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TITLE:

Three-Dimensional Angiogenesis Assay System Using Co-Culture Spheroids Formed by Endothelial Colony Forming Cells and Mesenchymal Stem Cells

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angiogenesis, co-culture spheroid, endothelial colony forming cells, mesenchymal stem cells, type I collagen gel, sprouting, bevacizumab

SUMMARY:

Three-dimensional co-culture spheroid angiogenesis assay system is designed to mimic the physiologic angiogenesis. Co-culture spheroids are formed by two human vascular cell precursors, ECFCs and MSCs, and embedded in collagen gel. The new system is effective for evaluating angiogenic modulators, and provides more relevant information to the in vivo study.

ABSTRACT:

Studies in the field of angiogenesis have been aggressively growing in the last few decades with the recognition that angiogenesis is a hallmark of more than 50 different pathological conditions, such as rheumatoid arthritis, oculopathy, cardiovascular diseases, and tumor metastasis. During angiogenesis drug development, it is crucial to use in vitro assay systems with appropriate cell types and proper conditions to reflect the physiologic angiogenesis process. To overcome limitations of current in vitro angiogenesis assay systems using mainly endothelial cells, we

developed a 3-dimensional (3D) co-culture spheroid sprouting assay system. Co-culture spheroids were produced by two human vascular cell precursors, endothelial colony forming cells (ECFCs) and mesenchymal stem cells (MSCs) with a ratio of 5 to 1. ECFCs+MSCs spheroids were embedded into type I collagen matrix to mimic the in vivo extracellular environment. A real-time cell recorder was utilized to continuously observe the progression of angiogenic sprouting from spheroids for 24 h. Live cell fluorescent labeling technique was also applied to tract the localization of each cell type during sprout formation. Angiogenic potential was quantified by counting the number of sprouts and measuring the cumulative length of sprouts generated from the individual spheroids. Five randomly-selected spheroids were analyzed per experimental group. Comparison experiments demonstrated that ECFCs+MSCs spheroids showed greater sprout number and cumulative sprout length compared with ECFCs-only spheroids. Bevacizumab, an FDA-approved angiogenesis inhibitor, was tested with the newly-developed co-culture spheroid assay system to verify its potential to screen anti-angiogenic drugs. The IC_{50} value for ECFCs+MSCs spheroids compared to the ECFCs-only spheroids was closer to the effective plasma concentration of bevacizumab obtained from the xenograft tumor mouse model. The present study suggests that the 3D ECFCs+MSCs spheroid angiogenesis assay system is relevant to physiologic angiogenesis, and can predict an effective plasma concentration in advance of animal experiments.

INTRODUCTION

Approximately 500 million people worldwide are expected to benefit from angiogenesis-modulating therapy for vascular malformation-associated diseases such as rheumatoid arthritis, oculopathy, cardiovascular diseases, and tumor metastasis[1]. Thus, the development of drugs that control angiogenesis has become an important research area in the pharmaceutical industry. During the drug development process, in vivo animal study is necessary to explore the effects of drug candidates on physiologic functions and systemic interactions between organs. However, ethical and cost issues have increased the concerns regarding animal experiments[2]. Therefore, improved in vitro assay systems are needed to obtain more accurate and predictable data leading to the better decision-making before animal experiments. Current in vitro angiogenesis assays usually measure proliferation, invasion, migration, or tubular structure formation of endothelial cells (ECs) seeded in two-dimensional (2D) culture plates[3]. These 2D angiogenesis assays are quick, simple, quantitative, and cost-effective, and have significantly contributed to the discovery of angiogenesis-modulating drugs. However, several issues remain to be improved.

Such 2D in vitro assay systems cannot reflect complex multi-step events of angiogenesis that occurs in in vivo physiologic conditions, leading to inaccurate results that cause discrepancies between in vitro assay data and clinical trial outcomes[4]. 2D culture conditions also induce the change of cellular phenotypes. For example, after proliferation in 2D culture plates, ECs have a weak cellular phenotype as manifested by reduced expression of CD34 and several signals that govern cellular responses[5,6]. To overcome the limitations of 2D culture-based angiogenesis assay systems, three-dimensional (3D) spheroid angiogenesis assay systems have been developed. Sprouting followed by tubular structure formation from spheroids formed by ECs reflect in vivo neo-vascularization processes[7,8]. Thus, the 3D spheroid angiogenesis assay has been considered an effective assay system for screening potential pro- or anti-angiogenesis drugs.

Most 3D spheroid angiogenesis assays utilize only ECs, mainly human umbilical vein endothelial cells (HUVECs) or human dermal microvascular endothelial cells (HDMECs) to focus on the cellular response of ECs during angiogenesis. However, blood capillaries are composed of two cell types: ECs and pericytes. Elaborating bi-directional interaction between ECs and pericytes is critical for proper vascular integrity and function. Several diseases, such as hereditary stroke, diabetic retinopathy, and venous malformation, are associated with altered pericyte density or decreased pericyte attachment to the endothelium[9]. Pericytes are also known as a key element of the angiogenic process. Pericytes are recruited to stabilize newly-formed vessel structures by ECs. In this regard, mono-culture spheroid angiogenesis assay does not incorporate pericytes[7,10]. Therefore, co-culture spheroids formed by ECs and pericytes may provide a valuable approach to more closely mimic physiologic angiogenic events.

The present study aimed to develop a 3D co-culture spheroid angiogenesis assay with a combination of human endothelial colony forming cells (ECFCs) and mesenchymal stem cells (MSCs) to more closely reflect in vivo angiogenesis. Co-culture spheroid system as an in vitro representation assembly of a normal blood vessel was first established by Korff et al. in 2001[11]. They combined HUVECs and human umbilical artery smooth muscle cells (HUSMCs), and demonstrated that co-culture of two mature vascular cells decreased the sprouting potential. Mature ECs (HUVECs) are known to progressively lose their ability to proliferate and differentiate, which negatively affects their angiogenesis responses[12,13]. Mature perivascular cells (HUSMCs) can cause endothelial cell inactivation through the abrogation of the vascular endothelial growth factor (VEGF) responsiveness[11]. The main difference between Korff's and our co-culture spheroid system is the cell types used. We applied two vascular precursors, ECFCs and MSCs, to establish a proper angiogenesis assay system to screen and investigate pro-or-anti-angiogenic agents. ECFCs are the precursor of ECs. ECFCs have robust proliferation capacity compared with mature ECs[14], which enable to overcome the limitation of ECs. ECFCs contribute to new vessel formation in many post-natal pathophysiologic conditions[15-17]. MSCs are pluripotent stem cells that have the capacity to differentiate into pericytes, thereby contributing to angiogenesis[18,19].

In previous reports, ECFCs and MSCs showed synergistic effects on in vitro tube formation[20], in vivo neo-vascularization[21,22], and improved reperfusion of ischemic tissues[23,24]. In the present study, ECFCs and MSCs were used to form co-culture spheroids and embedded in type I collagen gel to reflect an in vivo 3D environment. Collagen is considered as a major constituents of the extracellular matrix (ECM) surrounding ECs[25]. The ECM plays a critical role in regulating cell behavior[26]. The assay protocol proposed here can be easily and quickly carried out within two days using common laboratory techniques. For effective cell tracking during the sprouting process, each cell type can be fluorescently labeled and monitored using a real-time cell recorder. The newly-established 3D co-culture spheroid angiogenesis assay system is designed to increase sensitivity for evaluating potential angiogenic modulators and to provide predictable information in advance of in vivo study.

