JLA Journal of Lipid and Atherosclerosis

Original Article

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Differential Angiogenic Responses of Human Endothelial Colony-Forming Cells to Different Molecular Subtypes of Breast Cancer Cells

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Conflict of Interest
The authors have no conflict of interest to declare.

ABSTRACT

Objective: Triple negative breast cancer (TNBC) is one subtype of breast cancer. It is characterized by lack of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2. Compared with non-TNBC, TNBC is more aggressive, of higher grade, and frequently metastatic with poor prognosis, which is correlated with upregulated microvascular density. Endothelial colony-forming cells (ECFCs) mediate neovascularization, which is the crucial contributor to cancer growth and metastasis. The present study aimed to determine whether angiogenic responses of ECFCs are regulated differently by TNBC compared with non-TNBC.
Methods: MDA-MB-231 and MCF7 cells were utilized for TNBC and non-TNBC, respectively. Bone-marrow-derived human ECFCs were treated with a conditioned medium (CM) of cancer cells to investigate the paracrine effect on angiogenesis. Also, ECFCs were co-cultured with cancer cells to evaluate the angiogenic effect of direct cell-to-cell interaction. Angiogenic responses of ECFCs were evaluated by proliferation, migration, and tube formation. Gene expression profiles of pro-angiogenic factors were also analyzed.
Results: Migration and tube formation of ECFCs were increased by treatment with CM of MDA-MB-231, which correlated with a higher gene expression profile of pro-angiogenic factors in MDA-MB-231 compared to MCF7. Interestingly, ECFCs co-cultured with MDA-MB-231 showed further increase of tube formation, suggesting synergic mechanisms between the paracrine effect and direct interaction between the cells.
Conclusion: The angiogenic potential of ECFCs was enhanced by TNBC through both direct and indirect mechanisms. Therefore, the investigation of signaling pathways to regulate ECFC-mediated angiogenesis will be important to the discovery of anti-angiogenic therapies to treat TNBC patients.

Keywords: Triple negative breast cancer; Endothelial progenitor cells; Angiogenic effect; Conditioned medium; Cell-to-cell interaction

INTRODUCTION

Breast cancer is the most prevalent cancer and one of the leading causes of cancer-related deaths in women worldwide. Breast cancer can be categorized by histopathological type,

grade, tumor stage, and also the expression of receptors. These classification criteria are useful for establishing appropriate treatment strategies. Breast cancer can be divided into 4 different subtypes according to the expression profile of receptors such as the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2). Luminal A breast cancer is ER- and PR-positive, and HER2-negative. Luminal B breast cancer is ER- and/or PR-positive, and HER2-positive. HER2-enriched breast cancer is ER- and PR-negative, and HER2-positive. Triple negative breast cancer (TNBC) is ER-, PR-, and HER2-negative. Among these subtypes, TNBC accounts for about 15%–20% of all breast cancer types, and frequently affects young (less than 50 years old) African-American women.[1] Clinically, TNBC is characterized by aggressive behavior, higher grade (indicating a faster-growing cancer), frequent metastasis, and poor prognosis when compared with other molecular subtypes.[2,3] Due to the lack of receptors, TNBC has no FDA-approved targeted therapies other than conventional chemotherapeutics and radiation therapy.[4] Thus, TNBC has become a major focus of research to discover new therapeutic targets for patients of breast cancer.

Accumulated evidence indicates a critical role of angiogenesis in breast cancer growth and metastasis. Clinical data have demonstrated that higher microvascular density (MVD) is significantly associated with larger tumor size, distant metastasis, and poor prognosis.[5,6] Recent studies have shown that TNBC has higher MVD compared with non-TNBC subtypes.[7,8] Indeed, upregulated MVD is considered to be a direct contributor to the highly aggressive and invasive features of TNBC.[9] Typically, cancer cells secrete various pro-angiogenic factors, including vascular endothelial growth factors (VEGFs), fibroblast growth factor-2 (FGF-2), matrix metalloproteinases (MMPs), interleukins (ILs), transforming growth factor-alpha (TGF-α), TGF-β1, angiopoietin-1 (Ang-1), Ang-2, and platelet-derived growth factors (PDGFs). These factors induce angiogenesis to meet the oxygen and nutrient demands required for growth of cancer cells. Cancer cells also utilize newly formed blood vessels as a route for metastasis.

