Anti-angiogenesis triggers exosomes release from endothelial cells to promote tumor vasculogenesis

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ABSTRACT
Although anti-angiogenic therapies (AATs) have some effects against multiple malignancies, they are limited by subsequent tumor vasculogenesis and progression. To investigate the mechanisms by which tumor vasculogenesis and progression following AATs, we transfected microRNA (miR)-9 into human umbilical vein endothelial cells (HUVECs) to mimic the tumor-associated endothelial cells in hepatocellular carcinoma and simulated the AATS in vitro and in vivo. We found that administration of the angiogenesis inhibitor vandetanib completely abolished miR-9-induced angiogenesis and promoted autophagy in HUVECs, but induced the release of vascular endothelial growth factor (VEGF)-enriched exosomes. These VEGF-enriched exosomes significantly promoted the formation of endothelial vessels and vasculogenic mimicry in hepatocellular carcinoma and its progression in mice. Anti-autophagic therapy is proposed to improve the efficacy of AATs. However, similar effects by AATs were observed with the application of anti-autophagy by 3-methyladenine. Our results revealed that tumor vasculogenesis and progression after AATs and anti-autophagic therapies were due to the cross-talk between endothelial and tumor cells via VEGF-enriched exosomes.

Introduction
Anti-angiogenic therapies (AATs) which aim at starving tumors by suppressing tumor vascularization and destroying pre-existing tumor-associated blood vessels have been less successful than initially expected [1,2]. The benefits of AATs are transient, and the treated tumors have been shown to relapse faster through different forms of vasculogenesis, including vascular co-option, endothelial angiogenesis, and vasculogenic mimicry (VM) [3–7]. In addition, the tumor vasculature induced by the AAT has a leakier barrier, which makes it prone to tumor cell invasion and metastasis and drives acquired resistance to AATs in cancers such as hepatocellular carcinoma (HCC) [6,7], a leading cause of cancer-related death worldwide [8]. However, how the AATs result in tumor vasculogenesis remains a mystery [9].

MicroRNAs (miRNAs) have recently been shown to regulate gene expression associated with tumorigenesis and angiogenesis [10,11]. Oncogenic miR-9 is significantly elevated in HCC tissues and tumor-associated endothelial cells (ECs). High-level expression of miR-9 is closely related to the poor prognosis of HCC patients [10,12,13]. MiR-9 has been reported to increase vascular endothelial growth factor (VEGF) expression in tumor cells [14] and to promote tumor angiogenesis and progression in the lung and colorectal carcinoma. But these carcinomas were only moderately responsive to miR-9 antagonirs or anti-VEGF treatment [10,15]. Besides, the role of tumor-associated ECs overexpressing miR-9 in AATs is unknown.

MiR-9 has also been reported to promote autophagy [16], a cellular pathway involved in the clearance of protein aggregates, turnover of organelles, as well as recycling the by-products of autophagic degradation [17]. In particular, the induction of autophagy promotes angiogenesis including VEGF-induced angiogenesis, independent of nutritional or hypoxic stress [18,19]. However, whether miR-9 induces angiogenesis and whether miR-9-induced angiogenesis is affected by anti-autophagy treatment have not been fully explored.

In the past decade, extracellular vesicles (EVs) including apoptotic bodies, microvesicles (MVs) and exosomes
have emerged as important players in cell-to-cell communication in health and in disease [20]. Delivery of tumor-secreted miR-9 by MVs promotes EC migration [10]. Exosomes having a lipid-bilayer membrane and in the 30–150 nm size range [21,22] that allow genetic or molecular exchanges between cells through the transfer of angiogenic proteins, such as VEGF [23], and functional RNA molecules including mRNAs and miRNAs [21], have also been found to contribute to tumor progression and metastasis [22,24], although the role of exosomes in mediating tumor vasculogenesis, especially following AATs, is unclear.

To elucidate the mechanisms by which tumor vasculogenesis and progression following AATs and to address the above pertinent issues, we first transfected human umbilical vein endothelial cells (HUVECs) with miR-9 to mimic the tumor-associated ECs. We then examined the role of miR-9 in endothelial angiogenesis and in endothelial autophagy. Following that, we investigated the anti-angiogenic effects by a VEGFR2 (Flk1) inhibitor, vandetanib, and by an autophagy inhibitor, 3-methyladenine (3-MA). Finally, we discovered that the enhanced vasculogenesis and progression of HCC were due to the VEGF-enriched exosomes released by ECs overexpressing miR-9 in response to anti-angiogenic and anti-autophagic treatments.

Results

**Mir-9 induces angiogenesis in vivo**

To simulate the upregulation of miR-9 in tumor-associated ECs, we transfected miR-9-expressing lentivirus into HUVECs (Figure 1(a)). Murine Matrigel plugs mixed with HUVECs were previously used to evaluate angiogenesis in vivo based on the response of active angiogenesis to angiogenic factors in the Matrigel plug [25]. Platelet endothelial cell adhesion molecule 1 (PECAM1, or CD31) is a marker of endothelial cells [26]. The human progenitor cell antigen cluster of differentiation (CD34) is also expressed on HUVECs and acts as an endothelial marker for angiogenesis [27]. Through these specific EC makers, we located the microvessels formed in the Matrigel plug mixed with HUVECs overexpressing miR-9 in the absence of additional angiogenic factors (Figure 1(b, e, f)). The amount of angiogenic microvessels was significantly increased by miR-9 (33.8 ± 1.5/mm²) compared with NC (20.7 ± 0.2/mm²; Figure 1(c), P < 0.001) in 4 observed regions (each region is ~1326 μm × 998 μm large, see Supplementary Figure 1) in each animal and 4 animals for each group. The diameter of angiogenic vessels induced by miR-9 was 5–96 μm, with 31.4 μm the average.

In contrast, only small angiogenic vessels were found in the NC group (2.5–13 μm, with 5.2 μm the average; P < 0.001; Figure 1(b, e, f)). CD31 was also highly expressed on newly formed vessels (Figure 1(e)). Human CD31 positive ECs formed vessels were only observed in HUVECs overexpressing miR-9, while murine CD31 positive ECs formed vessel-like structures (without apparent lumen) were found in both NC and miR-9 groups, suggesting that miR-9 should promote the angiogenesis of HUVECs (Figure 1(e, f)). The vessel-like structures were most likely due to the recruitment of host endothelial cells in mice. Compared with the NC, miR-9 inhibited cell apoptosis (Figure 1(g)), enhanced phosphorylation of Flk1 (Figure 1(h)), and increased the level of the autophagic marker LC3B (Figure 1(i)). It was reported that CD34 expression in isolated ECs is inversely associated with apoptosis and positively associated with angiogenesis in vitro [28]. Consistent with the in vitro findings, we demonstrated that CD34 positivity in ECs was lost with the increase in apoptosis and autophagy in NC group.

