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Stem-like tumor cells involved in heterogeneous vasculogenesis in breast cancer

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Abstract

Sorafenib, a small-molecule tyrosine kinase inhibitor with antiangiogenic activity, has been used in liver cancer and kidney cancer treatments. However, clinical trials with sorafenib for breast cancer were stopped in phase III due to limited efficacy. The existence of heterogeneous vasculatures involving tumor cells, such as vessel-like structures formed by vasculogenic mimicry and mosaic vessels, and their resistance to antiangiogenic therapy are thought to be a possible reason for failure of sorafenib therapy. Nevertheless, the features and mechanism of vasculogenesis by tumor cells remain unclear. In the present study, we found that breast cancer stem-like cells (BCSLCs, ALDH1⁺ cells) were involved in vasculogenic mimicry and mosaic vessel formation in triple-negative breast cancer tissues. Further, only ALDH1⁺ BCSLCs sorted from MDA-MB-231 could exhibit the tube formation and angiogenesis ability. Sorafenib could inhibit vascularization from endothelial cells rather than that from ALDH1⁺ cells. α -SMA was identified as a key molecule in vascular formation of BCSLCs. Mechanistically, HIF-1 α enhanced the mRNA and protein levels of α -SMA by binding to the HRE element in the promoter directly and meanwhile increased the BCSLCs population. Interestingly, pigment epithelium-derived factor (PEDF), an endogenous angiogenesis inhibitor, could inhibit both endothelial cell-derived and tumor cell-derived angiogenesis by downregulating HIF-1 α in breast cancer. Our finding clarified the possible reason for the poor outcome of anti-angiogenesis therapy and PEDF may have the therapeutic potential.

Key Words

- ▶ stem-like tumor cells
- ▶ heterogeneous vasculogenesis
- ▶ breast cancer
- ▶ antiangiogenic therapy

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Introduction

Epidemiological studies have shown that breast cancer is the most common type of tumor and the leading cause of cancer-associated death in women worldwide (Torre *et al.* 2015). Similar to most tumors, breast cancer requires an effective microcirculation for growth, invasion, and metastasis (Folkman 2002). Targeting endothelial cells (ECs) have been a major focus of antiangiogenic therapeutics (Rayson *et al.* 1999). However, this strategy is encountering a bottleneck since most antiangiogenic therapeutics such as bevacizumab, endostatin and many tyrosine kinase inhibitors are not curative, likely because of common drug resistance during the late period of treatment. Sorafenib, a small-molecule tyrosine kinase inhibitor with antiangiogenic activity that targets vascular endothelial growth factor receptor (VEGFR) on ECs, is widely used in the clinical treatment of liver and kidney cancer (Wilhelm *et al.* 2008) and in preclinical studies of breast cancers. Unfortunately, sorafenib therapy can display limited efficacy in some cases, and phase III trials in breast cancer have been terminated (Gradishar 2012); additionally, drug resistance in liver and kidney cancer have been reported (Liang *et al.* 2013, Rudalska *et al.* 2014, Azzariti *et al.* 2016, Chen *et al.* 2016).

The growth and metastasis of solid tumors are dependent on neovascularization (Hanahan & Weinberg 2011). For years, angiogenesis and vasculogenesis have been considered the main mechanisms of tumor neovascularization. However, there are many sources and types of tumor neovascularization, leading to heterogeneity. Several new heterogeneous vascular patterns, including vessel-like structures formed by vasculogenic mimicry, mosaic vessels, and co-opted vessels, have been discovered (Jain 1988). Recent studies have found that the presence of heterogeneous vascularity is often accompanied by poor prognosis and resistance to antiangiogenic drugs (Yu *et al.* 2001). These heterogeneous vessels require the direct involvement of tumor cells; however, what confers tumor cells a special ability to form blood vessels is unknown. Cancer stem cells (CSCs) are a kind of pluripotent cells that are capable of self-renewal, multilineage differentiation, and indefinite proliferation, which are critical for tumor growth and drug resistance (Singh *et al.* 2003, Galli *et al.* 2004). Recent studies have suggested that the tumor endothelium might be derived from CSCs in various tumors including glioblastoma and breast cancer (Wang *et al.* 2010, Tang *et al.* 2014). Studies have shown that glioblastoma stem-like cells (GSCs)

can differentiate into ECs or pericytes to support vessel function and tumor growth (Wang *et al.* 2010, Cheng *et al.* 2013). However, the role of breast cancer stem-like cells (BCSLCs) in the formation of heterogeneous vasculature and the relationship between this role and drug resistance are still unclear. Therefore, we propose the hypothesis that tumor cells involved in heterogeneous vasculogenesis in breast cancer possess stem-like properties and that the resistance of stem-like cancer cells in heterogeneous vascularity to antiangiogenic therapy is a possible reason for treatment failure.

This present study was designed to demonstrate particular characteristics and angiogenic ability of tumor cells involved in heterogeneous vascularity in breast cancer and to determine the underlying reason and key molecule attributable for the poor curative effects of antiangiogenic therapy. The results of this study may provide new insights into the antiangiogenic treatment of breast cancer.

Materials and methods

Cell lines and cell culture

The human breast cancer cell lines MDA-MB-231 and MCF7 were kept by our own laboratory (Hong *et al.* 2014). The T-47D and MDA-MB-436 cells were donated by Prof. Erwei Song. All cells have been identified by STR probes and were 100% matched cell lines that found in ATCC and DSMZ data bank. The MDA-MB-231 cell line was in the 10–20 generation and the MCF7 cell line was in the 7–15 generation during the experiment. The T-47D and MDA-MB-436 cells were in the 5–10 generation during the experiment. Both cells were cultured in DMEM (HyClone, SH30021.01B) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco BRL). Insulin (0.01 mg/mL, Sigma) was added to the culture medium for MCF7 cells. The cells were incubated at 37°C in a humidified incubator with 5% CO₂ (normoxia) or 1% O₂ (hypoxia). Serum-free culture medium was used for treatments. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins following a previously described protocol (Jaffe *et al.* 1973). These cells were transferred on flasks coated with 0.2% gelatin and cultured with ECM medium containing 15% FBS (Gibco BRL), 15 mg/L ECGS (Upstate, NY, USA) and 100 U/mL streptomycin (Gibco BRL) in an atmosphere with 5% CO₂ at 37°C.