PROTOCOL

Human ECFCs were isolated from human peripheral blood as described in a previous report[27].

Briefly, the mononuclear cell layer was separated from the whole blood using the Ficoll-Paque Plus, and cultured in the proper medium until the endothelial-like colonies were appeared. Colonies were collected and ECFCs were isolated using CD31-coated magnetic beads. MSCs were isolated from the adherent mononuclear cell (MNC) fraction of human adult bone marrow. The study protocol was approved by the institutional review board of Duksung Women's University (IRB No. 2017-002-01).

1. Cell culture

1.1. Preparing ECFCs and MSCs medium and coat plates

1.1.1. Prepare endothelial cell growth medium MV2 (ECGM-MV2, except hydrocortisone) supplemented with 10% fetal bovine serum (FBS) and 1% glutamine-penicillin-streptomycin (GPS).

1.1.2. Prepare mesenchymal stem cell growth medium-2 (MSCGM-2) containing 10% FBS and 1% GPS.

1.1.3. For ECFC culture, coat the cell culture plates with 1% gelatin solution. To prepare 1% gelatin solution, dissolve 1 g of gelation powder in 500 mL of PBS with the magnetic stirrer, and sterilize with autoclave. Plates can be coated with 3 mL/60 mm, 5 mL/100 mm, or 15 mL/150 mm of 1% gelatin solution. Incubate coated plates for 15 min in a cell culture incubator (37 °C and 5% CO_2). After then, remove the remaining 1% gelatin solution by aspiration and wash the coated plates one time with PBS.

1.2. Growing ECFCs and MSCs

1.2.1. Seed $1x10^6$ ECFCs on 1% gelatin-coated 150 mm plates, and grow using ECGM-MV2 (10% FBS, 1% GPS) in a cell culture incubator (37°C and 5% CO_2) to 80-90% confluency. Use ECFCs at passage numbers 7-10 to obtain consistent results.

1.2.2. Seed $1x10^6$ MSCs on non-coated 150 mm plates, and grow using MSCGM-2 in a cell culture incubator (37°C and 5% CO_2) to 80-90% confluency. Use MSCs at passage numbers 7-10 to obtain consistent results.

2. Preparation of 1.2% w/v methylcellulose solution

2.1. Measure 6 g of methyl cellulose in a 500 mL glass bottle and sterilize in an autoclave.

2.2. Heat ECGM-MV2 (without FBS and GPS) at 60 °C for 20 min, and add 250 mL of heated ECGM-MV2 to sterilized methyl cellulose powder. To maintain sterile conditions, carry out the process inside the flow hood.

2.3. Add a sterilized magnetic stir bar and mix the solution for 20 min on the magnetic stirrer

until the methyl cellulose is thoroughly wetted and evenly dispersed with no lumps. Add 250 mL of cold (4 °C) ECGM-MV2 (without FBS and GPS) under the sterile condition and mix for an additional 10 min on the magnetic stirrer. Then, chill the solution in a refrigerator (4 °C) overnight.

NOTE: Methylcellulose is a carbohydrate polymer that dissolve well in cool temperatures due to swelling and subsequent hydration.

2.4. The next day, aliquot the solution in a 50 mL tube and centrifuge at 5000 x *g* for 3 h at 4 °C. Take the clear viscous supernatant solution and store at 4 °C until use for up to 3-6 months.

NOTE: The sediment may contain residual cellulose fiber, so gently take the supernatant solution leaving behind at least 5 mL of volume. This volume can be adjusted according to experimental conditions.

3. Generation and embedding of ECFCs-only, MSCs-only, and ECFCs-MSCs spheroids

Day 1

3.1. Preparing ECFCs and MSCs cell suspension

3.1.1. Wash the 80-90% confluent ECFCs and MSCs by adding phosphate buffered saline (PBS) without calcium and magnesium, and aspirate.

3.1.2. To detach ECFCs and MSCs from the culture plate, incubate them with 0.05% trypsin-EDTA for 3 min and 5 min in a cell culture incubator (37°C and 5% CO_2), respectively, in a cell culture incubator.

3.1.3. Inactivate trypsin by adding DMEM medium containing 10% FBS and 1% GPS. Pipette the cells up and down to create a single cell suspension.

3.1.4. Sediment the cells by centrifuging at 282 x *g* for 5 min at RT.

3.1.5. Remove the supernatant, and re-suspend the cells in the respective medium.

3.2. Labeling ECFCs and MSCs with fluorescent dye

NOTE: Perform cell membrane labelling of ECFCs with PKH67 (green) and MSCs with PKH26 (red) fluorescent dye following the manufacturer's instructions with slightly modifications as follows.

3.2.1. Wash the cells two times with serum free medium to remove FBS.

NOTE: Proteins and lipids in FBS reduces the effective dye concentration for labelling cells by binding.

3.2.2. Count ECFCs and MSCs using a hemocytometer under the microscope, and transfer 3 x 10^6 ECFCs and 2 x 10^6 MSCs into 2 mL micro-tubes.

NOTE: These are the optimum cell densities for labelling cells with dyes. Use of a large number of cells causes poor and heterogeneous staining, whereas use of too few cells yields poor cells recovery.

3.2.3. Centrifuge the tubes at 100 x *g* for 5 min to obtain a cell pellet. Carefully aspirate the supernatant leaving no more than 15-25 µL of residual volume.

3.2.4. Re-suspend ECFCs and MSCs in 250 µL of Diluent C from the dye kit. Gently pipette the cell suspension to ensure complete dispersion.

NOTE: Diluent C is a physiologic salt, and may reduce the staining efficiency of dye by forming micelles. Therefore, do not let cells stand in diluent C for long, and do not vortex the tubes.

3.2.5. Prepare the staining solution for ECFCs by adding 5 µL of PKH67 to 250 µL of Diluent C (final concentration of 20 µM) and for MSCs by adding 3 µL of PKH26 to 250 µL of Diluent C (final concentration of 12 µM).

NOTE: The final dye concentrations are different from the manufacturer' recommendations. High concentration would lead to cell clumping and cell toxicity. Low concentration would not be sufficient for staining.

3.2.6. Add 250 µL of ECFCs cell suspension to 250 µL of PKH67 dye solution, and 250 µL of MSCs to 250 µL of PKH26 dye solution. Immediately mix the cells with dyes by pipetting up and down and incubate for 5 min at room temperature. For better results, cover the tube with aluminum foil and place on a shaker to ensure sufficient mixing during staining.

3.2.7. Stop the staining process by adding 0.5 mL of FBS to the stained cell suspension, and then incubate for 1 min to allow FBS to bind excess unbound dye. Sediment the stained cells by centrifuging at 100 x *g* for 5 min.

NOTE: ECFCs and MSCs pellets are light yellow and pink, respectively, after staining.

3.2.8. Carefully remove the supernatant and wash the cells two times with complete medium to remove excess dye. Centrifuge the tubes at 100 x *g* for 5 min and re-suspend the pellet in 2 mL of each complete medium.

3.3. **Generating ECFC, MSC, or ECFC+MSC spheroids**

NOTE: Apply the hanging drop technique to generate spheroids as described previously[28]. The steps for the preparation of spheroids are given below.

