



## Two-Cell Spheroid Angiogenesis Assay System Using Both Endothelial Colony Forming Cells and Mesenchymal Stem Cells

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### Abstract

Most angiogenesis assays are performed using endothelial cells. However, blood vessels are composed of two cell types: endothelial cells and pericytes. Thus, co-culture of two vascular cells should be employed to evaluate angiogenic properties. Here, we developed an *in vitro* 3-dimensional angiogenesis assay system using spheroids formed by two human vascular precursors: endothelial colony forming cells (ECFCs) and mesenchymal stem cells (MSCs). ECFCs, MSCs, or ECFCs+MSCs were cultured to form spheroids. Sprout formation from each spheroid was observed for 24 h by real-time cell recorder. Sprout number and length were higher in ECFC+MSC spheroids than ECFC-only spheroids. No sprouts were observed in MSC-only spheroids. Sprout formation by ECFC spheroids was increased by treatment with vascular endothelial growth factor (VEGF) or combination of VEGF and fibroblast growth factor-2 (FGF-2). Interestingly, there was no further increase in sprout formation by ECFC+MSC spheroids in response to VEGF or VEGF+FGF-2, suggesting that MSCs stimulate sprout formation by ECFCs. Immuno-fluorescent labeling technique revealed that MSCs surrounded ECFC-mediated sprout structures. We tested vatalanib, VEGF inhibitor, using ECFC and ECFC+MSC spheroids. Vatalanib significantly inhibited sprout formation in both spheroids. Of note, the  $IC_{50}$  of vatalanib in ECFC+MSC spheroids at 24 h was  $4.0 \pm 0.40 \mu\text{M}$ , which are more correlated with the data of previous animal studies when compared with ECFC spheroids ( $0.2 \pm 0.03 \mu\text{M}$ ). These results suggest that ECFC+MSC spheroids generate physiologically relevant sprout structures composed of two types of vascular cells, and will be an effective pre-clinical *in vitro* assay model to evaluate pro- or anti-angiogenic property.

**Key Words:** Angiogenesis, Endothelial colony forming cells, Mesenchymal stem cells, Two-cell spheroid

### INTRODUCTION

Blood vessel formation relies on a highly controlled sequence of cellular events. Two fundamental processes by which blood vessels are formed are vasculogenesis and angiogenesis. Vasculogenesis refers to the *de novo* process of new vessel formation by migration and differentiation of endothelial progenitor cells (EPCs) into endothelial cells (ECs), whereas angiogenesis refers to the extension of a pre-existing blood vessels through ECs sprouting and subsequent stabilization by mural cells (Carmeliet, 2000). If either one or both of these processes are dysregulated, a number of pathological conditions can arise (Carmeliet and Jain, 2000).

Drugs that modify angiogenesis hold great promise as potential treatment options for vascular malformation-associated diseases. Many pharmaceutical companies and research institutes have spent considerable effort, time, and money on

discovering angiogenesis-modulating drugs. Irrespective of the efforts made, few drugs have entered into the clinical trials. This may be because the preclinical *in vitro* assay systems do not have sufficient sensitivity for identifying potential drug candidates that can effectively modify *in vivo* angiogenic events. Until now, only a handful of drugs, such as Bevacizumab (Avastin, Genentech-Roche, CA, USA) and Sunitinib (Sutent, Pfizer, NY, USA), have been approved for clinical use.

Pro- or anti-angiogenic properties are initially evaluated by *in vitro* assay systems that measure the degree of proliferation, invasion, migration, and tubular structure formation of ECs seeded in two-dimensional (2D) culture dishes. Although these assay systems have contributed significantly to the discovery of angiogenesis modulators, 2D culture systems have some limitations and drawbacks. One of the major limitations of 2D culture systems is loss of originality of cells. For example, 2D-cultured ECs progressively lose their differentiated

**Open Access** <https://doi.org/10.4062/biomolther.2018.134>

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Received Jul 19, 2018 Revised Aug 7, 2018 Accepted Aug 8, 2018

Published Online Sep 1, 2018

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phenotype as manifested by reduced expression of CD34 and several signals that govern cellular processes (Fina *et al.*, 1990; Delia *et al.*, 1993). Moreover, 2D-cultured cells cannot mimic the complex cascades involved in sprout formation *in vivo* (Lutolf *et al.*, 2009). Thus, 2D culture assay systems could provide misleading results, which might be responsible for the discrepancies between the effects of angiogenic-modifying drugs in clinical trial and what was expected based on *in vitro* assays. To address the issues associated with 2D culture systems and to mimic closely the complex angiogenesis process *in vivo*, 3-dimensional (3D) assay system has been gaining more attention among researchers (Lee *et al.*, 2016). 3D systems can provide an *in vivo*-like environment to the cells, which enable to generate data that bridge the gap between conventional 2D culture assay systems and *in vivo* animal models (Pampaloni *et al.*, 2007; Hutmacher, 2010).

