A novel approach for transforming breast cancer stem cells into endothelial cells

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Abstract. Tumor vascular endothelial cells play a pivotal in the tumor microenvironment, influencing the proliferation, invasion, and metastasis of tumor progression. The present study investigated a novel method for inducing the transformation of breast cancer stem cells into endothelial cells, providing a cellular model investigating anti-angiogenic mechanisms *in vitro*. The breast cancer cell line MCF-7 was used, and the expression of CD133 was initially detected using flow cytometry. $CD133^+$ breast cancer cells were purified using immunomagnetic bead sorting technology, yielding an MCF-7^{CD133+} subpopulation. The proliferation ability of these cells was assessed using an MTT assay, while their microsphere formation ability was evaluated using a microsphere formation assay. Post-transformation in an optimized endothelial cell culture medium, expression of endothelial cell markers CD31 and CD105 were detected using flow cytometry. Endothelial cell tube formation assays and DiI-labeled acetylated low-density lipoprotein (DiI-Ac-LDL) assays were employed to analyze the endothelial cell function of the MCF-7^{CD133+} cells. MDM2/CEN12 gene amplification was detected through fluorescence *in situ* hybridization (FISH). The MCF-7 breast cancer cell line exhibited 1.7±0.3% trace cells expressing the stem cell surface marker CD133. After anti-CD133 immunomagnetic bead sorting, MCF-7^{CD133+} and MCF-7^{CD133-} subpopulation cells were obtained, with CD133 expression rates of 85.6±2.8 and 0.18±0.08%, respectively. MTT assay results demonstrated that, after 7 days, the proliferation rate of MCF-7^{CD133+} cells was significantly higher compared with MCF-7^{CD133-} cells. MCF-7^{CD133+} subpopulation cells displayed strong stem cell characteristics, growing in suspension in serum-free media and forming tumor cell spheres. In contrast, MCF-7^{CD133-} cells failed to form microspheres. After culturing cells in endothelial cell differentiation and maintenance media, the percentage of MCF-7^{CD133+} cells before and after endothelial cell culture was 0.3±0.16 and 81.4±8.37% for $CD31^+$ cells and 0.2±0.08 and 83.8±7.24% for $CD105^+$ cells, respectively. Vascular-like structure formation and Ac-LDL phagocytosis with red fluorescence in the tube formation assays confirmed endothelial cell function in the MCF-7^{CD133+} cells. FISH was used to verify MDM2/CEN12 gene amplification in the induced MCF-7^{CD133+} cells, indicating tumor cell characteristics. The modified endothelial cell transformation medium effectively induced differentiated tumor stem cells to express vascular endothelial cell markers and exhibit endothelial functions, ideal for *in vitro* anti-angiogenesis research.

Introduction

Breast cancer represents a leading cause of cancer-associated mortality in women globally. Despite notable advancements in the early detection and treatment of breast cancer, including improved surgical techniques, chemotherapy, radiation therapy and targeted biological treatments, a subset of patients still experience recurrence and/or metastasis, resulting in the failure of conventional therapeutic strategies (1). Thus, there is an urgent need to understand the etiology of breast cancer and identify innovative therapeutic approaches to address this critical health concern.

The complex nature of breast cancer, characterized by its heterogeneity in molecular profiles, pathological features and response to treatment, underscores the necessity of a deeper understanding (2). Research is increasingly focusing on the genetic and molecular underpinnings of breast cancer, exploring the role of genetic mutations, epigenetic alterations and the tumor microenvironment in cancer progression and resistance to treatment (3). Furthermore, the emerging field of cancer stem cell biology is shedding light on a subset of cells within tumors that possess the ability to self-renew, differentiate and potentially drive tumor growth and metastasis.

In 1983, Mackillop *et al* (4) first proposed the tumor stem cell hypothesis, suggesting that tumors contain a small subpopulation of cells with stem cell-like properties. The significance of the tumor stem cell hypothesis lies in its implications for cancer treatment and resistance. Cancer stem cells (CSCs) have been implicated in the resilience of

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malignant tumors to chemotherapeutic agents and radiation therapy (5). Their stem-like properties allow them to survive traditional therapies, such as chemotherapy and radiotherapy, that primarily target rapidly dividing cells. CSCs thrive in hypoxic environments and exhibit high expression levels of free-radical scavenging mechanisms. This adaptive response results in the decreased intracellular accumulation of reactive oxygen species following exposure to radiation, which consequently gives rise to the development of a radioresistant phenotype (6). This survival advantage of CSCs is thought to contribute to the post-treatment recurrence and metastasis of tumors, as these residual CSCs can regenerate the tumor mass and facilitate its spread to distant sites (7-9). CD133, a 5-transmembrane (5_TM) glycoprotein, is a stem cell surface marker widely used as a biomarker in various solid tumors including brain (10), lung (11), gastric (12) and ovarian cancer (13).