3.3.1. Count the stained ECFCs and MSCs.

3.3.2. Suspend stained ECFCs, MSCs or ECFCs+MSCs in ECGM-MV2 containing 20% Methylcellulose solution and 5% FBS. Prepare at a cell density of 4 x 10^4 cells/mL for ECFCs or MSCs (25 µL of cell suspension contains 1,000 cells). For two cell suspension of ECFCs and MSCs, prepare at a cell density of 2 x 10^4 cells/mL ECFCs and 0.4 x 10^4 cell/mL MSCs (25 µL contains 500 ECFCs and 100 MSCs).

NOTE: Ratio of ECFCs to MSCs should not exceed 5:1. A larger number of MSCs could lead to excessive migration and irregular sprout morphology. A smaller number of MSCs could cause poor interaction between cells, yielding poor sprouting.

3.3.3. Prepare a hydration unit by adding 15 mL of PBS into the bottom of a 150 mm culture plate.

3.3.4. Transfer the cell suspension into the sterilized polystyrene rectangular reservoir for the use of multichannel pipette. Disperse the cells suspension evenly by pipetting.

3.3.5. Deposit 25 µL drops of cell suspension onto the cover of a 150 mm culture plate using a multi-channel pipette (approximately 100 drops/cover of the 150 mm plate). Invert the cover over a PBS-filled hydration unit and incubate in a cell culture incubator for 24 h.

NOTE: Methylcellulose solution provides a proper viscosity to the suspension solution. When the hanging drop method was performed without methylcellulose solution, drops were easily slipped down when the cover was inverted (**Supplemental Figure 1**). Furthermore, after overnight incubation, the spheroids were not perfectly formed without methylcellulose solution. This result indicate that proper viscosity by methylcellulose solution is necessary to form round shape of the spheroids.

Day 2

3.4. **Embeding spheroids in neutralized collagen gel**

3.4.1. Warm FBS and ECGM-MV2 (without FBS and GPS) in a water bath (37 °C). Place cold methylcellulose solution on a work bench to bring to room temperature.

3.4.2. Place a 24-well plate in a cell culture incubator (37 °C) for warming.

3.4.3. Cut the pointed 1 mL pipette tips 3-5 mm to aspirate spheroids and viscous suspensions, such as methylcellulose solution and type I collagen, comfortably and accurately.

3.4.4. Prepare 3 mg/mL neutralized type I collagen gel on ice following the type I collagen gel manufacturer's instruction.

NOTE: Neutralization should be performed on ice to avoid gelation of collagen at room temperature. For one well of a 24-well plate, 500 µL of neutralized collagen is needed. Always prepare 1 mL extra volume of collagen. Collagen gel can be used up to 2-3 h after neutralization if kept on ice.

3.4.5. Harvest the spheroids by rinsing the cover containing spheroids with 5 mL of PBS. Collect spheroid-suspended solution in a 50 mL conical tube. Re-rinse the cover with 5 mL of PBS to obtain the remaining spheroids.

NOTE: During harvest, closely observe to confirm the existence of spheroids. For more visual inspection, transfer about 50-100 µL of spheroid-suspended solution on the slide glass, and check the round shape of the spheroids under the microscope.

3.4.6. Sediment the spheroids by centrifuging at 282 x *g* for 5 min. Discard the supernatant carefully without disturbing the spheroids leaving behind not more than 100-200 µL of residual volume.

3.4.7. Gently tap on the wall of the tube so that spheroids are freely suspended in the remaining 100-200 µL residual solution.

3.4.8. Add ECGM-MV2 containing 5% FBS and 40% methylcellulose solution to the spheroid-containing tube. The volume added is determined based on approximately 100 spheroids/mL. Gently mix the spheroid suspension by pipetting with a blunt 1 mL pipette tip.

NOTE: Methylcellulose solution is widely used as a suspending agent that does not allow spheroids to sediment. FBS concentration should be 5%. Higher FBS concentration causes abnormal sprouting due to excessive growth factors. Lower FBS concentration cannot maintain healthy conditions for cells.

3.4.9. Mix spheroid-suspension solution and neutralized-type I collagen gel (3 mg/mL) at a 1:1 ratio on ice. Use a blunt 1 mL pipette tip to avoid breaking of spheroids.

NOTE: 1 mL of the spheroids-suspending collagen gel solution is required for one well of a 24-well plate. Make an extra mixed suspension solution if possible.

3.4.10. If the angiogenic properties of any agent(s) or chemical(s) need to be tested, transfer 1 mL of the spheroid-suspending collagen gel solution in a 1 mL micro-tube and add agent(s) or chemical(s) followed by gentle mixing with a blunt 1mL pipette tip.

NOTE:. The volume of agent and chemical can sum to 200 µL, which dilutes the type I collagen gel concentration from 1.5 mg/mL to 1.25 mg/mL, but does not affect type I collagen gel polymerization. Add same volume of vehicle to the control spheroids.

3.4.11. Add the spheroid-suspending collagen gel solution into wells of a pre-warmed 24-well

plate (0.9 mL/well) by pipetting, and then incubate for 30 min in a cell culture incubator for polymerization.

NOTE: During the spheroid embedding process, do not disturb the gel by agitating the plate.

3.4.12. Cover the spheroid-suspending collagen gel by adding 100 µL of ECGM-MV2 containing 2.5% FBS with/without VEGF. For ECFC-only spheroids, stimulate the cells with exogenous addition of VEGF (final concentration of 50 ng/mL) to form proper sprouts. ECFC+MSC spheroids do not require exogenous stimulation by any growth factors.

3.4.13. Place the plate in a real-time cell recorder installed in a cell culture incubator (37 °C and 5% CO_2), and randomly focus on 5-10 spheroids (10X objective lens). Monitor the sprouting formation of each fluorescence-labeled spheroids every 1 h for 24 h without any disturbance.

4. Quantitate spheroid sprouting

4.1. Import the image files to Image J software to quantitate the number of sprouts and measure the length of each sprout. For co-culture spheroids, label ECFCs with green fluorescent dye before making spheroids. Then, measure the number and length of sprouts stained with green fluorescence (**Supplemental Figure 2**). Five randomly-selected spheroids were quantified per experimental group.

NOTE: Sprouts are collaboratively elongated structures formed by several ECFCs extending out of spheroids. Sprout length is measured as the length from the point of sprouts origin from the surface of the spheroids to the tip of sprouts.

4.2. Express the values as means ± SEM of at least three independent experiments. Determine the statistically significant difference by one-way ANOVA for multiple comparisons or Student's *t*-test for paired comparisons.

NOTE: $P \leq 0.05$ is considered statistically significant.

REPRESENTATIVE RESULTS:

Comparison experiments were performed using mono-culture spheroids (ECFCs-only) and co-culture spheroids (ECFCs+MSCs) to examine whether MSCs play a considerable role in ECFCs-mediated angiogenesis. Sprouting formation from each spheroid was monitored for 24 h by a real-time cell recorder that could capture the progression of angiogenic sprouting from spheroids. Angiogenic potential was quantified by counting the number of sprouts and measuring the cumulative length of sprouts generated from individual spheroids. Five randomly-selected spheroids per experimental group were analyzed. For ECFCs+MSCs spheroids, number of sprouts and cumulative sprout length were significantly higher compared with those of ECFCs-only spheroids at all time-points (**Figure 1A-C**). Sprout number and length of ECFCs+MSCs spheroids increased continuously for 12 h, but number and length of ECFCs-only spheroids increased for 6 h and did not change at later time-points (**Figure 1B,C**). In addition, sprouts formed by

ECFCs+MSCs spheroids were thicker and more durable than those formed by ECFCs-only spheroids with/without VEGF treatment (**Figure 1A**, and **Supplemental Video 1A,B,D**). MSCs-only spheroids did not form sprouts but showed individual migration of MSCs outside of spheroids (**Figure 1A** and **Supplemental Video 1C**). These results demonstrate the significant contribution of MSCs, pericyte precursors, to the cellular angiogenesis of ECFCs. MSCs are known to secrete various growth factors[29] that may stimulate ECFCs to form sprouts and tubular structures.