Recently, 3D spheroid assay model has been recommended for angiogenic modulator screening before activating animal protocols (Friedrich *et al.*, 2009; Jaganathan *et al.*, 2014). Current 3D spheroid angiogenesis assay model is utilized mature ECs, mainly human umbilical vein endothelial cells (HUVECs), to focus on the behavior of ECs during angiogenesis. However, blood vessels are composed of two types of vascular cells: ECs and pericytes. During angiogenesis, ECs are responsible for sprout formation followed by the generation of new tubular structures, whereas pericytes are responsible for maturation of the nascent blood vessels by enveloping its surface. There needs to be a proper bi-directional interaction between the two cell types for the formation of functional blood vessels. To reflect the process of angiogenesis *in vivo*, some 3D angiogenesis assays have employed co-culture of ECs and vascular smooth muscle cells using scaffolds, micro-carriers or beads. The time required for assay varies from 4 to 15 days (Sanz-Nogues and O'Brien, 2016).

To improve current 3D assay model, we developed two-cell spheroid system by combining two vascular progenitors: endothelial colony forming cells (ECFCs) and mesenchymal stem cells (MSCs). ECFCs, also called late-EPCs, are circulating precursors of ECs. ECFCs have been reported to have robust proliferation capacity compared to mature ECs such as human umbilical vein endothelial cells (Melero-Martin *et al.*, 2007). MSCs belongs to the perivascular niche and are closely related to pericytes (Shi and Gronthos, 2003; Crisan *et al.*, 2008; Ayala-Cuellar *et al.*, 2018). Previous studies have reported that co-implantation of ECFCs and MSCs in immune-deficient mice results in the formation of perfused functional blood vessels *in vivo* (Foubert *et al.*, 2008; Melero-Martin *et al.*, 2008; Reinisch *et al.*, 2009). In the present study, ECFC+MSC spheroids were generated by the hanging drop spheroid formation method, which is relatively easy and do not require complex technique or materials to perform. This assay can be performed within two days. For effective tracking of complex-spout-formation events, we utilized a real-time cell recorder for continuous 24 h monitoring. Results demonstrated that sprouts from the two-cell spheroids were stable and durable, and may closely mimic the angiogenic sprouting *in vivo* compared with other angiogenesis assays based on one cell type.

## MATERIALS AND METHODS

### Isolation and culture of human ECFCs and MSCs

The study protocol was approved by the institutional review board of Duksung Women's University (IRB No. 2017-002-001). Human peripheral blood was provided from the national biobank. ECFCs were isolated from the adherent mononuclear cell (MNC) fraction using CD31-coated magnetic beads (Invitrogen, MA, USA) as described in the previous report (Melero-Martin *et al.*, 2008). The isolated ECFCs were expanded on 1% gelatin-coated plates (BD Biosciences, NJ, USA) using endothelial growth medium-2 (EGM-2; Lonza, MD, USA) without hydrocortisone supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, CO, USA) and 1% glutamine-penicillin-streptomycin (GPS; Gibco, MA, USA). In all experiments, ECFCs from passages 7 to 10 were used.

MSCs were obtained from the MNC fraction of human adult bone marrow (Lonza). MSCs were cultured in MSC growth medium (Lonza) containing 10% FBS and 1% GPS until 80% confluence was achieved. MSCs from passage numbers 5 to 8 were used.