We then analyzed the role of miR-9 in VEGF and Flk1 expression in HUVECs. Consistent with the angiogenic induction, miR-9 increased both mRNA and protein levels of VEGF and Flk1 in HUVECs (Figure 1(j–l)), as well as eliciting the release of VEGFA (Figure 3(e)), suggesting that miR-9 promote autocrine effects associated with VEGF signaling to induce angiogenesis.

**Mir-9 and anti-angiogenic vandetanib induce autophagy**

Treatment of ECs with angiogenesis inhibitors has been shown to induce autophagy [29]. To investigate if miR-9 induces autophagy and if anti-VEGFR2 (Flk1) treatment by vandetanib also induces autophagy, we evaluated changes in autophagy in HUVECs overexpressing miR-9 with and without vandetanib treatments (Figure 2). We observed numerous morphologically intact early/initial autophagic vacuoles (AVs) and late/degradative autophagic vacuoles (AVds) containing degraded cytoplasmic materials and lysosome-fusion components in HUVECs upon miR-9 transfection (Figure 2(a,b)). Vandetanib treatment significantly increased the AVds in HUVECs and further enhanced the increased AVds by miR-9, but abolished the increased AVs by miR-9 (Figure 2(a,b)). It also increased the size of AVs (Figure 2(c)) in HUVECs with and without overexpressing miR-9, as well as induced fusion of multivesicular bodies (MVBs) with the vacuoles (Figure 2(a)). Vandetanib treatment induced autophagic proteins LC3B and Beclin-1 expression (Figure 2(d, e, h, i)), conversion of LC3BI to LC3BII (Figure 2(h, i)),
Figure 1. MiR-9 induces angiogenesis.

(a). HUVECs overexpressing miR-9 after transfection with LV3-miR-9 (miR-9) and LV3-NC (NC). Mean ± standard deviation (SD), n = 4. ***P < 0.001. Student’s t-test was performed. (b). Investigation of angiogenesis by confocal microscopy in the mouse ventral subcutaneous region following transfer of a murine Matrigel plug mixed with lentivirus-infected HUVECs (GFP, green), CD34 (red), and nuclei (DAPI, blue) in cryosections. Vessel (yellow arrowhead), vascular content (blue arrow). The vascular structures were circled by the white dotted line. (c–f). Evaluation of vessel density (c) and diameter (d) and immunohistochemistry (IHC) for CD31 (e) and CD34 (f). Both human CD31 (hCD31) and murine CD31 (mCD31) were stained (e). The vessel density (c) and diameter (d) were calculated using CD34 images (f). Mean ± standard error of mean (SEM). The density was the number of vessels per unit area (c), averaged over four view fields (larger views in Supplementary Figure 1) per murine Matrigel plug, four animals per group, n = 4. Student’s t-test was performed for vessel density. Frequency distribution curve of diameter (interval 10 and range 0–100) was performed by ImageJ. Chi-squared test was performed for vessels distribution. *** P < 0.001 vs. NC. The green line enclosed area is the vessel lumen (f). Diameter (D) = (lumen area/n)^1/2×2. Small endothelial sprouts without apparent lumen were not calculated. At the upper left corner of (f), the enlarged views of the vessels are shown with the diameters for those indicated by the rectangular boxes. (g–i). IHC for TUNEL assay (g), p-Flk1 (h), and LC3B (i) in the mouse ventral subcutaneous region following transfer of a murine Matrigel plug mixed with lentivirus-infected HUVECs. Results shown are representatives of four experiments. (j–l). Quantitative reverse transcription polymerase chain reaction (j and k) and immunoblot results (l, 30 μg proteins) for VEGF and Flk1 in HUVECs overexpressing miR-9. Densitometric quantification (K, right). Mean ± SEM, n = 4. *P < 0.05; **P < 0.01; ***P < 0.001. Student’s t-test was performed.
consumption of p62 (Figure 2(h, i)) and autophagic vacuoles (Figure 2(g)) in both NC and miR-9 groups, indicating vandetanib treatment induced autophagy and also enhanced the autophagy by miR-9. We also evaluated the reverse effect of an autophagy inhibitor 3-methyladeneine (3-MA, 5 mM) on miR-9 and vandetanib induced autophagy (Figure 2(d–i)). Administration of 3-MA significantly abolished the miR-9 and vandetanib-induced autophagy by inhibiting LC3B and Beclin-1 expression (Figure 2(d–i)), conversion of LC3BI to LC3BII (Figure 2(h, i)) as well as consumption of p62 (Figure 2(h, i)). 3-MA also reduced the numbers of LC3B punctate dots (Figure 2(d–f)) and autophagic vacuoles (Figure 2(g)).

Vandetanib and 3-MA inhibit angiogenesis

Figure 3(a) demonstrates that miR-9-induced Flk1 expression and Flk1 phosphorylation were significantly suppressed in a concentration-dependent manner following administration of vandetanib for 60 min (P < 0.05 at 2 μM; P < 0.01 at 4 and 8 μM). We also found that administration of 4 μM vandetanib or 5 mM 3-MA for 60 min almost completely inhibited Flk1 phosphorylation or abrogated its relocalization in the cell membrane induced by miR-9 (Figure 3(b, c)). Although vandetanib did not change the VEGF mRNA expression in HUVECs, it reduced the miR-9-increased VEGF mRNA expression (Figure 3(d)). Differently,
3-MA reduced VEGF mRNA expression in HUVECs and completely abolished miR-9-increased VEGF mRNA expression (Figure 3(d)). Correspondingly, the miR-9-increased secretion of VEGFA was also abolished by 3-MA and vandetanib (Figure 3(e)). In contrast, vandetanib and 3-MA did not significantly alter the miR-9 levels in HUVECs with and without overexpressing miR-9 (Figure 3(f)).
We then investigated the roles of vandetanib and 3-MA in miR-9-induced angiogenesis. Treatments with either vandetanib or 3-MA significantly suppressed EC migration and invasion induced by miR-9 (Figure 3(g–i)). As a consequence, treatments with either vandetanib or 3-MA or both significantly decreased the tube formation, tube length and number of junctions in the network induced by miR-9 (Figure 3(j–l)). These results suggest that miR-9 promote angiogenesis not only by activation of Flk1 signaling but also by autophagy in HUVECs. Anti-angiogenesis and/or anti-autophagy abolished miR-9-induced angiogenesis (Figure 3(m)). Recombinant human VEGF 165 protein (50 ng/ml [30]; hVEGF) was used as a positive control. Similar to miR-9, inductions in human VEGF 165 protein (50 ng/ml [30]; hVEGF) was observed (Figure 3(g–i)).

