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Research article

CD133-containing microvesicles promote colorectal cancer progression by inducing tumor angiogenesis

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ABSTRACT

Angiogenesis is an indispensable mechanism in cancer progression, as cancer cells need to establish blood vessels to supply oxygen and nutrients. Extracellular vesicles (EVs) derived from cancer cells act as messengers in the tumor microenvironment and induce resistance to anti-angiogenic cancer treatment. EVs can be classified into two categories: exosomes and microvesicles (MVs). Although exosomes are involved in angiogenesis, the role of MVs in angiogenesis and cancer progression remains unclear. CD133 plays a key role in MV formation and oncoprotein trafficking. In this study, we investigated the role of CD133-containing MVs derived from colorectal cancer (CRC) in angiogenesis and cancer progression. CRC-derived MVs were incorporated into endothelial cells and increased the mesh area and tube length of endothelial cells. CD133-containing MVs also stimulate vessel sprouting in endothelial cell spheroids and mouse thoracic aortas. However, MVs derived from CD133-knockdown CRC cells exerted a limited effect on tube formation and vessel sprouting. CD133-containing MVs induced angiogenesis through p38 activation and angiogenesis induced by CD133-containing MVs was insensitive to the anti-vascular endothelial growth factor antibody bevacizumab. Survival analysis revealed that high expression level of CD133 correlated with poor prognosis in patients with metastatic CRC. These findings suggest that CD133-containing MVs act as key regulators of angiogenesis and are related to the prognosis of CRC patients.

1. Introduction

Colorectal cancer (CRC) ranks second in mortality and third in morbidity worldwide [1]. The mortality rate of CRC is decreasing owing to improved cancer treatment and increased cancer screening, but the 5-year survival rate of CRC sharply declines when malignant cancer spreads to distant sites [1,2]. Since hypoxia and rapid proliferation are common features of solid tumors, cancer cells require additional vasculatures to thrive. Angiogenesis, new blood vessel formation from the existing vasculatures, is an indispensable process for cancer progression to satisfy the demand for nutrients and oxygen [3]. Furthermore, angiogenesis and endothelial-to-mesenchymal transition play pivotal roles in the establishing metastatic niche [4].

Angiogenesis is induced by various growth factors, and vascular endothelial growth factor (VEGF) has a dominant role in angiogenesis [5]. Among several isoforms of VEGF, VEGFA acts as a key regulator of blood vessel growth [6]. VEGFA primarily binds to VEGF receptor 2 (VEGFR2) and transduces signals through the phosphorylation of VEGFR2 tyrosine residues [7]. The phosphorylation of VEGFR2 Tyr1175, followed by ERK and AKT pathway activation, promotes the proliferation, survival, and migration of endothelial

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cells as well as vascular permeability [8,9]. Another important phosphorylation site of VEGFR2 is Tyr1214, which is involved in activating the PAK/p38 MAPK pathway, thereby stimulating actin remodeling and endothelial cell migration [10]. The AKT and ERK signaling pathways play crucial roles in VEGF-induced angiogenesis; however, a recent study reported that endophilin-mediated VEGFR2 endocytosis promotes angiogenesis through PAK/p38 activation regardless of ERK stimulation [11]. It is widely known that various types of cancers, including CRC, induce tumor angiogenesis by secreting VEGF [6]. To block tumor angiogenesis and progression, multiple anti-VEGF and anti-VEGFR therapies have been developed and administered [12]. In 2004, the U.S. Food and Drug Administration approved bevacizumab, the first humanized monoclonal anti-VEGF antibody, for treating several solid tumors including CRC [13]. Bevacizumab, combined with chemotherapy, provided survival benefits for metastatic CRC patients [14]. However, after a fleeting period of clinical benefit, adaptive resistance developed, and the tumors began to regrow [15]. Given that tumor angiogenesis is a critical step in tumor progression, a broader understanding of pro-angiogenic mechanisms is required [16].

Extracellular vesicles (EVs) are generated by all types of cells and mediate intercellular communication by transporting proteins, lipids, and genetic materials [17]. EVs can be categorized into two groups depending on their size and biogenesis: exosomes and microvesicles (MVs) [17]. Exosomes are released from multivesicular bodies and are in a size range of 40–160 nm in diameter [18]. In contrast, MVs are generated by direct budding from the plasma membrane and have sizes ranging from 50 nm to 1 μm in diameter [19]. Although there are considerable differences between EV types, it is widely known that cancer-derived EVs in the tumor microenvironment (TME) promote cancer progression [17]. Recent studies have reported that cancer-derived exosomes induce angiogenesis and are insensitive to bevacizumab [20,21]. Ovarian cancer-derived exosomes induce angiogenesis via exosomal VEGF, which is insensitive to monoclonal VEGF antibodies [20]. Breast cancer-derived exosomes enriched with VEGF weaken the affinity for bevacizumab and promote angiogenesis [21]. There are also reports that cancer-derived exosomes induce angiogenesis via VEGF-independent mechanisms [22,23]. Exosomal ephrin type-A receptor 2 (EPHA2) promotes angiogenesis and is upregulated in highly metastatic breast cancer [22]. Truncated E-cadherin localizes to ovarian cancer-derived exosomes and promotes angiogenesis [23]. Considering that EVs transfer various molecules with high stability and enable intercellular interactions without the cell-to-cell contact, it is necessary to understand the diverse noncanonical signaling pathways that are stimulated by EVs. However, the exact mechanism underlying MV-induced angiogenesis is poorly understood.