The traditional theory of tumor angiogenesis states that tumor neovascularization primarily stems from two processes: Angiogenesis and vasculogenesis (14,15). Angiogenesis involves the sprouting of new blood vessels from pre-existing ones. In the context of tumors, angiogenic factors are released by cancer cells, which then stimulate the neighboring vascular endothelial cells to proliferate and form new vessel branches (16). Vascular endothelial growth factor (VEGF) is one of the most potent inducers of angiogenesis (17). In cancer, VEGF is produced and secreted by tumor cells, which is associated with tumor progression, invasiveness, metastasis and tumor recurrence (18). Fibroblast growth factor-2 (FGF2) exerts its effects on endothelial cells via a paracrine signaling after being released by tumor cells (19) Unlike angiogenesis, vasculogenesis refers to the formation of new blood vessels from endothelial progenitor cells (EPCs) that originate in the bone marrow. These EPCs are mobilized to the tumor site, where they differentiate into endothelial cells and contribute to the neovascular network (20).

In the absence of vascular endothelial cells, tumor stem cells within the tumor tissues can differentiate into vascular endothelial cells, promoting tumor angiogenesis (21,22). This process, a form of neovascularization distinct from traditional angiogenesis and vasculogenesis, involves the direct contribution of tumor stem cells to the tumor vasculature. These cells undergo endothelial differentiation, integrating into the developing vascular structure and thereby supporting the angiogenic process (23). Several studies have highlighted the link between tumor cell proliferation, invasion, metastasis, and angiogenesis (24-27). However, the role of tumor stem cells in inducing the formation of tumor blood vessels is not fully understood.

The present study utilized CD133 as a stem cell surface marker to isolate and purify tumor stem cells from breast cancer cell lines. By enhancing the differentiation of $CD133^+$ breast CSCs into vascular endothelial cells *in vitro*, this study aimed to establish a basis for studying anti-angiogenic mechanisms.

Materials and methods

Cells and cell culture. MCF-7 (cat. no. SCSP-531) were purchased from the Cell Bank of the Chinese Academy of Sciences and HUVECs (cat. no. iCell-h110) were purchased from Cellverse Bioscience Technology Co., Ltd. The MCF-7 breast cancer cell line was cultured in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. HUVECs were maintained in an ECM endothelial cell culture medium supplemented with 5% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. HUVECs are known to exhibit senescence after several passages, thus they were revived at cell passage 2 and consistently used at low passages, typically below passage 5. This practice was essential to maintain their physiological relevance and ensure the consistency of the results. Both cell types were incubated at 37˚C in a 5% CO_2 incubator. MCF-7 cells exhibiting adherent growth were deemed suitable for experimental use when adherent growth exceeded 80%. Before passaging or cryopreservation, cells were digested with trypsin.

Flow cytometry. Cells were digested using 0.25% trypsin, and digestion was halted by adding culture media. Subsequently, cells were washed with 0.01M PBS, resuspended, and centrifuged at 800 x g for 5 min at room temperature to remove the supernatant. The cells were prepared at a concentration of $1x10^6$ cells in 1 ml of 0.01 M PBS. Subsequently, 100 μl cell suspension was added into 5 ml flow tubes with phycoerythrin-labeled CD133 (1:20; cat. no. 12-1338-42; Thermo Fisher Scientific, Inc.), CD31 (1:40; cat. no. 12-0319-42; Thermo Fisher Scientific, Inc.), or CD105 antibodies (1:40; cat. no. MHCD10504; Thermo Fisher Scientific, Inc.). The mixture was thoroughly mixed and incubated at 4˚C in the dark for 10 min. Following incubation, cells were washed, resuspended, centrifuged at 800 x g for 5 min at 4˚C, and the supernatant was removed. Finally, cells were resuspended in 500 μl 0.01 M PBS, flow data were collected using BD FACSCanto II (Becton, Dickinson and Company) and analyzed using FLOWJO version 7.6.2 (flowjo.com/).

Isolation of CD133-positive cells. After culturing MCF-7 cells, they were digested and washed. Subsequently, cells were resuspended in 300 μl sorting buffer and combined with 100 μl anti-CD133 immunomagnetic beads (cat. no. 130-097-049; Miltenyi Biotec GmbH). The mixture was incubated at 4˚C in the dark for 30 min. After incubation, cells were washed with 0.01 M PBS and centrifuged at 800 x g for 10 min at 4˚C. The supernatant was discarded, and the cell pellet was resuspended in a sorting buffer. A pre-rinsed separation column with 500 μl sorting buffer was then loaded with the cell suspension and washed thrice with 0.01 M PBS. $CD133^+$ cells were eluted by washing the column with 1 ml sorting buffer using a syringe pump.

