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RESEARCH ARTICLE

# Inhibition of the PI3K–Akt and mTORC1 signaling pathways promotes the elongation of vascular endothelial cells

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## ABSTRACT

Endothelial cell morphology needs to be properly regulated during angiogenesis. Vascular endothelial growth factor (VEGF) induces endothelial cell elongation, which promotes sprouting of pre-existing vessels. However, therapeutic angiogenesis using VEGF has been hampered by side effects such as elevated vascular permeability. Here, we attempted to induce endothelial cell elongation without an overdose of VEGF. By screening a library of chemical inhibitors, we identified phosphatidylinositol 3-kinase (PI3K)–Akt pathway inhibitors and mammalian target of rapamycin complex 1 (mTORC1) inhibitors as potent inducers of endothelial cell elongation. The elongation required VEGF at a low concentration, which was insufficient to elicit the same effect by itself. The elongation also depended on Foxo1, a transcription factor indispensable for angiogenesis. Interestingly, the Foxo1 dependency of the elongation was overridden by inhibition of mTORC1, but not by PI3K–Akt, under stimulation by a high concentration of VEGF. Dual inhibition of mTORC1 and mTORC2 failed to induce cell elongation, revealing mTORC2 as a positive regulator of elongation. Our findings suggest that the PI3K–Akt–Foxo1 and mTORC1–mTORC2 pathways differentially regulate endothelial cell elongation, depending on the microenvironmental levels of VEGF.

**KEY WORDS: Endothelial cell, Embryonic stem cell, PI3K, Akt, mTOR, Foxo1, Cell elongation**

## INTRODUCTION

The process of forming new blood vessels through the sprouting of preexisting vessels is referred to as angiogenesis. Vascular endothelial growth factor (VEGF) promotes angiogenesis by driving widespread events in the involved endothelial cells. The cellular events involving VEGF include the tip cell selection for the initiation of sprouting, the proliferation of sprouting endothelial cells and the lumen formation in the elongated new vessels (Hellström et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Strilić et al., 2009; Suchting et al., 2007; Zeng et al., 2007a). Angiogenesis plays important roles not only in the physiological vascular development, but also in the therapeutic vascular formation in pathological settings.

The major objective in treating ischemic disorders is the reconstruction of damaged blood vessels. Administration of recombinant VEGF or overexpression of VEGF by gene transfer has been tested for the purpose of inducing therapeutic vascular growth. However, preclinical studies have shown that VEGF overexpression causes undesirable side effects, such as tissue edema due to elevated vascular permeability, as VEGF is known to be a potent vascular permeability factor (Rissanen et al., 2005; Senger et al., 1983). Although the tissue edema appeared to remain at the level of a temporal effect in clinical trials of VEGF gene therapy, this potential problem should be eliminated (Lee et al., 2000; Rajagopalan et al., 2003; Ylä-Herttuala et al., 2007).

In tissue engineering, the establishment of proper vascularization is key to the perfusion and survival of the grafted tissues. VEGF has also been used to achieve neovascularization in the engineered tissues (Jabbarzadeh et al., 2008; Chung and Shum-Tim, 2012). However, the microenvironmental level of VEGF has been shown to be crucial, as myoblasts secreting VEGF at a concentration higher than a certain threshold induce hemangiomas when transplanted into muscles (Ozawa et al., 2004).

Aberrant angiogenesis induced by high microenvironmental levels of VEGF is one of the major therapeutic targets in cancer treatment. VEGF mediates tumor angiogenesis by activating the phosphatidylinositol 3-kinase (PI3K) to Akt signaling pathway (Cross and Claesson-Welsh, 2001; Gerber et al., 1998). PI3K–Akt signaling also indirectly upregulates tumor angiogenesis through activation of the mammalian target of rapamycin complex 1 (mTORC1) (Jiang et al., 2002; Karar and Maity, 2011; Laughner et al., 2001; Okumura et al., 2012; Zhao et al., 2006). mTORC1 increases the level of hypoxia inducible factor 1α (HIF-1α) in tumor cells, thereby promoting VEGF secretion and the formation of leaky tumor vessels. Therefore, the normalization of aberrant vasculature to remedy the blood flow of tumors is now considered as a promising therapeutic strategy for cancer treatment (Carmeliet and Jain, 2011; Falcón et al., 2009; Tong et al., 2004). Based on the above, to therapeutically control angiogenesis, it is necessary to develop a method for promoting normal angiogenesis, without inducing excess proliferation of endothelial cells or hyper-permeability.

The cell biological functions of endothelial cells are dynamically regulated during the process of angiogenesis. These functions include cytoskeletal reorganization, and changes in cell morphology, cell–cell adhesion and migration (Arima et al., 2011; Guo et al., 2007; Jakobsson et al., 2010; Lampugnani et al., 2002; Geudens and Gerhardt, 2011). It has been reported that VEGF is a key regulator of endothelial cell morphology (Furuyama et al., 2004; Hirashima et al., 1999). VEGF stimulation induces the elongation of endothelial cells in an *in vitro* differentiation system of murine embryonic stem cells (ESCs). We have also demonstrated that the endothelial cell elongation depends on the function of forkhead box O1 (Foxo1), a forkhead transcription factor, as *Foxo1*-deficient endothelial cells fail to elongate in response to VEGF (Furuyama et al., 2004; Matsukawa et al., 2009). Foxo1 deficiency leads to embryonic lethality caused by impairment of angiogenesis (Furuyama et al., 2004; Hosaka et al., 2004), suggesting that endothelial cell elongation is an indispensable cellular event that drives angiogenesis.

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The key research question of this study was whether or not endothelial cell elongation can be induced even in the absence of strong stimulation of VEGF. Thus, this study set out to modulate signaling pathways in endothelial cells using chemical inhibitors, to promote cell elongation without excess VEGF stimulation. We show that inhibition of the PI3K–Akt or mTORC1 signaling pathway induces endothelial cell elongation under stimulation by a low level of VEGF, highlighting these pathways as candidate targets for the regulation of therapeutic angiogenesis.

## RESULTS

### PI3K, Akt and mTORC1 inhibitors induce the elongation of endothelial cells

Two fluorescent reporters, EGFP and DsRed.T4, were introduced into wild-type ESCs to examine the morphology of live endothelial cells and smooth muscle cells (SMCs) in culture. The expression of EGFP was driven by the F10-44 enhancer element of the *Mef2c* gene, to mark the endothelial cell lineage (De Val et al., 2008). The DsRed.T4 reporter was driven by the SMC-specific promoter of the *SM22* (also known as *Tagln*) gene (Akyurek et al., 2000). ESCs carrying the reporter genes were induced to differentiate into endothelial cells and SMCs by using OP9 stromal cells as feeder cells. Immunofluorescence staining showed that VE-cadherin-positive endothelial cells expressed EGFP, whereas desmin-positive SMCs expressed DsRed.T4 (Fig. 1A,B). Two ESC clones expressing these reporters, hereafter called F10-EGFP/SM22-DsRed.T4 ESCs, were isolated and both of them responded similarly to chemical inhibitors in the following screening experiments.

The two independent clones of F10-EGFP/SM22-DsRed.T4 ESCs were used to screen chemical inhibitors for their capacity to modulate endothelial cell morphology. We used the SCADS inhibitor kit that was provided by the Screening Committee of Anticancer Drugs, MEXT, Japan (http://scads.jfcr.or.jp/kit/index.html). The inhibitor kit consists of 335 chemical compounds whose targets have already been identified. The chemical inhibitors were added during the differentiation of endothelial cells and SMCs from ESC-derived Flk-1-positive vascular progenitor cells (VPCs). Live endothelial cell and SMC colonies were directly observed using fluorescent proteins as a probe. About 60% of the endothelial cell colonies were round and sheet-like in the DMSO-treated culture (Fig. 1C,D). The rest of the endothelial cell colonies were branched and thread-like, and were composed of elongated cells. The endpoint of the screening was the ability of an inhibitor to shift the morphology of endothelial cell colonies from sheet-like to thread-like. SMCs were monitored as a reference, to exclude non-specific effects on the cytoskeleton.

Each of the inhibitors influenced the proportion of thread-like endothelial cell colonies to varying degrees (Table S1). Some inhibitors exerted quite different effects on the two ESC clones. We selected four chemical inhibitors from the candidates that consistently increased thread-like endothelial cell colonies to the same degree as the VEGF-treated positive control in both clones. These inhibitors included LY294002 (a selective inhibitor of PI3K), Akt Inhibitor VIII (isozyme-selective Akti-1/2, a selective inhibitor of Akt1 and Akt2, hereafter called Akt inhibitor), rapamycin (a selective inhibitor of mTORC1) and everolimus (a rapamycin-derived selective inhibitor of mTORC1). These inhibitors increased the proportion of thread-like endothelial cell colonies without affecting the morphology of SMCs (Fig. 1C,D). Furthermore, the length of the branches in the thread-like colonies increased by twofold compared with the length of branches in the same type of colony in the DMSO-treated culture (Fig. 1E).

None of the four chemical inhibitors significantly affected the ratio between the number of endothelial cells and SMCs that were differentiated from the Flk-1-positive VPCs (Fig. 1F). Furthermore, these inhibitors did not influence the morphology of the OP9 feeder cells (Fig. S1).