VEGF-enriched exosomes are released from HUVECs following administration of either vandetanib or 3-MA

The disruption of intercellular communication networks associated with cell-cell junctions and exosomes is a prerequisite for angiogenesis [20,31]. Figure 4(a,b) show that miR-9 significantly enlarged the intercellular gaps from 147.0 ± 29.9 nm (NC) to 716.1 ± 44.8 nm although it did not change the number and size distribution of extracellular vesicles (EVs), which include exosomes and microvesicles (MVs) (Figure 4(c)). Surprisingly, vandetanib treatment (anti-VEGFR2) and 3-MA (autophagy inhibitor), which suppressed angiogenesis (Figure 3), not only further enlarged the intercellular gaps to 896.2 ± 94.3 nm and 1284.0 ± 357.2 nm, respectively (Figure 4(b)), but also increased the number of EVs and changed their size distribution (Figure 4(c)). Significantly more EVs with size of 30–150 nm were induced by either vandetanib or 3-MA treatment. HUVECs overexpressing miR-9 also have a larger cell body area (112.3 ± 5.8 μm, n = 4) compared to NC cells (85.9 ± 4.9 μm, n = 4, P < 0.05), vandetanib further increased the area to 136.9 ± 6.0 μm (n = 4, P < 0.01), whereas 3-MA reduced the area to 91.8 ± 5.3 μm (n = 4, P < 0.05).

We then isolated the exosomes from the culture media supernatant of HUVECs by ultracentrifugation. Immunoblotting was used to identify the exosomal markers TSG101, HSP70 and CD63 (Figure 4(d)). The purity of the isolated exosomes was confirmed by GRP94 that is absent in exosomes [32]. Exosomal proteins from 2.5 mL culture supernatants were loaded during western blotting (Figure 4(d)). The levels of these exosomal proteins were in the order: miR-9 + Van or miR-9 + 3-MA > miR-9 > NC (P < 0.05). The size (diameter) of the isolated exosomes ranged mostly from 30 nm to 150 nm (Figure 4(e)). The cup-shaped EVs in the inset of Figure 4(e) are exosomes (one is 30 nm and another 143 nm) [20]. Immunogold labelling of CD63 also confirmed the isolated vesicles were exosomes (Figure 4(f)). Using nanoflow cytometry, we further detected two groups of exosomes (exo1 and exo 2) that are VEGF-enriched from the supernatant of HUVECs overexpressing miR-9 and following administration of either vandetanib or 3-MA (Figure 4(g)). The size of these VEGF-enriched exosomes is less than 110 nm (Supplement Figure 2). However, we did not find VEGF-enriched MVs which are greater than 200 nm [33]. Although vandetanib or 3-MA increased the VEGF cargos in the isolated exosomes from the HUVEC culture supernatant, with a further increase from those with miR-9 overexpression (Figure 4(h–j)), neither of them had affected the miR-9 cargos in these exosomes (Figure 4(i)).

The measured VEGF levels in exosomes (lysed using radioimmunoprecipitation assay before ELISA detection, Figure 4(j)) were significantly higher than those in culture supernatants (without lysis, Figure 3(e)) and those in exosome-depleted media (Figure 4(k)), e.g. 12.47 ± 0.16 ng/mL in exosomes vs. 0.98 ± 0.06 ng/mL in culture supernatant and 0.17 ± 0.01 ng/mL in exosome-depleted media in miR-9 + vandetanib group, suggesting that VEGF mostly came from the HUVEC released exosomes following vandetanib or 3-MA treatments.

The above findings indicate that treatments with the VEGFR2 or autophagy inhibitor promote the release of VEGF-enriched exosomes from ECs, especially from those tumor-associated ECs (overexpressing miR-9) (Figure 4(l)).

VEGF-enriched exosomes promote colony formation and vascular mimicry by HCC cells in vitro

To explore the effect of VEGF-enriched exosomes on HCC development, we treated the malignant HCC cell lines Huh7 and SMMC-7721 with isolated exosomes from HUVECs overexpressing miR-9 and following vandetanib or 3-MA treatment, in the presence and absence of a VEGF neutralizing antibody Bevacizumab. Five μg of exosomes were used in the experiments since 5 μg isolated exosomes from HUVECs overexpressing miR-9 could significantly induce colony and tube formation. Figure 5(a–f) show that these VEGF-enriched exosomes significantly increased colony and tube formation in both Huh7 and SMMC-7721 cells, compared to miR-9-enriched exosomes from HUVECs overexpressing miR-9 in the absence of inhibitor treatment. hVEGF had a similar effect on the colony and tube formation as the VEGF-enriched exosomes. Bevacizumab significantly
attenuated the colony and tube formation induced by these VEGF-enriched exosomes, suggesting that VEGF is the main cargo in these exosomes that should be responsible for the failure of vandetanib to cause lasting remission of angiogenesis.

After inhibition of exosome release in HUVECs by GW4869, VEGF was significantly reduced in the cell culture supernatant (Figure 5(g)), the tube formations by the HCC cells co-cultured with HUVECs overexpressing miR-9 and following vandetanib or 3-MA Figure 4.

Figure 4. Inhibition of VEGFR (Flk1) or autophagy releases VEGF-enriched exosomes from HUVECs.