MTT assay. The MCF-7 cells were divided into two subpopulations, $MCF\text{-}7^{CD133+}$ and $MCF\text{-}7^{CD133-}$, and seeded into 96-well plates. Each well was filled with 0.2 ml serum-free media supplemented with 20 ng/ml basic fibroblast growth factor (bFGF; cat. no. AF-100-18B-500UG; Thermo Fisher Scientific, Inc.) and 20 ng/ml epidermal growth factor (EGF; cat. no. AF-100-15-500UG; Thermo Fisher Scientific, Inc.). The cells were cultured for 7 days, with the addition of 20 μl MTT reagent to each well daily. After 4 h of further incubation, the medium was removed and replaced with 150 μl DMSO. The absorbance at 490 nm was measured for each well to determine cell growth rates.

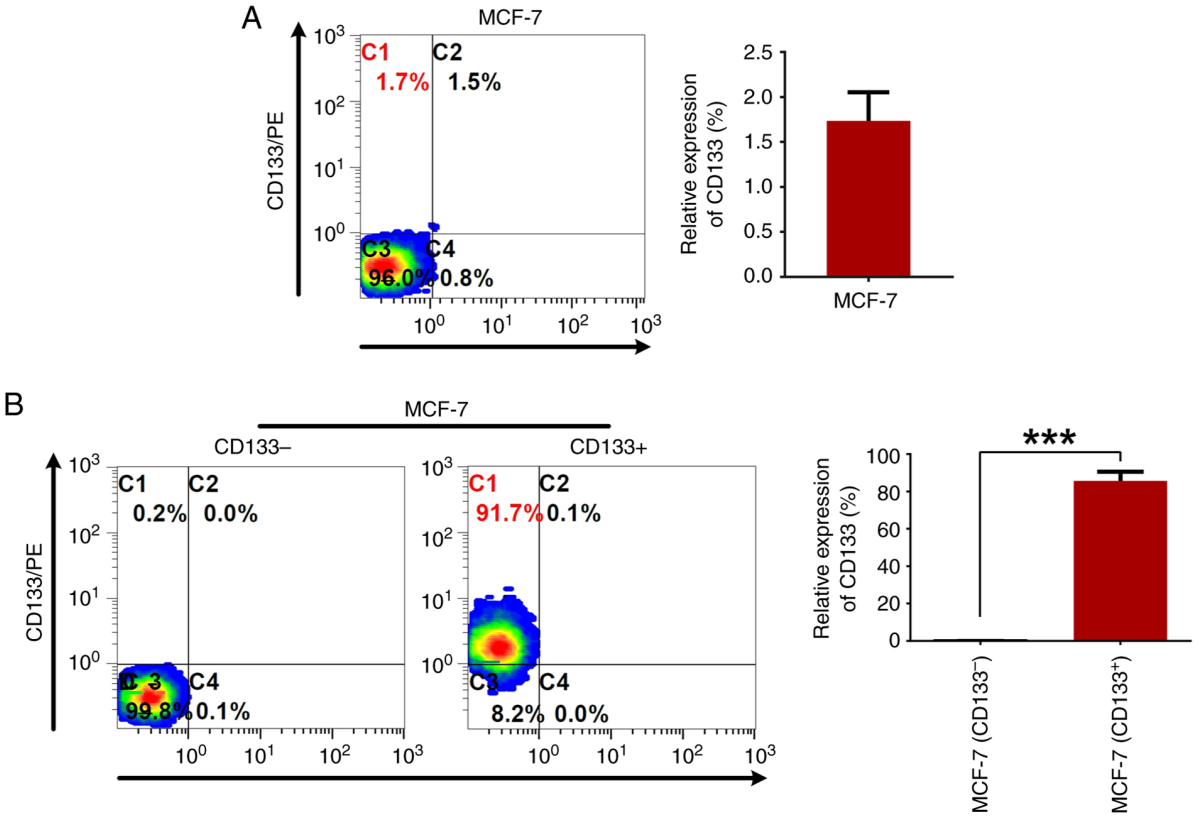

Figure 1. CD133 expression levels in MCF-7 cells pre- and post-immunomagnetic bead separation. (A) Prior to immunomagnetic bead separation, the expression levels of CD133 in the MCF-7 cell line was 1.7±0.3%. (B) Post immunomagnetic bead sorting, CD133 expression in MCF-7^{CD133+} cells was 85.6±2.8%, while in MCF-7^{CD133-} cells, only 0.18±0.08% expressed CD133. ***P<0.001. PE, phycoerythrin.

Spheroid formation assay. The MCF-7^{CD133+} and MCF-7^{CD133-} subpopulations were seeded at a density of 1,000 cells/ml in 12-well low-adhesion plates, each well contained 1 ml spheroid formation medium. This medium consisted of DMEM/F12 supplemented with 1x B27, 20 ng/ml bFGF, 20 ng/ml EGF, 5 g/ml insulin, and 1% penicillin-streptomycin. Spheroid formation was observed under a light microscope (x100 magnification) after 3 days.