### The elongation of endothelial cells depends on endogenous VEGF

ESC-derived endothelial cells are known to elongate in response to VEGF stimulation when co-cultured with OP9 cells (Furuyama et al., 2004; Matsukawa et al., 2009; Park et al., 2009). VEGF was not added exogenously in the above-mentioned screening. Nevertheless, the chemical-inhibitor-induced elongation of endothelial cells might depend on the low amount of VEGF secreted by OP9 cells. To investigate the involvement of the endogenous VEGF, we asked whether the Flt1 (also known as VEGFR1) chimera (Flt1-Fc), which sequesters VEGF, could block endothelial cell elongation. VPCs derived from F10-EGFP/SM22-DsRed.T4 ESCs were cultured on an OP9 cell layer in the presence of LY294002, Akt inhibitor, rapamycin or everolimus. The chemical inhibitors induced the elongation of VE-cadherin-positive endothelial cells, as indicated by the increase in thread-like colonies (Fig. 2). By contrast, the addition of Flt1-Fc completely blocked the endothelial cell elongation, suggesting the involvement of endogenous VEGF in endothelial cell elongation (Fig. 2).

### The elongation of endothelial cells depends on Foxo1

Foxo1 plays an indispensable role in the elongation of endothelial cells in response to VEGF (Furuyama et al., 2004; Matsukawa et al., 2009). To examine whether the chemical-inhibitor-induced elongation of endothelial cells depends on Foxo1, the response of *Foxo1*$^{-/-}$ endothelial cells to the chemical inhibitors was analyzed. EGFP-F/*Foxo1*$^{-/-}$ ESCs, which constitutively express a farnesylated form of EGFP, were used to derive endothelial cell colonies in the presence of the chemical inhibitors. F10-EGFP/SM22-DsRed.T4 ESCs were used as the wild-type control.

Wild-type endothelial cells formed thread-like colonies in the presence of the above-mentioned chemical inhibitors (Fig. 3A,B). The length of branches in the thread-like colonies increased more than twofold compared with that of the same type of colonies in the DMSO-treated cultures (Fig. 3C). In sharp contrast, none of the four chemical inhibitors induced the elongation of *Foxo1*$^{-/-}$ endothelial cells. Most of the endothelial cell colonies derived from *Foxo1*$^{-/-}$ ESCs were sheet-like even in the presence of the chemical inhibitors (Fig. 3A–C).

Foxo1 expression was then rescued by introducing an EGFP-tagged Foxo1, which was expressed under the control of the constitutive CAG promoter, into the *Foxo1*$^{-/-}$ ESCs. The forced expression of Foxo1–EGFP induced thread-like endothelial cell colonies even in the DMSO-treated control (Fig. 3D,E). Treatment of the chemical inhibitors showed only a minor effect on the proportion of thread-like colonies under the forced expression of Foxo1 (Fig. 3D,E). However, the average length of branches of the thread-like endothelial cell colonies significantly increased in response to the chemical inhibitors compared with control (Fig. 3F).

We have previously established a *Foxo1*$^{-/-}$ ESC clone in which Foxo1 expression is rescued using the endothelial-cell-specific promoter of the VE-cadherin gene (Furuyama et al., 2004). This ESC clone was used to confirm the cell autonomous requirement for Foxo1 in the chemical-inhibitor-induced endothelial cell elongation. The endothelial-cell-specific rescue of Foxo1 expression restored the formation of thread-like endothelial cell

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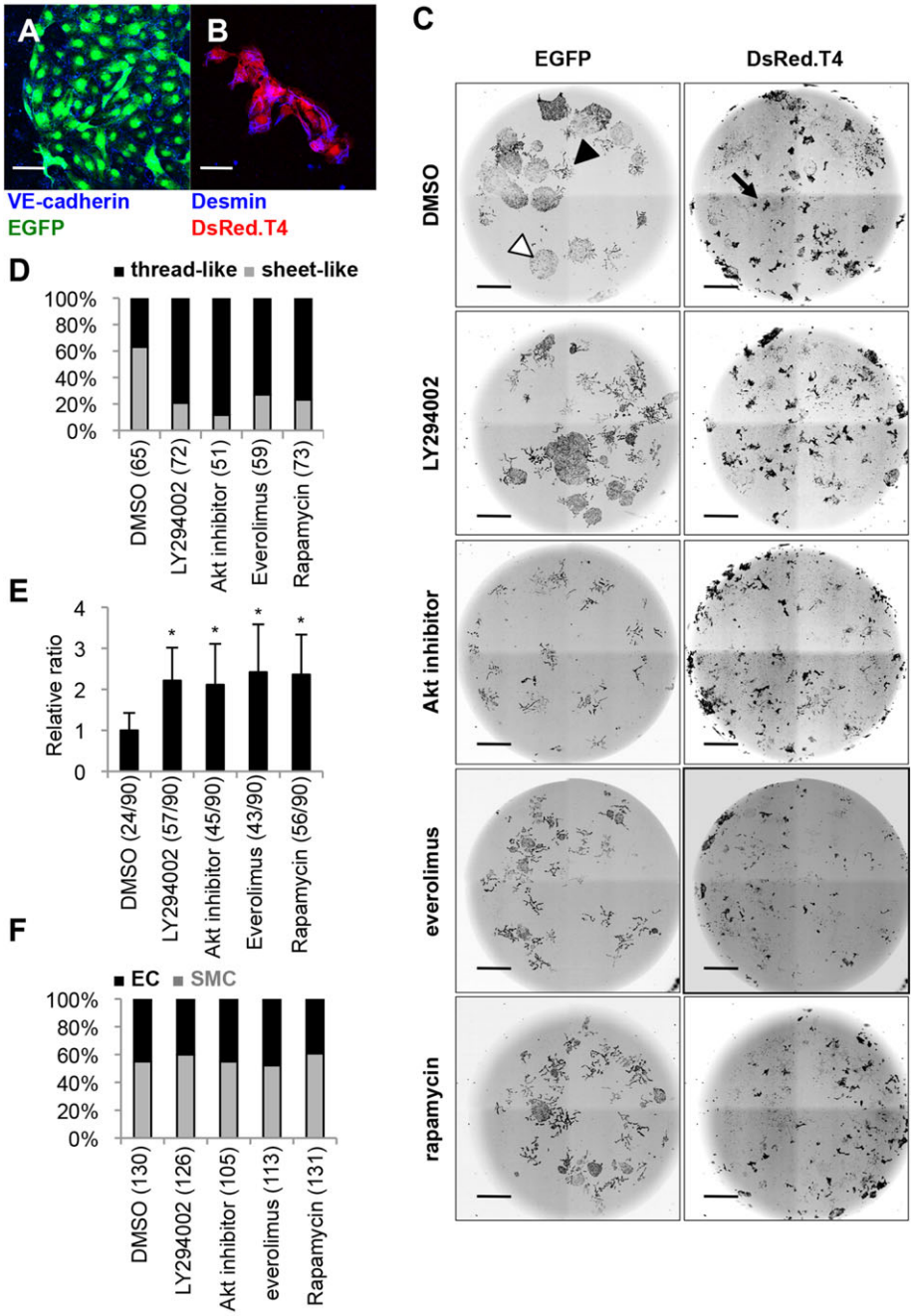


**Fig. 1. PI3K, Akt and mTORC1 inhibitors induce elongation of endothelial cells.** Flk1-positive VPCs derived from F10-EGFP/SM22-DsRed.T4 ESCs were seeded on an OP9 cell layer to induce the differentiation of endothelial cell and SMC lineages. At 1 day after seeding, the indicated chemical inhibitors were added at a concentration of 5 µM. DMSO was added as a control. Cells were further cultured for 3 days. (A) Immunostaining of endothelial cells (blue, VE-cadherin; green, EGFP) in DMSO control. Scale bar: 50 µm. Gamma settings were adjusted for clearness of images. Similar results were obtained from two independent ESC clones. (B) Immunostaining of SMCs (blue, desmin; red, DsRed.T4) in DMSO control. Scale bar: 50 µm. Gamma settings were adjusted for clearness of images. Similar results were obtained from two independent ESC clones. (C) Images of EGFP fluorescence (endothelial cells, left) and DsRed.T4 fluorescence (SMCs, right) in living cells. The images were stitched together to show the entire well. Scale bars: 1000 µm. The white arrowhead points to a sheet-like endothelial cell colony. The black arrowhead points to a thread-like endothelial cell colony. The arrow points to an SMC colony. Gamma settings were adjusted for clearness of images. Similar results were obtained from two independent ESC clones. (D) Proportion of the two types of endothelial cell colonies (thread-like and sheet-like) found in each group of cultures. Endothelial cell colonies generated from two independent ESC clones were analyzed. The total number of colonies examined is indicated in the brackets. (E) The length of branches of thread-like endothelial cell colonies in each group. Endothelial cell (EC) colonies generated from two independent ESC clones were analyzed. The total number of colonies and branches examined is indicated in the brackets (colonies/branches). The length is presented as a ratio relative to the DMSO control (mean±s.d.). *$P$<0.05, versus DMSO control (ANOVA, followed by Dunnett's test). (F) Proportion of the number of endothelial cell and SMC colonies found in each group. Colonies generated from two independent ESC clones were analyzed. The total number of colonies examined is indicated in the brackets.

colonies and the endothelial cell elongation induced by the chemical inhibitors (Fig. 3G–I).