### Generation of ECFC-only, MSC-only, and ECFC+MSC spheroids

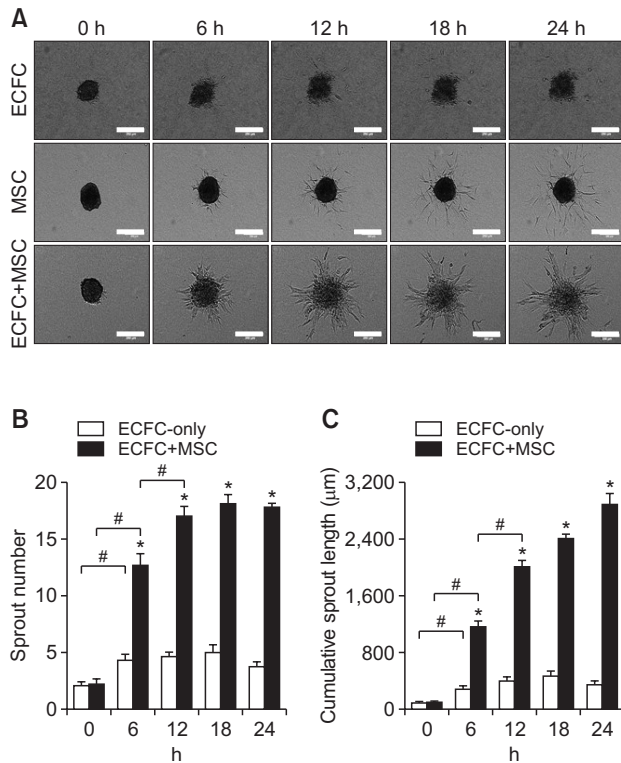
ECFCs and MSCs were trypsinized and suspended in the appropriate culture medium containing 20% methocel (Sigma, MO, USA). One-cell spheroids consisting of 600 cells/spheroid, and two-cell spheroids consisting of 500 ECFCs and 100 MSCs per spheroid were generated by the hanging drop spheroid formation method described previously (Korff and Augustin, 1998, 1999). In brief, 25  $\mu$ L of cell suspension was deposited on the lid of a 150 p (150 $\times$ 20 mm) culture dish followed by inversion of the lid onto the PBS-filled bottom chamber. Drops were incubated for 24 h at 37°C in a 5% CO<sub>2</sub> atmosphere with 95% relative humidity. Under these conditions, suspended cells were molded into standard spheroids with a defined size and cell number. These spheroids were harvested for experiments.

### *In vitro* 3D angiogenesis assay using ECFC, MSC, and ECFC+MSC spheroids

Spheroids comprising ECFCs, MSCs, or ECFCs+MSCs were collected, suspended in the corresponding basal medium with 5% FBS (Atlas Biologicals) and 40% methocel (Sigma, MO, USA) to avoid sedimentation of the spheroids. The spheroid suspension was mixed with neutralized collagen solution (Corning, NY, USA) and quickly seeded into pre-warmed 24-well plates followed by polymerization in an incubator. In some experiments, vatalanib was added to the spheroid-containing collagen gel just before seeding. After 30 min of polymerization, 0.1 mL of corresponding basal medium in the presence and absence of pro-angiogenic factors (VEGF or VEGF+FGF-2) was added to the top of the gel. The plate was placed on a real-time cell recorder (JuLI stage; NanoEnTek, Seoul, Korea), which took microscope images of spheroids every 1 h for 24 h automatically. Sprout formation was analyzed by the average number and cumulative length of sprouts from at least five spheroids per group.

### Live cell immuno-fluorescent labeling

Immuno-fluorescent labeling of live cell surfaces was performed as per the manufacturer's instructions (Sigma) with