Endothelial cell differentiation culture. The MCF-7^{CD133+} and MCF-7^{CD133-} subpopulations were cultured in stem cell maintenance medium, which was prepared by supplementing StemScale™ PSC medium (Gibco; Thermo Fisher Scientific, Inc.) with bFGF (20 ng/ml), EGF (20 ng/ml), and BMP4 (25 ng/ml; cat. no. 795604; BioLegend, Inc.). The cells were incubated for 2 days in low-adhesion dishes, and the media was replaced every 2 days. Subsequently, the cells were transferred to endothelial cell differentiation medium, which was composed of ECM medium (ScienCell) supplemented with VEGF (50 ng/ml; cat. no. AF-100-20-500UG; Thermo Fisher Scientific, Inc.) and bFGF (20 ng/ml). Cells were maintained in this differentiation culture media for 6 days, and the media was changed every 2 days.

Endothelial cell tube formation assay. Matrigel matrix gel was thawed at 4˚C and left undisturbed for 1 day. To initiate the experiment, refrigerated pipette tips and μ-Slide angiogenesis culture plates were used. A total of 10 μl Matrigel was added to each well of the μ-Slide plate. The μ-Slide plate was placed in an appropriately sized culture dish containing water-saturated absorbent paper to prevent moisture evaporation. The culture dishes were incubated for 30 min, allowing the gel to solidify. Cell suspensions (2x10^5 cells/ml) were prepared after cell digestion, and 50 μl of this suspension was added to the μ-Slide plate. The plates were covered and returned to the incubator for continuous culture. The cells were monitored using an optical microscope (x200 magnification) every 4-6 h.

DiI-labeled acetylated low-density lipoprotein (DiI-Ac-LDL) uptake assay. HUVECs, MCF-7^{CD133+}, or MCF-7^{CD133-} induced endothelial cells were seeded in 24-well plates and cultured for 48 h. Subsequently, the culture media was removed and cells were incubated in serum-free ECM endothelial cell medium for 3 h. DiI-Ac-LDL was prepared in serum-free EGM medium at a concentration of 10 μg/ml, added to cells, and incubated at 37˚C for 4 h. Following incubation, the media was discarded, and cells were washed three times in 0.01M PBS to eliminate any unbound DiI-Ac-LDL. Finally, supplemented culture medium was added and the cells were examined under a fluorescence microscope (x400 magnification).

MDM2/CEN12 fluorescent in situ hybridization (FISH) fluorescent probe detection. Cell preparation involved washing HUVEC, MCF-7^{CD133+}, and MCF-7^{CD133-}-induced endothelial cells with 0.01M PBS, dropping them onto a glass slide, denaturing at 73˚C for 2 min, hybridizing with a MDM2/CEN12 probe (cat. no. FG0020; Abnova) at 38˚C for 16 h, and washing