Angiogenesis is a highly-ordered multi-step process.[10] Pro-angiogenic factors such as VEGF-A, FGF-2, TGF-α, and TGF-β1 bind to their receptors on adjacent endothelial cells (ECs) to activate signal transduction pathways. MMPs released from cancer cells degrade the extracellular matrix, allowing activated ECs to migrate out of the pre-existing vessel wall toward the tumor region. ILs potentiate angiogenesis by stimulating the synthesis of pro-angiogenic factors such as MMPs and VEGFs.[11] Ang-2 binds to Tie-2 receptors on the ECs, resulting in vessel sprouting. Ang-1 and PDGFs promote vessel maturation and pericyte recruitment. During angiogenesis, not only ECs but also bone-marrow-derived circulating endothelial progenitor cells (EPCs) contribute to the formation of blood vessels. Endothelial colony-forming cells (ECFCs) represent one subpopulation of EPCs. ECFCs have been reported to have higher proliferative and greater angiogenic potential than mature ECs.[12] One study reported that 40% of ECs in tumor tissue were derived from ECFCs that originated in the bone marrow,[13] suggesting that ECFCs serve as another important brick during tumor angiogenesis.

Although the interactions between cancer cells and mature ECs have been investigated, few studies have demonstrated a differential response of ECFCs to the different subtypes of breast cancer, especially TNBC versus non-TNBC, in terms of angiogenesis. Thus, the purpose of the present study was to determine whether the angiogenic potential of ECFCs is regulated differently by TNBC in comparison with non-TNBC. The breast cancer cell lines MDA-MB-231 and MCF7 were utilized for TNBC cells and non-TNBC cells, respectively. Bone-marrow-derived ECFCs were isolated from human peripheral blood. ECFCs were treated with a conditioned medium (CM) of breast cancer cells to investigate the paracrine effect

to induce angiogenesis. In another set of experiments, ECFCs were co-cultured with cancer cells to evaluate the effect of direct cell-to-cell interaction. Our results showed a potentiated angiogenic capacity of ECFCs by TNBC, which was correlated closely with elevated gene expression profiling of pro-angiogenic factors produced by TNBC compared to non-TNBC.

MATERIALS AND METHODS

1. Culture of human ECFCs

The study protocol was approved by the Institutional Review Board (IRB) of Duksung Women's University (IRB No. 2017-002-001, 2018-007-006). ECFCs were isolated from the adherent mononuclear cell fraction of human peripheral blood using CD31-coated magnetic beads (Invitrogen, Waltham, MA, USA) as described previously.[14] Isolated ECFCs were expanded on 1% gelatin-coated plates (BD Biosciences, Franklin Lakes, NJ, USA) using EC growth medium MV 2 (EGM-MV 2 without hydrocortisone; Promocell, Heidelberg, Germany) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA) and 1% glutamine-penicillin-streptomycin (GPS; Gibco, Waltham, MA, USA). ECFCs obtained between passages 7 and 10 were used for all experiments.

2. Culture of human breast cancer cells

MDA-MB-231 and MCF7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). MDA-MB-231 cells were cultured in RPMI 1640 medium (Corning, NY, USA) supplemented with 10% FBS and 1% GPS. MCF7 cells were cultured in minimum essential medium eagle (Lonza, Basel, Switzerland) supplemented with 10% FBS, 1% GPS, and 20 μg/mL human insulin (Sigma-Aldrich, St. Louis, MO, USA).

3. Preparation of CM

MDA-MB-231 or MCF7 cells were seeded in a 90×20-mm cell culture dish at $2×10^6$ cells in each growth medium followed by stabilization for 24 hours. Cells were washed with Dulbecco's phosphate buffered saline (DPBS; Corning, NY, USA), and then incubated for 48 hours in EC basal medium MV 2 (EBM-MV 2) supplemented with 2% FBS and 1% GPS. After 48 hours, CM from each type of cancer cells was collected and centrifuged at 2,000 rpm for 10 minutes to clear off cell debris. The resultant supernatant was collected and stored at −80°C until experiments were performed.

4. Proliferation assay

The proliferation of ECFCs treated with/without cancer cell-derived CM was determined by the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). ECFCs were seeded in 96-well plates at $1×10^4$ cells/well in EGM-MV 2 (without hydrocortisone) supplemented with 10% FBS and 1% GPS, and then stabilized for 24 hours. Cells were washed with DPBS followed by incubation with EBM-MV 2 containing 2% FBS and 1% GPS (control medium) or CM with/without dilution. After incubation for 24 hours or 48 hours, control medium or CM was removed and replaced with 100 μL of EBM-MV 2 containing 2% FBS and 1% GPS; 10 μL of CCK-8 reagent was added into each well followed by incubation for 3 hours at 37°C. Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