### VEGF stimulation together with mTORC1 inhibition induces endothelial cell elongation independently of Foxo1

As the chemical-inhibitor-induced endothelial cell elongation appeared to depend on a low amount of VEGF, we assessed the effect of the inhibitors in the presence of a high concentration of VEGF. Flk-1-positive VPCs derived from F10-EGFP/SM22-DsRed.T4 ESCs were cultured on an OP9 cell layer in the presence of 10 ng/ml recombinant murine VEGF. The exogenous VEGF stimulated the outgrowth of elongated endothelial cells, thereby producing a net-like structure spread throughout a microscope field (Fig. 4A, DMSO-treated). In contrast, treatment with LY294002, Akt inhibitor, rapamycin or everolimus reduced the area occupied by endothelial cells by as much as 50% (Fig. 4A,B). A close observation revealed a fine and thin network of elongated endothelial cells formed in the presence of the chemical inhibitors and the exogenous VEGF (Fig. 4C, upper panels, D). The average length of branches was not significantly influenced by the presence of the inhibitors (Fig. 4E).

We next examined the involvement of Foxo1 in the elongation of endothelial cells induced by a high concentration of VEGF. In agreement with our previous reports (Furuyama et al., 2004; Matsukawa et al., 2009), endothelial cells derived from EGFP-F/*Foxo1*$^{-/-}$ ESCs failed to elongate in response to exogenous VEGF (Fig. 4C lower panels, D). The inhibition of PI3K or Akt by LY294002 or Akt inhibitor, respectively, did not influence the morphology of *Foxo1*$^{-/-}$ endothelial cells (Fig. 4C,D). Surprisingly, however, the inhibition of mTORC1 by rapamycin

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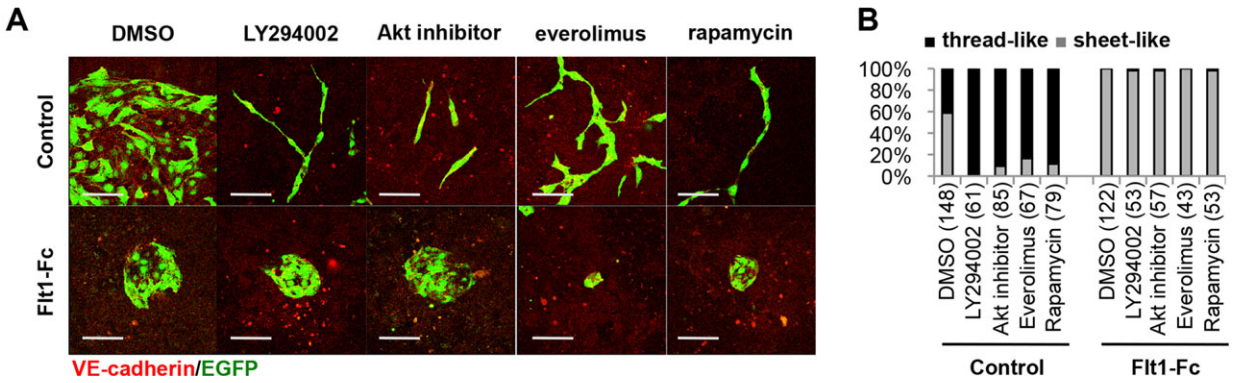


**Fig. 2. Elongation of endothelial cells depends on endogenous VEGF.** VPCs derived from F10-EGFP/SM22-DsRed.T4 ESCs were seeded on an OP9 cell layer and cultured in the presence or absence of Flt1 (also known as VEGFR1) chimeric protein (Flt1-Fc; 0.2 µg/ml). At 1 day after seeding, the indicated chemical inhibitors were added at a concentration of 5 µM. Cells were further cultured for 3 days. (A) Immunostaining of endothelial cell colonies (red, VE-cadherin; green, EGFP). Scale bars: 100 µm. Gamma settings were adjusted for clearness of images. The experiment was repeated three times with similar results. (B) Proportion of the two types of endothelial cell colonies (thread-like and sheet-like) found in each group of cultures. Endothelial cell colonies generated in three independent experiments were analyzed. The total number of colonies examined is indicated in the brackets.

or everolimus induced the elongation of *Foxo1*$^{-/-}$ endothelial cells under stimulation with a high concentration of VEGF (Fig. 4C–E).

We examined whether the Foxo1-independent endothelial cell elongation induced by mTORC1 inhibitors depends on PI3K–Akt signaling. As co-treatment with 5 µM inhibitors severely impaired endothelial cell growth, we used a combination of inhibitors at the concentration of 1 µM in this experiment. Treatment with everolimus or rapamycin alone induced elongation of *Foxo1*$^{-/-}$ endothelial cells in the presence of a high concentration of VEGF (Fig. S2). Co-treatment with LY294002 or Akt inhibitor together with the mTORC1 inhibitors did not influence the endothelial cell elongation (Fig. S2), suggesting that PI3K–Akt signaling is not involved in the Foxo1-independent endothelial cell elongation induced by mTORC1 inhibitors under stimulation with a high level of VEGF.

The Foxo1-independent elongation of endothelial cells induced by mTORC1 inhibition prompted us to investigate whether Foxo1 influences the expression of subunits and regulatory factors of the mTOR complexes. The gene expression profile of *Foxo1*$^{+/+}$ and *Foxo1*$^{-/-}$ endothelial cells that were cultured in the presence or absence of VEGF was analyzed by using a DNA microarray assay. There was little or no difference in the expression levels of the mTORC subunits and the regulatory factors in *Foxo1*$^{+/+}$ and *Foxo1*$^{-/-}$ endothelial cells regardless of the presence of VEGF (Fig. 4F, Gene Expression Omnibus number GSE76366). This result suggests that Foxo1 is not involved in the transcriptional regulation of these genes.

### Dual inhibition of mTORC1 and mTORC2 fails to induce elongation of endothelial cells

The inhibition of mTORC1 by rapamycin induces a compensatory or indirect activation of Akt, which is a target of mTORC2, suggesting that mTORC1 negatively regulates mTORC2 (Hong-Brown et al., 2011, 2012; Julien et al., 2010; O'Reilly et al., 2006; Shi et al., 2005). To examine whether mTORC1 interacts with mTORC2 in the elongation of endothelial cells, the effect of KU0063794, a dual inhibitor of mTORC1 and mTORC2, was compared with the effect of everolimus. In this experiment, ESC-derived Flk-1-positive VPCs were treated with the inhibitors for the indicated length of time (1–3 days, Fig. 5A).

In the absence of exogenous VEGF, everolimus induced the elongation of wild-type endothelial cells regardless of the duration of treatment (Fig. 5B). This effect was accompanied by an increase in the length of branches of the thread-like endothelial cell colonies (Fig. 5C). The length of branches further increased when VEGF was exogenously added to the culture (Fig. 5B,C). Inhibition of mTORC1 and mTORC2 by KU0063794 is known to block the cell cycle, cell growth and cell survival (Zhang et al., 2013; Ayub et al., 2015). Consistent with this notion, endothelial cell colonies that were formed in the presence of KU0063794 were smaller than the colonies observed in the control cultures (Fig. 5B). The exposure to KU0063794 for 1 day slightly increased the length of branches of the thread-like endothelial cell colonies under stimulation with a high concentration of VEGF (Fig. 5C). This effect might reflect a dominant inhibition of mTORC1 over mTORC2 during the short exposure. However, longer exposure to KU0063794 failed to induce endothelial cell elongation regardless of the concentration of VEGF (Fig. 5C). Although biochemical examination of the downstream signaling targets of mTORC2 in the inhibitor-treated endothelial cells might be important, it was extremely difficult to purify a high enough number of endothelial cells from the co-culture system with OP9 feeder cells. Moreover, immunostaining of the mTORC2 targets such as phosphorylated PKCα failed to detect any quantitative change because of the low expression of the molecule and/or the low detectability by this method (data not shown).

In contrast to the wild-type endothelial cells, *Foxo1*$^{-/-}$ endothelial cells failed to respond to everolimus, and thus formed sheet-like colonies in the absence of exogenous VEGF (Fig. 5D). Addition of VEGF together with everolimus, however, induced the elongation of *Foxo1*$^{-/-}$ endothelial cells (Fig. 5D,E). The elongation was more remarkable when cells were exposed to everolimus for 2 days. The Foxo1-independent endothelial cell elongation was not significantly induced when everolimus was replaced by KU0063794 (Fig. 5D,E).

These results indicate that the dual inhibition of mTORC1 and mTORC2 is unable to mimic the specific inhibition of mTORC1 in endothelial cell elongation.