CD133 is a penta-span transmembrane glycoprotein comprising an extracellular N-terminal domain, five transmembrane segments, and an intracellular C-terminal domain [24]. CD133 has been postulated as a cancer stem cell marker for various tumor types, including brain, colorectal, and lung cancers [25]. CD133 is also associated with a higher rate of cancer recurrence and distant metastasis in CRC [26]. Recent studies have reported that CD133 is incorporated into cancer-derived EVs and transferred to adjacent cells, thereby increasing the phosphorylation of intracellular proteins involved in cell migration and proliferation [27]. The number of CD133-containing EVs is higher in patients with advanced CRC than in healthy donors [28]. Furthermore, CD133 plays a key role in MV formation and budding in CRC [29]. CD133-containing MVs derived from KRAS mutant CRC cells transport KRAS oncoproteins to adjacent cells and induce cell motility, proliferation, and anti-epidermal growth factor receptor drug resistance in CRC [29]. However, the role of CD133-containing MVs in angiogenesis remains unclear. In this study, we investigated the role of CD133-containing MVs in angiogenesis and their effect on cancer progression.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) medium 1640, and Optimized Minimum Essential Medium (Opti-MEM) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT, USA). Myco-guard™ was purchased from Biomax (Seoul, South Korea). SB203580 was purchased from Tocris Biosciences (Bristol, UK). Human recombinant VEGF 165 ($hVEGFA_{165}$) was purchased from R&D Systems (Minneapolis, MN, USA). Mouse monoclonal antibodies recognizing β-actin, AKT, ERK, hVEGFA, and flotillin-2 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse monoclonal antibodies recognizing phospho-p44/42 ERK and rabbit monoclonal hVEGFR2, p38, phospho-p38, and phospho-AKT antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibodies recognizing CD133 were purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). Anti-mouse and anti-rabbit peroxidase-conjugated secondary antibodies were obtained from Pierce (Madison, WI, USA). Bevacizumab (avastin) was obtained from Genentech (San Francisco, CA, USA).

2.2. Cell culture and stable cell line generation

HCT116 and EA.hy926 cells were purchased from American Type Culture Collection (Manassas, VA, USA). All the cell lines were authenticated using the short tandem repeat (STR) method before used in the experiments and regularly mixed with Myco-guard (Biomax, Seoul, South Korea) to prevent mycoplasma contamination. To establish Mock short hairpin RNA (shMock) and CD133 short hairpin RNA (shCD133) HCT116 stable cell lines, HCT116 cells were transfected with pSliencer 2.1-Zeo or pSliencer 2.1-CD133-Zeo plasmid for 24 h and zeocin was used as the antibiotic for selection. shMock and shCD133 HCT116 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 100 μg/ml zeocin (Thermo Fisher Scientific). EA.hy926 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a 37 °C humidified incubator containing 5% CO_2.

2.3. MV purification

shMock and shCD133 HCT116 cells were cultured at a density of 6×10^6 cells/plate on 150 mm plates. After 48 h, cells were cultured in serum-free RPMI 1640 medium, and the cells were incubated for 24 h. The media were collected and centrifuged at 500×*g* for 5 min and at 3000×*g* for 10 min. Supernatants were filtered through a 0.8 μm Supor membrane filter (Pall, Washington, NY, USA) and centrifuged at 20,000×*g* for 1 h. Pellets were resuspended in suitable medium and used for experiments.

2.4. Western blot analysis

shMock and shCD133 MVs were isolated at a density of 6×10^6 cells/plate on 150 mm plates (a total of 8 plates per experiment). Proteins of cells were extracted using RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris-HCl, pH 7.4, and protease inhibitors). Equal amount of protein was resolved in 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with specific antibodies at 4 °C overnight. β-actin or flotillin-2 was used as control. Protein bands were detected using a western blot detection kit (YoungIn Frontier, Seoul, South Korea).

2.5. Fluorescence microscopic analysis

For MV detection in recipient cells, EA.hy926 cells were plated on confocal dishes (SPL Life Sciences, Pocheon, South Korea) at a density of 3×10^4 cells/well. Isolated MVs (a total of 4 plates, 6×10^6 cells/plate on 150 mm plates) were stained with 1 μg/ml wheat germ agglutinin (WGA)-488 (Thermo Fisher Scientific) for 10 min and washed twice with PBS. Cells were incubated with stained MVs for 16 h. After fixation using 4% paraformaldehyde for 15 min, cells were stained with 200 ng/ml DAPI for 10 min at 25 °C and washed twice with PBS. Fluorescence intensity was captured using an LSM800 Meta confocal microscope (Carl Zeiss, Jena, Germany).

2.6. Tube formation assay

Growth factor-reduced Matrigel (Corning, Bedford, MA, USA) was plated in 24-well plates and incubated for 1 h at 37 °C. shMock and shCD133 MVs were isolated at a density of 6×10^6 cells/plate on 150 mm plates (a total of 8 plates per experiment). EA.hy926 cells were seeded on Matrigel-coated wells at a density of 1.5×10^5 cells/well in 5% DMEM with or without MVs and SB203580. Following incubation for 13 or 16 h, the capillary tube structures were visualized under an Axiovert 100 inverted microscope (Carl Zeiss, Jena, Germany). For bevacizumab treatment analysis, Matrigel was plated in 96-well plates, and EA.hy926 cells were seeded at a density of 3×10^4 cells/well in serum-free DMEM with or without MVs (a total of 4 plates, 6×10^6 cells/plate on 150 mm plates), $hVEGF_{165}$, and bevacizumab. Following incubation for 16 h, the capillary tube structures were visualized using an IX73 inverted microscope (Olympus, Tokyo, Japan). Five random fields were analyzed for each experiment. The results were quantified using the angiogenesis analyzer plugin in the ImageJ software, measuring the number of tubes and meshes, total length of tubes, and total mesh area in each field.

2.7. Mouse aortic ring assay

The animal study protocol was approved by the Institutional Animal Care and Use Committee of Korea University. Animal studies were performed in accordance with the guidelines for the use and care of laboratory animals. C57/BL6 mice were maintained at 22 ± 2 °C under a 12-h light-dark regimen. Thoracic aortas excised from 10-week-old female C57/BL6 mice were cut into 0.5 mm long cross sections. The aortic rings were placed in rat tail collagen type I (Corning)-coated wells and incubated for 30 min at 37 °C to allow the collagen to polymerize. Opti-MEM supplemented with 2% FBS, 1% penicillin/streptomycin, and 30 ng/ml hVEGFA was added to the wells. After 48 h, aortic rings were incubated with MVs derived from shMock and shCD133 HCT116 cells (a total of 4 plates, 6×10^6 cells/plate on 150 mm plates) for 3 days. Sprouts were observed on day 5 using an IX73 inverted microscope (Olympus). Vascular outgrowth was quantified by counting all the sprouts from a single aortic ring. All the assays were performed in duplicate, and each experiment was repeated three times.