ECFCs were labelled with green-fluorescent dye and MSCs were labeled with red-fluorescent dye before combining to generate ECFCs+MSCs spheroids. Together with real-time recording , this live cell fluorescence labeling technique can track the cellular movements of ECFCs and MSCs during sprout formation. Fluorescent imaging showed that ECFCs-mediated sprout structures were covered with MSCs (**Figure 2** and **Supplemental Video 2**). This suggest that combined MSCs function as perivascular cells during sprout formation, which enhance sprout stability and durability by the tight association between two vascular cells.

The newly-developed co-culture spheroid assay system was tested with bevacizumab, an FDA-approved angiogenesis inhibitor, to verify its potential to screen anti-angiogenic drugs. ECFCs+MSCs spheroids pretreated with bevacizumab showed decreased sprout number and cumulative sprout length in a dose-dependent manner compared with control ECFCs+MSCs spheroids (**Figure 3A,B**). In parallel experiments, ECFCs-only spheroids pretreated with bevacizumab followed by stimulation with VEGF (50 ng/mL) also showed decreased VEGF-induced sprout number and cumulative sprout length in a dose-dependent manner (**Figure 3C,D**). Of note, the IC_{50} values of bevacizumab for inhibiting cumulative sprout length in ECFCs+MSCs spheroids was 46 times greater than that in ECFCs-only spheroids (**Table 1**). This result strongly implies that higher concentration is needed to inhibit physiologically-relevant vascular formation by ECFCs and MSCs compared with the concentration needed to inhibit only EC-mediated vascular formation.

Next, a xenograft tumor mouse model was performed with bevacizumab treatment to reveal which spheroid system provide predictable data correlating with in vivo effective plasma concentration. Human-derived glioblastoma U87MG-Red-FLuc cell line was subcutaneously injected to immune-deficient mice. After confirming tumor formation at 1 week, mice were randomly divided into 3 groups that received different treatments: control (0 mg/kg), low dose (1 mg/kg), and high dose (30 mg/kg). Tumor growth was significantly inhibited in the 30 mg/kg-treated group compared with control group (**Supplemental Figure 3A**). The 1 mg/kg-treated group showed a tendency for tumor decrease, but there was no statistical difference compared with control group. Mouse plasma concentration at 3 week of bevacizumab treatment was 568.0±40.62 μg/mL at 30 mg/kg and 38.1±0.72 μg/mL at 1 mg/kg (**Supplemental Figure 3B**). Notably, plasma concentration of bevacizumab showing effective inhibition (568.0±40.62 μg/mL at 30 mg/kg for 3 week treatment) was closely achieved by ECFCs+MSCs spheroids (1261.5±214.49 μg/mL) but not ECFCs-only spheroids (27.0±9.97 μg/mL). Thus, the ECFCs+MSCs spheroid angiogenesis assay system can be considered as a suitable assay system for predicting effective plasma concentration in advance of animal experiments.

FIGURE AND TABLE LEGENDS:

Figure 1: Sprout formation from ECFCs-only, MSCs-only, and ECFCs+MSCs spheroids. (A) Representative images of sprout formation from ECFCs-only, MSCs-only and ECFCs+MSC spheroids embedded in type I collagen gel at 0, 6, 12, 18, and 24 h (scale bar = 100 μm). **(B)** Quantitative graph of sprout number formed from ECFCs-only and ECFCs+MSCs spheroids (mean±SEM, n = 3). **(C)** Quantitative graph of cumulative sprout length formed from ECFCs-only and ECFCs+MSCs spheroids (mean±SEM, n = 3). * indicates significant difference between ECFCs-only and ECFCs+MSCs spheroids at the same time-points ($p \leq 0.05$). # indicates significant difference between groups indicated by a bracket ($p \leq 0.05$). This figure has been modified from a previous pubication[30].

Figure 2: Localization of ECFCs and MSCs in sprout structures. Representative images showing locations of two cell types in sprout structures after 24 h. ECFCs and MSCs were fluorescently labelled with PKH67 (green) and PKH26 (red), respectively, and performed 3D ECFCs+MSCs spheroid angiogenesis assay. Arrows shows that MSCs were covered with ECFCs-mediated sprout structures (scale bar=100 μm).

Figure 3: Inhibitory effect of bevacizumab on sprout formation from ECFCs+MSCs and ECFCs-only spheroids. ECFCs+MSCs and ECFCs-only spheroids were treated with bevacizumab, and sprout formation was monitored for 24 h. **(B)** Quantitative graph of cumulative sprout length from ECFCs+MSCs spheroids treated with bevacizumab (mean±SEM, n = 3). **(C)** Quantitative graph of sprout number from VEGF-stimulated ECFCs-only spheroids treated with bevacizumab (mean±SEM, n = 3). **(D)** Quantitative graph of cumulative sprout length from VEGF-stimulated ECFCs-only spheroids treated with bevacizumab (mean±SEM, n = 3). * indicates significant difference from control group (white bar) ($p \leq 0.05$). # indicates significant difference from VEGF-treated group (black bar) ($p \leq 0.05$).

Table 1: IC_{50} values of bevacizumab for inhibition of cumulative sprout length in either ECFCs-only or ECFCs+MSCs spheroids. ECFCs-only spheroids were treated with bevacizumab followed by stimulation with VEGF (50 ng/mL), which is required for sprout formation from ECFCs-only spheroids. ECFCs+MSCs spheroids were treated with bevacizumab without VEGF stimulation. Both spheroids embedded into type I collagen gel, and sprout formation from each spheroid was observed for 24 h using a real-time cell recorder. Data are represented as mean ± SEM (n = 3).

DISCUSSION:

The present study introduce an improved in vitro angiogenesis assay system utilizing co-culture spheroids formed by two human vascular cell progenitors, ECFCs and MSCs. Co-culture spheroid system can mimic in vivo vascular sprout formation, which is accomplished by interaction and incorporation between endothelial cells and pericytes. Compared to other in vitro angiogenesis assays that reflect only ECs-mediated angiogenesis, this co-culture assay system is more representative of the multistep cascade of physiologic angiogenesis including cellular interaction, sprouting, tube formation, and vessel maturation. This newly-established assay system also resembles the in vivo extracellular microenvironment by seeding spheroids into type I collagen gel. We suggest that the 3D co-culture spheroid angiogenesis assay system is reliable, repeatable,

easily quantifiable, and most importantly physiologically relevant.

While performing the co-culture spheroid assay, it is essential to embed spheroids into gel with appropriate concentration of neutralized type I collagen and FBS. The best final concentration of neutralized type I collagen is 1.5 mg/mL, and the percentage of FBS is 2.5%. In the preliminary experiments, higher concentrations of collagen resulted in stiff gel and hampered the quality and quantity of sprouts originating from spheroids. Lesser concentrations of collagen yielded soft and fragile gel that could not maintain the integrity of spheroids. Similarly, higher amounts of FBS caused plethoric sprouting from spheroids at the basal level irrespective of angiogenic factor stimulation. Lower FBS concentration led to poor conditions of cells. For consistent and reproducible results, a proper number of spheroid should be embedded (about 50 spheroids/well). More than 50 spheroids/well could lead to close proximity of spheroids within the well, which may abnormally affect the quality and quantity of sprouts generated.