### The elongation of endothelial cells depends on the Rho–ROCK pathway

The Rho–ROCK pathway regulates the actin cytoskeleton of endothelial cells, thereby controlling angiogenesis (Chen et al., 2014; van Nieuw Amerongen et al., 2003; Zhao et al., 2006). In line with the proposed role of the Rho–ROCK pathway, inhibition of

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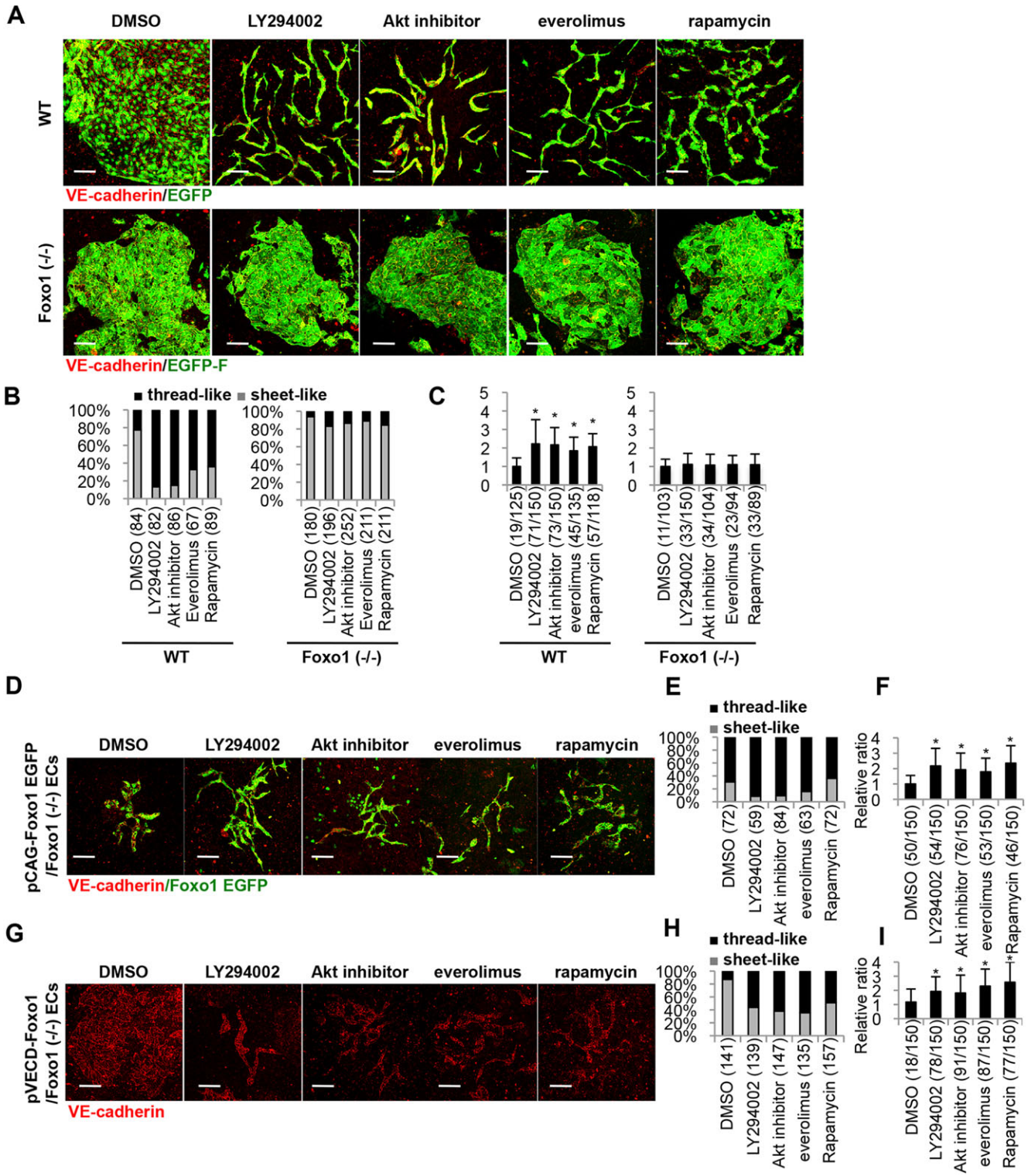


**Fig. 3.** See next page for legend.

ROCK activity by the specific inhibitor, thiazovivin, blocked the elongation of endothelial cells. Thiazovivin diminished the elongation of wild-type endothelial cells induced by Akt inhibitor and everolimus in the absence of exogenous VEGF (Fig. 6A). In addition, thiazovivin also blocked the elongation of *Foxo1*$^{-/-}$ endothelial cells induced by everolimus under stimulation with exogenous VEGF (Fig. 6B).

### Inhibition of mTORC1 induces vessel-like structures independently of Foxo1

To rule out the possibility that the chemical inhibitors influenced the OP9 cells and thereby indirectly modulated endothelial cell morphology, we assessed the effect of the inhibitors using an OP9-free culture system. Flk1$^{+}$ VPCs derived from *Foxo1*(+/+) or *Foxo1*$^{-/-}$ ESCs were aggregated and cultured in a type I collagen

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**Fig. 3. Elongation of endothelial cells depends on Foxo1.** VPCs derived from F10-EGFP/SM22-DsRed.T4 ESCs (WT) or EGFP-F/*Foxo1*$^{-/-}$ ESCs [Foxo1(-/-)] were seeded on an OP9 cell layer to induce endothelial cell differentiation. At 1 day after seeding, the indicated chemical inhibitors were added at a concentration of 5 µM. Cells were further cultured for 3 days. (A) Immunostaining of wild-type endothelial cell colonies derived from F10-EGFP/SM22-DsRed.T4 ESCs (red, VE-cadherin; green, EGFP) and *Foxo1*$^{-/-}$ endothelial cell colonies derived from EGFP-F/*Foxo1*$^{-/-}$ ESCs (red: VE-cadherin, green: EGFP-F). Scale bars: 100 µm. Gamma settings were adjusted for clearness of images. The experiment was repeated three times with similar results. (B) Proportion of the two types of endothelial cell colonies (thread-like and sheet-like) found in each group of cultures. Endothelial cell colonies generated in three independent experiments were analyzed. The total number of colonies examined is indicated in the brackets. (C) The length of branches of thread-like endothelial cell colonies in each group. Endothelial cell colonies generated in three independent experiments were analyzed. The total number of colonies and branches examined is indicated in the brackets (colonies/branches). The length is presented as a ratio relative to the DMSO control (mean±s.d.). **P*<0.05 versus DMSO control (ANOVA followed by Dunnett's test). (D) Immunostaining of endothelial cell (EC) colonies derived from *Foxo1*$^{-/-}$ ESCs that express Foxo1–EGFP under the control of the CAG promoter (red, VE-cadherin; green, Foxo1–EGFP). Scale bars: 100 µm. Gamma settings were adjusted for clearness of images. The experiment was repeated three times with similar results. (E) Proportion of the two types of endothelial cell colonies (thread-like and sheet-like) found in each group of cultures. Endothelial cell colonies generated in three independent experiments were analyzed. The total number of colonies examined is indicated in the brackets. (F) The length of branches of thread-like endothelial cell colonies in each group. Endothelial cell colonies generated in three independent experiments were analyzed. The total number of colonies and branches examined is indicated in the brackets (colonies/branches). The length is presented as a ratio relative to the DMSO control (mean±s.d.). **P*<0.05 versus DMSO control (ANOVA followed by Dunnett's test). (G) Immunostaining of endothelial cell colonies derived from a *Foxo1*$^{-/-}$ ESC clone that expresses Foxo1 under the control of the VE-cadherin promoter and enhancer (red, VE-cadherin). Scale bars: 100 µm. Gamma settings were adjusted for clearness of images. The experiment was repeated three times with similar results. (H) Proportion of the two types of endothelial cell colonies (thread-like and sheet-like) found in each group of cultures. Endothelial cell colonies generated in three independent experiments were analyzed. The total number of colonies examined is indicated in the brackets. (I) The length of branches of thread-like endothelial cell colonies in each group. Endothelial cell colonies generated in three independent experiments were analyzed. The total number of colonies and branches examined is indicated in the in the brackets (colonies/branches). The length is presented as a ratio relative to the DMSO control (mean±s.d.). **P*<0.05 versus DMSO control (ANOVA, followed by Dunnett's test).

gel in the presence of 10 ng/ml VEGF. *Foxo1*(+/+) VPCs generated a vessel-like structure consisting of endothelial cells and SMCs (Fig. 7A and data not shown). The endothelial cells had an elongated shape and showed fine actin filaments accumulated at the edge of the cells (Fig. 7A). As we have previously demonstrated (Park et al., 2009), *Foxo1*$^{-/-}$ VPCs formed a significantly shortened vessel-like structure consisting of short polygonal endothelial cells (Fig. 7Ba,C). The *Foxo1*$^{-/-}$ endothelial cells showed a dot-like accumulation of F-actin scattered over the cytoplasm (Fig. 7Bb,c).

The inhibition of PI3K–Akt signaling by LY294002 or Akt inhibitor did not influence the abnormal morphology and F-actin distribution in the *Foxo1*$^{-/-}$ endothelial cells (Fig. 7Bd–i). The inhibition of mTORC1 by rapamycin or everolimus restored the elongation of *Foxo1*$^{-/-}$ endothelial cells, thus inducing elongated vessel-like structures comparable with *Foxo1*$^{+/+}$ endothelial cells (Fig. 7Bj,m). The filamentous distribution of F-actin was also restored by these inhibitors (Fig. 7Bk,l,n,o). However, the positive effect of mTORC1 inhibition was impaired by inhibition of ROCK (Fig. 7Bv–x). In contrast to the mTORC1 inhibition, the dual inhibition of mTORC1 and mTORC2 by KU0063794 failed to restore the morphology of *Foxo1*$^{-/-}$ endothelial cells (Fig. 7Bp–r).

These findings mostly resembled the effects of the chemical inhibitors on the endothelial cells cultured on an OP9 cell layer.