2.8. Spheroid-based sprouting assay

EA.hy926 cells were suspended in a 4:1 mixture of 10% FBS DMEM and Methocel (1% w/v methylcellulose in serum-free DMEM). EA.hy926 cell spheroids containing 1×10^3 cells were created using the hanging drop method at 37 °C in a humidified incubator containing 5% CO_2. After 24 h of incubation, spheroids were collected and centrifuged at 200×*g* for 5 min. The spheroids were then resuspended in a mixed solution containing 20% FBS, 3 mg/ml collagen type I, 10 × DMEM, and 0.2 N NaOH to adjust the pH to 7.4. Fifty spheroids (0.7 ml of the mixed solution were added to individual wells of a pre-warmed 24-well plate and polymerized for 30 min in a 37 °C humidified incubator containing 5% CO_2. After 30 min, shMock and shCD133 MVs (a total of 4 plates, 6×10^6 cells/plate on 150 mm plates) in 300 μl of DMEM were plated on a polymerized collagen mixture. After 48 h, the spheroids were observed using an Axiovert 100 inverted microscope (Carl Zeiss). The number and cumulative length of sprouts per spheroid were quantified from five spheroids for each condition using the ImageJ software.

2.9. Human angiogenesis antibody array

Cell lysates were obtained from EA.hy926 cells treated with or without shMock or shCD133 MVs (a total of 8 plates, 6×10^6 cells/plate on 150 mm plates) for 16 h. The human angiogenesis antibody array was performed using the RayBio Human Angiogenesis Array G1000 (RayBiotech Life, Norcross, GA, USA), according to the manufacturer's protocol.

2.10. Survival analysis

Survival analysis was performed using the Human Protein Atlas database (https://www.proteinatlas.org/). The Human Protein Atlas database contains survival and cancer progression data of 747 CRC patients. The log-rank $p < 0.05$ was considered statistically significant.

2.11. Statistical analysis

Data are presented as means ± SEM. Statistical analyses were performed using GraphPad Prism Software 5 (GraphPad Software, La Jolla, CA, USA). For the two-tailed Student's *t*-test, $p < 0.05$ was considered statistically significant.

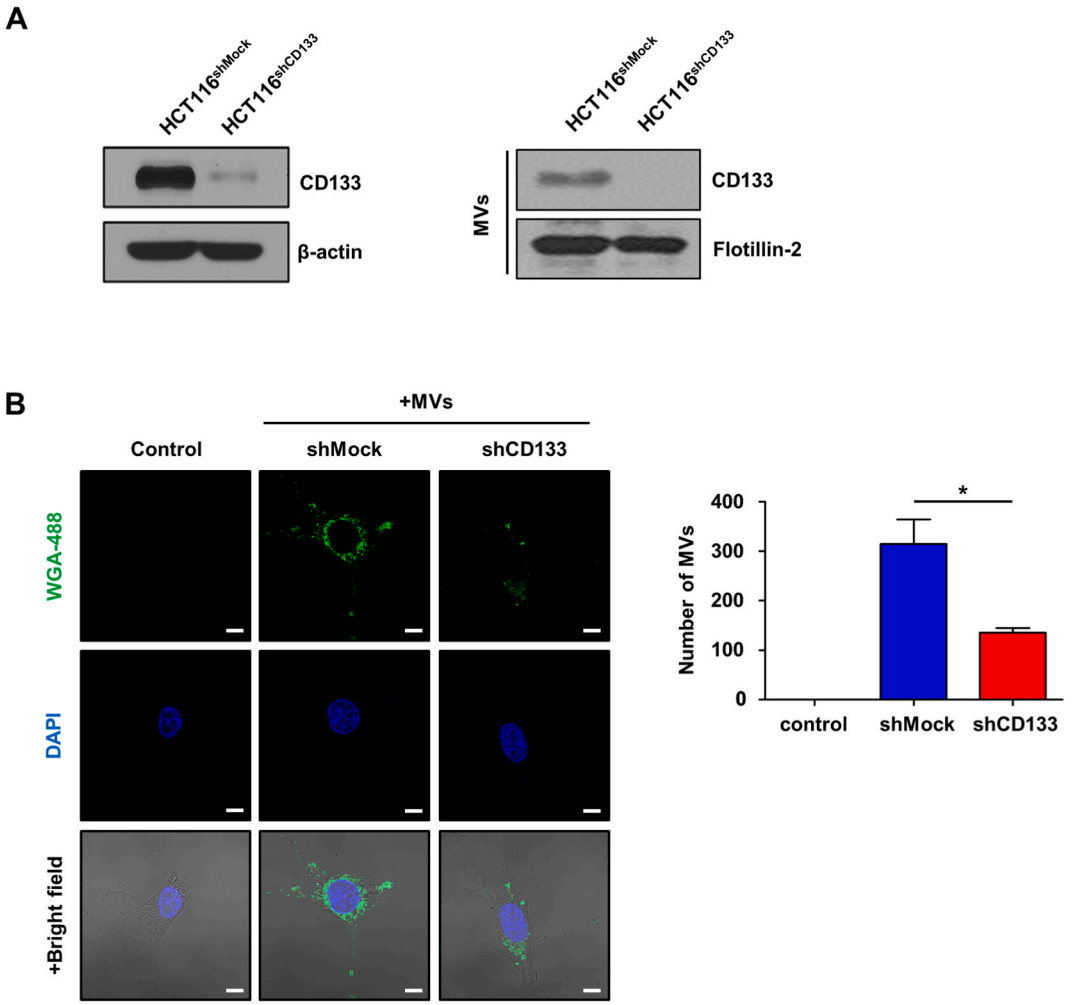

Fig. 1. CD133-containing MVs are incorporated into endothelial cells. (A) Stable shMock and shCD133 HCT116 cells were generated. MVs were isolated from shMock and shCD133 HCT116 cells. Protein level was determined using western blotting. Full-length blot images are shown in Fig. S1 (B) shMock and shCD133 MVs were stained with WGA-488. EA.hy926 cells were incubated with stained MVs for 16 h. Images were captured by confocal laser scanning microscopy (green, WGA-488; blue, DAPI). Scale bar = 10 μm. The number of MVs were counted by imageJ. All *p* values were obtained using unpaired two-tailed Student's *t*-test. $^*p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