Animal experiments

Female athymic mice (BALB/c nu/nu; Guangdong Medical Laboratory Animal Center) weighing 15–18 g were purchased at 5 weeks of age. All animals were maintained in a specific pathogen-free environment at the Laboratory Animal Center of Sun Yat-sen University. All the animal experiments were carried out with the approval of the Animal Care and Use Committee of Sun Yat-sen University (approval ID: SCXK 2015-0107). Breast cancer cells MDA-MB-231 (3×10^6) were inoculated into the mammary fat pads of mice at 6 weeks of age. When the tumor size reached 100 mm³ on day 9, the mice were randomly divided into four groups receiving the following treatments ($n=11$ /group). Recombinant PEDF was expressed and purified as described previously (Yang *et al.* 2009) and was dissolved in PBS. Intraperitoneal injection of PBS or pigment epithelium-derived factor (PEDF) (5 mg/kg) every day. These two groups are used to observe the therapeutic effect of PEDF. Sorafenib (Nexavar, 200 mg/tablets) was dissolved in Cremophor EL/95% ethanol (50:50; Sigma) and diluted with water before use. Lavage solvent control or sorafenib (30 mg/kg) every day to verify the efficacy of sorafenib in breast cancer. After 15 days, three mice were randomly selected from each group to examine the tumors halfway through the experiment. On day 43 after cells were injected, the experiment was terminated, and the remaining mice were killed. The maximum diameter (a) and minimum diameter (b) of the tumors were measured with calipers every 3 days to determine the tumor volume. The tumor volume was calculated by the following formula: $V=a \times b^2 \times 0.5$. Paraffin sections (4 μ m) and frozen sections (4.5 μ m) of harvested organs were used for immunohistochemistry (IHC) and immunofluorescence (IF) assessment.

Tissue immunohistochemistry and double-staining methods

This study was approved by the Medical Ethics Committee of Sun Yat-sen University for Medical Sciences, Guangzhou. All clinical tissue sections (from the Third Affiliated Hospital and Cancer Center of Sun Yat-sen University, Guangzhou) or paraffin sections from the animals were deparaffinized by baking slides at 60°C for 40 min. After deparaffinization and rehydration with graded alcohol, the slides were pretreated with 0.01 M citrate buffer (pH 6.0) for 2 min at 100°C in an autoclave and then allowed to cool naturally. Endogenous peroxidase activities were quenched by 3% H₂O₂ in distilled water

for 30 min, and nonspecific background was blocked in normal goat serum for 1 h at room temperature. Three washes were performed in PBS Tween-20 (PBST) (pH 7.2) after each step. Next, the slides were incubated with anti-CD31 antibody (1:100, Neomarkers, USA) at 4°C overnight in a humidified chamber. Following a brief rinse in PBST, the sections were incubated with a biotin-conjugated second antibody (DAKO, K5007) for 30 min at room temperature and developed in DAB for 20 s. Some sections were treated with a Periodic Acid-Schiff Staining Kit (Baso Diagnostic Inc., BA-4080A) according to the manufacturer's instructions. After rinsing in distilled water, the sections were counterstained with hematoxylin. For negative control, slides were incubated in PBS in the place of primary antibody. For animal samples, all quantifications were blindly performed in at least three randomly chosen sections from each mouse.

Immunofluorescence

Triple-negative breast cancer (TNBC) tissues and animal tissue paraffin sections were pretreated as for IHC. Frozen animal tissue sections were fixed in ice-cold 4% paraformaldehyde following permeabilization for 20 min with 0.01% Triton X-100 in PBS. The sections were subsequently blocked with normal donkey serum at room temperature for 1 h. We used rabbit anti-aldehyde dehydrogenase (ALDH)-1A1 polyclonal antibody (1:100, Abcam, ab52492) or mouse anti-ALDH1 polyclonal antibody (1:50, BD, 611194) to detect BCSLCs. Mouse anti-CD31 MAB (1:100, CST, 3528S) or rabbit anti-CD31 polyclonal antibody (1:100, Neomarkers) was used to detect ECs. Goat anti- α -SMA polyclonal antibody (1:100 dilution, Abcam ab21027) or mouse anti- α -SMA polyclonal antibody (1:200 dilution, Sigma, A2547) was used to detect pericytes. To detect cancer cells, we used rabbit anti-NuMA polyclonal antibody (1:100, Abcam, ab97585). After overnight incubation at 4°C, the slides were incubated with appropriate fluorescent secondary antibodies (1:200 dilution in blocking buffer, 1 h, 37°C). After immunolabeling, the slides were washed and then stained with 4,6-diamino-2-phenylindole (DAPI) (Sigma) for 5 min at room temperature. Cells were visualized under a confocal microscope (Axio Observer Z1, ZEISS).

Flow cytometry and isolation of cells

For CD44 and CD24 flow cytometry, cells were harvested using 0.25% trypsin-EDTA (Invitrogen), and single cells were resuspended in 1% FBS-PBS (1×10^6 cells/400 μ L)

containing FITC-conjugated anti-CD44 (BD Biosciences) and PE-conjugated anti-CD24 (BD Biosciences) antibodies. Subsequently, the cells and their respective controls were incubated on ice in the dark for 45 min. After incubation, the stained cells were washed with PBS and suspended in 500 μ L 1% FBS-PBS and detected by a flow cytometer (Gallios, Beckman Coulter Inc.). Kaluza software (Beckman Coulter Inc.) was used for data analysis.

The ALDEFLUOR assay was performed as described by the manufacturer (Stem Cell Technologies, Vancouver, British Columbia, Canada). Briefly, cells were harvested and resuspended in assay buffer at 1×10^6 cells/mL and added to a tube containing activated ALDEFLUOR substrate BODIPY amino acetaldehyde (BAAA). Half of each sample was transferred to a tube containing 5 μ L/500 μ L ALDH1 inhibitor diethylamino benzaldehyde (DEAB). The samples were incubated at 37°C for 45 min. The cells were resuspended in assay buffer and assayed with a FACS Canto II flow cytometer. ALDH1 bright region was based on the control DEAB sample that was gated to represent less than 0.5 percent of cells. Based on cell-surface marker phenotype and ALDH1 activity, MDA-MB-231 cells were isolated into ALDH1⁺ and ALDH1⁻ subsets by a FACS Vantage/Diva cell sorter (BD Biosciences). The sorted cells were immediately used for functional *in vitro* experiments and Western blotting.

Tube formation assays

Matrigel (BD Biosciences, San Jose, CA, USA) was thawed at 4°C overnight, and 100 μ L of Matrigel was evenly added to each well of a pre-cooled 24-well plate. The plate was incubated at 37°C for 30 min. Cells (2×10^5) in 1 mL suspension were added on top of the matrix in 24-well plates and incubated at 37°C for varying durations. For co-culture experiments, different cells and culture medium were divided in half. Wells were imaged using an inverted fluorescence microscope (Olympus 1 $\times$ 71).