It is critical to maintain type I collagen in chilled conditions during neutralization and mixing steps with the spheroid suspension because collagen can clot at room temperature, resulting in an irregular matrix. While mixing the spheroid suspension with collagen solution, it is recommended to use 1 mL pipette tip with 3-5 mm cut at the end to make wide hole. This enables easy handling of the viscous collagen solution and protects spheroids from rupture. Agitation of the plate after embedding spheroid-suspended collagen solution could disturb the integrity of the gel and result in breakage that may hamper normal sprout generation.

In co-culture spheroid assay using ECFCs and MSCs, 5:1 ratio should be maintained. Use of a larger number of MSCs causes spreading around the spheroid due to the migratory phenotype of MSCs, which can affect ideal sprout generation. A smaller number of MSCs is insufficient to stimulate ECFCs sprouting and cover the sprouts properly. In addition, it is essential to check cell conditions during growth. If poor sprouting is observed, it is recommended to use another healthy passages of the cells. For better outcome, use of ECFCs and MSCs at passage less than 10 is strongly recommended.

Here, we present an improved 3D angiogenesis assay system using co-culture spheroids that closely capture in vivo angiogenesis. Compared with 2D single cell assay systems, this co-culture spheroid assay system reflect more faithfully cellular responses between two vascular cell types to form tubular structures in physiologic conditions. However, this system remains oversimplified compared to the complex multi-step process of in vivo angiogenesis. Vascular generation in vivo usually occurs through multiple interaction of various other cell types, including epithelial cells, fibroblasts, immune cells, and also abundant ECM proteins. Future applications of this new system include introduction of other cell types and the ECM to more precisely reflect the physiological and/or pathological angiogenesis.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1.

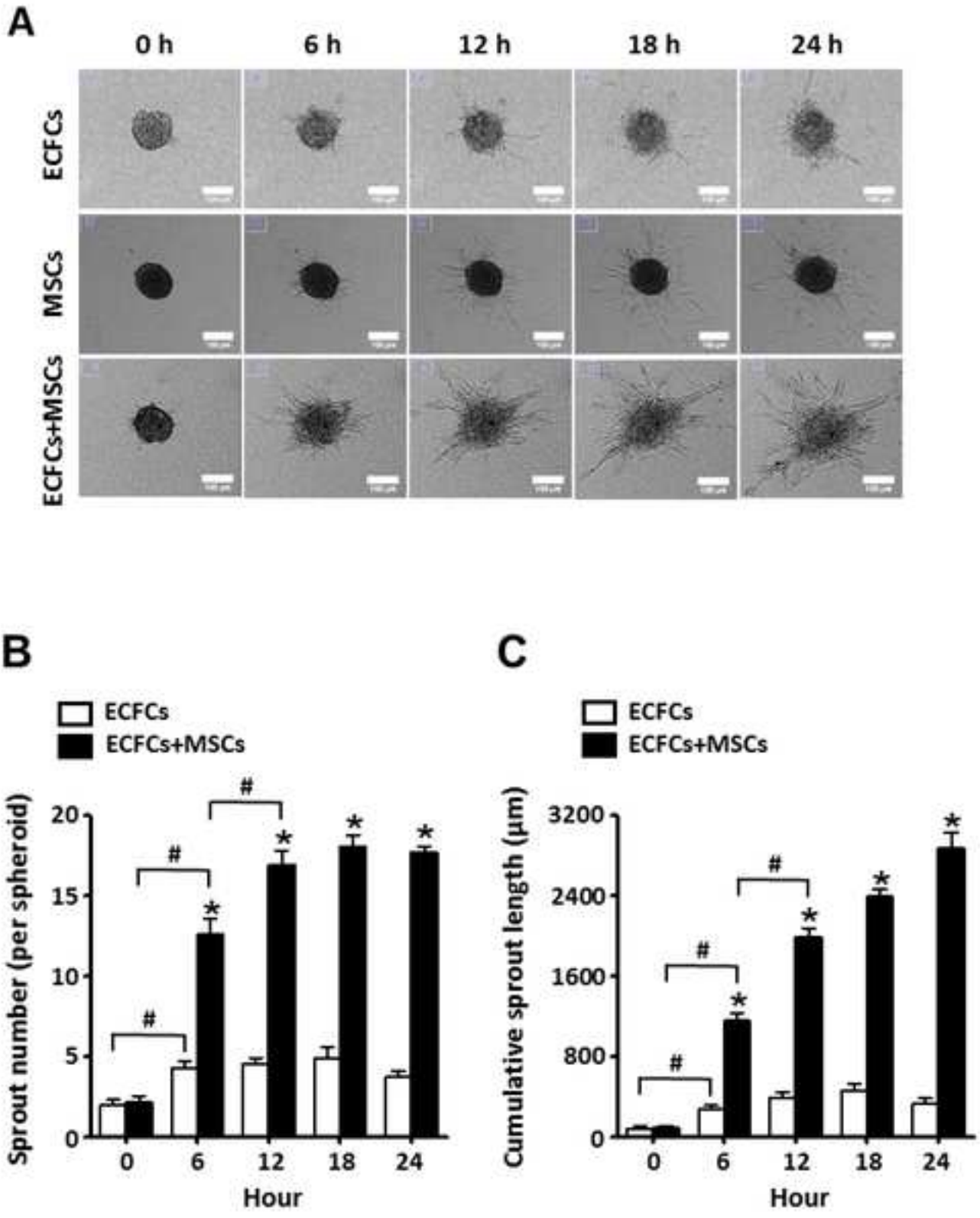

Figure 2.