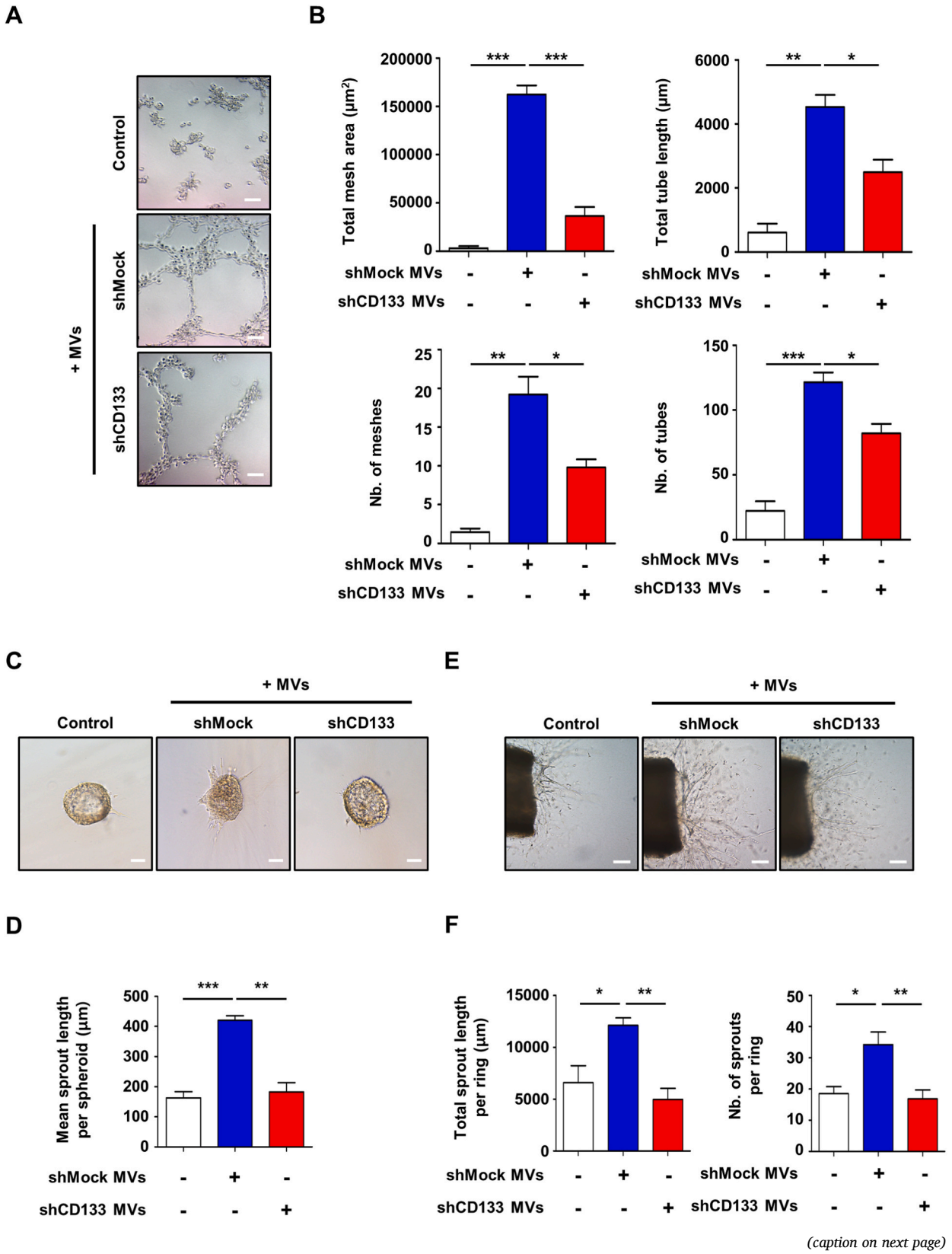

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Fig. 2. CD133-containing MVs promote tube formation and vessel sprouting in endothelial cells. (A) EA.hy926 cells were treated with MVs isolated from shMock and shCD133 HCT116 cells for 16 h. Scale bar = 100 μm. (B) Meshes and tubes were measured from five random fields in each well. (C) EA.hy926 cell spheroids embedded in 3D collagen gels were treated with shMock or shCD133 MVs for 48 h. Representative images of endothelial spheroids were shown. Scale bar = 100 μm. (D) Five random spheroids were measured in each well. (E) Mouse aortic rings were embedded in 3D collagen gels and stimulated with 2% FBS media supplemented with VEGF (30 ng/mL). After 48 h, rings were washed twice, and media were changed with 2% FBS containing shMock or shCD133 MVs and incubated for 3 days. Representative images of mouse aortic rings were shown. Scale bar = 200 μm. (F) All the sprouts of each ring were measured. Mean ± SEM of $n = 3$ independent experiments. All p values were obtained using unpaired two-tailed Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$.

3. Results

3.1. CD133-containing MVs are incorporated into endothelial cells

CD133 is associated with cancer metastasis, which is a major cause of deaths in CRC [2]. A recent study revealed that CD133-containing MVs derived from CRC cells are incorporated into adjacent cells and promote cell motility [29]. Angiogenesis is a crucial process in cancer progression. Therefore, we investigated the effect of CD133-containing MVs on angiogenesis. We first examined whether CD133-containing MVs were incorporated into endothelial cells in the TME of CRC. CD133 knockdown (shCD133) and mock (shMock) HCT116 stable cells were generated using short hairpin RNA, and MVs were isolated from cells using differential centrifugation. shCD133 cells showed decreased CD133 expression compared to shMock cells (Fig. 1A). CD133 expression was also lower in MVs isolated from shCD133 cells (shCD133 MVs) than in MVs from shMock cells (shMock MVs) (Fig. 1A). Next, we examined the transport of CD133-containing MVs to endothelial cells. The shMock and shCD133 MVs were stained with the plasma membrane indicator WGA-488 and incubated with EA.hy926 cells. The EAhy926 cell line is a human cell line displaying various features of vascular endothelial cells, and has been widely used in the study of angiogenesis. Confocal microscopy analysis revealed that shMock and shCD133 MVs were incorporated into endothelial cells (Fig. 1B). The number of MVs incorporated into endothelial cells was much lower in shCD133 MVs than in shMock MVs (Fig. 1B). These results indicate that CD133-containing MVs are incorporated into endothelial cells.