(a). Transmission electron microscopic view of control HUVEC and those overexpressing miR-9 and treated with vandetanib or 3-MA. Released extracellular vesicles (EVs) (red arrowhead) and intercellular junctions (blue arrowhead). At the upper left corner of each image, there is an enlarged view of likely EVs/EV-like structures indicated by the rectangular box. Their diameter values are shown in red. (b). Comparison of the intercellular gap distance under each case. Mean ± SEM, n = 10 gaps for each case. ***P < 0.001 vs. NC; #P < 0.05, ##P < 0.01 vs. miR-9; @P < 0.05 vs. miR-9 + Van. (c). Comparison of the amount of extracellular vesicles (EVs) under each case. Mean ± SEM, n = 4. EVs were observed by TEM and frequency distribution curve of diameter (interval 20 and range 0–1000) was performed by ImageJ. Chi-squared test was performed for EVs distribution. *P < 0.05 vs. NC; #P < 0.01 < 0.001 vs. miR-9. (d). Immunoblot for exosomal markers TSG101, HSP70 and CD63 in exosomes isolated from 2.5 mL culture supernatants from about 1 × 10⁷ HUVECs (left). The purity of exosome was assessed by GRP94. Densitometric quantification (right). Mean ± SEM, n = 4. *P < 0.05, **P < 0.01 vs. NC; ###P < 0.01 vs. miR-9. (e, f). Transmission electron microscopic view of isolated exosomes (e, average size 63 ± 15 nm, Mean ±SD; 78% vesicles in the range of 50–150 nm) and immunogold labelling of CD63 (f). (g). Nano-flow cytometry analysis of VEGF-positive exosomes released from HUVECs overexpressing miR-9 and following administration of vandetanib or 3-MA (exo1, left; exo2, right). (h). Immunoblot for VEGF in exosomes (10 µg proteins) isolated from HUVEC culture supernatant in the presence or absence of miR-9 overexpression and following administration of vandetanib or 3-MA (bottom). CD63 was used as a reference. Densitometric quantification (top). Control, HUVECs in the absence of treatment. Mean ± SEM, n = 3. *P < 0.05, **P < 0.01 vs. control; ###P < 0.001 vs. miR-9. (i). MiR-9 mRNA levels in exosomes. U6 snRNA and cel-miR-39 were used as endogenous and exogenous controls. Mean ± SEM, n = 3, ***P < 0.001 vs. NC. (j, k). Enzyme-linked immunosorbent assay of exosomal VEGF and VEGF in exosome-depleted media. Exosomal proteins were extracted using radioimmunoprecipitation assay lysis. Data was normalized to the volume of culture supernatants. Mean ± SEM, n = 4, *P < 0.05, **P < 0.01, and ***P < 0.001 vs. NC; #P < 0.05; ###P < 0.01, ##P < 0.01 vs. miR-9; @@ P < 0.01 vs. miR-9 + 3-MA. (l). Schematic representation of induction of release of VEGF-enriched exosomes by Flk1 inhibitor and anti-autophagy.
treatment, were also significantly suppressed (Figure 5 (h–j)), confirming that the effects on the tube formation were due to the released exosomes which are enriched with VEGF.

Above results demonstrate that VEGF-enriched exosomes promote HCC proliferation and cancer cell vascular mimicry (VM). VM is some uncanny ability of tumor cells to acquire endothelial-like properties and
generate vascular patterned networks similar to host endothelial blood vessels [34].

**VEGF-enriched exosomes promote tumor vasculogenesis and HCC progression in vivo**

To examine the role of VEGF-enriched exosomes in HCC progression *in vivo*, we injected Matrigel mixed with SMMC-7721 cells and exosomes derived from HUVECs subcutaneously into the ventral region of athymic nude mice. We observed that tumor growth was significantly increased in mice receiving Matrigel mixed with exosomes derived from HUVECs either in the presence of miR-9 (miR-9-enriched exosome, without vandetanib or 3-MA treatment) or absence (NC-exosome) at 10 days and 17 days (P < 0.05), respectively, compared to those receiving Matrigel mixed with phosphate-buffered saline (PBS) (Figure 6(a–c)). However, the larger tumors were formed in the mice receiving Matrigel mixed with VEGF-enriched exosomes from HUVECs overexpressing miR-9 following 3-MA treatment. Consistent to that more VEGF-enriched exosomes were induced by vandetanib than by 3-MA (Figure 4(g, h)), even larger tumor formed as expected when using exosomes from HUVECs overexpressing miR-9 following vandetanib treatment (Figure 6(a–c)).

To investigate the microvessel proliferation associated with exosome transfer from the HUVECs, we quantified EC vasculogenesis and VM in the tumor section by immunohistochemical (IHC) staining for CD34 (red in Figure 6(d), endothelial vessel indicator) and periodic acid-Schiff (PAS, pink in Figure 6(d), VM indicator) staining, respectively (Figure 6(d–g)). Exosomes derived from miR-9-overexpressed HUVECs (without vandetanib or 3-MA treatment) did induce more formation of endothelial vessels (P < 0.001 for vessel number and P < 0.01 for vessel diameter) compared to PBS and NC-exosomes, but no more formation of VM. Whereas VEGF-enriched exosomes not only induced even more formation of endothelial vessels (Figure 6(d–f)) but also more formation of VM (Figure 6(d–g)), suggesting that VEGF-enriched exosomes specifically promote VM.

Subsequently we verified the increases in the protein levels of VEGF (Figure 7(a)), p-Flk1 (Figure 7(b)), and Flk1 (Figure 7(c)) in tumor cells receiving miR-9- and VEGF-enriched exosomes. These increases were further confirmed by western blot (Figure 7(d, e)). No changes in apoptosis were observed in tumor cells receiving PBS, and in those receiving NC-, miR-9- and VEGF-enriched exosomes.

Combining the results from Figures 3, 4(a–k), 5, 6 and 7(a–e), we concluded that anti-angiogenesis (e.g. by vandetanib) and/or anti-autophagy (e.g. by 3-MA) initiate the release of VEGF-enriched exosomes from the tumor-associated ECs (overexpressing miR-9), which increase the formation of endothelial vessels and VM, especially the formation of VM in the tumor tissue, and consequently promote tumor growth and progression, as illustrated in Figure 7(f).

**Discussion**

VEGF binding to VEGFRs, such as VEGFR2 (Flk1), initiates a tyrosine kinase signaling cascade to promote angiogenesis for tumor growth and progression [35,36]. Targeting tumor vasculature via VEGFR inhibitors (e.g. vandetanib), or anti-angiogenic therapies (AATs), however, has not resulted in ideal outcomes for most cancer patients [37–39]. Administration of anti-angiogenic agents together with conventional radio- or chemo- therapies has only produced modest survival benefits [40]. Although it has long been recognized that the mechanisms involved in the failure of AATs include the upregulation of alternative proangiogenic pathways, vascular co-option, and resistance to hypoxia [41], how these alternative proangiogenic pathways are developed is a mystery. The results from our study should shed light on it. The finding that anti-VEGFR 2 by vandetanib successfully suppressed the angiogenesis of tumor-associated ECs (overexpressing miR-9) and their secretion of VEGFA explains the short-term benefits of AATs. However, vandetanib triggered the release of VEGF-enriched exosomes from ECs, which increased the colony and vascular network formation in HCC *in vitro*, enhanced endothelial vasculogenesis and VM of HCC and tumor progression *in vivo*, as well as elevated levels of VEGF, p-Flk1 and Flk1 in the tumor tissue. These findings suggest how the later resistances to AATs are developed. The high level of VEGF also suggests that the newly formed endothelial vessels in the tumor tissue have a leaky wall which is susceptible to tumor invasion and metastasis [42,43].

