 11, 179–194
10. Dirix, A. E., Oude Egbrink, M. G., Wagstaff, J., and Griffioen, A. W. (2006) Monocyte/macrophage infiltration in tumors. Modulators of angiogenesis. J. Leukoc. Biol. 80, 1183–1196
11. Bartel, D. P. (2004) MicroRNAs. Genomics, biogenesis, mechanism, and function. Cell 116, 281–297
12. Ambros, V. (2004) The functions of animal microRNAs. Nature. 431, 350–355
13. He, L., and Hannon, G. J. (2004) MicroRNAs. Small rnas with a big role in gene regulation. Nat. Rev. Genet. 5, 522–531
14. Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., Lötvall, J. O. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat. Cell Biol. 9, 654–659
15. Zhang, Y., Liu, D., Chen, X., Li, J., Li, L., Bian, Z., Sun, F., Lu, J., Yin, Y., Cai, X., Sun, Q., Wang, K., Ba, Y., Wang, Q., Wang, D., Yang, J., Liu, P., Xu, T., Yan, Q., Zhang, J., Zen, K., and Zhang, C. Y. (2010) Secreted mononuclear miR-150 enhances targeted endothelial cell migration. Mol. Cell. 39, 133–144
16. Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., Matsuki, Y., and Ochiya, T. (2010) Secretory mechanisms and intercellular transfer of microRNAs in living cells. J. Biol. Chem. 285, 17442–17452
17. Mittelbrunn, M., Gutiérrez-Vázquez, C., Villarroya-Beltri, C., González, S., Sánchez-Cabo, F., González, M. Á., Bernad, A., and Sánchez-Madrid, F. (2011) Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. Nat. Commun. 2, 282
18. Malinda, K. M. (2009) In vivo Matrigel migration and angiogenesis assay. Methods Mol. Biol. 467, 287–294
19. Auerbach, R., Lewis, R., Shinners, B., Kubai, L., and Akhtar, N. (2003) Angiogenesis assays. A critical overview. Clin Chem. 49, 32–40
20. Lin, Y. C., Kuo, M. W., Yu, J., Kuo, H. H., Lin, R. J., Lo, W. L., and Yu, A. L. (2008) C-myc is an evolutionary conserved miR-150 target and miR-150/c-myc interaction is important for embryonic development. Mol. Biol. Evol. 25, 2189–2198
21. Xiao, C., Calado, D. P., Galler, G., Thai, T. H., Patterson, H. C., Wang, J., Rajewsky, N., Bender, T. P., and Rajewsky, K. (2007) Mir-150 controls b cell differentiation by targeting the transcription factor c-myc. Cell 131, 146–159
22. Wang, S., and Olson, E. N. (2009) AngiomiRs. Key regulators of angiogenesis. Curr. Opin. Genet. Dev. 19, 205–211