3.2. CD133-containing MVs promote tube formation and vessel sprouting in endothelial cells

To investigate whether the incorporation of CD133-containing MVs was involved in angiogenesis, we performed a tube formation assay in EA.hy926 cells. Results revealed that shMock MV-treated cells showed increased capillary formation compared to the untreated control cells (Fig. 2A) However, shCD133 MVs exerted a little effect on capillary formation (Fig. 2A). shMock MV-treated cells exhibited an increased mesh area, tube length, and number of meshes and tubes compared to those of control cells, whereas shCD133 MVs had little effect on the formation of meshes and tubes (Fig. 2B). Sprout formation in endothelial cells is the initial step in pathological angiogenesis [3]. To investigate whether CD133-containing MVs induced endothelial sprouting, we performed spheroid-based sprouting and mouse aortic ring assays in a 3D cell culture environment. The results of the spheroid-based sprouting assay revealed that shMock MV-treated spheroids exhibited increased endothelial sprouting compared with control spheroids, whereas shCD133 MVs did not affect endothelial sprouting (Fig. 2C). The average sprout length was 4-fold higher in shMock MV-treated spheroids than that in control spheroids, whereas shCD133 MVs did not affect sprout length (Fig. 2D). The results of the mouse aortic ring assay revealed that shMock MV-treated mouse aortic rings showed enhanced endothelial sprouting compared to the untreated mouse aortic rings (Fig. 2E). However, shCD133 MVs had little effect on endothelial sprouting (Fig. 2E). shMock MVs increased the total sprout length per ring and the number of sprouts by 39- and 33.4-fold, respectively (Fig. 2F). However, shCD133 MVs did not show a significant increase (Fig. 2F). These results indicate that CD133-containing MVs promote tube formation and sprouting in endothelial cells.

3.3. CD133-containing MVs induce angiogenesis through the p38 MAPK signaling pathway

A variety of pro-angiogenic molecules, such as VEGF, regulate angiogenesis through phosphorylation of protein kinases [30]. The phosphorylation of ERK, AKT, and p38 promotes the proliferation, migration, and permeability of endothelial cells, leading to angiogenesis [8,10]. To investigate the mechanism of angiogenesis induced by CD133-containing MVs, we first examined whether protein kinase phosphorylation was involved in MV-induced angiogenesis. CD133-containing MVs did not affect AKT or ERK phosphorylation (Fig. 3A). However, shMock MV-treated cells showed elevated phosphorylation levels of p38 MAPK compared with those of control cells, whereas shCD133 MVs had little effect on p38 phosphorylation (Fig. 3A and B). To investigate whether p38 MAPK acts as a major regulator of MV-induced angiogenesis, EA.hy926 cells were treated with the p38 MAPK inhibitor SB203580 and a tube formation assay was performed. In the presence of SB203580, shMock MVs were unable to induce formation of intact tubes (Fig. 3C). p38 MAPK inhibition abrogated the shMock MV-induced increase in mesh area, tube length, and number of meshes and tubes (Fig. 3D). These results indicate that CD133-containing MVs induce angiogenesis through the p38 MAPK signaling pathway.

3.4. CD133-containing MV-induced angiogenesis is not mediated by the canonical angiogenesis pathways

To identify the proteins involved in angiogenesis among molecules transported to endothelial cells by CD133-containing MVs, an

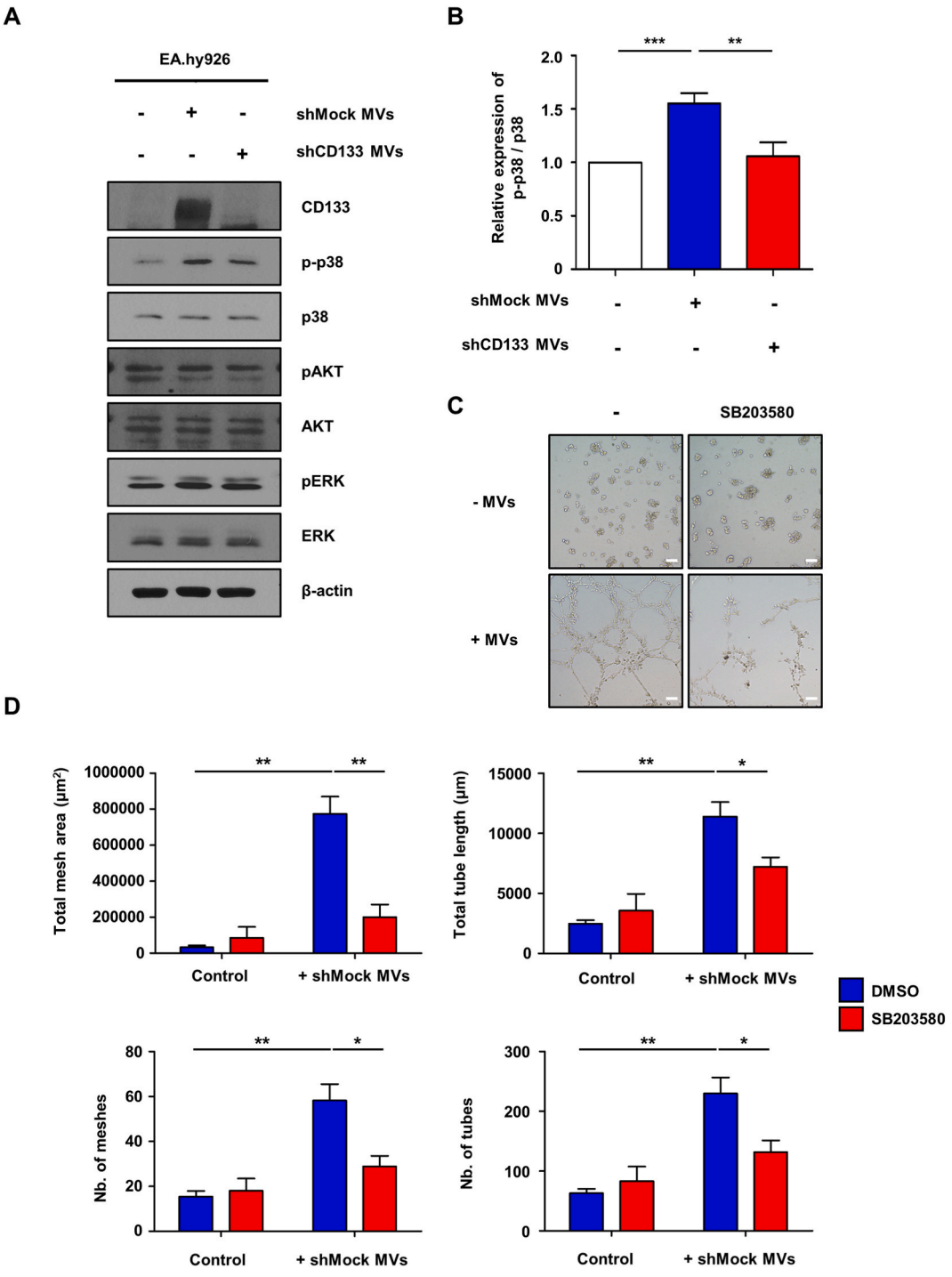

Fig. 3. CD133-containing MVs induce angiogenesis through the p38 MAPK signaling pathway. (A) EA.hy926 cells were treated with shMock and shCD133 MVs for 16 h. Protein level was determined using western blotting. p-p38, phosphorylated p38; pAKT, phosphorylated AKT; pERK, phosphorylated ERK. Full-length blot images are shown in Fig. S2 (B) Quantification of the p-p38 level. (C) EA.hy926 cells were pre-treated with SB203580 (20 μM) for 2 h and treated with shMock MVs in the presence of SB203580 for 13 h. Representative images of tube formation were shown. Scale bar = 100 μm. (D) Meshes and tubes were measured from five random fields in each well. Mean ± SEM of $n = 3$ independent experiments. All p values were obtained using an unpaired two-tailed Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