It has been well established that the “soil” at distant metastatic sites should be first enabled by the factors, e.g. tumor-secreted factors and tumor-shed EVs including exosomes, from the primary tumors, to form suitable microenvironments to the survival and outgrowth of circulating tumor cells (CTCs) before their arrival at these sites [pre-metastatic niches] [44]. It was found that vandetanib induced a significant increase in circulating VEGF and VEGF expression in tumor tissues in HCC patients and xenograft mouse models [39,45,46]. The VEGF-enriched exosomes released from the primary tumor-associated ECs following AATs (by vandetanib) can be a new candidate other than the primary tumor cell-initiated factors to precondition the distant metastatic organs by increasing the permeability of their
microvessels for the adhesion and extravasation of CTCs [47–49], as well as for the nutrients supply for tumor growth. Detecting VEGF-enriched exosomes from the blood can be a novel approach for metastatic cancer diagnosis after AATs. Targeting tumor-associated ECs may be as critical as targeting tumors in cancer therapies.

An important difference between AATs and other targeted cancer therapies is that anti-angiogenic agents are usually given to unselected patients for inhibiting the angiogenesis commonly existing in tumor tissues [50]. Oncogenic miR-9 is significantly elevated in HCC tissues and HCC-associated ECs, our findings

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**Figure 6.** VEGF-enriched exosomes induce tumorigenesis and promote tumor vasculogenesis.

(a–c). In vivo tumorigenesis of SMMC-7721 cells (1.0 × 10^7 in 500 μL Matrigel) inoculated with phosphate-buffered saline alone or with exosomes (12.5 μg/μL, a total of 100 μg) collected from HUVEC culture supernatant. Images of mice with tumors and harvested tumors after 21 days from SMMC-7721 cells implant (a). Red arrowheads indicate tumors. Tumor volume vs time (b) and tumor weight at 21 days after tumor cell implantation (c). Mean ± SEM, n = 4. *P < 0.05; **P < 0.01; ***P < 0.001 vs. PBS; #P < 0.05, ##P < 0.01 vs. miR-9; @@P < 0.01 vs. miR-9 + 3-MA. (d). Endothelial vessels labeled with CD34 (yellow arrowhead) and VM vessels labeled with PAS (blue arrowhead). Data are representatives of three experiments with four animals per group. (e–g). Evaluation of number of endothelial vessels (e), and diameter (f). Area percentage of VM vessels (g). Mean ± SEM, six fields in 0.32 mm^2 for each animal, four animals per group, n = 4. *P < 0.05; **P < 0.01; ***P < 0.001 vs. PBS; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. miR-9; @@P < 0.01 vs. miR-9 + 3-MA.
show that anti-VEGFR2 by vandetanib predominantly increased the release of VEGF-enriched exosomes from miR-9 overexpressed ECs, suggesting that AATs should also be given to selected patients to avoid developing resistances.

We simulated the upregulation of miR-9 in tumor-associated ECs and found that miR-9 promoted EC angiogenesis through activation of VEGF/Flk1 signaling and released miR-9-enriched exosomes which may participate in promoting endothelial vasculogenesis in HCC xenografts in nude mice. Anti-VEGFR2 by vandetanib successfully abolished miR-9 induced EC angiogenesis but triggered the ECs to release VEGF-enriched exosomes.

Since the sensitivity of Apogee A60 Micro Plus Flow Cytometer is 80 nm for the particle size, the exosomal VEGF were further confirmed by WB and ELISA as VEGF-enriched exosomes are in the range of 30–150 nm (Figure 4(e,f)). It has been demonstrated that...
VEGF exists on the lipid-bilayer membrane of the exosomes from the blood sample of glioblastoma multiforme patient [23]. Like cells, lipid-bilayer membrane should be lysed for ELISA detection. In Treps et al’s study [23], EVs were lysed by a 72°C SDS incubation. In our study, we lysed the exosomes using radioimmunoprecipitation assay. The lysed lipid-bilayer membrane released the VEGF from the exosomes and thus led to a higher VEGF level in the isolated exosomes than that in the culture supernatant. In the future, we may seek a better method to isolate the membrane protein from exosomes.

Autophagy is a dynamic process of subcellular degradation and recycling that is essential for cell survival under nutrient-deprived conditions. Autophagy can be tumor-promoting and tumor-suppressing, which depends on the tumor types and treatment methods [51]. It has been demonstrated that miR-9 inhibited autophagy in A549 cells to enhance cisplatin sensitivity [52]. It has also been demonstrated that miR-9 activated autophagy to promote neuronal differentiation [16]. The controversial roles of miR-9 in autophagy depend on the cell type. Our results showed that miR-9 suppressed EC apoptosis but induced autophagy. Induction of autophagy has been shown in AATs-treated ECs [29]. Anti-VEGFR2 by vandetanib abolished miR-9-induced angiogenesis but promoted miR-9-induced autophagy, similarly to other angiogenesis inhibitors such as kringle 5 and endostatin [19].

The induction of autophagy by miR-9 might be associated with multiply target genes. It was demonstrated that miR-9 increases autophagy by regulating histone deacetylases activity in lymphoma cells [53]. Histone deacetylases inhibitors induce autophagy in cells through Forkhead box protein O1 (FOXO1)-dependent pathway [54]. MiR-9 can also directly target on FOXO1 to enhance the invasion and migration of cervical carcinomas [55]. MiR-9 directly targets on Cullin4A (CUL4A) to promote gastric cancer proliferation and invasion by upregulating the Hippo signaling [56] while phosphorylation of LC3 by the Hippo kinases is essential for autophagy [57]. MiR-9 targets on the inhibitors of β-catenin signaling pathway CK1α and GSK3β [58], which contribute to the disruption of the autophagic flux in HCC cells [59]. MiR-9 targets on neurogenic locus notch homolog protein 2 (Notch2) [60], whose pathway is closely associated with autophagy induction in neural progenitor cells [61] and plays an important role in neurogenesis. MiR-9 targets on cyclin D1 [62] that restraints oncogene-induced autophagy [63]. Furthermore, miR-9 directly targets on monocyte chemoattractant protein-induced protein 1 (MCPIP1) to promote proliferation of neuronal progenitors by activation of the nuclear factor-kappa B (NF-κB) and cAMP response element-binding protein (CREB) pathways [64]. The suppression of autophagy in pulmonary endothelial cells might be regulated by suppressing NF-κB signaling [55]. Thus, miR-9 may induce autophagy via targeted suppression of FOXO1, CUL4A, CK1α, GSK3β, Notch2, cyclin D1, and MCPIP1. Efforts will be made in future studies to clarify the signaling pathway involved in miR-9-induced autophagy in ECs.

Anti-VEGFR2 and anti-autophagy inhibited the miR-9-induced Flk1 expression and phosphorylation, suggesting that vandetanib inhibits phosphorylation of Flk1, which then is cleared by autophagy rather than recycled, whereas miR-9 increases the recycle of Flk1 by autophagy and the phosphorylation of Flk1 to activate angiogenesis.