Western blot analysis

Samples for Western blot analysis were prepared as follows: Lysates from cultured cells were prepared using a 1 $\times$ SDS buffer. Protein concentrations were determined using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories) according to the manufacturer's protocol. Lysates with equal amounts of protein (50 ng) were loaded and separated by 10% SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore Corp.) for 3 h. After blocking

with 7% milk in Tris-buffered saline Tween-20 (TBST) for 1.5 h, the membranes were incubated overnight at 4°C with primary antibodies against HIF-1 α (1:1000, BD), CD44 (1:4000, Proteintech, 15675-1-AP), ALDH1 (1:1000, Abcam, ab52492), α -SMA (1:1000, Sigma, A2547), PEDF (1:1000, Millipore, MAB1059), PDGFR (1:1000, CST, 3169S), DESMIN (1:1000, Santa Cruz, sc-365130), NG2 (1:1000, Millipore, MAB5384), VE-cadherin (1:1000, Abcam, ab33168), or CD31 (1:1000, Neomarkers). The blots were washed three times with 1 $\times$ TBST and incubated with their respective secondary antibodies for 4 h at 4°C. The membranes were again washed with TBST, and then immunoblots were developed with ECL reagents from Pierce. To confirm equal sample loading, we probed the blots with an antibody specific to β -actin (1:5000, Sigma).

siRNA transfection

For knockdown experiments, siRNA oligonucleotides matching selected regions of α -SMA sequence and control siRNA were purchased from RiboBio Company (Shanghai, China). Transfection was performed at approximately 60% confluency using HiPerfect reagent (Qiagen) with siRNA oligonucleotides at a final concentration of 100 nM according to the manufacturer's instructions. Briefly, siRNAs and transfection reagent diluted in Opti-MEM (Invitrogen) were mixed and incubated at room temperature for 20 min. Next, the siRNA-reagent complex was added to cells in 1.5 mL (six-well plate) serum-free medium. The knockdown of protein expression was confirmed by Western blotting 36 h later.

Chromatin immunoprecipitation (ChIP) assay

Hypoxia-inducible factor 1 α (HIF-1 α) is a transcription factor that can bind to hypoxia-responsive elements (HREs; 5'-A/G CGT G/CC-3') to regulate the expression of its target genes. Several predicted HREs are located upstream of the *ACTA2* promoter, and ChIP assays were carried out to investigate whether HIF-1 α protein binds to these HREs under hypoxic conditions. To this end, we used a ChIP assay kit (Millipore Corp Cat. No. 17-10461) essentially against it according to the manufacturer's specifications. MDA-MB-231 cells were cultured with 1% oxygen at 37°C for 48 h and fixed with 1% formaldehyde for 15 min. The cross-linking reaction was quenched with glycine. Cell lysates were then sonicated to shear cross-linked DNA to ~200- to 1000-bp fragments. Precleared lysates were subjected to overnight

immunoprecipitation (IP) of cross-linked protein/DNA with rabbit anti-HIF-1 α antibody (10 g/mL) (14179S, Cell Signaling Technologies (CST)) or normal rabbit IgG (10 g/mL) (negative control). ChIP DNA enrichment was evaluated through quantitative real-time PCR (qRT-PCR) using the following primers: *ACTA2* first set of primer sequences are 5'-CGGGAGCCAGTCTCCAACGC-3' and 5'-AGCTGGAGCTGCTTCACAGG-3'. *ACTA2* second set of primer sequences are 5'-GCAAGTCCTCCAGCGTTCTG-3' and 5'-GGGGATAGGCAAAGTGGGGC-3'. The positive control VEGF primer sequences are 5'-CGCACGTAACCTCACTTCC-3' and 5'-CAATGAAGGGGAAGCTCGAC-3'. Pre-immunoprecipitated samples (input) were also included as controls for PCR.

Luciferase reporter assay

To verify the binding sites for HIF-1 α protein on the *ACTA2* gene promoter, we amplified the sequences of the *ACTA2* gene promoter by PCR from MDA-MD-231 cells. The amplified DNA sequences were inserted into the pGL3-basic vector (OBIO, Shanghai, China) to generate *ACTA2* promoter luciferase reporter vectors. Mutated *ACTA2* promoter luciferase reporter vectors were generated using standard PCR techniques to validate the following predicted binding site on the *ACTA2* promoter: 5'-CGCCTCCCGCAGCAGCCCGCCGCTCCCAGGTGCCC**GTGCGC**CGCTGCC-3'. For luciferase reporter assays, MDA-MD-231 cells were seeded in 96-well plates prior to transfection. Then, 0.2 μ g of *ACTA2* promoter reporter construct was transfected into cells together with 0.02 μ g Renilla luciferase reported construct, and 0.2 μ g indicated si-HIF-1 α plasmids following incubation in 1% oxygen at 37°C for 48 h. Firefly luciferase activity and Renilla luciferase activity were measured using the Dual-Luciferase Reporter system (Promega) using a luminometer (Berthold Centro LB 960, Germany) according to manufacturer's instructions. Luciferase activities were normalized to that of Renilla luciferase, and a negative control vector, pGL3-basic, was added at identical amount to normalize the expression as fold increase in relative light units.

Statistical analysis

Student's *t*-tests were used for comparisons between two groups. SPSS version 13.0 software was used for all analyses. $P < 0.05$ was considered statistically significant. Kaplan–Meier actuarial survival curves were used for survival analyses.

Results

Sorafenib has no effect on heterogeneous vascularity formed by breast cancer cells *in vivo* or *in vitro*

Sorafenib is an oral multikinase inhibitor, approved for antiangiogenic treatment of advanced renal cell carcinoma and hepatocellular carcinoma (HCC) targeted at ECs (Llovet *et al.* 2008, Yang *et al.* 2009). However, studies have found that long-term treatment with sorafenib can even enhance the metastatic potential of HCC, non-small-cell lung cancer (Adjei *et al.* 2011), and renal cell carcinoma (Hutson *et al.* 2010). In breast cancer, a phase III clinical trial with sorafenib was terminated because of limited efficacy. To identify the possible reasons for the poor therapeutic role of sorafenib in breast cancer, we performed several *in vivo* and *in vitro* experiments.

In our xenograft model, tumor size in the sorafenib group was significantly smaller than that in the control groups on day 24, but there was no difference on day 43 (Supplementary Fig. 1B and C, see section on [supplementary materials](#) given at the end of this article). Four mice were euthanized in the control group, two on day 38, one on day 39 and one on day 41. Three mice were euthanized in the sorafenib group, one on day 39, one on day 40 and one on day 41. We found that sorafenib did not improve overall survival (Supplementary Fig. 1D).