angiogenesis antibody array was performed in EA.hy926 cells treated with shMock or shCD133 MVs. Levels of urokinase plasminogen activator receptor (uPAR, *PLAUR*) and matrix metalloproteinase-1 (MMP-1) were slightly higher in shMock MV-treat cells than those in the untreated cells (Fig. 4A). However, none of the angiogenesis-related proteins showed a significant increase in shMock MV-treated cells compared with untreated cells (Fig. 4A). A recent study revealed that cancer-derived exosomes contain membrane-bound VEGF [21]. Therefore, we investigated whether CD133-containing MVs transferred VEGF or VEGFR to endothelial cells. The results showed that CD133-containing MVs did not carry either VEGF or VEGFR2 (Fig. 4B). These results indicate that CD133-containing MV-induced angiogenesis is not mediated by the canonical angiogenesis pathways.

3.5. Angiogenesis induced by CD133-containing MVs is resistant to bevacizumab and CD133 overexpression predicts poor prognosis in metastatic CRC patients

Because CD133-containing MVs induce VEGF-independent angiogenesis, we investigated whether the angiogenesis induced by CD133-containing MVs was insensitive to anti-angiogenic drugs targeting the canonical VEGF signaling. Bevacizumab, a humanized monoclonal antibody against VEGF, was the first approved drug to inhibit tumor angiogenesis as the first-line treatment for metastatic CRC patients [13]. However, the clinical benefit of bevacizumab is not as promising as previously expected [31]. It has been reported that EVs derived from cancer cells induce angiogenesis through the bevacizumab-insensitive VEGF or VEGF-independent pathways [20,23]. Therefore, we performed a tube formation assay in endothelial cells treated with VEGF or shMock MVs, in the presence or absence of bevacizumab. Capillary tube formation was increased by VEGF and shMock MV treatments (Fig. 5A). Bevacizumab effectively inhibited VEGF-induced tube formation; however, shMock MV-induced tube formation was unaffected by bevacizumab treatment (Fig. 5A). VEGF-induced tube formation decreased in the presence of bevacizumab, but shMock MV-induced angiogenic

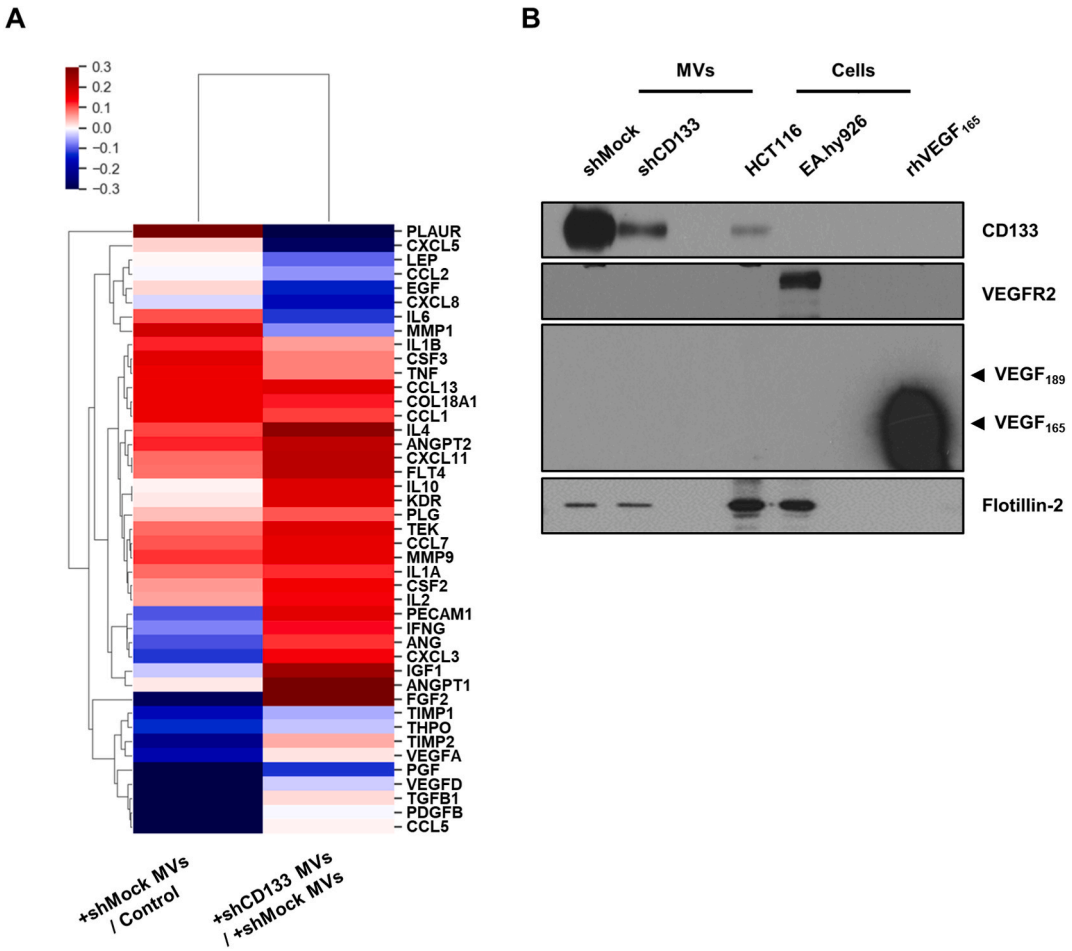

Fig. 4. CD133-containing MV-induced angiogenesis is not mediated by the canonical angiogenesis pathways. (A) Angiogenesis antibody array was performed using EA.hy926 cells treated with shMock MVs or shCD133 MVs. Red represents high protein expression levels, and blue represents low protein expression levels. (B) Protein expression was determined using western blot analysis. Human recombinant VEGF165 protein was used as a control. Full-length blot images are shown in Fig. S3. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