Tumor vasculogenesis includes both VM and endothelial vasculogenesis [5,65]. Previous studies suggest that induction of autophagy might be associated with tumor vasculogenesis in some tumors [66,67] but the formation of VM is independent of VEGF-driven angiogenesis through the autophagy pathway and activation of Flk1 [67,68]. Our findings indicate that inhibition of autophagy by 3-MA abolished miR-9-induced EC angiogenesis, similar to that following vandetanib treatment. Therefore, anti-autophagy can be an adjuvant strategy for AATs. Both anti-VEGFR2 and anti-autophagy abolished miR-9-induced angiogenesis although anti-VEGFR2 also promoted autophagy. Anti-autophagy by 3-MA abolished anti-VEGFR2-induced autophagy but did not alter the anti-angiogenic effect induced by anti-VEGFR 2. Future studies will be conducted to identify the relevant molecular mechanisms by which anti-autophagy and anti-VEGFR2 contribute to AATs.

On the other hand, inhibition of angiogenesis by vandetanib or 3-MA significantly induced the secretion of VEGF-enriched exosomes from the miR-9 overexpressing ECs with a higher level secretion after vandetanib treatment. Exosomes are intraluminal vesicles released upon fusion of MVBs with the plasma membrane [69]. Inhibition of autophagy may promote EC exosome secretion to remove cellular waste through a fused autophagosome–MVB intracellular compartment [70–72]. However, it was a surprise that inhibition of angiogenesis also promoted ECs to release VEGF-enriched exosomes. But how the VEGF-enriched exosomes are generated and released by anti-angiogenesis and anti-autophagy remains to be found in the future study. Nevertheless, our current results clearly showed the effects of exosomes in tumor vasculogenesis, which resulted from the alternate function of exosomes as intercellular messengers through the delivery of cargos to target other cells, thereby promoting tumor progression and metastasis [20,22,24]. VEGF
from these EC released exosomes should be responsible for the failure of vandetanib to cause lasting remission of angiogenesis.

In conclusion, we have discovered that VEGF-enriched exosomes released from the tumor-associated ECs after anti-angiogenesis or anti-autophagy treatments are responsible for the cross-talk between tumor cells and ECs to promote tumor vasculogenesis. Our finding provides an interpretation for how the alternative proangiogenic pathways are developed following AATs. Our study also found that anti-autophagy is as effective as anti-angiogenesis but triggers less release of VEGF-enriched exosomes, suggesting that anti-autophagy is a promising adjuvant to or even a preferred replacement for AATs. Our findings further suggest that control of exosome release or alteration of exosome cargo composition to inhibit tumor vasculogenesis may serve as a novel strategy to augment the long-term efficacy of anti-angiogenic and anti-autophagic therapies for tumors.

Materials and methods

Cell culture

HUVECs (Allcells, Shanghai, China) were cultured in HUVEC medium (HUVEC-004; Allcells) as previously described [73,74]. We used HUVECs at passages three through six. Human HCC cell lines SMMC-7721 and Huh7 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Dulbecco's modified Eagle medium high glucose medium (Hyclone; GE Healthcare, Logan, UT, USA) with 10% fetal bovine serum (FBS; Hyclone). Cells were maintained at 37°C with 5% CO₂.

MiR-9 mimic (5'-TCTTTGGTTATCTAGCTGTA TGA-3') was inserted into the LV3-pGLV-H1-GFP/puro-lentiviral vector (LV3-miR-9; Cat. No. 150611AZ; GenePharma, Shanghai, China). A lentiviral vector (LV3-NC; Cat. No. E23BZ; GenePharma) was used as NC. After DNA sequencing, recombinant lentiviruses (miR-9 mimics and NC) were produced by co-transfection of 293T cells with the lentiviral vectors pGag/Pol, pRev, and pVSV-G and used to infect HUVECs according to the manufacturer’s instructions. Fluorescence-activated cell sorting based on GFP expression was performed after 72 h, and miR-9 overexpression was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). To investigate the roles of Flk1 and autophagy in tumor-cell migration, invasion, angiogenesis, and the release of exosomes, the Flk1 inhibitor vandetanib (4 μM, S1046, Selleck, Shanghai, China) and autophagy inhibitor 3-MA (5 mM, M9281, Sigma-Aldrich, St. Louis, MO, USA) were administered to pretreated HUVECs for 60 min and subsequently added to the experimental medium (HUVEC basal medium, HUVEC-004B; Allcells). Recombinant human VEGF 165 Protein (50 ng/ml; hVEGF; 293-VE, R&D system, Minneapolis, MN, USA) was used as a positive control. VEGF antibodies (4 μg/ml; Bevacizumab, HY-P9906, MCE, Monmouth Junction, NJ, USA) were used for the in vitro angiogenesis assay and colony formation assay for HCC cells.

Isolation and purification of exosomes

Exosomes were isolated from the cell culture supernatant. Briefly, the lentivirus-infected HUVECs were cultured as monolayers for 48 h in respective complete medium under an atmosphere of 5% CO₂ at 37°C. Then, cells were pretreated with or without vandetanib (4 μM) or 3-MA (5 mM) for 60 min, and cultured for 48 h. Exosomes were isolated by differential centrifugation at 300 g (Centrifuge 5804R; Eppendorf, Hamburg, Germany) at 4°C for 20 min and 3000 g at 4°C for 30 min to remove dead cells and cell debris, followed by a 30 min centrifugation at 10,000 g (Centrifuge 5804; Eppendorf) at 4°C to pellet the MVs. The supernatant was filtered with a 0.22 μm filter (Millipore, Billerica, MA, USA). Exosomes in the supernatant were subsequently pelleted by ultracentrifugation (Optima L-80 XP, Beckman Coulter, Brea, CA, USA) for 2 h at 100,000 g. The final EV pellet was resuspended in 100 μL PBS and stored at 4°C.

Nano-flow cytometry analysis of exosomes

To detect specific surface proteins, exosomes were isolated from 50 mL culture supernatants of about 5 × 10⁷ HUVECs and incubated with anti-VEGF antibody (1:200; ab52917; Abcam, Cambridge, MA, USA) for 30 min at 25°C, and then incubated with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (1:2000; H&L; ab150081; Abcam) for 10 min at 25°C. Rabbit IgG (1:2000; monoclonal; ab172730; Abcam) and unlabeled sample were used as isotype control and blank control, respectively. Then, exosomes were analyzed on an Apogee A60 Micro Plus Flow Cytometer (Apogee Flow Systems, Northwood, UK) that was specially developed for the analysis of nanoparticles (http://www.apogeeflow.com/products.php).