## DISCUSSION

The purpose of the current study was to explore a signaling molecule that negatively regulates the elongation of endothelial cells. We established a culture system that could assess the morphology of live endothelial cells derived from ESCs. The endothelial cells were expected to elongate in the presence of chemical inhibitors that block the functions of the negative regulators of endothelial cell elongation. By screening a standardized library of chemical inhibitors, we found that the PI3K–Akt inhibitors (LY294002 and Akt inhibitor) and mTORC1 inhibitors (rapamycin and everolimus) induced endothelial cell elongation. These inhibitors did not influence the cytoskeleton of SMCs and OP9 cells at the same concentration, indicating that the effect of inhibitors was specific to endothelial cells. A limitation of this study is that the results rely mostly on the specificity of the chemical inhibitors. However, as the two different inhibitors of each signaling pathway showed the same effect on endothelial cell morphology, the possibility of non-specific effects might be excluded as a cause.

The ESC culture system with OP9 feeder cells has been reported to contain a low concentration of endogenous VEGF (38.5 pg/ml) that maintains endothelial cell colonies as sheet-like (Hirashima et al., 1999). However, endothelial cells have been shown to undergo morphological transformation to an elongated shape in response to exogenously added VEGF at a concentration higher than 3 ng/ml (Hirashima et al., 1999; Furuyama et al., 2004; Matsukawa et al., 2009; Tsuji-Tamura et al., 2011). It appears that the VEGF-dependent regulation of cell morphology is one of the characteristic features of endothelial cells. Interestingly, the PI3K–Akt inhibitors and the mTORC1 inhibitors induced endothelial cell elongation in the absence of exogenous VEGF. However, the sequestration of VEGF by a soluble decoy receptor diminished the endothelial cell elongation, indicating that the elongation still depended on the endogenous VEGF. An intrinsic physiological mechanism in endothelial cells was probably involved in the elongation induced by the chemical inhibitors. We showed that treatment with mTORC1 inhibitor for 1 day was enough to induce endothelial cell elongation. By using time-lapse imaging, it has been demonstrated that VEGF-induced endothelial cell elongation takes less than 20 h to complete (Matsukawa et al., 2009). These observations might indicate that the phenomenon does not involve differentiation or proliferation of endothelial cells. Thus, our results suggest that PI3K–Akt and mTORC1 negatively regulate endothelial cell elongation.

We have previously reported that Foxo1 is an essential transcription factor for the elongation of endothelial cells (Furuyama et al., 2004; Matsukawa et al., 2009). The requirement for Foxo1 in the endothelial cell elongation induced by PI3K/Akt and mTORC1 inhibition was confirmed, as *Foxo1*$^{-/-}$ endothelial cells failed to elongate in response to the inhibitors. It is well established that the PI3K–Akt signaling induces the phosphorylation of Foxo1, thereby suppressing its transcriptional activity (Brunet et al., 1999; Kau et al., 2004; Takaishi et al., 1999). Thus, inhibition of PI3K–Akt probably activates Foxo1 and induces the Foxo1-dependent elongation of endothelial cells. Although the signaling pathway that leads to the activation of the PI3K–Akt pathway was not identified in this study, the elongation of endothelial cells caused by PI3K–Akt inhibition is a predictable consequence.



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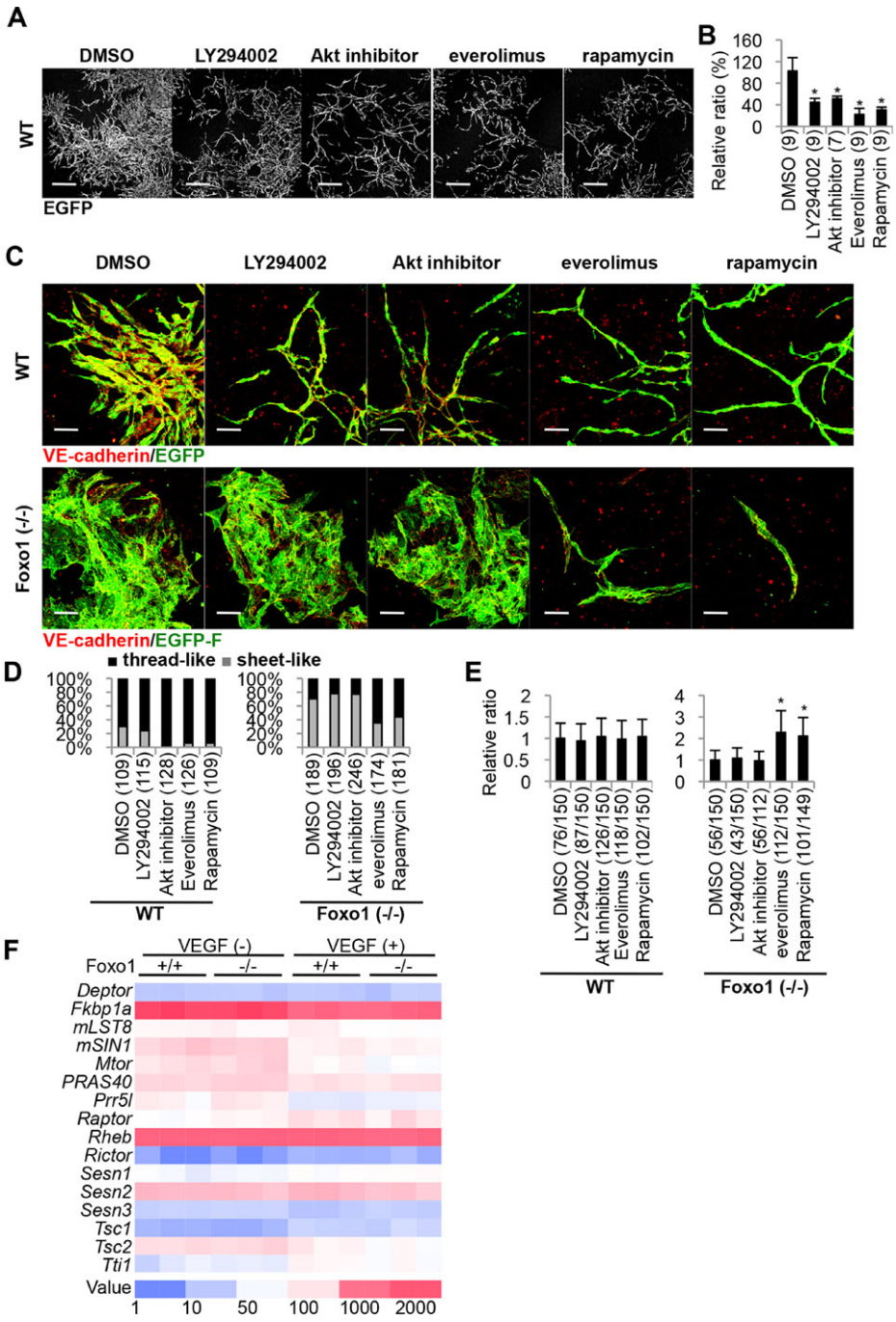


**Fig. 4. VEGF stimulation together with inhibition of mTORC1 induces endothelial cell elongation independently of Foxo1.** VPCs derived from F10-EGFP/SM22-DsRed.T4 ESCs (WT) or EGFP-F/*Foxo1*$^{-/-}$ ESCs [Foxo1(-/-)] were seeded on an OP9 cell layer and cultured in the presence of VEGF (10 ng/ml). At 1 day after seeding, the indicated chemical inhibitors were added at a concentration of 5 µM. Cells were further cultured for 3 days. (A) Immunostaining of wild-type endothelial cell colonies (gray, EGFP). Scale bars: 500 µm. Gamma settings were adjusted for clearness of images. The experiment was repeated three times with similar results. (B) Quantification of endothelial cell area in the field. The area is presented as a ratio (%) relative to the DMSO control. Endothelial cell cultures obtained in three independent experiments were analyzed. The total number of wells examined is indicated in the brackets. The data are mean±s.d. **P*<0.05 versus DMSO control (ANOVA followed by Dunnett's test). (C) Immunostaining of wild-type endothelial cell colonies (red, VE-cadherin; green, EGFP) and *Foxo1*$^{-/-}$ endothelial cell colonies (red, VE-cadherin; green, EGFP-F). Scale bars: 100 µm. Gamma settings were adjusted for clearness of images. The experiment was repeated three times with similar results. (D) Proportion of the two types of endothelial cell colonies (thread-like and sheet-like) found in each group of cultures. Endothelial cell colonies generated in three independent experiments were analyzed. The total number of colonies examined is indicated in the brackets. (E) The length of branches of thread-like endothelial cell colonies in each group. Endothelial cell colonies generated in three independent experiments were analyzed. The total number of colonies and branches examined is indicated in the brackets (colonies/branches). The length is presented as a ratio relative to the DMSO control (mean±s.d.). **P*<0.05 versus DMSO control (ANOVA followed by Dunnett's test). (F) Expression profiles of mTOR-related genes in endothelial cells derived from *Foxo1*(+/+) and *Foxo1*$^{-/-}$ ESCs. Endothelial cells were generated in the presence or absence of VEGF (10 ng/ml). Each row represents a gene, whereas each column represents an individual sample (*n*=3). Values of gene expression are depicted with a color scale, where blue represents a lower level of expression and red represents a higher level of expression (see the bottom row).