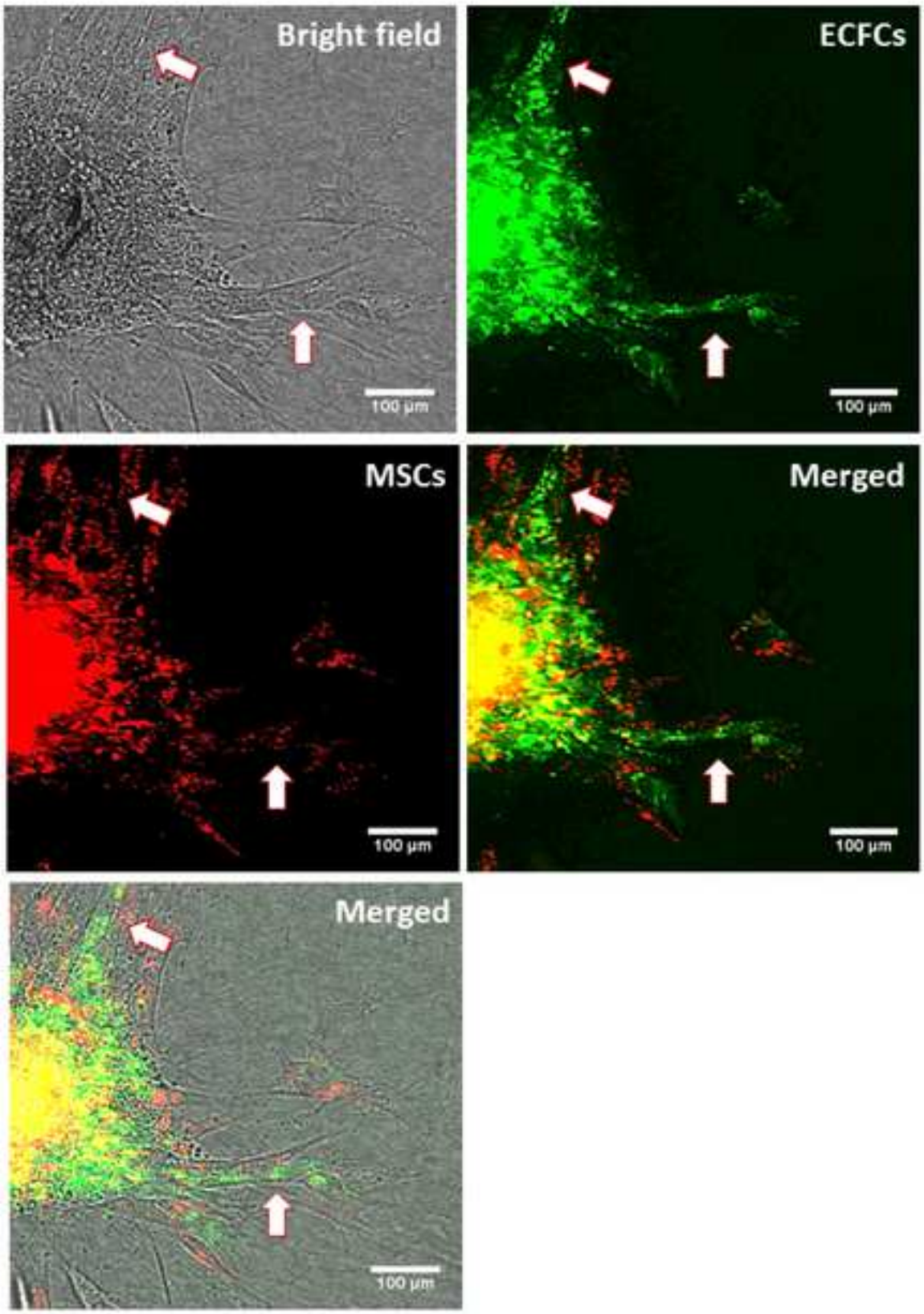

Figure 3.

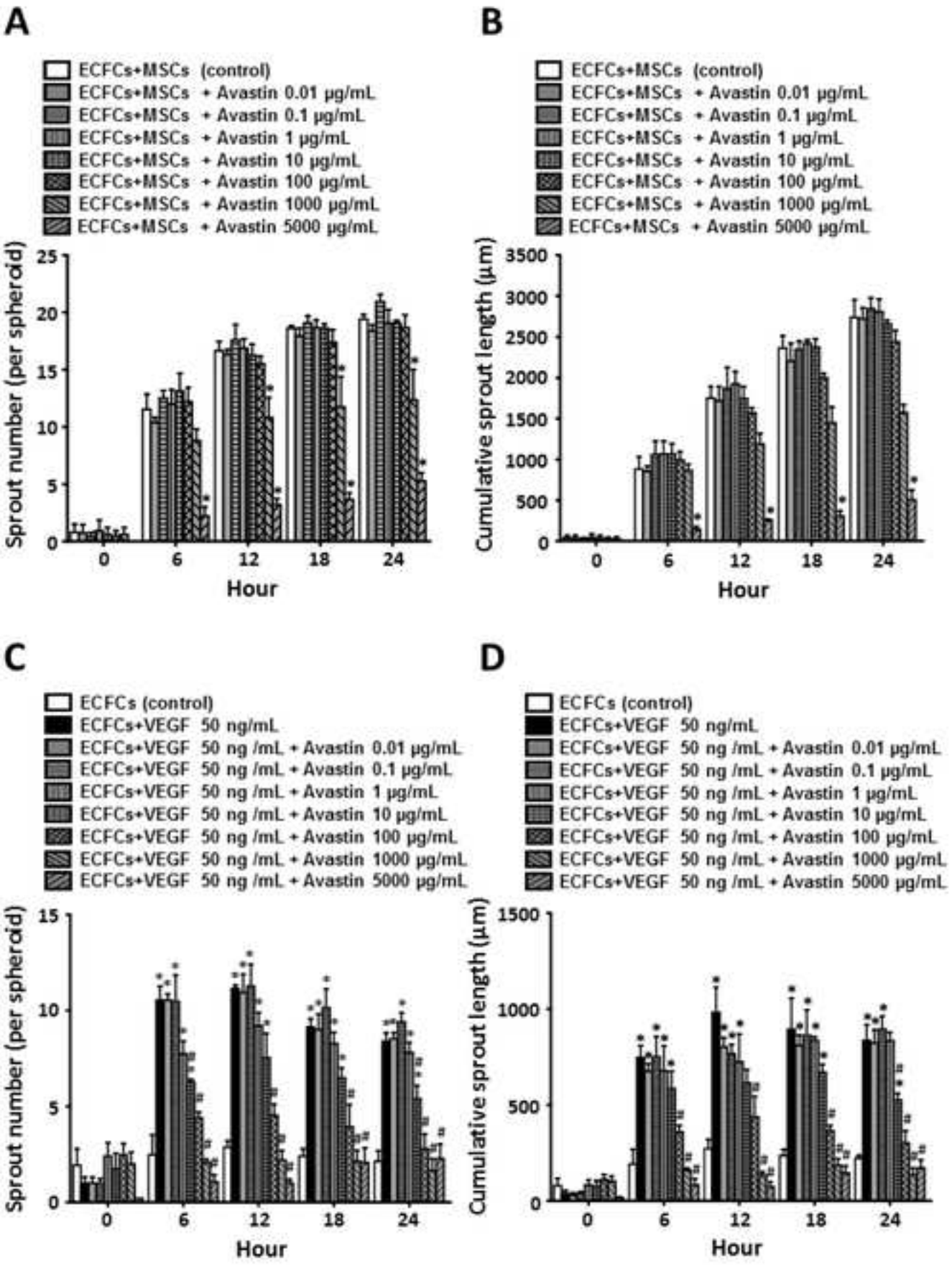

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Table 1

Time (hour)	IC_{50} (μg/mL) of Avastin		p-value
	ECFCs-only spheroid	ECFCs+MSCs spheroid	
6	94.62 ± 38.53	3058.21 ± 373.31	0.003
12	58.61 ± 17.80	2006 ± 484.73	0.015
18	83.38 ± 54.54	1509.51 ± 483.88	0.042
24	27.04 ± 9.97	1261.51 ± 214.49	0.0045

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0.05 % Trypsin EDTA (1X)	Gibco	25300-054	
Bevacizumab	Roche	NA	Commercial name: Avastin
Dulbecco Modified Eagle Medium	Gibco	11885-084	DMEM
Dulbeco's Phosphate buffered saline (10X)	Gibco	21600-010	PBS (10X)
Dulbeco's Phosphate buffered saline (1X)	Corning	21-031-CVR	PBS (1X)
Endothelial cell Growth medium MV2 kit	Promocell	C-22121	ECGM-MV2
Fetal bovine serum (FBS)	Atlas	FP-0500A	FBS
Gelatin	BD Sciences	214340	
L-Glutamine–Penicillin–Streptomycin	Gibco	10378-016	GPS
Mesenchymal stem cell growth medium-2	Promocell	C-28009	MSCGM-2
Methyl cellulose	Sigma-Aldrich	M0512	
PKH26 Fluorescent Cell Linker Kits	Sigma-Aldrich	MINI26	PKH26
PKH67 Fluorescent Cell Linker Kits	Sigma-Aldrich	MINI67	PKH67
Sodium Hydroxide	Sigma-Aldrich	S5881	
Type I collagen gel	Corning	354236	
Vascular endothelial growth factor A	R&D	293-VE-010	VEGF