There are many sources and types of tumor neovascularization, leading to heterogeneity. Several new heterogeneous vascular patterns, including vessel-like structures formed by vasculogenic mimicry, mosaic vessels, and co-opted vessels, have been discovered (Jain 1988). Vasculogenic mimicry is a phenomenon by which aggressive tumor cells form vascular structures to obtain nutrients without the participation of ECs (Basu *et al.* 2006). Moreover, mosaic tumor vessels are known as transitional heterogeneous vascularity made of both ECs and tumor cells (di Tomaso *et al.* 2005). Heterogeneous vascularity was detected by staining the tumor specimens with anti-CD31 antibody to detect ECs and periodic acid-Schiff (PAS) to detect glycogen. Notably, microvascular density (MVD, CD31⁺) was dramatically decreased in the sorafenib-treated group at all time points, and compared to that in mice in the control group vasculogenic mimicry (CD31⁺/PAS⁺) was observed in mice in the sorafenib group at a later stage (Supplementary Fig. 1E and F). We speculated that at later stages, vasculogenic mimicry, a type of heterogeneous vascularity constituted by aggressive tumor cells form vascular structures to obtain

nutrients without the participation of ECs (CD31-/PAS+) (Basu *et al.* 2006), gives rise to predominant vascular structures in tumors (full of blood cells) and that sorafenib is ineffective against this type of blood vessels.

Finally, to verify our *in vivo* observations with sorafenib, we performed 3D *in vitro* experiments. In agreement with our previous study *in vivo*, sorafenib suppressed tubular network formed by HUVECs in a concentration-dependent manner but was unable to affect the structures formed by MDA-MB-231 cells (Supplementary Fig. 1G).

These results suggested that sorafenib can suppress vascular structures composed of ECs rather than those formed by vasculogenic mimicry of breast cancer cells, and this may be the likely cause of the lack of efficacy of sorafenib in breast cancer.

ALDH1⁺ breast cancer cells exhibit tube-forming ability

Formation of capillary-like structures in Matrigel is a commonly used functional test for tube formation. To verify whether BCSLCs can generate heterogeneous vasculature *in vitro*, we initially chose two different breast cancer cell lines, namely, MCF7 and MDA-MB-231, to investigate their tube-forming ability. Among these cell lines, only MDA-MB-231, a TNBC cell line, could form typical vessel-like structures under the same 3D culture conditions. In contrast, MCF7 cells, which are less invasive, could not form vascular networks *in vitro* (Fig. 1A). Moreover, flow cytometry was used to assess the expression of BCSLC markers in MCF7 and MDA-MB-231 cells. As illustrated in Fig. 1B and C, the proportion of CD44⁺CD24⁻ and ALDH1⁺ cells were significantly higher in MDA-MB-231 cells than in MCF7 cells. To further clarify the role of BCSLCs in heterogeneous vasculogenesis *in vitro*, we isolated ALDH1⁺ and ALDH1⁻ MDA-MB-231 cells by a FACS sorter and cultured them in a 3D matrix to examine VM formation for 3 days. Interestingly, we found that only BCSLCs, ALDH1⁺ MDA-MB-231 cells, exhibited tube-forming ability *in vitro*, while ALDH1⁻ cells lacked this ability (Fig. 1D). The similar results were confirmed in T-47D and MDA-MB-456 cell lines (Supplementary Fig. 2). Furthermore, we co-cultured BCSLCs with HUVECs and found that BCSLCs could form more capillary-like structures in Matrigel than ALDH1⁻ MDA-MB-231 cells, and the co-cultures led to the formation of mosaic vessels made of both HUVECs and tumor cells (di Tomaso *et al.* 2005) *in vitro* (Supplementary Fig. 3).

These results indicated that BCSLCs directly participated in heterogeneous vasculogenesis (both VM and mosaic vessels) in breast cancer. However, the mechanisms underlying the ability of BCSLCs to mimic a capillary wall with ECs are not yet clear.

α -SMA is a critical molecule in tube formation by BCSLCs

It is known that highly aggressive cancer cells can initiate the formation of vascular channels by mimicking the behavior of ECs (Du *et al.* 2014). We wondered whether BCSLCs could do the same. To this end, we performed molecular analysis of specific vascular markers using Western blotting. A high level of expression of pericyte marker α -SMA was observed in ALDH1⁺ MDA-MB-231 cells capable of tube formation. However, none of the breast cancer cells expressed EC markers (Fig. 2A). However, α -SMA is not only a pericyte marker but also a marker of epithelial-to-mesenchymal transition (EMT). Latest research indicates that EMT endows cancer cells with pericyte properties and is indispensable for vascular stabilization and sustained tumor growth (Shenoy *et al.* 2016). To understand whether EMT enables BCSLCs to participate in heterogeneous vasculogenesis in tumors, we also examined several EMT markers in BCSLCs by Western blotting. Interestingly, α -SMA was the only observed upregulated mesenchymal marker in ALDH1⁺ MDA-MB-231 cells, although both ALDH1⁺ and ALDH1⁻ cells exhibited EMT (Fig. 2B). In other words, not all cells undergoing EMT, such as ALDH1⁻ cells, can form tubular structures, and only stem-like property enables tumor cells to form tube-like structures. Furthermore, to explore whether EMT and the pericyte marker α -SMA play a critical role during tube formation, we silenced the expression of α -SMA protein in MDA-MB-231 cells using siRNA technology. The reduction in α -SMA expression was confirmed using Western blot analysis (Fig. 2C). Observably, the tube-forming ability of MDA-MB-231 was inhibited (Fig. 2C). Moreover, the tube-forming capacity was higher in α -SMA-transfected MCF7 cells than in vector-transfected cells (Fig. 2D). Furthermore, the tubular structures were closer to normal blood vessels and more stable in MDA-MB-231 cells co-cultured with HUVECs than in cells cultured alone (Fig. 2E and F). Thus, we believe that α -SMA is a critical functional molecule during tube formation by BCSLCs. This important ability of α -SMA in tube formation was also confirmed by *in vitro* experiment in the T-47D and MDA-MB-456 cell lines (Supplementary Fig. 4).