Abdelnour-Berchtold et al. have reported that mTORC1 inhibition by rapamycin enhances the phosphorylation of Akt proteins in human colon cancer cell lines (Abdelnour-Berchtold et al., 2010). The activated Akt induced the phosphorylation and inactivation of Foxo1, thereby attenuating the anti-proliferative effect of rapamycin. Their report has indicated the presence of an mTORC1–Akt–Foxo1 axis that regulates the proliferation of cancer cells. The action of the mTORC1–Akt–Foxo1 axis contradicts our finding that the rapamycin-induced endothelial cell elongation depended on Foxo1. We have previously reported that the proliferative activity of *Foxo1*$^{-/-}$ endothelial cells was comparable to that of wild-type endothelial cells, suggesting that Foxo1 is dispensable for the regulation of endothelial cell proliferation (Furuyama et al., 2004). If the major role of the mTORC1–Akt–Foxo1 axis is to regulate cell proliferation, this axis has a negligible impact on endothelial cells.

Conversely, PI3K–Akt signaling is known to activate mTORC1 (Song et al., 2012; Zoncu et al., 2011). Activated Akt phosphorylates tuberous sclerosis 2 (TSC2) and blocks the TSC1–TSC2–TBC1D7 complex from activating the GTPase activity of the small GTPase Rheb (Dibble et al., 2012; Inoki et al., 2002; Shimobayashi and Hall, 2014; Tee et al., 2003). As the GTP-bound form of Rheb binds mTOR and activates mTORC1, inhibition of TSC2 results in mTORC1 activation. Furthermore, Cao et al. have reported that phosphorylated Foxo1 directly associated with TSC2, thereby negatively regulating the function

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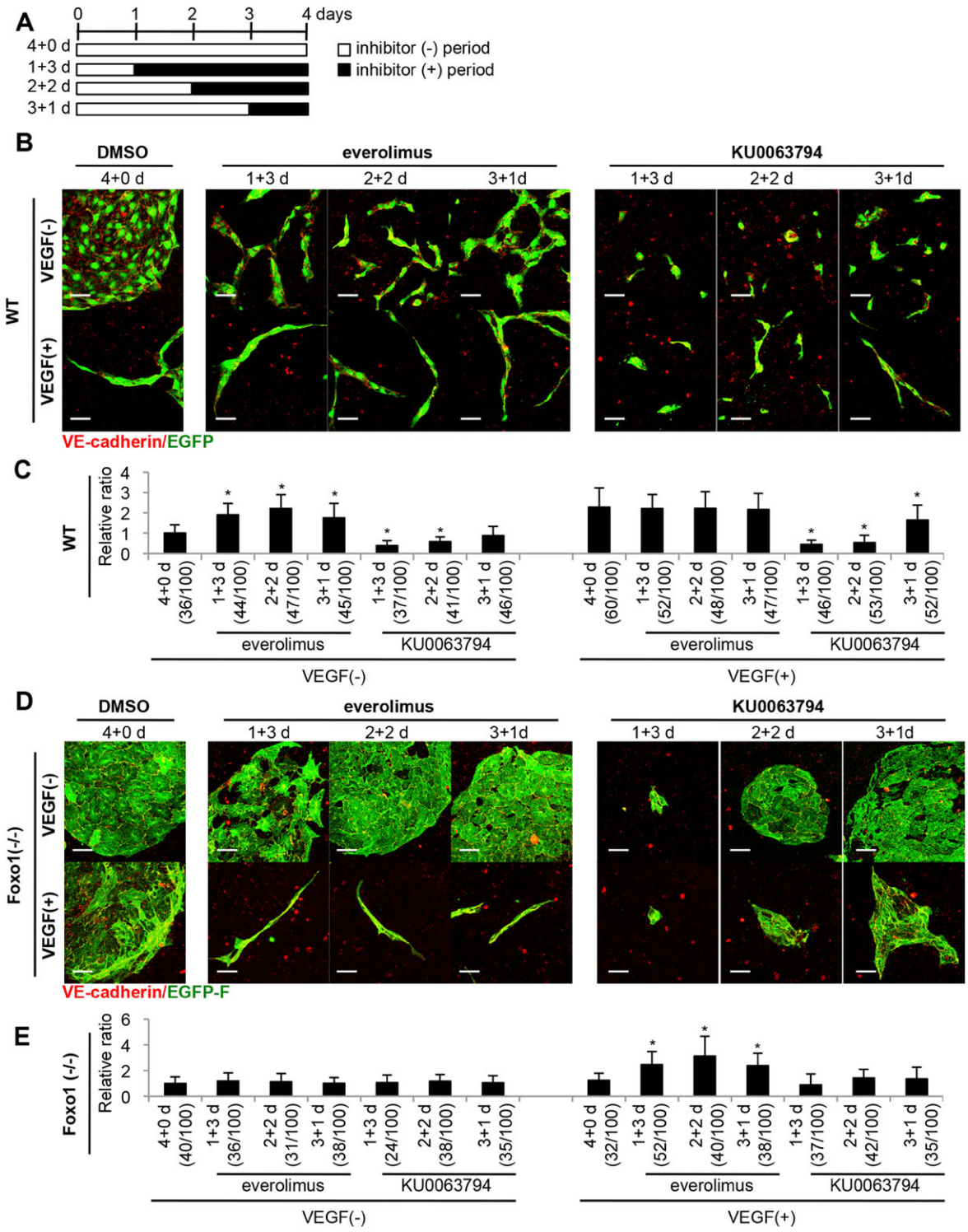


**Fig. 5. Dual inhibition of mTORC1 and mTORC2 fails to induce elongation of endothelial cells.** VPCs derived from F10-EGFP/SM22-DsRed.T4 ESCs (WT) or EGFP-F/*Foxo1*$^{-/-}$ ESCs [Foxo1(-/-)] were seeded on an OP9 cell layer and cultured in the presence or absence of VEGF (10 ng/ml). At 1 to 3 days after seeding, everolimus or KU0063794 was added at a concentration of 5 µM. Cells were cultured for a total of 4 days. (A) Experimental schedule of the treatment with the inhibitors. DMSO was added as a control. (B) Immunostaining of wild-type endothelial cell colonies (red, VE-cadherin; green, EGFP). Scale bars: 50 µm. Gamma settings were adjusted for clearness of images. The experiment was repeated two times with similar results. (C) The length of branches of thread-like endothelial cell colonies in each group. Endothelial cell colonies generated in two independent experiments were analyzed. The total number of colonies and branches examined is indicated in the brackets (colonies/branches). The length is presented as a ratio relative to the DMSO control (mean±s.d.). **P*<0.05 versus DMSO control (ANOVA followed by Dunnett's test). (D) Immunostaining of *Foxo1*$^{-/-}$ endothelial cell colonies (red, VE-cadherin; green, EGFP-F). Scale bars: 50 µm. Gamma settings were adjusted for clearness of images. The experiment was repeated two times with similar results. (E) The length of branches of thread-like endothelial cell colonies in each group. endothelial cell colonies generated in two independent experiments were analyzed. The total number of colonies and branches examined is indicated in the brackets (colonies/branches). The length is presented as a ratio relative to the DMSO control (mean±s.d.). *P*<0.05 versus DMSO control (ANOVA followed by Dunnett's test).

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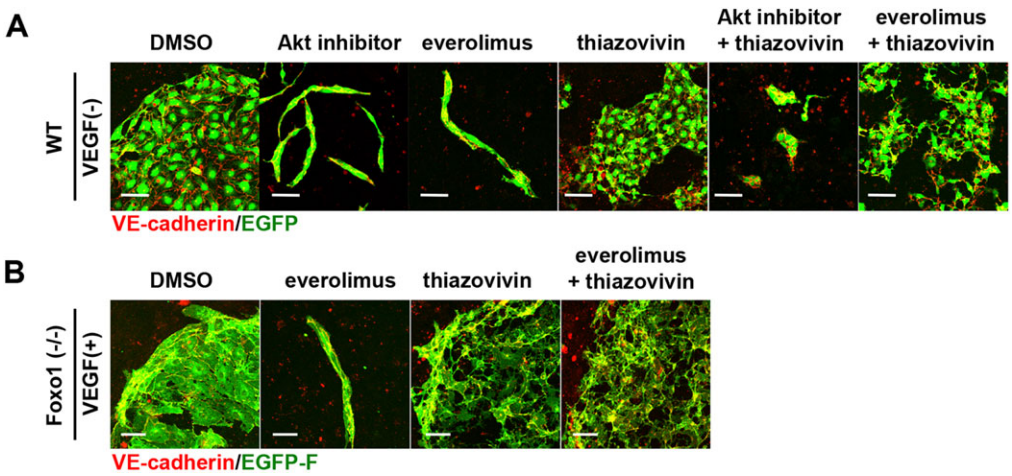


**Fig. 6. Elongation of endothelial cells depends on the Rho/ROCK pathway.** VPCs derived from F10-EGFP/SM22-DsRed.T4 ESCs (WT) or EGFP-F/*Foxo1*$^{-/-}$ ESCs [Foxo1 $^{-/-}$] were seeded on an OP9 cell layer and cultured in the presence or absence of VEGF (10 ng/ml). At 1 day after seeding, the indicated chemical inhibitors were added at a concentration of 5 µM. Cells were further cultured for 3 days. The experiment was repeated three times with similar results. (A) Immunostaining of wild-type endothelial cell colonies (red, VE-cadherin; green, EGFP). Scale bars: 50 µm. Gamma settings were adjusted for clearness of images. (B) Immunostaining of *Foxo1*$^{-/-}$ endothelial cell colonies (red, VE-cadherin; green, EGFP-F). Scale bars: 50 µm. Gamma settings were adjusted for clearness of images.

of TSC2 (Cao et al., 2006). The activation of mTORC1 is thus linked to the inactivation of Foxo1. Taken together, the activation of PI3K–Akt and mTORC1 signaling is suggested to impose an unfavorable influence on the Foxo1-dependent cellular events such as the elongation of endothelial cells.