5. Migration assay

The migratory function of ECFCs was evaluated by the scratch wound migration assay. ECFCs were seeded on 1% gelatin-coated 24-well plates at $30×10^4$ cells/1 mL/well with EGM-MV 2

(without hydrocortisone) supplemented with 10% FBS and 1% GPS. Cells were incubated for 16 hours at 37°C to promote cell attachment. After 16 hours, the medium was changed to EBM-MV 2 (2% FBS and 1% GPS) as the control or CM (2:1 diluted with EBM-MV 2 containing 2% FBS and 1% GPS). After 12 hours of incubation, the ECFC monolayers were scraped to generate scratch wounds. Scratch wounds were made across the middle of the well by a 200-μL pipette tip. Cells were rinsed with EBM-MV 2 (2% FBS and 1% GPS) or CM to remove the detached cell debris, and were pre-incubated for 10 minutes for stabilization. Images were taken using the real-time cell history recorder (JuLI Stage; NanoEnTek, Seoul, Korea) every 30 minutes for 48 hours. The magnitude of migration was evaluated by measuring the area covered by migrated cells using JuLI STAT software (NanoEnTek).

6. Tube formation assay

The capability of ECFCs to form tubule-like structures was determined using a tube formation assay with modifications of previous procedures.[15] The 24-well plates were coated with ice-cold Matrigel solution (Phenol Red-Free; BD Biosciences) and incubated at 37°C for 30 minutes to allow the Matrigel to solidify. ECFCs were harvested, suspended in EBM-MV 2 (2% FBS and 1% GPS) or CM (1:1 dilution with EBM-MV 2 containing 2% FBS and 1% GPS), and added to the Matrigel-coated 24-well plates at 15×10^4 cells/well. For the co-culture experiment, ECFCs were mixed with MDA-MB-231 or MCF7 in a ratio of 3:2 and added to the Matrigel-coated 24-well plates at 15×10^4 cells/well. Cells were pre-incubated for 30 minutes at 37°C to promote cell attachment. Sequential images of tube formation were taken using a real-time cell recorder (JuLI Stage; NanoEntek) every 1 hour over 48 hours. Four contiguous image positions of the 4× object lens were identified using the software, and the real-time cell recorder then acquired images automatically at the same positions every hour. Image-stitching software was used to obtain a larger observation area. The 2×2 stitched area from the 4× object lens was used to quantify tube number and length using the angiogenesis analyzer of Image J software (NIH, Bethesda, MD, USA).

7. RNA preparation and RNA sequence analysis

Total RNA from MDA-MB-231 or MCF7 cells was isolated using TRIzol reagent (Invitrogen). For control and test RNAs, a library was constructed using QuantSeq 3′mRNA-Seq Library Prep Kit (Lexogen, Inc., Vienna, Austria) according to the manufacturer's instructions. In brief, 500 ng of RNA were prepared and an oligo-dT primer containing an Illumina-compatible sequence at its 5′ end was hybridized to the RNA and reverse transcription was performed. After degradation of the RNA template, second-strand synthesis was initiated by a random primer containing an Illumina-compatible linker sequence at its 5′ end. The double-stranded library was purified by using magnetic beads to remove all reaction components. The library was amplified to add the complete adapter sequences required for cluster generation. The finished library was purified from PCR components. High-throughput sequencing was performed as single-end 75 sequencing using NextSeq 500 (Illumina, Inc., San Diego, CA, USA).

8. Statistical analysis

All values are expressed as the mean±standard error of mean of at least 3 independent experiments. The data were analyzed using analysis of variance followed by Fisher's least significant difference *post hoc* test for multiple comparisons or the Student's *t*-test for paired comparisons using Origin software (OriginLab, Northampton, MA, USA). A value of *p*≤0.05 was considered statistically significant.

RESULTS

1. ECFC proliferation after treatment with breast cancer cell-derived CM

The steps of angiogenesis include EC activation, sprouting, proliferation, migration, tube formation, vessel fusion and pruning, and maturation by pericyte recruitment. ECFCs, circulating endothelial progenitors, are also known to contribute to tumor angiogenesis.[16] Thus, we performed experiments to determine whether 2 different molecular subtypes of breast cancer cells affect ECFC proliferation similarly or not. MDA-MB-231 and MCF7 cell lines were used for TNBC cells and non-TNBC cells (luminal A type), respectively. ECFCs were treated with CM of MDA-MB-231 or MCF7, and proliferation was analyzed at 24 hours and 48 hours. Interestingly, ECFC proliferations were not different between CMs of MDA-MB-231 and MCF7 (**Fig. 1A and B**). This may be due to the robust proliferation capacity of stem-like ECFCs regardless of exogenous stimulation. Unexpectedly, undiluted CMs attenuated ECFC proliferation at both 24 hours and 48 hours. It has been reported that cancer cells-derived inflammatory cytokines can inhibit proliferation of ECs. IL-1β, interferon-γ (IFN-γ), and TNF-α have been considered to possess an anti-proliferative effect. For example, a combination of IFN-γ and TNF-α markedly suppress EC proliferation, which appeared to be due to a cytotoxic effect on ECs.[17] Thus, attenuation of ECFC proliferation might be due to a cytotoxic effect of high concentration of inflammatory cytokines within the undiluted CMs.