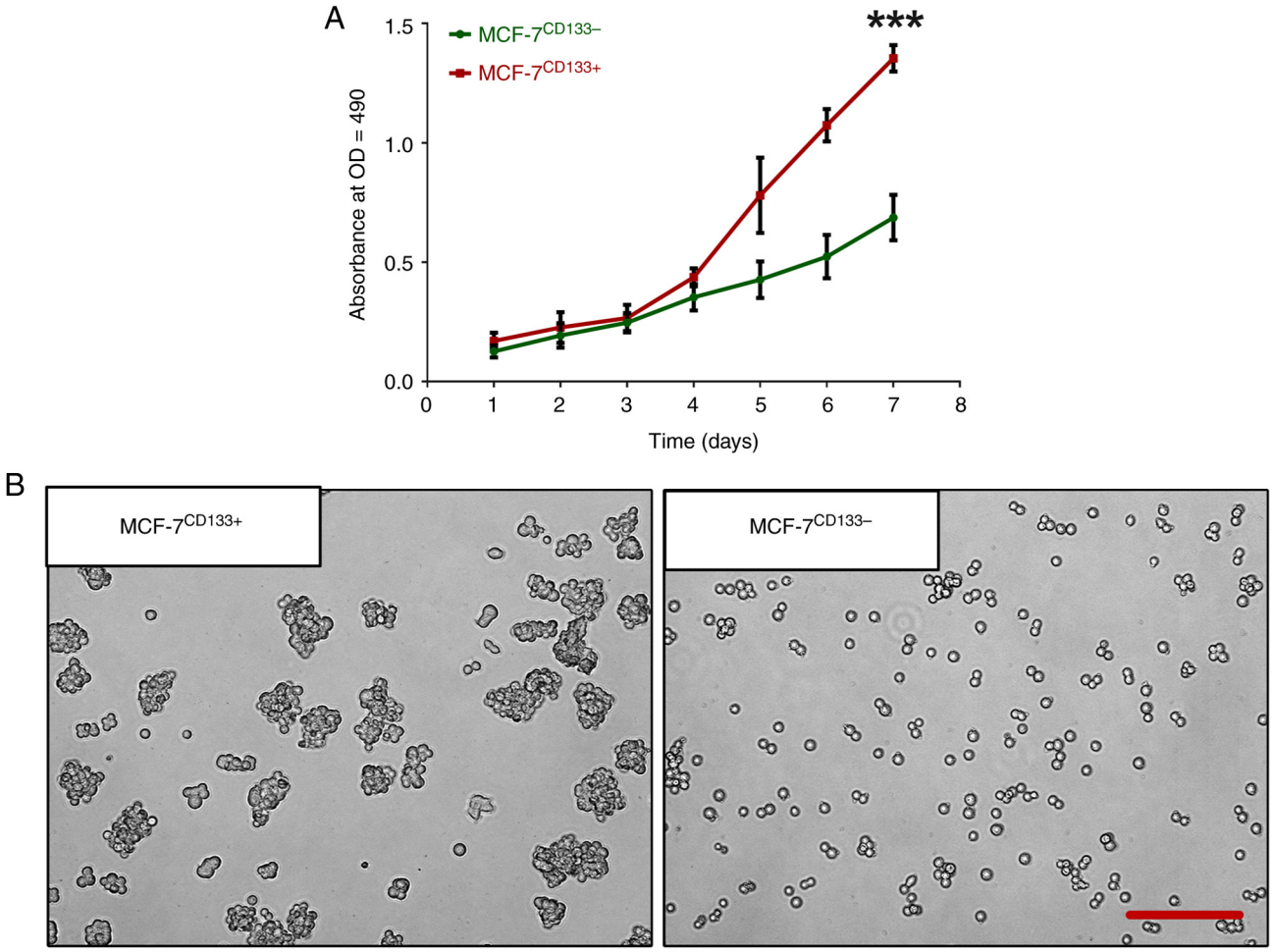

Figure 2. *In vitro* proliferation and spheroid formation capabilities in MCF-7 cell subpopulations. (A) Comparison of the proliferation of MCF-7^{CD133+} and MCF-7^{CD133-} cells using MTT assays. ***P<0.001. (B) Evaluation of spheroid formation capacity of the MCF-7^{CD133+} and MCF-7^{CD133-} cells using a spheroid formation assay, Scale bar, 100 μm. OD, optical density.

with 0.3% NP-40/SSC and 0.1% NP-40/SSC. The slides were sequentially dehydrated in 70, 90 and 100% ethanol for 2 min each. Finally, the cell nuclei were counterstained for 5 min at room temperature with DAPI and observed under a fluorescence microscope (x1,000 magnification).

Statistical analysis. Data were analyzed using SPSS version 18.0 (SPSS Inc.), all data are presented as the mean ± SD. Differences between groups were compared using an unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

CD133 expression levels in MCF-7 pre- and post-immunomagnetic bead separation. Flow cytometry was used to assess CD133 expression in the MCF-7 breast cancer cells, and it was found that only 1.7±0.3% of the cells exhibited CD133 expression (Fig. 1A). Post anti-CD133 immunomagnetic bead sorting, two subpopulations, MCF-7^{CD133+} and MCF-7^{CD133-}, were isolated. Flow cytometry was used to measure the proportions of CD133+ cells in these subpopulations, revealing expression rates of 85.6±2.8% for MCF-7^{CD133+} and 0.18±0.08% for MCF-7^{CD133-} (Fig. 1B).

MCF-7 $^{CD133+}$ cells exhibit increased in vitro proliferation and spheroid formation capabilities compared with the MCF-7 $^{CD133-}$ cells. Compared with the MCF-7^{CD133-} cells, the MCF-7^{CD133+} cells exhibited increased *in vitro* proliferation capacity, most notably on day 7 (Fig. 2A). The spheroid formation assay highlighted the cancer stem cell characteristics of both MCF-7^{CD133+} and MCF-7^{CD133-} cells. After 3 days in low-attachment culture plates, MCF-7^{CD133+} cells displayed increased differentiation and growth capacity, resulting in a significantly higher number of spheroids compared with the MCF-7^{CD133-} (Fig. 2B).

Assessment of endothelial cell marker expression pre- and post-endothelial cell induction culture. MCF-7^{CD133+} and MCF-7^{CD133-} cells were cultured in stem cell maintenance media for 4 days, followed by 6 days in endothelial cell induction media, to induce tumor stem cells towards endothelial differentiation (Fig. 3A). Flow cytometry was used to assess the expression levels of endothelial cell surface markers CD31 and CD105 before and after induction. In the MCF-7^{CD133+} cells, the CD31^{+} proportions were 0.3±0.16% pre-induction and 81.4±8.37% post-induction, and the CD105^{+} proportions were 0.2±0.08% pre-induction and 83.8±7.24% post-induction (Fig. 3B). In MCF-7^{CD133-} cells, the CD31^{+} proportions were