Rapamycin and its derivatives are generally accepted as specific inhibitors of mTORC1. However, several reports have shown that prolonged treatment with rapamycin also inhibited the mTORC2 signaling in some cell types (Sarbassov et al., 2006; Zeng et al., 2007b). The mTORC2 signaling is known to phosphorylate and inactivate Foxo1 and Foxo3 through activation of Akt signaling (Guertin et al., 2006; Sarbassov et al., 2005; Zoncu et al., 2011). As the rapamycin-induced endothelial cell elongation depended on Foxo1, it is possible that the elongation was caused not only by the inhibition of mTORC1, but also by the inhibition of mTORC2. This possibility, however, is unlikely for two reasons. First, the major role of the mTORC2–Akt–Foxo1 axis has been proposed to be the regulation of cell proliferation and survival (Sarbassov et al., 2005; Zoncu et al., 2011). As Foxo1 plays only a minor role in the regulation of endothelial cell proliferation (Furuyama et al., 2004), the mTORC2–Akt–Foxo1 axis might not be well developed in the ESC-derived endothelial cells. Second, and more importantly, we found that the dual inhibition of mTORC1 and mTORC2 by treatment with KU0063794 failed to promote endothelial cell elongation. This finding suggests that mTORC2 needs to be active for the induction of endothelial cell elongation. The inactivation of mTORC1 by treatment with rapamycin or everolimus is known to induce the compensatory activation of mTORC2 in renal cell carcinoma (Rini and Atkins, 2009). In line with the inverse correlation between mTORC1 and mTORC2, Julien et al. have reported that the activation of ribosomal protein S6 kinase 1 (S6K1) by the mTORC1 signaling induced phosphorylation of rapamycin-insensitive companion of mTOR (Rictor), thereby inhibiting the mTORC2 signaling (Julien et al., 2010). Therefore, it is possible that the inhibition of mTORC1 leads to the activation of mTORC2 in the ESC-derived endothelial cells.

Several studies have explored the role of mTORC2 in the regulation of the cytoskeleton. mTORC2 has been reported to be necessary for F-actin polymerization and cell spreading of fibroblasts (Jacinto et al., 2004). Activation of the Rho GTPase Rac appears to mediate F-actin polymerization and lamellipodia formation downstream of mTORC2 signaling. Disruption of mTORC2 signaling has been shown to inactivate protein kinase Cα (PKCα) and enhance the formation of stress fibers in HeLa cells (Sarbassov et al., 2004). In contrast, inactivation of mTORC2 in endothelial cells leads to the inhibition of stress fiber formation and migration mediated by VEGF (Moss et al., 2010). Inhibition of endothelial cell migration appears to depend on an increase in the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (also known as CDKN1B), which blocks the activation of RhoA. Therefore, the function of mTORC2 in the regulation of the cytoskeleton might vary according to cell types. Recently, Wang et al. have demonstrated that *Rictor*-deficient endothelial cells fail to assemble and generate a network structure in response to VEGF in Matrigel cultures (Wang et al., 2015). They have also reported that the endothelial cell-specific deletion of the *Rictor* gene leads to embryonic lethality, suggesting that mTORC2 plays a crucial role in embryonic angiogenesis.

Taken together, mTORC2 appears to regulate angiogenesis by controlling the cytoskeletal organization in endothelial cells. Thus, we suggest that the elongation of endothelial cells induced by inhibition of mTORC1 is mediated by the compensatory activation of mTORC2 (Fig. 8).

In the current study, we found that stimulation with a high concentration of VEGF influenced the morphological response of *Foxo1*$^{-/-}$ endothelial cells to mTORC1 inhibitors. The VEGF stimulation together with the mTORC1 inhibition induced the elongation of *Foxo1*$^{-/-}$ endothelial cells, which would otherwise fail to elongate in the absence of one of them. The dual inhibitor of mTORC1 and mTORC2 did not induce the elongation of *Foxo1*$^{-/-}$ endothelial cells even under stimulation with a high dose of VEGF. Therefore, the elongation of *Foxo1*$^{-/-}$ endothelial cells seemed to also depend on mTORC2.

Several studies have proposed a possible regulation of mTORC2 expression by Foxo1. A constitutively active form of Foxo1 has been reported to increase the mRNA and protein levels of Rictor, thereby enhancing the mTORC2 activity in fibroblasts (Chen et al.,

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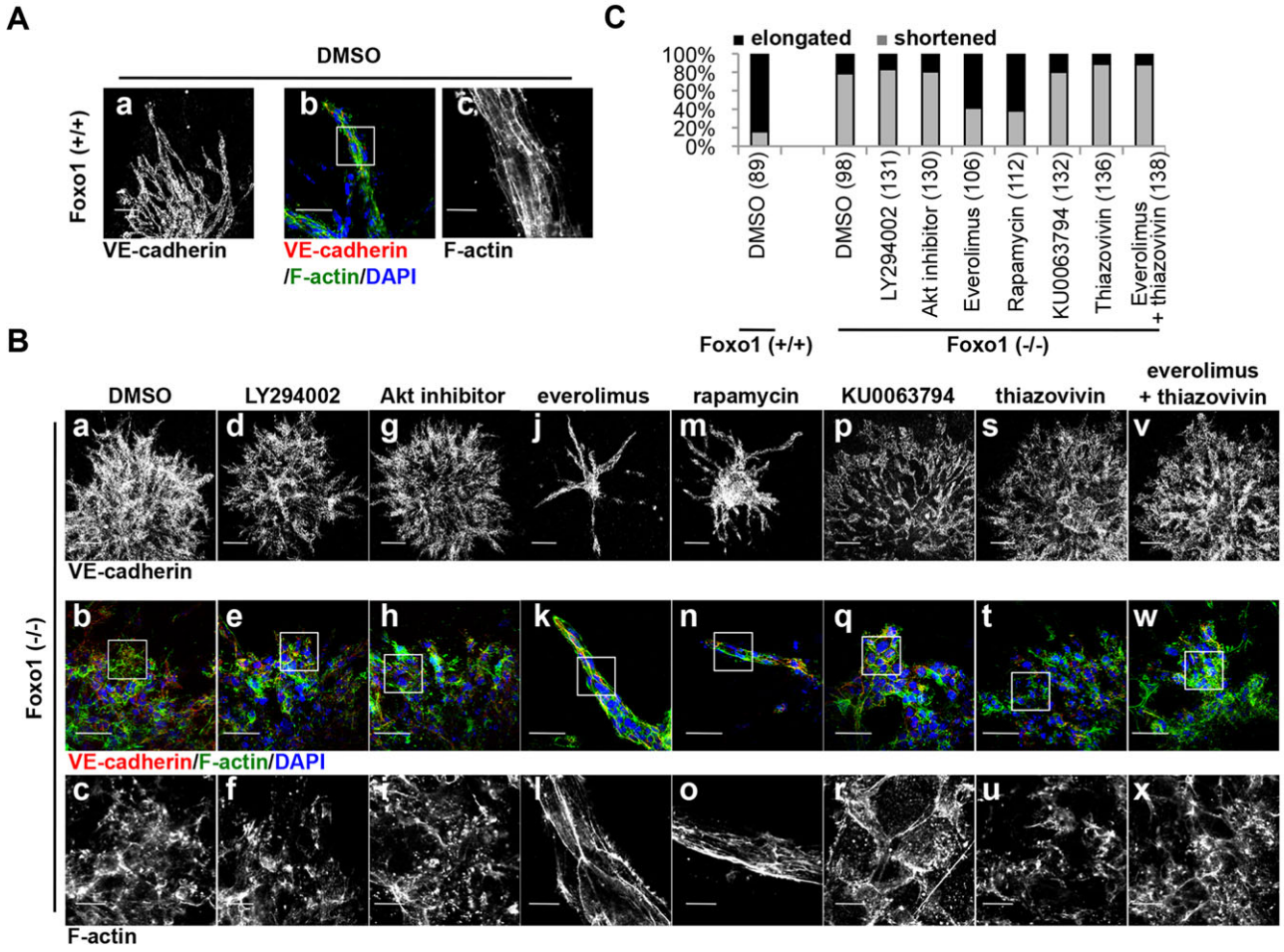


**Fig. 7. Inhibition of mTORC1 induces vessel-like structures independently of Foxo1.** VPCs derived from *Foxo1*$^{+/+}$ or *Foxo1*$^{-/-}$ ESCs were aggregated and cultured in type I collagen gel in the presence of VEGF (10 ng/ml). At 1 day after seeding, the indicated chemical inhibitors were added at a concentration of 0.5 µM. Cells were further cultured for 3 days. (A) Elongated vessel-like structures generated from wild-type VPCs. Cultures were stained with VE-cadherin antibody (gray in left panel, red in middle panel) and phalloidin (green in middle panel, gray in right panel) together with DAPI (blue in middle panel). The higher magnification of the boxed area in b is shown in c. Scale bars: 100 µm (a); 50 µm (b); 10 µm (c). Gamma settings were adjusted for clearness of images. The experiment was repeated three times with similar results. (B) Vessel-like structures generated from *Foxo1*$^{-/-}$ VPCs in the presence of inhibitors. Cultures were stained with anti-VE-cadherin antibody (gray in upper panels, red in middle panels) and phalloidin (green in middle panels, gray in lower panels) together with DAPI (blue in middle panels). The higher magnification of the boxed area in the middle panels is shown in the bottom panels. Note that the vessel-like structures generated in the presence of everolimus (j–l) or rapamycin (m–o) consist of elongated endothelial cells, whereas others consist of short polygonal endothelial cells. Scale bars: 100 µm (top panels); 50 µm (middle panels); 10 µm (bottom panels). Gamma settings were adjusted for clearness of images. The experiment was repeated three times with similar results. (C) Proportion of the two types of vessel-like structures (elongated and shortened) found in each group of cultures. Colonies generated in three independent experiments were analyzed. The total number of colonies examined is indicated in the brackets.