2. ECFC migration after treatment with breast cancer cell-derived CM

EC migration is essential to the angiogenic process. Activated ECs migrate toward a gradient of soluble chemoattractant such as VEGF-A and FGF-2. The migratory function of circulating ECFCs has also been reported to play a critical role during neovascularization in many cancers.[18] To compare the effect of TNBC- and non-TNBC-derived secretory factors on ECFC migration, ECFCs were treated with CM of MDA-MB-231 or MCF7, and the magnitude of migration was evaluated by measuring the area covered by migrated ECFCs every 30 minutes for 48 hours using a real-time cell history recorder. As shown in **Fig. 2A and B**, CM of MDA-MB-231 enhanced ECFC migration significantly compared to control medium, whereas CM of MCF7 reduced ECFC migration significantly compared to control medium, suggesting that the secretory factors from only MDA-MB-231 enhance ECFC migration.
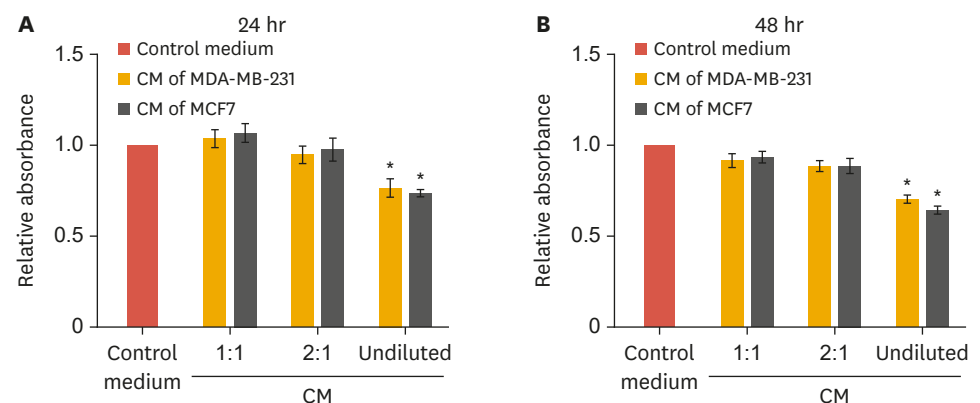
Fig. 1. Proliferation of human ECFCs after treatment with CM derived from MDA-MB-231 or MCF7. (A) Bar graph of relative proliferation rate of ECFCs treated with CM derived from MDA-MB-231 or MCF7 after 24 hours. (B) Bar graph of relative proliferation rate of ECFCs treated with CM derived from MDA-MB-231 or MCF7 after 48 hours. Each bar represents the mean±standard error of mean (n=4).
CM, conditioned medium; ECFC, endothelial colony-forming cell.
*Indicates significant difference from control ($p<0.05$).
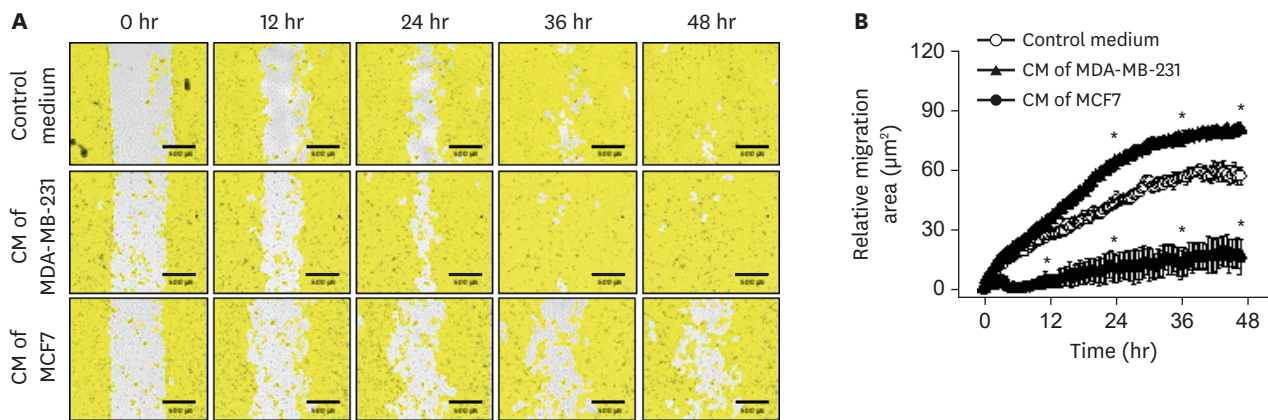
Fig. 2. Migration of human ECFCs after treatment with CM derived from MDA-MB-231 or MCF7. (A) Representative images of the area covered by migrated ECFCs (yellow color analyzed by JuLI STAT software) treated with CM derived from MDA-MB-231 or MCF7 over 48 hours (scale bar=500 μm). (B) Graph of the migration area at each time point over 48 hours. Each point represents the mean±standard error of mean (n=3).
CM, conditioned medium; ECFC, endothelial colony-forming cell.
*Indicates significant difference from control ($p < 0.05$).