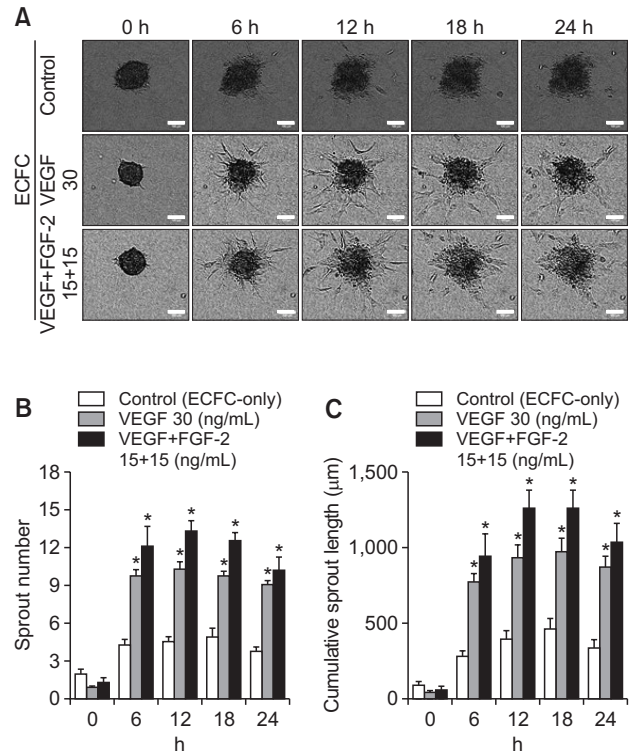


**Fig. 1.** Comparison of sprout formation between ECFC-only, MSC-only, and ECFC+MSC spheroids. ECFC-only spheroids, MSC-only spheroids, or ECFC+MSC spheroids were embedded in collagen gel and sprout formation was observed by real-time cell recorder every 1 h for 24 h. (A) Representative images of sprouts formed by ECFC-only, MSC-only, or ECFC+MSC spheroids at 0, 6, 12, 18, and 24 h time points (scale bar=250 μm). (B) Analysis of sprout number of ECFC-only or ECFC+MSC spheroids. (C) Analysis of cumulative sprout length of ECFC-only or ECFC+MSC spheroids. \*Significant difference ( $p \leq 0.05$ ) between groups. #Significant difference ( $p \leq 0.05$ ) between groups indicated by a bracket ( $n=3$ ).

slight modifications. Prior to spheroid generation, single cell suspensions of ECFCs and MSCs were washed once with the corresponding basal medium. ECFCs and MSCs were incubated with PKH67 (green) or PKH26 (red), respectively, for 5 min at room temperature. Dye uptake by cells was terminated by addition of FBS, and then cells were washed twice in their corresponding medium. Labeled ECFCs and MSCs were used for spheroid generation as described above. Fluorescent-labeled sprouting from spheroids were observed using a real-time cell recorder every hour for 24 h.

**Statistical analysis**

Values are expressed as means ± SEM of at least three independent experiments. Statistically significant differences were determined by one-way ANOVA followed by Fisher's least significant difference (LSD) *post hoc* test for multiple comparisons or Student's *t*-test for paired comparisons (OriginLab, MA, USA). A value of  $p \leq 0.05$  was considered statistically significant.

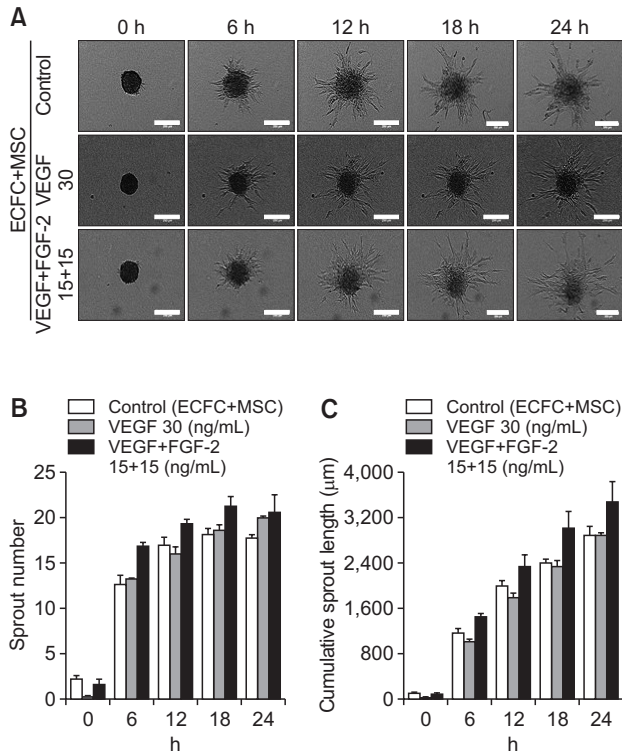


**Fig. 2.** Effect of exogenous angiogenic factors on sprout formation by ECFC-only spheroids. ECFC-only spheroids were treated with VEGF (30 ng/mL) or VEGF (15 ng/mL)+FGF-2 (15 ng/mL) followed by seeding in a plate. (A) Representative images of sprouts formed by ECFC-only spheroids in the presence and absence of angiogenic factors at 0, 6, 12, 18, and 24 h (scale bar=100 μm). (B) Analysis of sprout numbers of ECFC-only spheroids with/without angiogenic factors. (C) Analysis of cumulative sprout length of ECFC-only spheroids with/without angiogenic factors. \*Significant difference ( $p \leq 0.05$ ) from control group (not treated with angiogenic factors) ( $n=3$ ).