The reference ApogeeMix beads with a refractive index of 1.59 (mixture of 110 and 500 nm green fluorescent latex beads; Cat#1493; Apogee Flow Systems) and non-fluorescent silica beads (180, 240, 300, 590, 880, and 1300 nm; http://www.apogeeflow.com/products.php) with a refractive index of 1.43 were used to assess the
flow cytometer performance and exosome size distribution. PBS was used as a background control. Default settings were used for the reference beads and exosome samples. Large angle light scatter (LALS) was used for particle sizing using a sample flow rate of 1.5 μL/min and a total volume of 130 μL. The sample flow rate thresholds for the laser (488-LALS) were set as 30 V and 350 V, and those for the 488-Grn (green fluorescence) laser were 17 V and 475 V. After gating the background, events in the region of interest were calculated using Apogee Histogram software (v255.0.0.68; Apogee Flow Systems).

**In vitro angiogenesis assay and colony formation assay**

When plated on Matrigel, HUVECs undergo differentiation into capillary-like tube structures in vitro. A tube-formation assay was used to evaluate angiogenesis in vitro as previously described [75]. The 24-well plates were coated with Matrigel (300 μL/well; BD Biosciences, San Jose, CA, USA) without introducing air bubbles. After gelling of the Matrigel, 5 × 10^4 lentivirus-infected HUVECs were plated into each Matrigel-coated well along with 200 μL of HUVEC basal medium containing 10% FBS. To evaluate roles for exosomes derived from HUVECs in VM of malignancy, 5 × 10^4 HCC cells (Huh7 or SMMC-7721) were plated into each Matrigel-coated well along with 200 μL of Dulbecco’s modified Eagle high glucose medium containing 10% FBS and 5 μg exosomes. The amount of exosomes was detected by measuring the total protein content using a protein determination kit (Cayman Chemical Company, Ann Arbor, MI, USA). After 6 h (HUVEC angiogenesis) or 8 h (VM by HCC cells) incubation at 37°C in a 5% CO₂/95% air incubator, the medium was gently aspirated from each well and incubated with Diff-Quick fixative (Dade Behring, Deerfield, IL, USA) for 30 s and subsequently stained with solution II for 2 min. Tube structures were observed and imaged by microscopy. The pseudovascular organization of cells was analyzed by ImageJ software (version 1.51s; National Institutes of Health, Bethesda, MD, USA) using the Angiogenesis Analyzer plugin (written by Gilles Carpentier, 2012. The plugin is available at [http://imagej.nih.gov/ij/macros/toolsets/Angiogenesis%20Analyzer.txt](http://imagej.nih.gov/ij/macros/toolsets/Angiogenesis%20Analyzer.txt)).

The crosstalk between HUVECs and HCC cells were performed by Transwell (BD Biosciences). Cells were seeded at a density of 1 × 10^4 HUEVCs per Transwell insert. After pretreatment with culture media containing 0.001% dimethyl sulfoxide (DMSO) and 10 μM GW4869 (dissolved in DMSO; D1692, Sigma-Aldrich, USA) in the presence of vandetanib or 3-MA for 48 h, the Transwell inserts with HUVECs were co-cultured with 5 × 10^4 HCC cells on the 24-well plates for 48 h. Tube structures were observed and imaged by microscopy.

For the colony formation assay, HCC cells were seeded in a 35-mm cell culture dish at a density of 2 × 10^4 cells/well and treated with 5 μg of exosomes for 14 days to form colonies. Cells were stained with crystal violet (0.5%, w/v), photographed by microscopy, and quantified using ImageJ software.

**In vivo angiogenesis assay**

The murine Matrigel plug was used to evaluate angiogenesis in vivo [76]. Use of animals was in accordance with animal care guidelines, and all animal experiments were approved by the Animal Care and Use Committee of Sichuan University, China. Matrigel (500 μL) was subcutaneously injected either alone or mixed with 1.0 × 10^7 lentivirus-infected HUVECs into the ventral region of three athymic BALB/c Nu/Nu 6-week-old nude mice (Guangdong Medical Lab Animal Center, Guangdong, China) to form a Matrigel plug. Mice were sacrificed at 7-days after injection, and the Matrigel plug was removed, fixed with formalin, and embedded in paraffin. The level of angiogenesis was determined by hematoxylin and eosin and immunohistochemistry (IHC) staining of CD34 and CD31. The amount and size of vascular vessels were analyzed using ImageJ software. Apoptotic cells were detected by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay.

**In vivo tumorigenesis assay**

Matrigel (500 μL) was mixed with a total of 1.0 × 10^7 SMMC-7721 cells and 100 μg of exosomes derived from HUVECs (NC lentivirus-infected HUVECs or miR-9-mimic lentivirus-infected HUVECs in the presence or absence of 3-MA or vandetanib), and subcutaneously injected into the right flank of three 6-week-old Balb/c athymic nude female mice. Matrigel mixed with SMMC-7721 cells and PBS was used as a control. Tumor sizes were measured weekly, and growth curves were plotted. Three weeks later, the mice were euthanized, the dissected tumors were collected and weighted, and tumor volumes were assessed via caliper measurement. Vasculogenesis was assessed by IHC staining and western blot detection of CD34-PAS, VEGF, Flk1, and p-Flk1.
Cell migration and invasion assays

After pretreatment, lentivirus-infected HUVECs were digested with 0.25% EDTA trypsin and resuspended in HUVEC basal medium with or without 4 μM vandetanib, 5 mM 3-MA or 50 ng/mL hVEGF. Cells were seeded at a density of 1 × 10⁵ cells per Transwell (BD Biosciences), and HUVEC basal medium containing 10% FBS was added to the lower chamber. After 48 h, cells were fixed with 4% paraformaldehyde for 10 min, followed by washing twice with PBS and staining with crystal violet (0.5%, w/v). Non-invading cells were removed using a cotton swab, and cells that had migrated through the membrane were quantified [77]. The Transwell membrane was precoated with Matrigel for invasion and not precoated for migration.