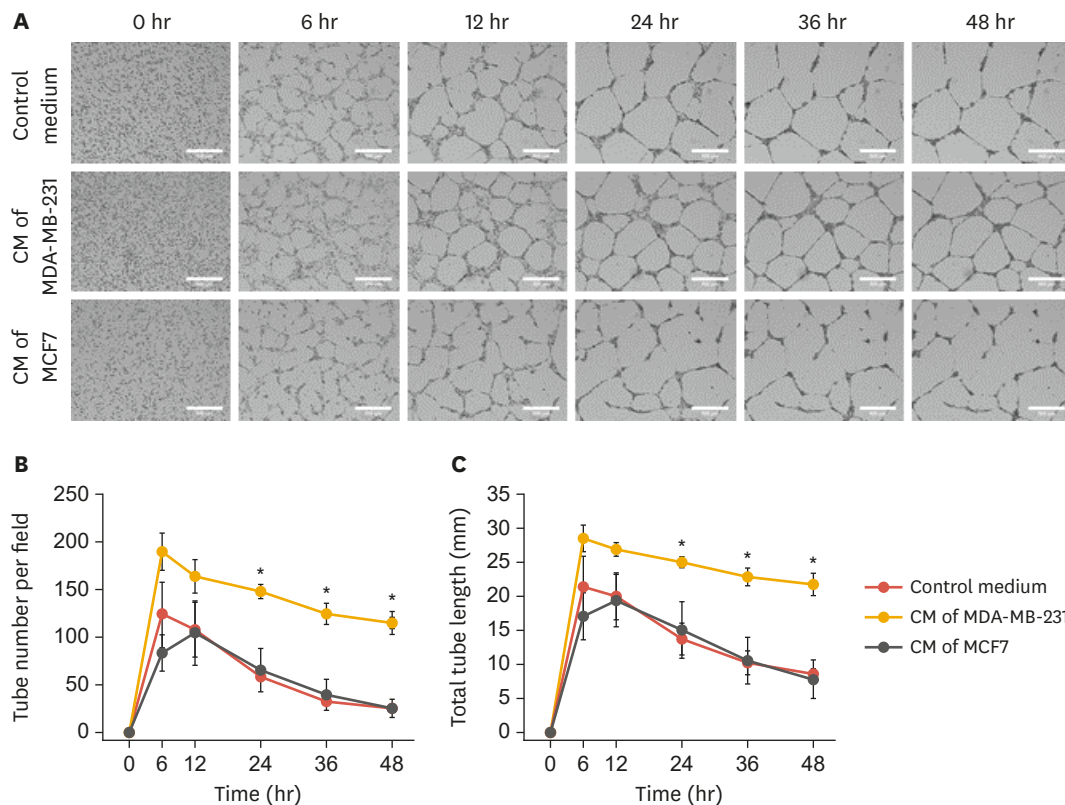### 3. ECFC tube formation after treatment with breast cancer cell-derived CM

ECFCs are recruited from peripheral circulation into the tumor region, migrate to the sites requiring new vessel formation, incorporate into the intima, and finally participate in the formation of tubular structures. To measure tube formation of ECFCs treated with each CM, we used a real-time cell history recorder to take sequential images at the same position every 1 hour for 48 hours. Video clips made by these sequential images demonstrated the dynamic progression of tube formation from a single-cell suspension in which tubular structures are formed, elongated, and degraded in a time-dependent manner (**Supplementary Video 1**). Treatment with CM of MDA-MB-231 significantly increased the number and length of tubes formed by ECFCs compared with control medium and CM of MCF (**Fig. 3**). This result suggests that pro-angiogenic factors secreted from MDA-MB-231 potentiated ECFC angiogenesis more than pro-angiogenic factors secreted from MCF7.