Figure 1

ALDH1⁺ MDA-MB-231 cells exhibit tube-forming ability. (A) Matrigel tube formation assays were used to detect the ability of tube formation in a TNBC cell line MDA-MB-231 and poorly invasive cell line MCF7. (Original magnification, ×200). (B) CD44⁺CD24⁻ stem-like cells were detected by flow cytometry. The corresponding quantified data by FACS analysis ($n = 3$). (C) ALDH1⁺ stem-like cells labeled with the ALDEFLUOR™ assay kit (Stem Cell). The gate for ALDH1⁺ cells was determined in relation to the DEAB control (first column DEAB⁺) and showed the brightly fluorescent ALDH1 population versus the side scatter population. The number shown in each panel reflects the percentage of ALDH1⁺ MDA-MB-231 and MCF7 cells. The corresponding quantified data by FACS analysis ($n = 3$). (D) FACS was used to isolate ALDH1⁺ and ALDH1⁻ subsets from MDA-MB-231 cells for tube formation assays over 3 days. The expression of BCSLC marker ALDH1 was identified before tube formation assays. Representative micrographs with different magnification showing the formation of capillary-like structures in Matrigel. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0054>.

Heterogeneous vascularity is found in TNBC specimens

Vasculogenic mimicry is a phenomenon by which aggressive tumor cells form vascular structures to obtain nutrients without the participation of ECs (Basu *et al.* 2006). It is a newly defined type of tumor vasculature discovered by Maniotis *et al.* (1999). After initial observations in melanoma (Hendrix *et al.* 2003), evidence

for vasculogenic mimicry has been discovered in other cancers, including glioblastoma (El Hallani *et al.* 2010), breast cancer (Shirakawa *et al.* 2001), HCC (Sun *et al.* 2010), and prostate cancer (Sharma *et al.* 2002). Vasculogenic mimicry is negatively correlated with disease prognosis. Moreover, mosaic tumor vessels are known as transitional heterogeneous vascularity made of both ECs and tumor cells (di Tomaso *et al.* 2005). Although few in number, these two types of heterogeneous vascularity composed

Figure 2

α -SMA is a critical molecule in tube formation by cancer cells. (A) Western blot analysis of vascular markers (A) and EMT markers (B) expressed by breast cancer cells MDA-MB-231 and MCF7 (β -actin, control protein). HUVECs were used as a positive control for EC markers. (C) siRNA interference effect was validated by Western blotting. siRNA interference technology was used to knock down α -SMA in MDA-MB-231 cells before the formation of capillary-like structures in Matrigel. (D) Matrigel tube formation assay in breast cancer MCF7 cells transiently transfected with vector/ α -SMA for 24 h. (E) Representative micrographs showing the formation of capillary-like structures in Matrigel formed by MDA-MB-231 cells and HUVECs and their co-cultures. (F) Stability of these three tubular structures. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0054>.

of tumor cells are thought to be possible reasons for the poor curative effects of antiangiogenic therapy. CSCs are similar in the sense that they are few in number but can drive tumor progression. Thus, we speculated that not all tumor cells can form tube-like structures and that only CSCs have the ability to form tubes. Therefore, we examined 40 surgically resected breast cancer specimens for evidence of vasculogenic mimicry and mosaic vessels using IF. We used anti-CD31 antibody to identify ECs, anti- α -SMA antibody to detect pericytes and anti-ALDH1 antibody to identify BCSLCs.

In breast cancer, CD44⁺/CD24⁻ and ALDH1⁺ cells have been reported as tumor-initiating cells (Al-Hajj *et al.* 2003, Ginestier *et al.* 2007, Nakanishi *et al.* 2010). ALDH1 is a kind of intracellular enzyme that can oxidize aldehyde into acetic acid; it is highly expressed in a variety of CSCs and is closely correlated with poor prognosis in breast cancer (Ginestier *et al.* 2007). There are reports that ALDH1 is a better clinical indicator for relapse of invasive ductal breast cancer than the CD44⁺/CD24⁻ phenotype (Zhong *et al.* 2014). Therefore, we selected ALDH1 as a marker for BCSLCs to determine the location of BCSLCs and to investigate their relationship with heterogeneous vascularity in breast cancer.

We found that most vessels in breast cancer specimens were adorned with CD31⁺ cells with typical pericytic location (α -SMA⁺/CD31⁺/ALDH1⁻) which is known as angiogenesis in cancer (Fig. 3A). In TNBC specimens, we also found that some vessels were composed of BCSLCs, stained by ALDH1 together with α -SMA or CD31. We considered this kind of vessels as mosaic tumor vessels (Fig. 3A). Surprisingly, in a few TNBC specimens (5%, two specimens), some cancer cells that highly expressed ALDH1 and α -SMA but were negative for CD31 organized themselves into vessel-like structures similar to vasculogenic mimicry (α -SMA⁺/ALDH1⁺/CD31⁻) (Fig. 3A), and these vessels were histologically different from breast ducts (Supplementary Fig. 5). These results indicated the presence of heterogeneous vascularity in breast cancer tissues and showed that the cells involved in these vessels were BCSLCs rather than normal tumor cells.

MDA-MB-231 expressing α -SMA participate in the formation of various heterogeneous vascularity in xenograft models

Our previous results confirmed the presence of heterogeneous vascularity in TNBC specimens (Fig. 3)

Figure 3

MDA-MB-231 cells expressing α -SMA participate in the composition of various heterogeneous vasculatures *in vivo*. (A) IF staining of a pericyte marker α -SMA (green), an EC marker CD31 (red) and a BCSLC marker ALDH1 (pink) in TNBC. Nuclei were stained with DAPI (blue); the arrow points to the corresponding vessel. Scale bar: 20 μ m. (B, C and D) IF staining of CD31 (an EC marker) and α -SMA (a pericyte marker) in xenografts. Nuclei were stained with DAPI. NuMA was used for identifying human breast cancer cells. Arrows show heterogeneous vascularity (NuMA⁺ and α -SMA⁺).

and the important functional role of α -SMA, an EMT and pericyte marker, in the formation of capillary-like structures (Fig. 2). Next, we performed IF to verify different types of microcirculation patterns in xenografts in nude mice.

Most tumor vessels in the xenografts were adorned with CD31⁺ cells with typical pericytic (α -SMA⁺ cells) location and morphology of vessels (Fig. 3B). These α -SMA⁺ cells were completely negative for the human cell-specific nuclear antigen NuMA, confirming that these pericytes were derived from the nude mice. As illustrated in Fig. 3C, in vessels that were only composed of α -SMA⁺ cells, the α -SMA⁺ cells also expressed NuMA, indicating that these cells were derived from the human MDA-MB-231 cells. We called the process of formation of capillary-like structures vasculogenic mimicry. The third kind of vessels (Fig. 3D) are the mosaic vessels. Tumor vessels were adorned with α -SMA⁺ cells that were mostly derived from nude mice (NuMA⁻), but a fraction of cells was derived from human MDA-MB-231 (NuMA⁺).