**RESULTS**

**Comparison of the sprouting potential of ECFC-only and ECFC+MSC spheroids**

Spheroids comprised of ECFCs and MSCs at the ratio of 5:1 were generated for the purpose of developing *in vitro* two-cell spheroid angiogenesis assay system. To compare sprout formation, ECFC-only spheroids and MSC-only spheroids were also generated using the same harvested cells. Sprout formation from spheroids was monitored for 24 h by real-time cell recorder, which could capture the progressions of cellular angiogenic sprouting from tip cells to capillary-like sprout structures. As shown in Fig. 1, numerous capillary-like sprouts were formed by ECFC+MSC spheroids. The number and cumulative length of the sprouts from ECFC+MSC spheroids were significantly greater than those of ECFC spheroids (Fig. 1A, 1B, 1C). Sprouts formed by ECFC+MSC spheroids appeared to be thicker and longer than sprouts from ECFC spheroids. Moreover, the number and length of ECFC+MSC sprouts increased for 12 h in a time-dependent manner (Fig. 1B, 1C). This suggests that MSCs may improve the stability and durability of newly-formed sprouts in ECFC+MSC spheroids. MSC spheroids did not produce sprouts but some indi-



**Fig. 3.** Effect of exogenous angiogenic factors on sprout formation by ECFC+MSC spheroids. ECFC+MSC spheroids were treated with VEGF (30 ng/mL) or VEGF (15 ng/mL)+FGF-2 (15 ng/mL). (A) Representative images of sprouts formed by ECFC+MSC spheroids in the presence and absence of angiogenic factors at 0, 6, 12, 18, and 24 h (scale bar=250 μm). (B) Analysis of sprout number of ECFC+MSC spheroids with/without angiogenic factors. (C) Analysis of cumulative sprout length of ECFC+MSC spheroids with/without angiogenic factors (n=3).

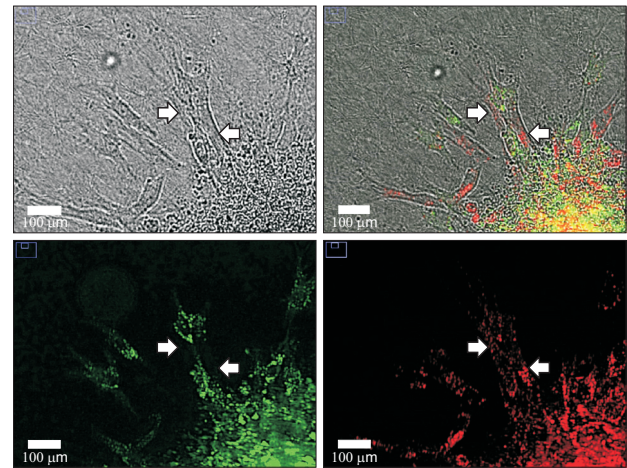
vidual cells migrated (Fig. 1A).

### Effect of exogenous angiogenic factors on sprout formation in ECFC-only and ECFC+MSC spheroids

To determine the sprout formation ability of spheroids, spheroids were treated with angiogenic growth factor, vascular endothelial growth factor (VEGF), and fibroblast growth factor-2 (FGF-2). In ECFC spheroids, the number and length of sprouts increased significantly upon treatment with VEGF as well as VEGF+FGF-2 compared with the control group (Fig. 2A, 2B, 2C). ECFC+MSC spheroids were also treated with angiogenic growth factors. As shown in Fig. 3, VEGF or VEGF+FGF-2 treatment did not result in a further increase in sprout number or length compared with the control group (Fig. 3A, 3B, 3C). This suggests that ECFCs and MSCs interact, thereby MSCs induce robust sprout formation by ECFCs.

### Localization of ECFCs and MSCs in sprout structures

ECFCs are endothelial progenitors, while MSCs are multipotent stem cells that can differentiate into pericytes during angiogenesis. Live cell immuno-fluorescent labeling of ECFCs with green dye and MSCs with red dye was conducted followed by two-cell spheroid generation to determine the location of ECFCs and MSCs in sprouts. Fluorescent images revealed



**Fig. 4.** Localization of ECFCs and MSCs in sprout structures. Live cell fluorescent staining of ECFCs and MSCs was performed before spheroid formation using PKH67 (green) and PKH26 (red), respectively. Representative image was taken at the 24 h time point using a real-time cell recorder. Arrows indicate MSCs beside ECFCs in sprout structures (scale bar=100 μm).