4. ECFC tube formation by co-culture with breast cancer cells

To determine if direct cell-to-cell interactions between cancer cells and ECFCs alter tube formation, ECFCs were co-cultured with MDA-MB-231 or MCF7 to assess tube formation. Both number and length of tubes formed by ECFCs co-cultured with MDA-MB-231 were greater than either ECFCs only (control) or ECFCs co-cultured with MCF7 (**Fig. 4**, **Supplementary Video 2**). Interestingly, ECFCs co-cultured with MDA-MB-231 induced further increases in both number and length of tubes when compared with ECFCs treated with CM of MDA-MB-231 (**Fig. 3B and C**, **Fig. 4B and C**), which suggests synergic effects between paracrine action and direct interaction between MDA-MB-231 and ECFCs. However, ECFCs co-cultured with MCF7 did not induce tube formation (**Fig. 4**). This result may be due to the low angiogenic and migratory capacity of MCF7 in *in vitro* culture conditions.[19]

5. Gene expression level of pro-angiogenic factors in breast cancer cells

During the angiogenesis process, ECFCs were stimulated to form new blood vessels by many pro-angiogenic factors released from cancer cells. Total RNA from MDA-MB-231 or MCF7 cells was isolated to evaluate gene expression levels of pro-angiogenic factors. As shown in **Fig. 5**, the most important pro-angiogenic factors, such as the VEGF family, were highly expressed in MDA-MB-231 compared to MCF7, which correlates well with increased tube formation of ECFCs treated with CM of MDA-MB-231 as well as co-cultured with MDA-MB-231.
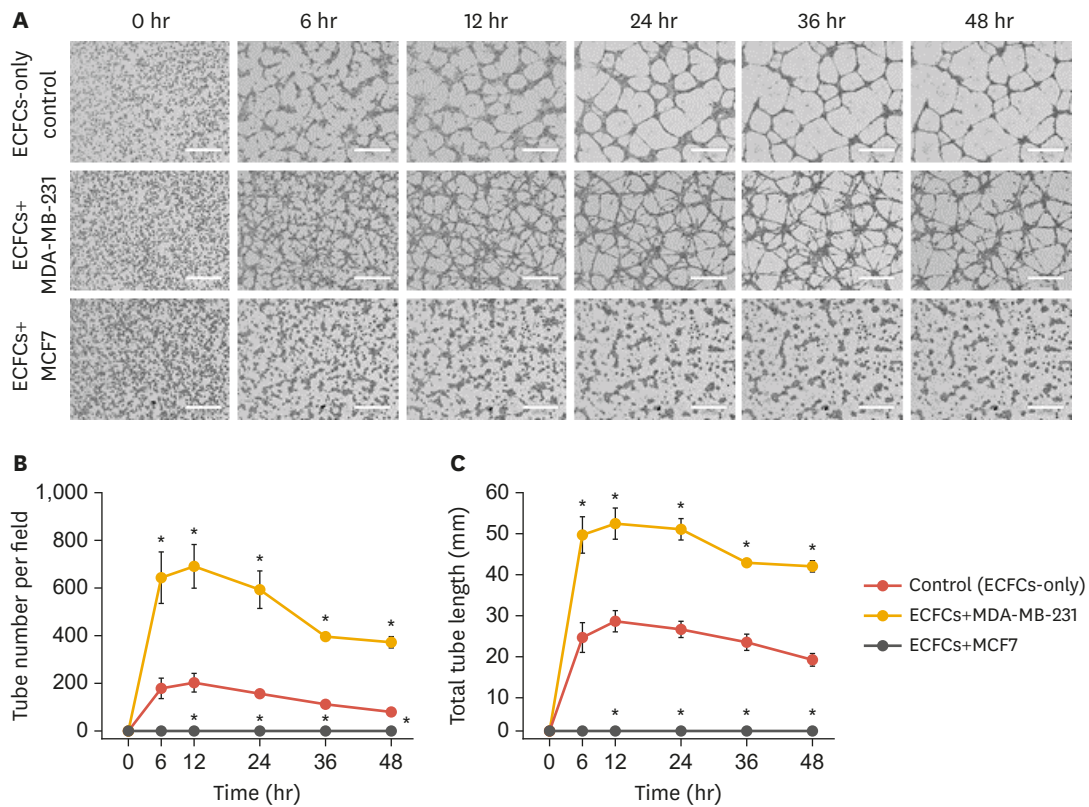
Fig. 3. Tube formation of human ECFCs after treatment with CM derived from MDA-MB-231 or MCF7. (A) Representative images of the tubular structures formed by ECFCs treated with CM derived from MDA-MB-231 or MCF7 over 48 hours (scale bar=500 μm). (B) Graph of the tube number at each time point over 48 hours. (C) Graph of total tube length at each time point over 48 hours. Each point represents the mean±standard error of mean (n=4).
CM, conditioned medium; ECFC, endothelial colony-forming cell.
*Indicates significant difference from control ($p < 0.05$).