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■ 아 래 ■

- 논문 제목: Two-Cell Spheroid Angiogenesis Assay System Using Both Endothelial Colony Forming Cells and Mesenchymal Stem Cells. Biomol Ther (Seoul). 26 (5), 474-480 (2018)
- 저자 정보: Sajita Shah and Kyu-Tae Kang
- 재 사 용 : Three-dimensional angiogenesis assay system using co-culture spheroids formed by endothelial colony forming cells and mesenchymal stem cells. Journal of visualized experiments (in rivision)

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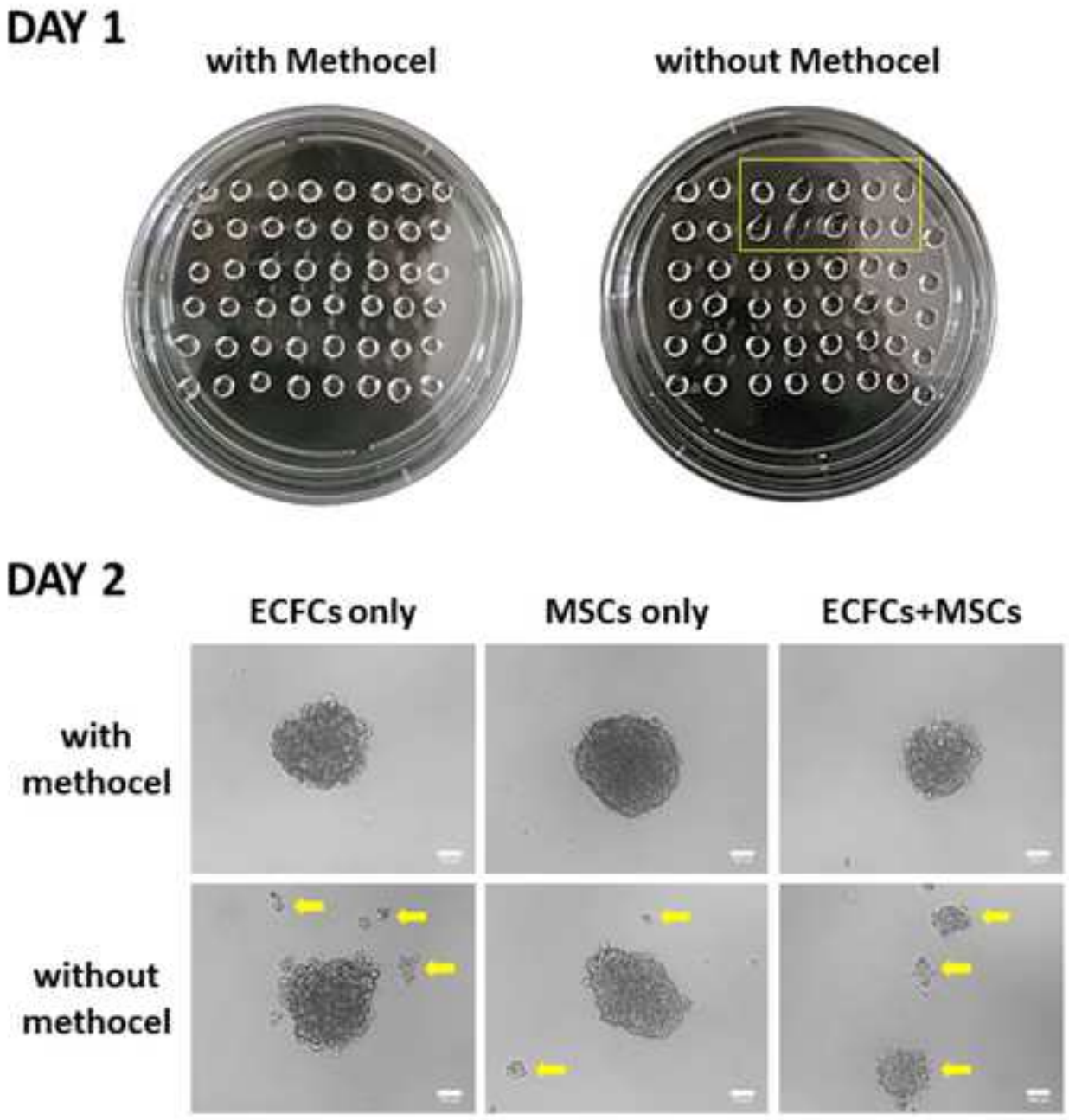
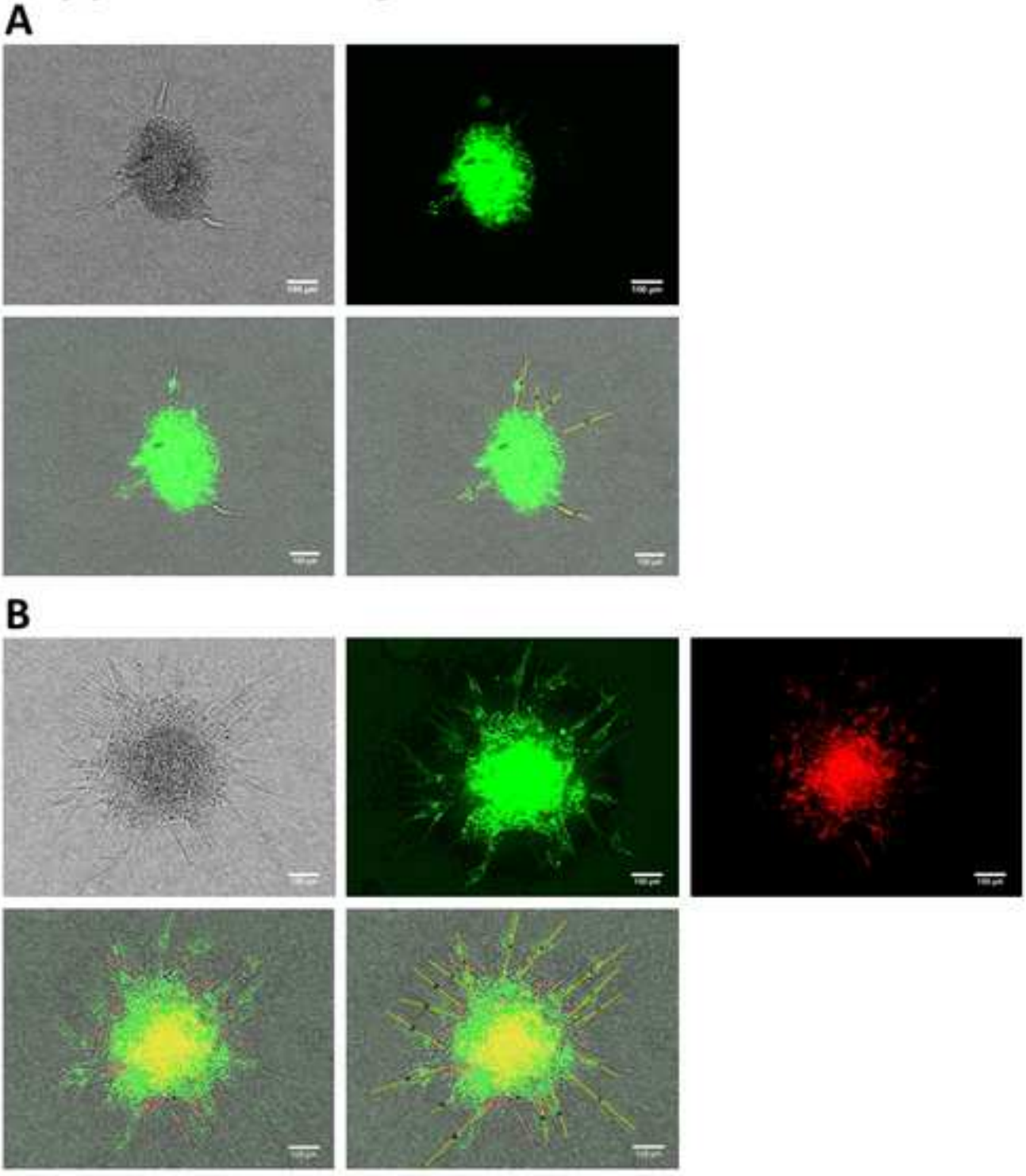
Supplemental Figure 1.