Collectively, these data provide direct evidence that MDA-MB-231 cells expressing α -SMA participate in tube formation *in vivo*. However, why the TNBC cell line MDA-MB-231 has a high proportion of BCSLCs and highly express α -SMA are not yet known.

HIF-1 α upregulates α -SMA transcription by binding to the HRE on the ACTA2 promoter

To verify the regulatory mechanism of α -SMA, we assessed the expression of HIF-1 α and α -SMA in breast cancer cell lines by Western blot analysis. A high level of expression was found in MDA-MB-231 cells, with absent or very low expression in MCF7 cells (Fig. 4A). Moreover, we inhibited the expression of the HIF-1 α protein in MDA-MB-231 cells using 2-methoxyestradiol (2ME2) which is a small-molecule inhibitor of HIF-1. Both the mRNA and protein levels of ACTA2 (the gene encoding α -SMA) significantly decreased upon 2ME2 treatment (Fig. 4D and E). In contrast, α -SMA protein and mRNA expression was significantly increased in MCF7 cells cultured under hypoxia (Fig. 4B and C).

Since HIF-1 α is a transcription factor that can bind to HREs (5'-A/G CGT G/CC-3') to regulate the expression of its target genes, we speculated that the ACTA2 promoter is directly regulated by HIF-1 α . To this end, we first predicted several HREs located upstream of the ACTA2 promoter using a series of bioinformatics software. Next, we carried out ChIP assays to investigate whether HIF-1 α proteins bind to these HREs in MDA-MB-231 cells after hypoxia conditioning. ChIP DNA enrichment was

Figure 4

HIF-1 α regulates the levels of α -SMA by binding to the HRE on the promoter. (A) α -SMA and HIF-1 α expression in MCF7 and MDA-MB-231 cells by Western blot analysis (β -actin, control protein). Western blot and RT-PCR analysis for α -SMA expression in MCF7 cells after hypoxic conditioning for 24 h (B and C) and in MDA-MB-231 cells treated with 2ME2 (D and E). (F and G) ChIP-PCR validation of the increase in α -SMA expression in MDA-MB-231 cells after hypoxia conditioning. (H) Luciferase activity of the ACTA2 promoter after the inhibition of HIF-1 α in MDA-MB-231 cells under hypoxia was determined as specified in the Methods section. (I) The luciferase activity of the wild-type and mutated ACTA2 promoter reporter in MDA-MB-231 cells under hypoxia (Mean $\pm$ s.d., * P < 0.05, *** P < 0.001 compared with untreated control). A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0054>.

evaluated through qRT-PCR, which indicated the binding between HIF-1 α protein and the ACTA2 promoter after 48 h of hypoxia conditioning (Fig. 4F and G). ACTA2 promoter luciferase reporter vectors were generated by OBIO company (Shanghai, China) and co-transfected into MDA-MB-231 cells with indicated si-HIF-1 plasmids or neutral control vectors. Luciferase activity was restored by si-HIF-1 plasmids in the presence of 1% oxygen at 37°C for 48 h (Fig. 4H). Moreover, to verify the binding sites for HIF-1 α on the ACTA2 gene promoter, we mutated the following predicted binding site on the ACTA2 promoter: 5-CGCCTCCCGCAGCAGCCCGCGCTCCCAGGTGC CCGCGTGC GCGCGCTGCC-3 to generate a mutated ACTA2 promoter the fluorescence could also be restored by the mutation promoter report vectors (Fig. 4I). These results indicated that HIF-1 α regulates α -SMA by directly binding to the HRE on its promoter.

Hypoxia induces heterogeneous vasculogenesis by increasing the proportion of stem-like cells

Oxygen is very important for tumor angiogenesis and metastasis and cell proliferation and apoptosis (Zhong *et al.* 1999). HIF-1 α is the major transcription factor involved in the adaptive response to hypoxia. It combined with a constitutively expressed β -subunit (HIF-1 β) to compose HIF-1 transcription factors.

HIF-1 α protein is crucial factor for vasculogenic mimicry in several tumor types (Sun *et al.* 2007, 2012, Du *et al.* 2014) and increases cancer stemness (Takubo *et al.* 2010, Lu *et al.* 2015). Hence, we hypothesized that sorafenib can exacerbate hypoxia to increase the proportion of BCSLCs, which results in more heterogeneous vascularity and drug resistance.

First, we assessed the mRNA and protein expression level of HIF-1 α in MDA-MB-231 and MCF7 cells. HIF-1 α expression was high in MDA-MB-231 cells; in contrast, the level of HIF-1 α in MCF7 cells was significantly lower (Fig. 5A and B). The functional role of HIF-1 α in the formation of vessel-like tubes *in vitro* was determined by treatment with of MDA-MB-231 and MDA-MB-436 cells with 2ME2 (a selective HIF-1 α inhibitor) and hypoxia conditioning in MCF7 and T-47D cells. Compared with the controls, 2ME2 significantly reduced the proportion of stem-like cells (Fig. 5C, E and G) and inhibited vascular channel formation (Fig. 5I) in MDA-MB-231 cells as well as MDA-MB-436 cells (Supplementary Fig. 6C and D). In addition, after hypoxia conditioning, the proportion of the stem-like cells increased significantly (Fig. 5D, F and H) in MCF7 cells and resulted in the formation of similar vessel-like structures (Fig. 5J). The similar results were confirmed in T-47D (Supplementary Fig. 6A and B). Therefore, hypoxia promotes the expression of HIF-1 α , which is a critical mediator of tube formation by

Figure 5

Hypoxia induces channel formation by increasing the proportion of stem-like cells. (A and B) HIF-1 α mRNA and protein expression level in MDA-MB-231 and MCF7 cells (* $P < 0.05$). (C, D, E and F) The proportion of stem cells detected by flow cytometry after the addition of the HIF-1 α inhibitor 2ME2 to MDA-MB-231 cells and hypoxic conditioning in MCF7 cells. (G and H) Western blotting was used to examine the protein levels of CD44 after the addition of 2ME2 (DMSO was used as a negative control) in MDA-MB-231 cells and hypoxic conditioning in MCF7 cells. (I and J) Variation in tube-forming ability after the addition of the HIF-1 α inhibitor 2ME2 to MDA-MB-231 cells and hypoxic conditioning in MCF7 cells. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0054>.

BCSLCs. As a key transcription factor, HIF-1 α can regulate many downstream target genes by binding to HREs on their promoter. However, whether HIF-1 α can directly regulate the functional level of α -SMA via transcriptional modulation is still unclear.