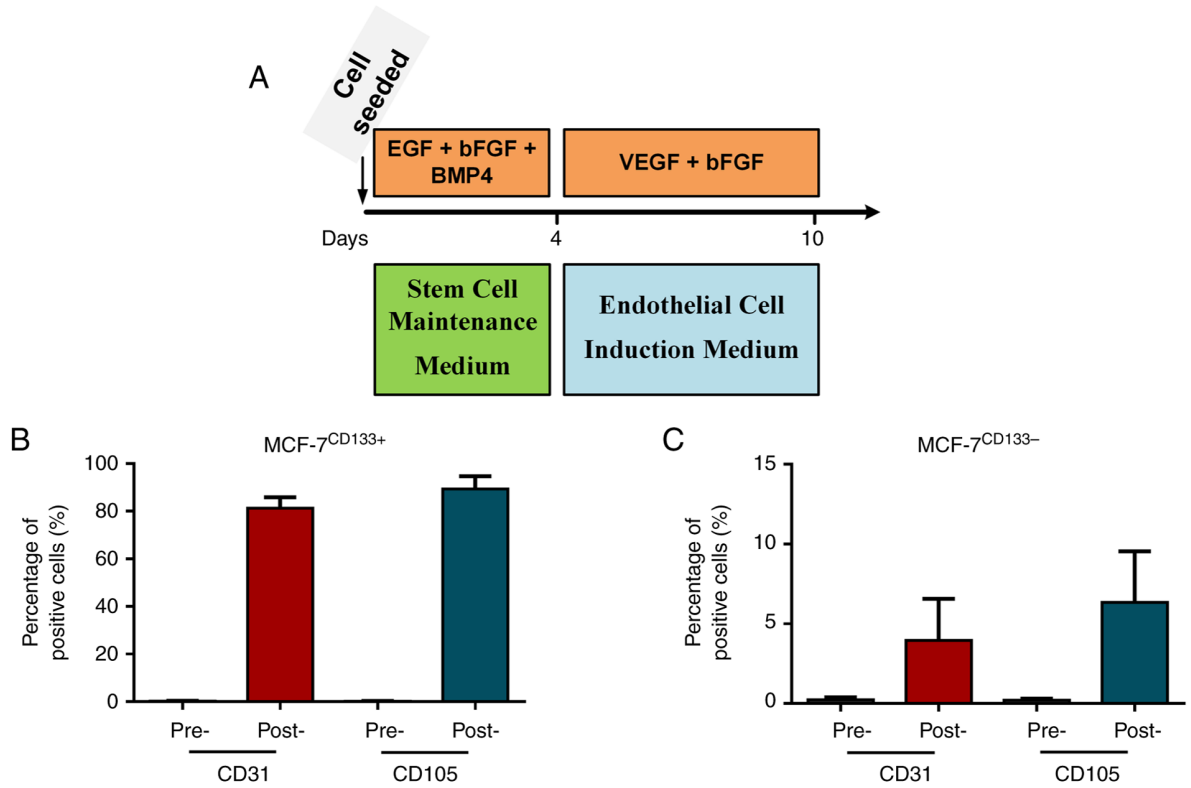

Figure 3. Evaluation of endothelial cell surface markers pre and post-culture with endothelial cell induction media. (A) Method for endothelial cell transformation. (B) Flow cytometry analysis of CD31 and CD105 expression in the MCF-7^{CD133+} cells pre- and post-endothelial cell induction. (C) Flow cytometry analysis of CD31 and CD105 expression in the MCF-7^{CD133-} cells pre- and post-endothelial cell induction. EGF, epidermal growth factor; bFGF, basic fibroblast growth factor.

0.23±0.12% pre-induction and 3.95±2.1% post-induction, and the CD105^{+} proportions were 0.26±0.04% pre-induction and 6.3±2.6% post-induction (Fig. 3C).

Evaluation of endothelial cell function and gene amplification. In the endothelial cell tube formation assay, both the positive control HUVECs and MCF-7^{CD133+} cells formed lumen-like structures. In contrast, the MCF-7^{CD133-} cells did not form these structures (Fig. 4A). In the endothelial cell uptake assay, both positive control HUVECs and MCF-7^{CD133+} cells emitted red fluorescence after staining with DiI-Ac-LDL (Fig. 4B). FISH experiments indicated that after induction, MCF-7^{CD133+} cells still exhibited amplification of the MDM2/CEN12 gene in the cell chromosomes (Fig. 4C).

Discussion

Breast cancer, a heterogeneous malignant tumor, is influenced by various risk factors including diet, environment, genetics, and epigenetics. Current data suggest that the 5-year survival rates for stage II and III breast cancer patients are 75 and 61%, respectively. However, 20-30% of cases still exhibit recurrence and/or metastasis (28). Therefore, finding effective strategies to prevent the recurrence and metastasis of breast cancer is crucial.