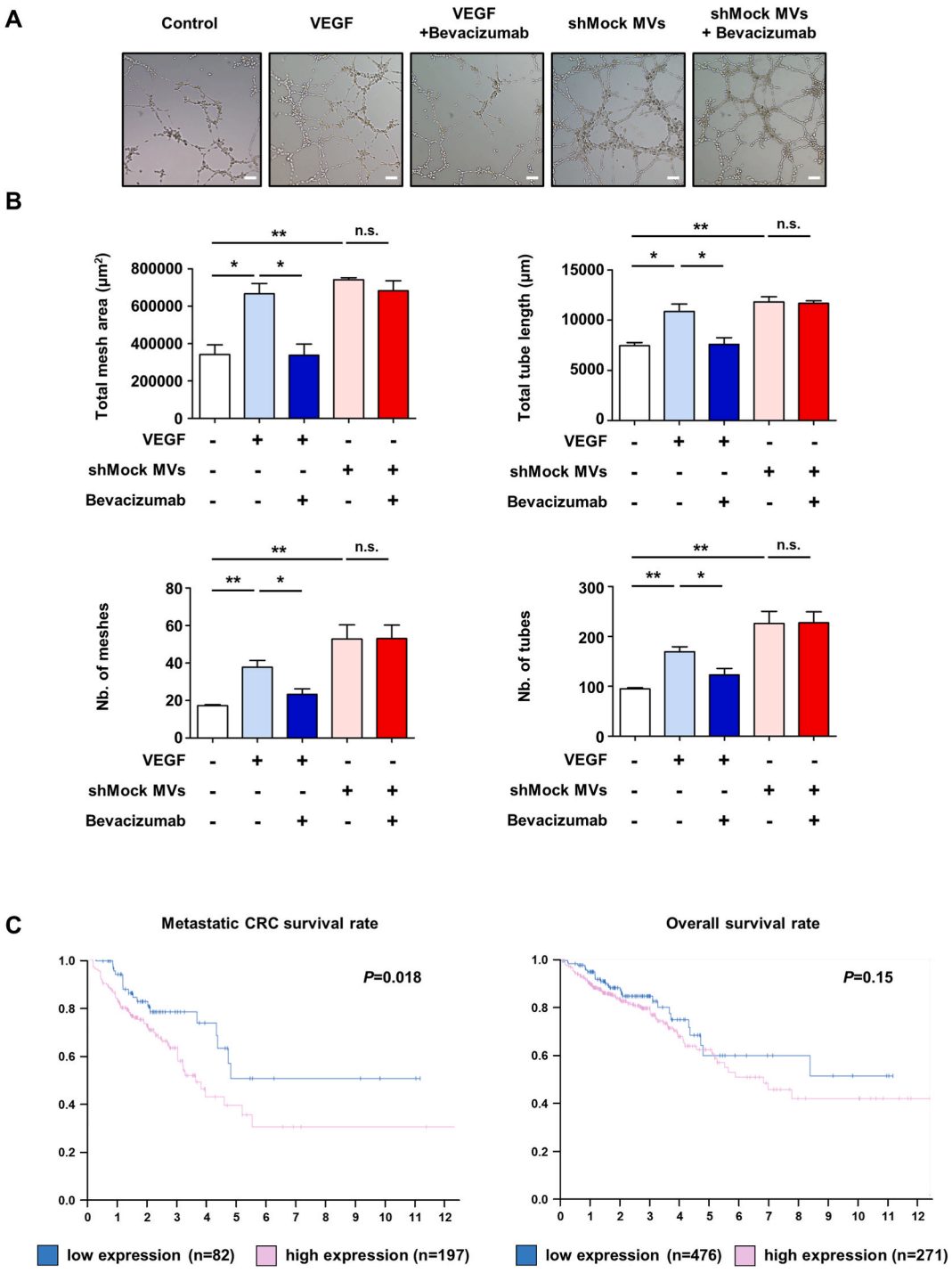

Fig. 5. Angiogenesis induced by CD133-containing MVs is resistant to bevacizumab and CD133 overexpression predicts poor prognosis in metastatic CRC patients. (A) EA.hy926 cells were treated with VEGF (200 ng/mL) or shMock MVs in the presence or absence of bevacizumab (100 μg/mL) for 16 h. Representative images of tube formation. Scale bar = 100 μm. (B) Meshes and tubes were measured from five random fields in each well. Mean ± SEM of $n = 3$ independent experiments. All p values were obtained using an unpaired two-tailed Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (C) The correlation between CD133 mRNA expression level and patient survival rate was analyzed. Kaplan-Meier plots were obtained from the Human Protein Atlas database. The metastatic CRC survival rate represents patients in stages III and IV. The overall survival rate represents CRC patients in all stages.

responses were not affected by bevacizumab (Fig. 5B). Since angiogenesis is associated with cancer progression and metastasis in CRC, we investigated whether CD133 expression is associated with poor prognosis in metastatic CRC. Results from the Human Protein Atlas database revealed that the survival rate of metastatic CRC patients (stages III and IV) was significantly reduced in patients with high CD133 expression compared to that in patients with low CD133 expression (Fig. 5C). However, there was no correlation between the expression level of CD133 and survival rate in all CRC patients (Fig. 5C). These results indicate that angiogenesis induced by CD133-containing MVs is resistant to bevacizumab, and CD133 overexpression predicts poor prognosis in metastatic CRC patients.

4. Discussion

Cancer cells need to establish blood vessels to supply oxygen and nutrients [32]. Therefore, cancer cells induce tumor angiogenesis by secreting a variety of pro-angiogenic molecules [33]. In addition, tumor angiogenesis develops leaky vessels and provides the principal route by which cancer cells exit the primary tumor site and enter circulation [4]. EVs derived from cancer cells promote tumor progression and metastasis by mediating intercellular communication [34]. Exosomes derived from various tumors induce angiogenesis by transporting miRNAs and proteins [21]. However, the role of MVs in angiogenesis is unclear. A recent study revealed that CD133 is involved in MV budding and oncoprotein trafficking in CRC [29]. Furthermore, MVs are incorporated into adjacent cells and increase cell motility in a CD133 dependent manner [29]. Consistent with a previous study, our results showed that both shMock and shCD133 MVs were incorporated into endothelial cells. shMock MVs increased tube formation by endothelial cells and promoted endothelial sprouting *in vitro* and *ex vivo*. However, shCD133 MVs had little effect on angiogenesis compared to shMock MVs. These results suggest a novel function for CD133-containing MVs in angiogenesis. However, these analyses do not mimic intact biological systems that are affected by various factors such as blood flow or mural cells [35]. Therefore, further studies are required to determine the function of CD133-containing MVs in angiogenesis induction using an *in vivo* angiogenesis analysis system.