DISCUSSION

Angiogenesis is a required process for tumor growth and progression because the blood vessels that develop deliver nutrients and oxygen to cancer cells. Newly formed tumor vasculature also serves as an invasion route from the primary organ to other organs for metastasis. ECFCs are bone-marrow-derived endothelial progenitors that circulate in the peripheral blood vessels and home in to the tumor site to participate in new blood vessel formation. Breast cancer can be divided into several molecular subtypes, which reflect differential biological activity, clinical behavior, and response to treatment. Among these subtypes, TNBC is characterized clinically by high proliferation, more aggressiveness, and frequent metastasis with a reduced survival rate, whereas non-TNBC (luminal A) is less aggressive, non-invasive, and normally has low metastatic potential.[20] Thus, in the present study, we investigated the differential effect of TNBC and non-TNBC on ECFC-mediated angiogenesis.

ECFC-mediated angiogenesis is a complex process, including mobilization from the bone marrow into the peripheral circulation, recruitment, adhesion, and migration to the sites requiring neovascularization, incorporation into the intima, and participation in the assembly of newly forming blood vessels. The potential of MDA-MB-231 (TNBC cells) and MCF7 (luminal A cells) to induce ECFC-mediated angiogenesis was evaluated by several methods to measure proliferation, migration, and tube formation. We found that treatment with MDA-MB-231-

Fig. 4. Tube formation of human ECFCs after co-culture with MDA-MB-231 or MCF7. (A) Representative images of the tubular structures formed by ECFCs co-cultured with MDA-MB-231 or MCF7 over 48 hours (scale bar=500 μm). (B) Graph of the tube number at each time point over 48 hours. (C) Graph of total tube length at each time point over 48 hours. Each point represents the mean±standard error of mean (n=2–4).
ECFC, endothelial colony-forming cell.
*Indicates significant difference from control ($p<0.05$).
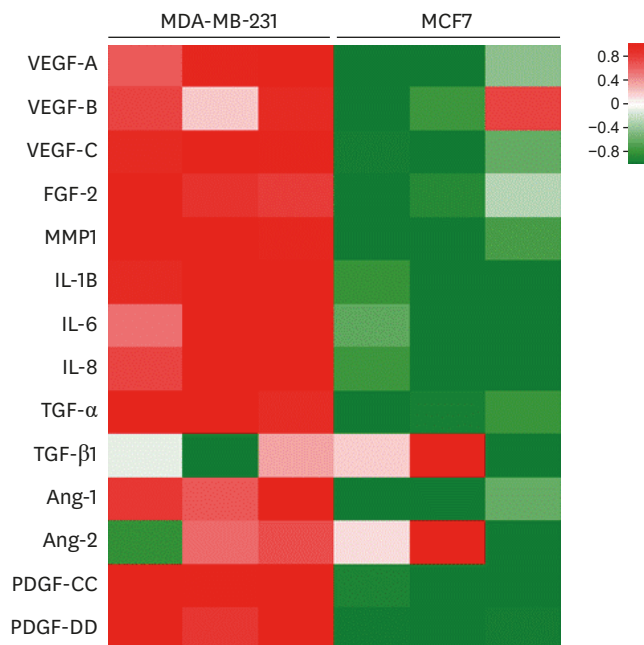
Fig. 5. Comparison of gene expression level of pro-angiogenic factors between MDA-MB-231 and MCF7. Gene clustering was analyzed using MeV 4.9.0. Hierarchical cluster analyses were carried out with Euclidean distance correlation as the distance measurement with average linkage. Clusters and heat maps were visualized via MeV 4.9.0.
VEGF, vascular endothelial growth factor; FGF-2, fibroblast growth factor-2; MMP-1, matrix metalloproteinase-1, IL, interleukin; TGF, transforming growth factor; Ang, angiopoietin; PDGF, platelet-derived growth factor.