Contemporary theories suggest the presence of tumor stem cells in tumor patients, cells with characteristics akin to embryonic stem cells. These cells have unlimited proliferation, self-renewal, and multilineage differentiation capabilities. Additionally, they exhibit chemoresistance and radioresistance, contributing to recurrence and metastasis despite comprehensive anti-tumor therapies (29-31). CD133, also known as Prominin-1, a member of the Prominin family, is a five-transmembrane domain glycoprotein predominantly located on cell membrane surface protrusions and is recognized as a key biomarker for CSCs (32,33). In the present study, we initially identified a minor population of CD133-expressing cells within the MCF-7 breast cancer cell line. Utilizing anti-CD133 immunomagnetic bead separation, MCF-7^{CD133+} cells with a high CD133 positivity rate of approximately 85% were isolated, as determined by flow cytometry. It is important to note that the proportion of CSCs within cancer cell lines can indeed change over time and with continuous passaging. Thus, the proportion of stem cells and cancer cells within the cell lines across different passages was monitored to assess this variability. In comparison with MCF-7^{CD133-} cells, the MCF-7^{CD133+} cells exhibited a significantly enhanced proliferative capacity. An actively proliferating subset of CSCs may play a crucial role in the growth and progression of tumors. These cells can give rise to more differentiated tumor cells while maintaining the CSC population, allowing the tumor to grow and potentially spread. When cultured in serum-free media enriched with growth factors, MCF-7^{CD133+} cells rapidly formed spheroids, exhibiting growth in suspension, and robust proliferation, while MCF-7^{CD133-} cells showed a significantly reduced capacity for spheroid formation. These findings indicate that the MCF-7^{CD133+} cells were enriched in stem cells.

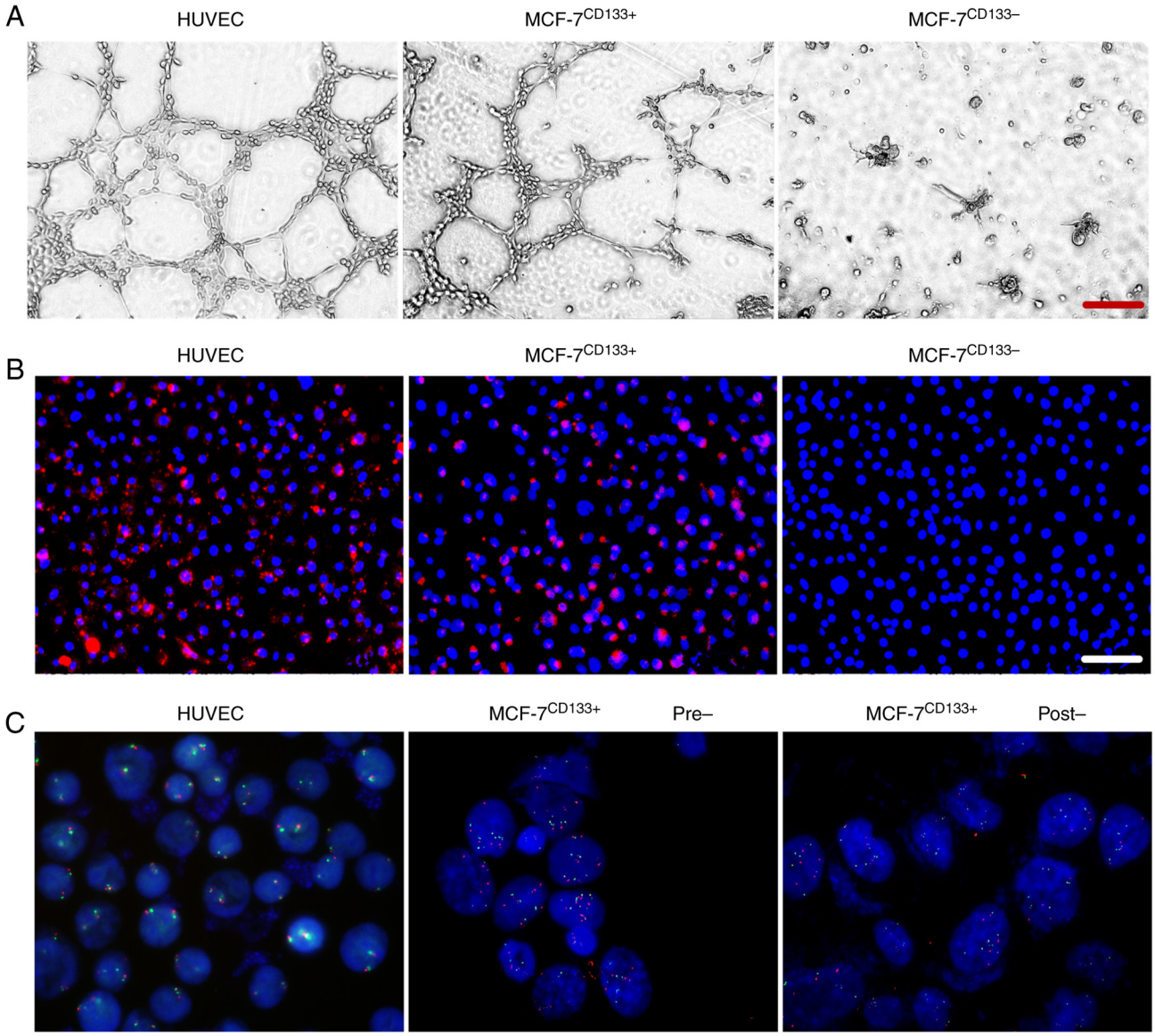

Figure 4. Evaluation of endothelial cell function and gene amplification. (A) Endothelial tube formation was visualized under a light microscope. Scale bar, 100 μm. (B) Endothelial cell uptake was observed under a light microscope. Scale bar, 50 μm. (C) Fluorescence *in situ* hybridization assay for MDM2/CEN12 gene amplification.