PEDF inhibits breast tumor growth by suppressing the formation of heterogeneous vascularity

Based on the above results, we believe that the development of new antiangiogenic therapeutic agents is urgently needed for breast cancer treatment. PEDF is a kind of endogenous multifunctional glycoprotein with prominent antiangiogenic effects (Ek & Dass *et al.* 2006, Zhang *et al.* 2006, Yang *et al.* 2009). Previous studies have reported that PEDF can inhibit vasculogenic mimicry in melanoma (Orgaz *et al.* 2009). However, the exact mechanism has not been thoroughly elucidated. Additionally, the effect of PEDF on heterogeneous vascularity in breast cancer has not yet been reported.

Our results showed high PEDF expression in MCF7 cells and significantly lower PEDF expression in MDA-MB-231 cells (Fig. 6A). To explore whether high PEDF inhibits the tube-forming ability of MCF7 cells, we knocked down PEDF in MCF7 cells with siRNA and assessed their capability of tube formation (Fig. 6C). Tube formation was lower in MDA-MB-231 cells transfected with Ad-PEDF than in cells transfected with Ad-GFP (Fig. 6B). Next, we conducted 3D *in vitro* experiments and found that PEDF can dramatically disrupt tube formation by HUVECs and MDA-MB-231 cells (Fig. 6D).

In vivo, we measured tumor volume every 3 days and found that tumor size in the PEDF group was obviously smaller than that in the control group (Fig. 6F and G). One mouse in the PBS group was euthanized on days 27, 31, 38 and 41, while one mouse in the PEDF group was euthanized on days 38 and 42. By day 43 of the experiment, we found that PEDF could effectively improve the survival rate (Fig. 6H). We collected further tumors at different time points by CD31/PAS staining. Consistent with the *in vitro* results, PEDF could suppress blood vessel formation and vasculogenic mimicry (Fig. 6I).

These results indicated that the endogenous factor PEDF can effectively inhibit tumor growth by suppressing heterogeneous vasculogenesis. Mechanistically, the effect is likely dependent on HIF-1 α / α -SMA (Fig. 6J and Supplementary Fig. 7).

Hence, we propose a molecule, PEDF, that can effectively suppress angiogenesis and significantly inhibit heterogeneous vasculogenesis, opening new opportunities for the clinical treatment of breast cancer.

Mechanism summary

In breast cancer, the antiangiogenic drug sorafenib can decrease MVD by targeting ECs. However, this therapeutic

Figure 6

Effect of PEDF on breast cancer *in vivo* and *in vitro*. (A) Western blot analysis for PEDF expression in breast cancer cells MDA-MB-231 and MCF7. β -Actin was used as a loading control. (B) Representative micrographs showing the formation of capillary-like structures in Matrigel by MDA-MB-231 cells transfected with Ad-GFP and Ad-PEDF. (C) Verification of the effect of siRNA-mediated interference of PEDF promoting tube formation by MCF7 cells. (D) HUVECs and MDA-MB-231 cells were seeded into 24-well plates coated with Matrigel. After 8 h, the cells were treated with different doses of PEDF. Formation of tube-like structures was examined by phase contrast microscopy 12 h later. (Objective magnification, $\times 200$). (E) Schematic representation showing the strategy for animal experiments. (F and G) Tumor size in mice bearing MDA-MB-231 breast cancer cells treated with PBS ($n = 3$) and PEDF ($n = 3$) at two time points. (H) Survival analysis of the PBS and PEDF groups. Kaplan-Meier actuarial survival curves were used to compare the PBS group ($n = 7$) with the PEDF group ($n = 7$). The PEDF group tended to have a higher overall survival than the PBS group. (I) Paraffin sections of tumors at two time points were subjected to CD31/PAS double staining. White arrow shows CD31⁺ ECs. Black arrow shows CD31⁺/PAS⁺ structures formed by vasculogenic mimicry with red blood cells. (J) HIF-1 α and α -SMA expression by Western blot analysis in MDA-MB-231 cells transfected with vector and PEDF. A full colour version of this figure is available at <https://doi.org/10.1530/ERC-19-0054>.

strategy may lead to aggravated anoxia in the tumor (Conley *et al.* 2012). The hypoxic environment will promote the expression of HIF-1 α as an adaptive response. HIF-1 α can subsequently increase the proportion of BCSLCs and enhance the expression of α -SMA by directly binding to the HRE on the ACTA2 promoter. The increased expression of functional α -SMA enables BCSLCs to participate in the heterogeneous vasculogenesis. PEDF can suppress both heterogeneous vasculogenesis and traditional angiogenesis by targeting BCSLCs and ECs to effectively starve tumors (Supplementary Fig. 8).

Discussion

Heterogeneous vascularity was once considered a possible reason for the poor curative effects of antiangiogenic therapy. The molecular basis underlying the pathogenesis of heterogeneous vascularity in breast cancer is not completely understood. The present study, for the first time, demonstrated that only BCSLCs have the capability to participate in the formation of blood vessels. Mechanistically, we have illustrated that (1) α -SMA, a marker of EMT and pericytes, was a key functional molecule contributing to capillary structure formation by BCSLCs; (2) HIF-1 α increased BCSLC population and enhanced the mRNA and protein levels of α -SMA by directly binding to the HRE on the ACTA2 promoter; (3) the endogenous angiogenesis inhibitor PEDF could inhibit not only traditional angiogenesis but also heterogeneous vasculogenesis by downregulating HIF-1 α and α -SMA in breast cancer. Our study not only suggested a new mechanism in which HIF-1 α -driven BCSLCs participate in heterogeneous vascularity in tumors, but also provided an effective approach to suppress these specific vessels. These findings further enrich our knowledge on the properties of heterogeneous vascularity and provide a promising candidate drug for the treatment of breast cancer.

Previously, tumor angiogenesis was considered to mainly arise from normal blood vessel sprouting. However, accumulated evidence indicates that vascular perfusion is temporally and spatially heterogeneous and involves cancer cells participating in various vessel-like structures including co-opted vessels, mosaic vessels, and tubular structures formed by vasculogenic mimicry (Jain 1988), suggesting that targeting ECs alone is not sufficient for effective tumor control. We have detected a variety of microcirculation models including vasculogenic mimicry and mosaic vasculogenesis in TNBC specimens (Fig. 3A). In addition, we provided evidence both *in vitro*

and *in vivo* that sorafenib could inhibit tube formation or vascularization by ECs but not by breast cancer cells (Supplementary Fig. 1). However, what determines the features and mechanisms of vascular formation by these tumor cells remained unknown. Previously, highly malignant and aggressive tumor cells were shown to participate in vasculogenic mimicry by expressing endothelial maker VE-cadherin or endothelial phenotypes (Hendrix *et al.* 2000). Elvin Wagenblast proposed that enforced expression of SERPINE2 and SLPI in human breast cancer cell lines also programmed them for vascular mimicry (Wagenblast *et al.* 2015). Moreover, recent studies have found that EMT endows cancer cells with pericyte properties and is indispensable for vascular stabilization and sustained tumor growth (Shenoy *et al.* 2016). In our study, we confirmed that only the stem-like ALDH1⁺ tumor cells in breast cancer had tube-forming ability. Although the ALDH1⁻ cells isolated from MDA-MB-231 cells expressed most mesenchymal markers, they could not form tube-like structures (Fig. 1).