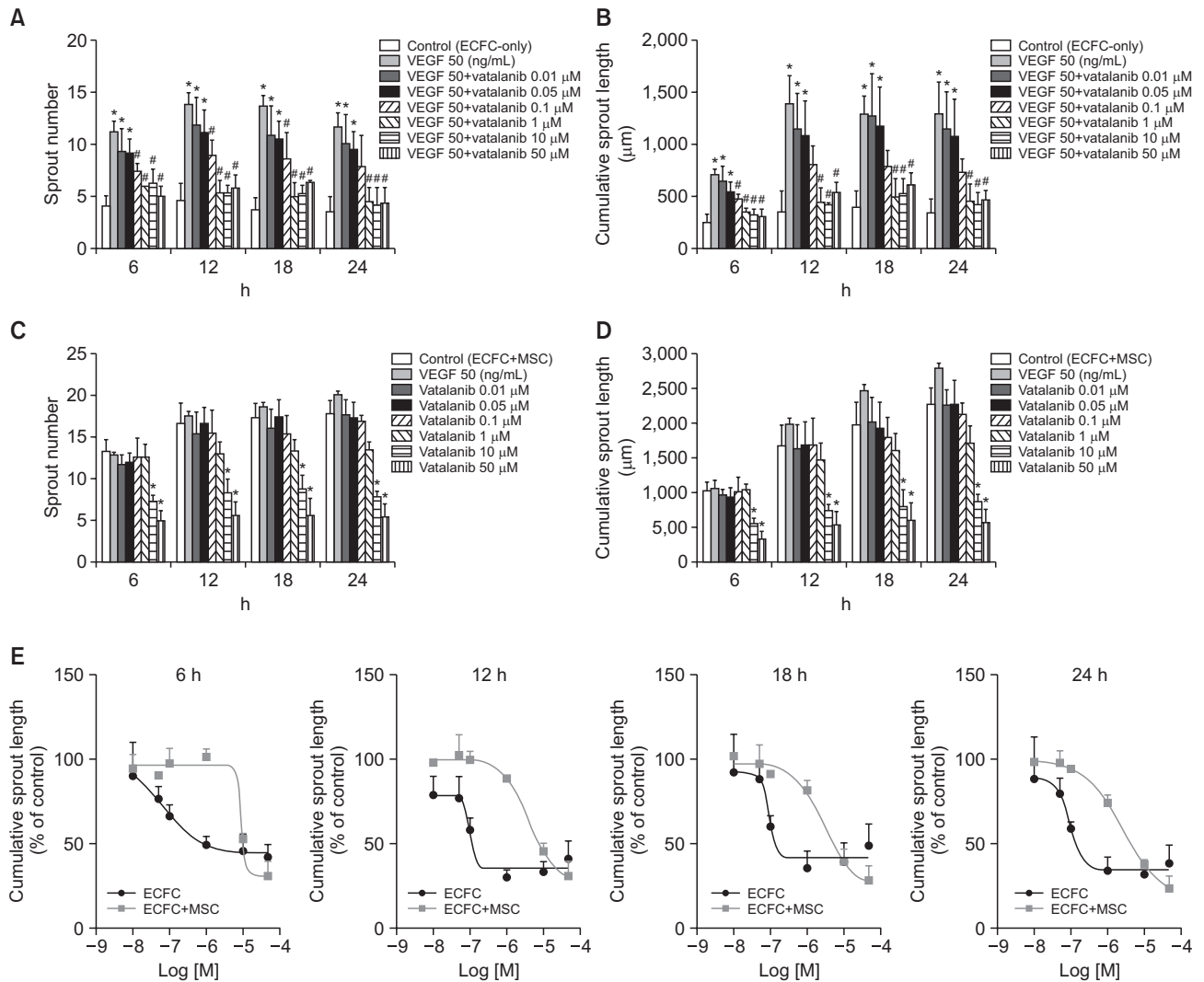
that MSCs surrounded the ECFC-mediated sprout structures (Fig. 4). This result is consistent with a previous study that human MSCs were found in regions adjacent to ECFC-mediated luminal structures when these two cells were co-injected subcutaneously into immune-deficient mice (Melero-Martin *et al.*, 2008). This suggests that MSCs function as perivascular cells and may contribute to sprout stability and durability.

### Inhibitory effect of vatalanib on sprout formation by ECFC-only and ECFC+MSC spheroids

To examine the ability of our newly-developed two-cell spheroid assay system to screen anti-angiogenic drug candidates, spheroids were treated with vatalanib, a VEGF inhibitor. ECFC spheroids were treated with vatalanib followed by stimulation with VEGF (50 ng/mL), which is required for sprout formation from ECFC-only spheroids. Vatalanib significantly decreased ECFC spheroid sprout number and length starting at 0.1 μM compared with VEGF-treated group (Fig. 5A, 5B). Vatalanib also treated to ECFC+MSC spheroids without VEGF stimulation. Vatalanib significantly inhibited sprout number and length of ECFC+MSC spheroids starting at 10 μM compared with control group (Fig. 5C, 5D). We assessed IC<sub>50</sub> values of vatalanib for inhibition of sprout length in both spheroids (Table 1 and Fig. 5E). Of note, IC<sub>50</sub> values of vatalanib was significantly greater for ECFC+MSC spheroids than ECFC-only spheroids at all-time points (Table 1). These results suggest that two-cell spheroid forms physiologically relevant sprouts composed of two vascular cells, thereby two-cell spheroid system may be more reasonable assay model to evaluate pro- or anti-angiogenic efficiency on *in vivo* angiogenic events.