derived CM increased migration (**Fig. 2**) and tube formation of ECFCs (**Fig. 3**) compared to MCF7-derived CM, which correlates well with the increased gene expression level of pro-angiogenic factors in MDA-MB-231 (**Fig. 5**), whereas ECFC proliferation was not increased by treatment with both CMs. This may be due to the characteristics of ECFCs, especially fast growth capacity. ECFCs are considered to be stem/progenitor cells derived from bone marrow that give rise to mature ECs. In 1997, Asahara et al.[21,22] first reported the existence of circulating EPCs that contribute to various types of postnatal pathophysiological angiogenesis, such as wound healing, myocardial ischemia, limb ischemia, ischemic stroke, and cancer. EPCs were then studied extensively regarding their origin, character, and function in angiogenesis. Accumulating data has confirmed 2 main subpopulations of EPCs. Early EPCs have features of hematopoietic cells, and play a role in angiogenesis by secreting large amounts of pro-angiogenic factors.[23] Late EPCs, which are referred to as ECFCs, participate in the process of angiogenesis by providing structural support via differentiation into mature ECs.[24] ECFCs are reported to maintain robust proliferation and differentiation capacity compared with mature ECs.[12] Thus, exogenous stimulation by treatment with CM of cancer cells may not affect the proliferation rate of ECFCs.

Folkman[25] revealed that both direct and indirect interaction between cancer cells and ECs could influence the growth and progression of tumors through stimulating tumor angiogenesis. To determine whether direct interaction with breast cancer cells potentiates ECFC-mediated angiogenesis, ECFCs were co-cultured directly with MDA-MB-231 or MCF7 to assess tube formation. Tube formation of ECFCs was enhanced noticeably by co-culture with MDA-MB-231 compared to ECFCs alone (control, **Fig. 4**). Interestingly, co-culture with MDA-MB-231 induced ECFC tube formation more than treatment with CM of MDA-MB-231 (**Figs. 3** and **4**). Furthermore, ECFC tube formation was increased significantly at earlier time points (6 hours and 12 hours) when co-cultured with MDA-MB-231, whereas ECFC tube formation was not increased significantly at the same time points when treated with CM of MDA-MB-231. Our results suggest synergic effects between paracrine action and direct interaction between MDA-MB-231 and ECFCs. Previous work indicated that the complex cell-to-cell interaction between cancer cells and ECs causes modifications in gene expression levels of ECs and their activation to promote angiogenesis.[26] Adhesion receptors are also essential not only for the adhesive process but also for the direct interaction between cancer cells and ECs. Chen et al.[27] previously demonstrated that JAGGED1 transmembrane protein expressed in breast cancer cells can activate the NOTCH receptor in ECs and trigger an angiogenic cascade. Thus, ECFCs can be activated more by both indirect and direct interaction when co-cultured with MDA-MB-231. In contrast, tube formation was reduced significantly in ECFCs co-cultured with MCF7 compared with ECFCs only (control). This result may be due to the less amount of pro-angiogenic factors within CM of MCF7 compared to MDA-MB-231. MCF7 cells was found to express lower levels of mRNA for VEGFs than MDA-MB-231 in our result (**Fig. 5**) as well as previous studies.[28] Indeed, MCF7 have shown a poor angiogenic potential and less tumorigenicity.[29] Furthermore, MCF7 cells form compact colonies with typical epithelial polygonal shape in close contact with each other,[30] and do not usually migrate and invade,[20] which characters may reduce cell-to-cell interaction between MCF7 and neighboring ECFCs under the co-culture condition.

In conclusion, angiogenesis is a direct contributor to the highly aggressive and invasive features of TNBC and its poor prognosis.[9] ECFCs are bone marrow-derived stem cells that contribute to new blood vessel formation. ECFC-mediated angiogenesis was enhanced by TNBC through both direct and indirect mechanisms. Therefore, the investigation of

a signaling pathway to regulate ECFC-mediated angiogenesis will be important to the discovery of anti-angiogenic therapies to treat TNBC patients.

SUPPLEMENTARY MATERIALS

Supplementary Video 1

Video clip of dynamic progression of tube formation from human ECFCs with treatment of (A) control medium, (B) CM of MDA-MB-231, and (C) CM of MCF7. Video clip was generated with 1-hour interval images taken by real-time cell history recorder (NanoEnTek, Seoul, Korea) for 48 hours (scale bar=500 μm).

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Supplementary Video 2

Video clip of dynamic progression of tube formation from human ECFCs in co-culture with (A) none (=ECFCs-only control), (B) MDA-MB-231, (C) MCF7. Video clip was generated with 1-hour interval images taken by real-time cell history recorder (NanoEnTek, Seoul, Korea) for 48 hours (scale bar=500 μm).

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