The perivascular region is recognized as a niche for stem-like cancer cells in several head and neck cancers (Ritchie & Nor 2013). Vascular cells including ECs and pericytes can help maintain CSC phenotypes in a paracrine manner. However, it was unknown how CSCs contribute to tumor vessel formation. Recent studies have proposed that CSCs have the potential to generate pericytes and ECs to form blood vessels in breast cancer and glioblastoma (Wang *et al.* 2010, Cheng *et al.* 2013, Tang *et al.* 2014). These studies showed that CSCs had the ability to contribute to the tumor vasculature formation by promoting EC and pericyte differentiation through the production of directional differentiation factors. Nevertheless, our data suggest for the first time that tumor cells involved in heterogeneous vasculogenesis in breast cancer possess stem-like properties owing to their ability to express α -SMA without differentiation. Furthermore, we confirmed that α -SMA plays an important role in the formation of vessel-like structures by BCSLCs (Figs 2 and 3).

α -SMA has been recognized as an important marker for pericytes, EMT and cancer-associated fibroblasts (CAFs). Tumor cells undergoing EMT or CAFs are reported to promote tumor metastasis (Kalluri 2016, Shenoy *et al.* 2016). However, the functions of α -SMA have not been thoroughly investigated in these cells. In this study, we provide evidence that α -SMA is a crucial functional molecule enabling the participation of BCSLCs in heterogeneous vasculogenesis *in vivo* and *in vitro* (Figs 2 and 3). However, the regulatory mechanism of α -SMA

is unclear at present. The suppression of Flk-1 has been reported to be associated with a corresponding reduction in α -SMA expression (Francescone *et al.* 2012). Phosphate can decrease the level of α -SMA in vascular smooth muscle cells (VSMCs) via the Wnt/ β -catenin pathway (He *et al.* 2018). Our recent study showed that high glucose increased the expression of the mesenchymal marker α -SMA in Müller cells (Zhou *et al.* 2017). In this article, we demonstrated for the first time that HIF-1 α , an important transcription factor that can regulate many target genes in hypoxia, upregulates the expression of α -SMA by directly binding to the HRE on the ACTA2 promoter (Fig. 4).

The lack of effect on BCSLC-derived vessels might be the reason for the poor outcomes of antiangiogenic therapy. In November 2011, the FDA announced the cancellation of bevacizumab (Avastin®) use for breast cancer. The results of a phase III clinical trial on sorafenib for HER-2-negative breast cancer showed that sorafenib combined with capecitabine cannot improve progression-free survival relative to placebo but results in harmful side effects (Bergh *et al.* 2012, Bertucci *et al.* 2016, Ribatti *et al.* 2016). The therapeutic strategy may lead to aggravated anoxia in the tumor. Hypoxia plays a critical role in the evolution of the tumor stromal microenvironment and can inhibit tumor cell differentiation and promote CSC maintenance (Ezashi & Das *et al.* 2005, Gustafsson *et al.* 2005). Accumulating evidence shows that culturing glioma cells in 2% oxygen rather than in standard 20% oxygen increases the CD133⁺ cell population and enhances tumor sphere formation of stem cell populations by activating HIF-1 α (Morrison *et al.* 2000, Blazek & Foutch *et al.* 2007). Knockdown of HIF-1 α abrogates the expansion of CD133⁺ GSCs under hypoxia (Soeda *et al.* 2009). Consistent with these reports, we found that HIF-1 α can increase the proportion of BCSLCs and induce capillary structure formation (Fig. 5). Therefore, we inferred that BCSLCs participate in tumor vascular formation in a HIF-1 α / α -SMA-dependent manner, which is insensitive to sorafenib. However, PEDF can significantly inhibit this pathway (Supplementary Fig. 8).

PEDF is an endogenous antiangiogenic factor that plays an important role in inhibiting tumor growth and metastasis (Yang *et al.* 2009), suppressing EMT in tumor cells (Zhang *et al.* 2017), and inducing tumor cell apoptosis (Li *et al.* 2014). PEDF can inhibit vasculogenic mimicry in melanoma (Orgaz *et al.* 2009). Our recent study demonstrated that PEDF can inhibit HIF-1 α expression in nasopharyngeal carcinoma CNE-2 cells (Xu *et al.* 2011). The present study demonstrated for the first time that PEDF could inhibit vasculogenic mimicry by

downregulating HIF-1 α and BCSLC proportions in breast cancer. These results suggested that PEDF might be a powerful antiangiogenic drug because of its dual effects on angiogenesis and heterogeneous vasculogenesis in breast cancer.

In summary, we identified a critical role of BCSLCs in heterogeneous vasculogenesis in tumors. Activation of HIF-1 α enhanced BCSLC proportion and α -SMA expression, and heterogeneous vasculatures formed by BCSLCs might be the reason for the poor curative effects of sorafenib. Mechanistically, HIF-1 α / α -SMA could potentially be exploited as novel therapeutic targets for the treatment of heterogeneous tumor vascularity. Additionally, PEDF may be a promising candidate for the treatment of breast cancer.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-19-0054>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

All authors have contributed significantly to this manuscript and each author has contributed to the manuscript as follows: Yuling Mao and Guoquan Gao designed the project. Yuling Mao, Liqing Zhu and Chuanghua Luo collected clinical samples and performed the histology experiments and analyzed the data. Yuling Mao and Liqing Zhu performed mainly cell experiments and immunofluorescent assay, as well as analyzed the results. Yuling Mao and Weiwei Qi did the PEDF protein production and analyzed the data. Yuling Mao and Zhijian Huang

performed the tube-formation assay and analyzed the data. Zhijian Huang carried out the additional experiments. Xia Yang, Guoquan Gao and Yuling Mao wrote the manuscript. Weiwei Qi, Zhonghan Yang and Ti Zhou revised the manuscript.

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