## DISCUSSION

All blood vessels are formed by two types of vascular cells, namely endothelial cells and pericytes. During angiogenesis, precise cell-cell interactions between these two cell types are



**Fig. 5.** Inhibitory effect of vatalanib on sprout formation by ECFC-only spheroids or ECFC+MSC spheroids. ECFC-only spheroids or ECFC+MSC spheroids were treated with vatalanib, a VEGF inhibitor, followed by seeding in a plate. (A) Analysis of number of sprouts formed by ECFC-only spheroids with/without vatalanib treatment. (B) Analysis of cumulative length of sprouts formed by ECFC spheroids with/without vatalanib treatment. (C) Analysis of number of sprouts formed by ECFC+MSC spheroids with/without vatalanib treatment. (D) Analysis of cumulative length of sprouts formed by ECFC+MSC spheroids with/without vatalanib treatment. (E) Comparison of IC<sub>50</sub> values of vatalanib for inhibition of sprout length of ECFC-only spheroids and ECFC+MSC spheroids at 6, 12, 18 and 24 h. \*Significant difference ( $p \leq 0.05$ ) from control group (not treated with angiogenic factors), #Significant difference ( $p \leq 0.05$ ) from VEGF-treated group ( $n=3$ ).

needed for functional blood vessels to form. To mimic vascular sprout formation *in vivo*, we created spheroids comprising two vascular progenitors: ECFCs and MSCs. The one-cell angiogenesis assays commonly use human umbilical vein endothelial cells or human dermal microvascular endothelial cells because of their ready availability (Arnaoutova and Kleinman, 2010). However, these mature ECs progressively lose their proliferative phenotype as well as differentiation potential, which dampens their angiogenic responses (Delia *et al.*, 1993). Mature ECs also possess considerable organ- and tissue-specific heterogeneity (Gumkowski *et al.*, 1987). This weakness can be overcome by using ECFCs, also known as late-EPCs. EPCs were first recognized by Asahara *et al.* (1997). Since then, several subpopulations of EPCs have been identified. The consensus so far is that early-EPCs promote

neovascularization by secreting pro-angiogenic factors rather than differentiating into endothelium (Fadini *et al.*, 2012). In contrast, late-EPCs, here called ECFCs, have been defined in *in vitro* and *in vivo* assays to have proliferative and functional potential for neovascularization (Ingram *et al.*, 2004). It is now recognized that ECFCs contribute to new blood vessel formation in many post-natal pathophysiological conditions (Kwon *et al.*, 2012). For instance, circulating ECFCs are recruited into sites such as ischemic tissues for vascular regeneration, where they are incorporated into the vascular endothelial lining and differentiate *in situ* into endothelial cells (Murasawa and Asahara, 2005). It has also been reported that 40% of endothelial cells in tumor tissue are derived from ECFCs that originate from bone marrow (Rafii *et al.*, 2002). Therefore, use of ECFCs in *in vitro* angiogenesis assay systems can provide

**Table 1.** IC<sub>50</sub> values of vatalanib for inhibition of cumulative sprout length of either ECFC or ECFC+MSC spheroids at 6, 12, 18, and 24 h

Time (h)	IC <sub>50</sub> (μM) of vatalanib		p-value
	ECFC spheroid	ECFC+MSC spheroid	
6	0.6 ± 0.23	14.80 ± 0.70	0.00005
12	0.2 ± 0.10	8.9 ± 1.57	0.02200
18	0.1 ± 0.02	5.9 ± 0.29	0.00060
24	0.2 ± 0.03	4.0 ± 0.40	0.00073

ECFC spheroids were treated with vatalanib followed by stimulation with VEGF (50 ng/mL), which is required for sprout formation from ECFC spheroids. ECFC+MSC spheroids were treated with vatalanib without VEGF stimulation. Both spheroids embedded into the collagen gel, and sprout formation from each spheroid was observed for 24 h by real time cell recorder. Data are represented as mean ± SEM (n=3).

valuable insights into the pro- and anti-angiogenic properties of drug candidates. As a source of pericytes, MSCs are multipotent stem cells that have self-renewal and differentiation capacity and that participate in angiogenesis. ECFCs and MSCs were found to have a synergistic effect on new vessel formation (Melero-Martin *et al.*, 2008) and ischemic tissue repair (Kang *et al.*, 2013). Moreover, promising results were obtained when these cells were used in several clinical studies (Lara-Hernandez *et al.*, 2010; Gupta *et al.*, 2013). Based on these previous findings, we employed two vascular progenitors, ECFCs and MSCs, to generate two-cell spheroid model to closely mimic *in vivo* sprouting angiogenesis.