The comparison of hanging drop methods in the presence and absence of Methocel (1.2% w/v methylcellulose solution)

The hanging drop methods were performed with/without Methocel. After pipetting cell-suspension solution on the cover, some drops without Methocel were easily slipped down (yellow box) when the cover was inverted over the PBS- filled hydration unit for incubation. Furthermore, after overnight incubation, the spheroids were not perfectly formed without Methocel and small aggregates were found. These results indicate that the proper viscosity with Methocel is necessary to form round shape of spheroids (scale bar=100 μm).

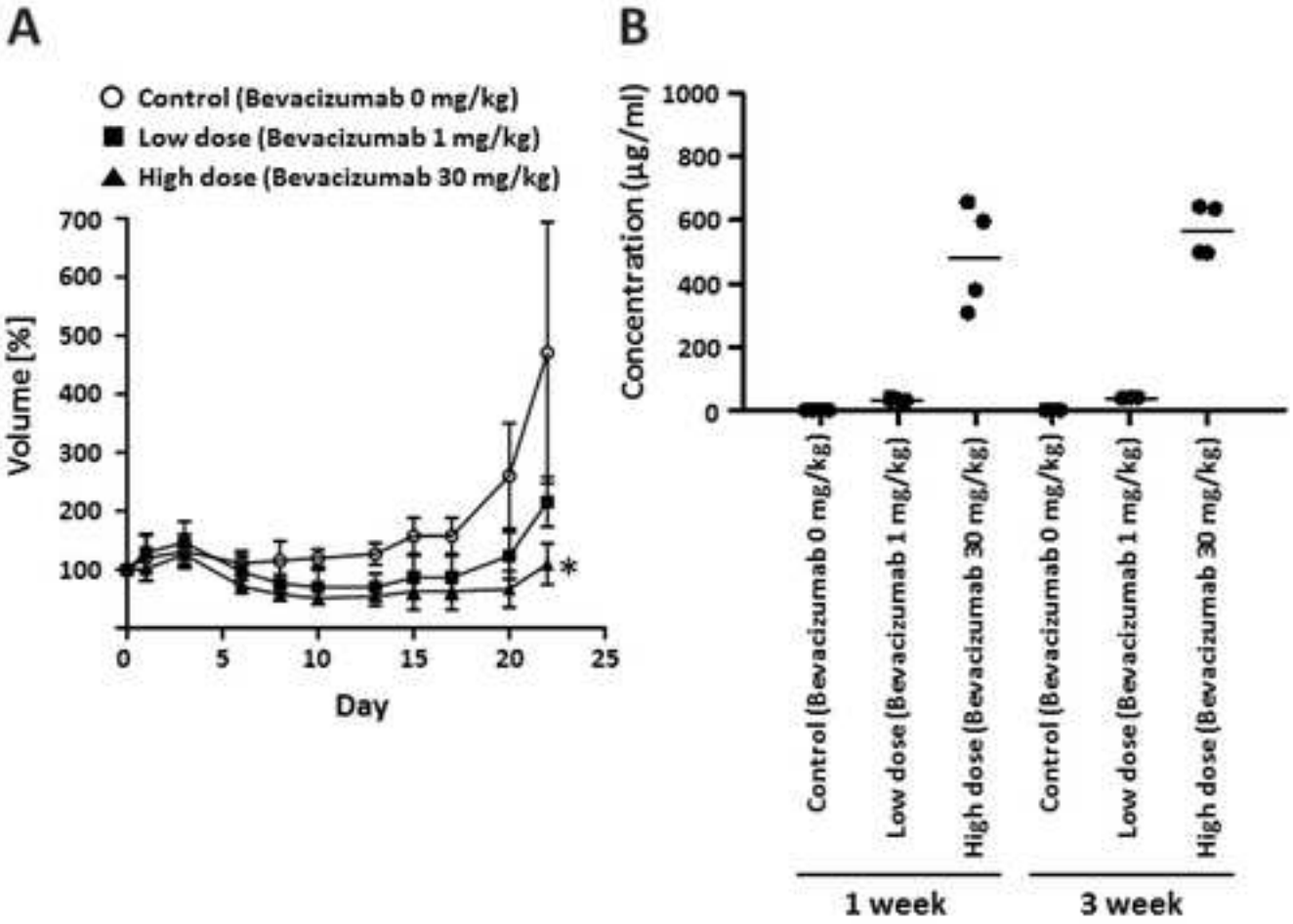
Supplemental Figure 2.



Representative images of the sprouts for analysis.

Live cell fluorescence labeling of ECFCs and MSCs was performed before spheroid formation using PKH67 (green) and PKH26 (red), respectively. **(A) Analysis of ECFC-only spheroids.** The sprout structures emerging out of the spheroid are confirmed with green fluorescence and analyzed by using the mage J software. **(B) Analysis of ECFC+MSC spheroids.** The merged image of bright field, green fluorescence, and red fluorescence was taken, and the sprout structure formed by green-labelled ECFCs are selected for analysis (scale bar=100 μm).

Supplemental Figure 3.



Xenograft tumor mouse model with bevacizumab treatment.

This experiment was performed after obtaining approval from the Institutional Animal Care and Use Committee (IACUC) of the Duksung Women's University (Approval No. 2018-003-012). 6 weeks-old male Balb/c nude mice with body weight of 19 to 24 g were used to establish an xenograft tumor mouse model. Human-derived glioblastoma U87MG-Red-Fluc cells (Perkin Elmer, MA) were injected to mouse subcutaneously (1X107 cells/100μL per mouse). After confirming tumor formation at 1 week, mice were randomly divided into 3 groups followed by bevacizumab treatment (qdX5, ip): control (0 mg/kg), low dose (1 mg/kg), and high dose (30 mg/kg). Tumor volume were measured every 3 days, and normalized with the volume at day 0. Plasma concentration of bevacizumab were measured with human bevacizumab ELISA kit (abcam, UK) at 1 week and 3 weeks after treatment. **(A) Quantitative graph of tumor volume (%) for 3 weeks (n=3-8).** Tumor growth was significantly inhibited at 30 mg/kg treated group compared with control. Whereas, 1 mg/kg treated group showed a tendency to decrease, but there was no statistical difference when compared with control. * indicates significant difference from control group ($p \leq 0.05$). **(B) Mouse plasma concentration after treatment of bevacizumab (n=4).** High dose treatment (30 mg/kg) showed significantly higher plasma concentration than low dose treatment (1 mg/kg) after 1 and 3 week of bevacizumab treatment.