Enzyme-linked immunosorbent assay (ELISA) detection of VEGF

Lentivirus-infected HUVECs were pretreated with vandetanib and/or 3-MA for 60 min, and VEGF levels in cell-culture supernatant, exosome-depleted media and exosomes were determined using an ELISA kit (Catalog#PDVE00; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. For exosome pellets, proteins were extracted by radio-immunoprecipitation assay lysis.

qRT-PCR

RNA was extracted from cells, tissues, and exosome pellets using TRIzol (Invitrogen, Carlsbad, CA, USA), and qRT-PCR assays were performed using a SYBR Premix Ex Taq kit (TaKaRa, Shiga, Japan) as previously described [73]. The primer sequences were as follows: VEGF, 5′-CTGACGGACAGACACAGACACACC-3′ (forward) and 5′-AGCCCGAAGTGGACGAAA-3′ (reverse); Flk1, 5′-ACCTGGGAAATCAGACGA CAA-3′ (forward) and 5′-GGTTCCCATCCTCTCA ATACAAT-3′ (reverse); and β-actin, 5′-ATCGTG CGTGCACATTAAGGAGA-3′ (forward) and 5′-AGGAAGGGAGGCTGGAAG-3′ (reverse). All primers and probes were obtained from TaKaRa. Gene expression was normalized to that of β-actin using the 2^−ΔΔCT method, and data are presented as expression relative to the indicated controls. The stem-loop primers and probes for mature miR-9 and U6 small nuclear (sn)RNA were as follows: hsa- miR-9-5p, 5′-ACACTCCAGCTGTTCTTGGGTA TCTAG-3′ (forward) and 5′-CTCAACTGTTGTC GTGGAGTGCGCAATCTAGTTCATACAG-3′ (reverse); and U6 snRNA 5′-CTCGTTCGGCA GCACA-3′ (forward) and 5′-AACGCTTACGGAATT TGCGT-3′ (reverse). The relative expression level of miR-9 was normalized to that of U6 snRNA and shown as a ratio relative to the expression level in the control. For exosomal miR-9 detection, the synthetic miRNA Caenorhabditis elegans miR-39 (cel-miR-39; 5 fmol/μL; Sequence: 5′-UCACCCGGUGUAACUCACUUUG-3′; Qiagen, Hilden, Germany) was added to the isolated RNAs and was used as an exogenous control. Data are representative of three independent experiments.

Western blotting

Proteins were extracted from cells, tissues, and exosome pellets using radioimmunoprecipitation assay lysis buffer containing a protease-inhibitor cocktail (Beyotime, Beijing, China). After determination of protein concentration using a protein determination kit (Cayman Chemical Company, USA), equal amounts (20–30 μg for cells, 10 μg for exosome pellets, and 50 μg for tissues) of protein samples or exosomal proteins from 2.5 mL culture supernatant were size fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, electro-transferred onto a polyvinylidene fluoride membrane (Millipore), blocked with 5% non-fat milk in PBS, and hybridized with antibodies against VEGF (TA500289; Origene, Rockville, MD, USA), Flk1 (#9698; Cell Signaling Technology, Danvers, MA, USA), p-Flk1 (Tyr1175; #19A10; Cell Signaling Technology), LC3B/II (ab192890; Abcam), TSG101 (14,497–1-AP; Proteinteck; Rosemont, IL, USA), HSP70 (10,995–1-AP; Proteinteck), CD63 (ab134045; Abcam), and GRP94 (ab3674; Abcam) at 4°C overnight. A 1:1000 dilution of the antibodies was used for detection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The blots were incubated with the corresponding horse-radish peroxidase (HRP)-conjugated secondary antibodies (1:5000; Beyotime), and enhanced chemiluminescence was performed using an Immobilon western chemiluminescent HRP substrate (WBKLS0050; Millipore) to visualize the bands. Densitometric quantification was performed using ImageJ software.

Immunostaining and confocal microscopy

Immunofluorescence staining and quantitative analysis of LC3BII and p-Flk1 were performed as previously described [78,79]. After treatment, HUVECs were immediately fixed with 2% paraformaldehyde/0.1% glutaraldehyde for 30 min, permeabilized with 0.1% Triton X-100 (T-8787; Sigma-Aldrich) for 5 min, blocked with 2% goat serum (Invitrogen) for 30 min, stained with antibodies against p-Flk1 (1:100; Tyr1175;
Acridine orange (AO) staining of autophagosomes

AO (Sigma-Aldrich) was used to evaluate the formation of acidic vesicular organelles during autophagy. After treatment, HUVECs were washed with PBS once and incubated with AO (1 µg/mL) for 15 min at 37°C in the dark. Cells were then washed with PBS and observed under an inverted fluorescence microscope (Olympus IX71; Olympus, Tokyo, Japan).

Transmission electron microscopy

After treatment, HUVECs were immediately fixed with 2% electron-microscopy-grade glutaraldehyde (Polysciences, Warrington, PA, USA) in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min. Cells were then washed once with sodium cacodylate buffer, post-fixed using 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA, USA) and dehydrated in a graded ethanol series before being embedded in Araldite. Thin sections were cut and stained with lead citrate and uranyl acetate before being observed under a Hitachi H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).
USA) in 0.1 M sodium cacodylate buffer for 1 h, and en bloc-stained with 1% uranyl acetate (Polysciences) in 70% ethanol for 1.5 h. After dehydration in ascending grades (50–100%) of ethanol, samples were embedded in an epoxy resin mixture of Epon 812 and Araldite M (Sigma-Aldrich). Ultrathin sections (40 nm) were analyzed using a transmission electron microscope (Tecnai G2 Spirit; BioTWIN; FEI; Thermo Fisher Scientific). For isolated exosomes, the resuspended pellets (10 μL) were absorbed onto formvar/carbon-coated copper grids for 5 min at 25°C, contrasted with 10 μL of 1% phosphotungstic acid for 5 min at 25°C, and examined using transmission electron microscopy. For gold labelling, exosomes were fixed with 2%PFA for 30 min and absorbed onto formvar/carbon-coated copper grids for 20 min at 25°C, followed by washing in 50 mM glycine for 3 min and PBS. The exosomes were blocked with 5%BSA for 10 min, washed with PBS and incubated with CD63 rabbit monoclonal antibody (Ab134045, Abcam) for 30 min, 1% glutaraldehyde for 3 min, washed in distilled water and finally embedded in 2% phosphotungstic acid.

**Statistical analysis**

Statistical significance was determined by student’s t-test or one-way analysis of variance with either the least significant difference test or Tamhane’s T2 test (depending on Levene’s statistic for homogeneity of variance) using SPSS software (v24.0; IBM, Armonk, NY, USA). P < 0.05 was considered statistically significant.

**Acknowledgments**

The authors would like to thank Bio-Gene Technology Ltd., Hong Kong for help with exosomes analysis using the Apogee A60 Micro Plus Flow Cytometer.

**Funding**

National Natural Science Foundation of China (Grant no. 11402153), and the Excellent Young Scientist Foundation (No. 2015SCU04A38) and the Talent Introduction Scientific Research Projects Funded Start-Up Funds (No.2082204174089) of Sichuan University.

**Author contributions**

Y.Z. and B.M.F. designed the studies. Y.Z., X.H.Y., and Z.P. Y. performed and analyzed the experiments. Y.Z. and B.M. F. wrote the manuscript. X.H.Y. and B.M.F. assisted with the flow cytometry, data analysis and collection of the samples. X.L. H. and J.W. helped with tissue staining. X.H.L., L.L., and X.J. L. contributed to study design. Y.Z. and B.M.F. oversaw all data analysis.

**Disclosure of interest**

The authors report no conflicts of interest.

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