Most *in vitro* angiogenesis assays developed to date are based on monoculture of ECs grown in 2D cell culture dishes. However, ECs grown on artificial plastic surfaces do not have sufficiently cell-cell and cell-matrix interactions, thereby yielding less physiologically relevant information. In addition, 2D *in vitro* assay systems reflect part of angiogenesis such as proliferation, migration, or invasion (Gautier *et al.*, 2011; Hada *et al.*, 2012). Thus, *in vitro* assay systems that can reflect the complete cascade of angiogenesis would be more appropriate systems for screening pro- or anti-angiogenic compounds. To generate an *in vivo*-like 3D environment for the cells, we embedded ECFC+MSC spheroids in collagen type I gels. The extracellular matrix has been shown to play a crucial role in regulating cell behavior (Lu *et al.*, 2012). Collagen is one of the main constituents of the matrix around ECs (Detry *et al.*, 2011; Paupert *et al.*, 2011).

In the present study, we found that both the number and length of sprouts from ECFC+MSC spheroids were significantly greater than those of sprouts from ECFC-only spheroids. Previous studies have shown that MSCs provide a strong angiogenic stimulus to ECFCs by secreting growth factors such as VEGF-A (Melero-Martin *et al.*, 2008; Grellier *et al.*, 2009). ECFCs can also function as paracrine mediators prior to the establishment of blood perfusion, modulating the regenerative potential of MSCs (Lin *et al.*, 2014). Therefore, bi-directional angiogenic stimulation of ECFCs by MSCs in our two-cell spheroids might be sufficient for sprout formation. Angiogenic cross-stimulations between ECFCs and MSCs is supported by the observation of no significant differences in the sprouting potential of ECFC+MSC spheroids when treated with VEGF and FGF-2 or not. In contrast, ECFC spheroids

required exogenous growth factors for sprout formation. Results from live cell fluorescent labeling of ECFCs and MSCs also suggest that MSCs contribute to the sprout formation process in a pericyte-like manner because they were found in the surrounding area of ECFC-mediated sprout structures. Future studies will focus on the integrated cross-talk between ECFCs and MSCs, which will provide the novel insight into the pro- and anti-angiogenic drug discovery.

We verified our newly developed two-cell spheroid assay system by performing an inhibitor study using vatalanib. Vatalanib is a potent inhibitor of VEGF receptor tyrosine kinases (Banerjee *et al.*, 2009). We found that the IC<sub>50</sub> value of vatalanib for inhibition of sprout length was 4.0 ± 0.40 μM for ECFC+MSC spheroids and 0.2 ± 0.03 μM for ECFC spheroids after 24 h. IC<sub>50</sub> values of vatalanib was significantly greater in two-cell spheroid assay system compared with conventional one-cell spheroid system (Table 1). ECFC+MSC spheroids generate stable and durable sprouts which are composed of two vascular cell types. Interestingly, IC<sub>50</sub> value from two-cell spheroid assay system is closer to the previous *in vivo* data. In the previous report, oral administration of vatalanib 50 mg/kg/day inhibited more than 50% of DUI45 carcinoma tumor growth, and plasma concentration of vatalanib was >1 μM 8 h after administration of 50 mg/kg (Wood *et al.*, 2000). Further studies are necessary to prove whether ECFC+MSC spheroid assay system have considerable sensitivity to provide predictive data for *in vivo* studies.

To summarize, we developed an advanced 3D angiogenesis assay system by utilizing spheroids comprising two types of vascular cells, ECFCs and MSCs, to mimic physiological vascular formation. We used a real-time cell recorder to observe angiogenic sprouting over time. The results from our new assay system confirm that MSCs play an important support role in angiogenesis by activating endothelial cells and by covering the outer surface of newly-formed sprout structures. Moreover, our new assay system provided more relevant inhibitory concentration data to that obtained from *in vivo* studies than one-cell assay system. Therefore, the two-cell spheroid assay system described here may be a useful *in vitro* assay system for more accurately evaluating pro- or anti-angiogenic property.

## ACKNOWLEDGMENTS

This research was supported by a grant (17172MFDS215) from Ministry of Food and Drug Safety in 2017, the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (2017R1A2B4005463), and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016R1A6A1A03007648).

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