Pro-angiogenic molecules such as VEGF can phosphorylate and activate AKT and ERK, which act as key regulators of angiogenesis [7]. Activated AKT and ERK induce angiogenesis by promoting endothelial cell survival, proliferation, and vessel maturation [9,30]. We observed that CD133-containing MVs induced the phosphorylation of p38 MAPK, but did not induce the phosphorylation of AKT and ERK. Tube formation induced by CD133-containing MVs was inhibited by p38 inhibitor, indicating that p38 MAPK functions as an essential mediator of CD133-containing MV-induced angiogenesis. It has been reported that p38 MAPK has a dual function in angiogenesis [36,37]. p38 negatively regulates endothelial cell migration and survival during FGF-2-stimulated angiogenesis [36]. In contrast, endothelial p38 activation and subsequent signaling trigger actin remodeling and increase endothelial cell migration to promote angiogenesis [37]. Recent studies have also demonstrated that p38 MAPK is activated by the phosphorylation of VEGFR2 and promotes angiogenesis [10,11]. Therefore, the role of p38 MAPK in endothelial cells is dependent on the upstream stimulators. As several recent studies have reported that p38 MAPK promotes endothelial cell migration, p38 MAPK is more likely to act as a pro-angiogenic stimulator under VEGF signaling. We found that CD133-containing MVs induced angiogenesis through p38 MAPK activation, indicating that p38 MAPK plays a role in angiogenesis and cancer progression in CRC [37].

To investigate the upstream signaling pathway of p38 MAPK, an angiogenesis antibody array was conducted in endothelial cells treated with CRC-derived MVs. The expression of uPAR and MMP-1 was slightly increased, but the increase was not significant. Since VEGF is the main regulator of angiogenesis and VEGFR2 tyr1214 is an upstream molecule of p38 MAPK, we examined whether CD133-containing MVs transport VEGF or VEGFR2 to endothelial cells. However, CRC-derived MVs did not carry VEGF or VEGFR2. A recent study reported that VEGFR2 signaling is tightly regulated by ligand and receptor expression as well as by the presence of co-receptors

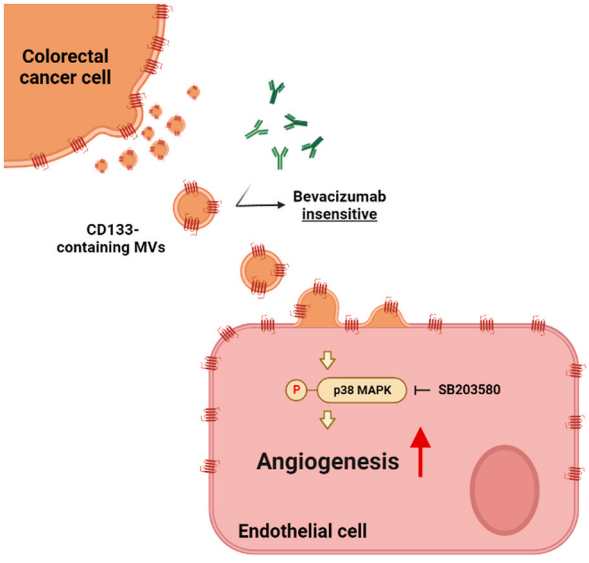

Fig. 6. Schematic diagram regarding the role of CD133-containing MVs derived from CRC cells in angiogenesis and CRC progression.

and accessory proteins, such as integrins and neuropilins [7]. For example, crosstalk between VEGFR2 and integrin $\alpha V\beta_3$ is important for transducing VEGF signals to the p38 pathway [38]. In addition, endophilin-A2 has been suggested as a mediator of VEGFR2 endocytosis, which is controlled by SLIT2-ROBO1 to promote angiogenesis through p38 MAPK [11]. Given that CD133-containing MVs do not carry VEGF and VEGFR2, p38 activation induced by CD133-containing MVs may be due to the transport of co-receptors or accessory proteins. Further studies are required to determine whether CD133-containing MVs carry VEGFR2 partner proteins.

Bevacizumab is a humanized monoclonal VEGF antibody that has been approved as an anti-cancer drug targeting angiogenesis. It has been used for various types of cancers, including CRC [13]. However, the efficacy of bevacizumab is transient, and tumors relapse frequently [15]. Recent studies have reported that EVs induce angiogenesis through VEGF-independent signaling pathways [22,23]. Furthermore, CRC-derived exosomes promote angiogenesis through heparin-bound and bevacizumab-insensitive VEGF [20]. We found that angiogenesis induced by CD133-containing MVs was resistant to bevacizumab. These results suggest that CD133-containing MVs may contribute to resistance against bevacizumab and the malignant progression of CRC.

Up to 60% of CRC patients suffer from distant metastasis within 5 years, and the mortality rate of patients with distant metastasis has sharply increased [2]. A recent study reported a correlation between blood circulating CD133-containing EV concentration and poor prognosis in metastatic CRC patients [28]. Since CD133-containing MVs induce angiogenesis and are resistant to bevacizumab treatment, we investigated whether the expression level of CD133 correlates with the patient survival rate according to CRC stage [39]. Analysis of the Human Protein Atlas demonstrated that high CD133 expression in metastatic CRC correlates with a low survival rate. These findings suggest that CD133-containing MVs may be responsible for the reduced survival rate in patients with metastatic CRC by inducing tumor angiogenesis.

Taken together, these results indicate that CD133-containing MVs induce angiogenesis via p38 MAPK (Fig. 6). Angiogenesis induced by CD133-containing MVs is insensitive to bevacizumab treatment. Elevated CD133 expression in patients with late-stage CRC is closely associated with low survival. CD133 can be a target molecule that inhibit MV-induced angiogenesis in metastatic CRC patients.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Beomsu Kim: Writing – original draft, Investigation. **Suhyun Kim:** Supervision, Data curation. **Sungyeon Park:** Validation, Data curation. **Jesang Ko:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29292.

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