Tumor activities such as proliferation, invasion, and metastasis are closely associated with angiogenesis; however, the relationship between tumor stem cells and tumor vascular formation remains unclear and necessitates further investigation. In the present study, MCF-7^{CD133+} cells were isolated using immunomagnetic bead separation, and these cells were subjected to endothelial cell-inducing and maintenance media to transform the cells. Subsequently, the MCF-7^{CD133+} subpopulation cells formed luminal-like structures in the Matrigel matrix, akin to those formed by HUVECs (the positive control). Conversely, the MCF-7^{CD133-} cells failed to form similar structures in endothelial cell transformation culture. In the endothelial cell phagocytosis assay, both the transformed MCF-7^{CD133+} cells and HUVECs internalized DiI-Ac-LDL and emitted red fluorescence, corroborating related studies (34,35). FISH was used to confirm that MCF-7^{CD133+} cells retained their tumor cell characteristics following endothelial cell culture conversion. These findings suggest that a distinct subpopulation of cells with stem cell-like properties, capable of differentiating into endothelial cells, exists in breast cancer.

The complex molecular mechanisms driving the differentiation of CSCs into vascular endothelial cells remain elusive. Previous research has shown that in hypoxic conditions, CSCs secrete VEGF, a powerful factor stimulating their transformation into endothelial cells, thus enhancing their propensity to differentiate under oxygen-deprived conditions (36). Alvero *et al* (37) found that CSCs from ovarian cancer possess the ability to differentiate into progenitors of vascular endothelial cells and form vascular-like structures in xenograft tumor inhibition models. In the present study, by enriching the culture medium with stimulatory factors such as bFGF, EGF, BMP4, and VEGF, transformation and maintenance cultivation of MCF-7^{CD133+} and MCF-7^{CD133-} cells was successfully achieved. Subsequent endothelial cell tube formation and phagocytosis experiments suggested that the

transformed MCF-7^{CD133+} cells exhibited endothelial cell functionality. These findings support the notion that CSCs can transform into vascular endothelial cells.

The tumor microenvironment plays a pivotal role in the onset and progression of malignant tumors (38). Modulating the tumor microenvironment can mitigate or inhibit tumor growth (39). Currently, modulating changes in the tumor microenvironment is challenging, but indirectly delaying or suppressing the formation of the tumor microenvironment by adjusting the functions of relevant cells within it, may serve as a treatment method for malignant tumors. Recent research has suggested that targeting tumor vascular endothelial cells is a promising direction for anti-tumor drug development. Anti-angiogenic drugs target various aspects of the angiogenic process. This includes inhibiting growth factors such as VEGF and its receptors (VEGFR), which are key drivers in the formation of new blood vessels. Drugs such as Bevacizumab (Avastin), an antibody that binds to VEGF, prevents it from activating VEGFR on endothelial cells. Tyrosine kinase inhibitors such as Sunitinib target VEGFR directly. The partial failure of anti-VEGF strategies in controlling cancer can be attributed to two major factors. First, the precise molecular mechanisms of cancer neo-angiogenesis are incompletely understood. Additionally, the abrogation of blood supply also restricts drug delivery to the tumor, reducing its effectiveness and promoting drug resistance (40). Correspondingly, a paradox in using anti-angiogenic drugs has emerged from recent findings. By inhibiting new blood vessel formation, anti-angiogenic drugs can increase the level of hypoxia (oxygen deprivation) within the tumor. This hypoxic environment can lead to the selection of more aggressive tumor cells that are better adapted to survive in low oxygen conditions (41). Several studies suggest that anti-angiogenic therapy might stimulate the tumor to become more invasive (42-45). In an attempt to access more blood supply, cancer cells might begin to invade surrounding tissues or spread to other parts of the body (46-51). These suggest that strategies aimed at normalizing tumor vessels, rather than eradicating blood supply, could enhance the delivery of therapeutic agents to cancer cells, thereby improving the efficacy and limiting cancer cell spread (52).

In conclusion, the present study induced the transformation of breast CSCs, and these transformed cells provided a more representative model for studying anti-angiogenesis *in vitro* than HUVECs. This approach addresses the challenges of the low availability and difficulty of separation of tumor vascular endothelial cells *in vivo*. It also lays the groundwork for more comprehensive and targeted research for understanding and combating tumor vascularization.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QQM and XCJ contributed to the conception and design of the research. JNZ, WFT, QQM and SCZ performed the experiments and collected and interpreted the data. The first draft of the manuscript was written by QQM. SCZ and XCJ confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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