2010). Foxo1 has also been shown to induce the expression of sestrin 3, which inhibits mTORC1 activity (Chen et al., 2010; Nogueira et al., 2008). The inhibition of mTORC1 might, in turn, lead to the activation of mTORC2 in fibroblasts. It was thus possible that the expression of mTORC2 components is compromised in the *Foxo1*$^{-/-}$ endothelial cells, hence the endothelial cells failed to elongate. VEGF stimulation might somehow restore the expression of mTORC2, thereby giving endothelial cells the ability to elongate. However, our microarray analysis excluded this possibility, as *Foxo1*$^{-/-}$ endothelial cells expressed almost the same levels of mTORC1 and mTORC2 components as wild-type endothelial cells regardless of VEGF stimulation. Accordingly, our results suggest that the combined activation of VEGF and mTORC2 signaling triggers a new Foxo1-independent mechanism for the control of endothelial cell elongation (Fig. 8). The molecular basis of the mechanism was not addressed in this study and remains to be elucidated in future research.

In conclusion, we demonstrated that treatment with PI3K–Akt pathway or mTORC1 inhibitors induced the elongation of endothelial cells that were differentiated from ESCs. The cell elongation promoted the formation of a thread-like structure of endothelial cells that resembles angiogenic sprouting. This morphological response was induced without excessive VEGF stimulation, thus an overgrowth of endothelial cells could be avoided. Our findings thus have implications in settings where it is possible to induce physiological angiogenesis by stimulation with a minimal level of VEGF. Further studies need to be carried out in order to validate the effect of PI3K–Akt and mTORC1 inhibition using *in vivo* models such as retinal vascular development. In addition, PI3K–Akt or mTORC1 inhibition suppressed excessive endothelial cell proliferation and yet still induced endothelial cell elongation in the presence of a high dose of VEGF. In cancer treatment, the maturation and normalization of abnormal tumor vessels, rather than the inhibition of angiogenesis, has been proposed

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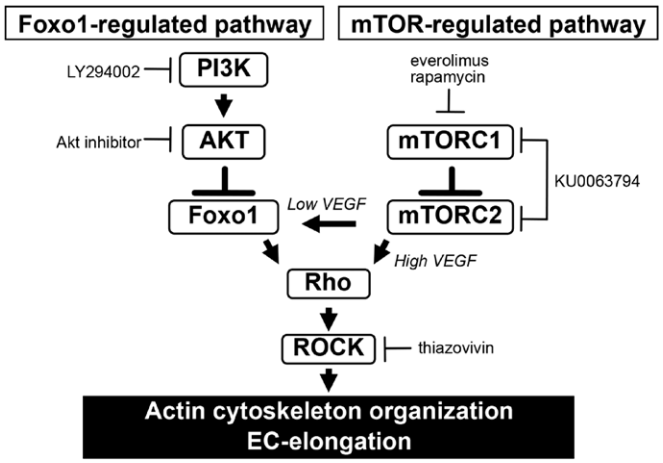


**Fig. 8. Schematic model of endothelial cell elongation regulated by the Foxo1 and mTOR pathways.** Foxo1 positively regulates the elongation of endothelial cells. As PI3K–Akt signaling inactivates Foxo1, treatment with PI3K–Akt inhibitors (LY294002, Akt inhibitor) induces endothelial cell elongation through Foxo1 activation (the Foxo1-regulated pathway). In the presence of a low level of VEGF (endogenous VEGF produced by OP9 feeder cells), mTORC2 induces endothelial cell elongation in a Foxo1-dependent manner. As mTORC1 is known to suppress the function of mTORC2, the inhibition of mTORC1 by treatment with everolimus or rapamycin induces endothelial cell elongation through a possible activation of mTORC2. KU0063794 treatment does not induce endothelial cell elongation, because it inhibits mTORC2 as well as mTORC1. It is not known whether Foxo1 is a direct downstream target of mTORC2 signaling. In the presence of a high level of VEGF (exogenously-added VEGF; 10 ng/ml in this study), mTORC2 induces endothelial cell elongation independently of Foxo1 (the mTOR-regulated pathway). In both pathways, Rho–ROCK signaling regulates the organization of the actin cytoskeleton, thereby promoting the endothelial cell (EC) elongation.

as a novel target for chemotherapy. Formation of functional and stable vessels can improve the delivery of antitumor drugs into tumor cells (Carmeliet and Jain, 2011; Goel et al., 2012). In this context, mTORC1 and mTORC2 would be candidate molecular targets for vessel normalization in the abundance of microenvironmental VEGF. It should also be noted that angiogenesis is not driven solely by endothelial cell elongation. Proliferation and migration of endothelial cells also play important roles in angiogenesis. Therefore, it is an important issue to develop an effective means to control proliferation, migration and morphological response of endothelial cells in a coordinated manner.

## MATERIALS AND METHODS

### Cells

KTPU8 ESCs (Nakahara et al., 2013), *Foxo1*$^{-/-}$ ESCs and parental *Foxo1*$^{+/+}$ ESCs (Furuyama et al., 2004) were used in this study. For ESC differentiation, the OP9 stromal cell line (Kodama et al., 1994) was used as feeder cells. Undifferentiated ESCs and OP9 cells were maintained as described previously (Park et al., 2009).

### Chemical inhibitors

The SCADS inhibitor kit was supplied by the Screening Committee of Anticancer Drugs, which was supported by the Scientific Support Programs for Cancer Research, the Ministry of Education, Culture, Sports, Science and Technology, Japan (http://scads.jfcr.or.jp/kit/index.html). LY294002 (PI3K inhibitor) and KU0063794 (mTOR inhibitor) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Akt inhibitor VIII, isozyme selective (Akti-1/2, an Akt1 and Akt2 inhibitor), was purchased from Calbiochem (La Jolla, CA, USA). Everolimus (mTOR inhibitor) was purchased from Selleck Chemicals (Houston, TX, USA). Rapamycin was purchased from AdipoGen Inc. (San Diego, CA, USA). Thiazovivin (ROCK inhibitor) was purchased from StemRD Inc. (Burlingame, CA, USA).

### Plasmid construction and transfection

An expression vector that carries the gene encoding the farnesylated form of enhanced green fluorescent protein (EGFP-F) was constructed as follows. An NheI/HindIII DNA fragment encoding EGFP-F was isolated from the pEGFP-F plasmid (Clontech, Palo Alto, CA, USA). The fragment was subcloned into the pPyCAGIRESzeocinpA expression vector, which consists of the CAG promoter (Niwa et al., 1991), an internal ribosome entry site (IRES), the zeocin resistance gene, and a globin poly(A) site. The EGFP-F expression vector was electroporated into *Foxo1*$^{-/-}$ ESCs. Zeocin-resistant clones (EGFP-F/*Foxo1*$^{-/-}$ ESCs) were expanded and screened for the expression of EGFP-F.

To construct an expression vector that carries a gene encoding a Foxo1–EGFP fusion protein, full-length coding sequences of murine Foxo1 and EGFP were amplified using primers designed for in-frame joining of the two genes. The primer sequences used were 5′-CTCGAGCCACCATGGCCG-AAGCGCCCCAGGTG-3′ (*Foxo1* forward), 5′-TCTAGAGCCTGACAC-CCAGCT-3′ (*Foxo1* reverse), 5′-TCTAGA GTGAGCAAGGGCGAGG-3′ (*EGFP* forward) and 5′-CCACCGCGGTGGTTACTTGTACAGCTCGT-CCATG-3′ (*EGFP* reverse). The joined *Foxo1–EGFP* cDNA was inserted into a cloning site of pCAGIPuro vector, which carries the CAG promoter, an IRES and the puromycin resistance gene (Niwa et al., 1991). The vector was electroporated into *Foxo1*$^{-/-}$ ESCs, followed by screening for the expression of EGFP in puromycin-resistant clones (Foxo1–EGFP-expressing *Foxo1*$^{-/-}$ ESCs).

An expression vector that carries the EGFP reporter under the control of the endothelial cell-specific F10-44 enhancer of the *Mef2c* gene (De Val et al., 2008) was constructed as follows. A synthesized oligonucleotide encoding the F10-44 enhancer element was ligated to the basal promoter of the *Hspa1b* gene, the EGFP cDNA and the SV40 poly(A) signal sequence by subcloning into pBlueScript (Stratagene, La Jolla, CA, USA). This F10/EGFP expression vector was co-transfected with pCAGIPuro into KTPU8 ESCs by electroporation. Puromycin-resistant clones (F10-EGFP ESCs) were induced to differentiate as described later. Expression of EGFP in endothelial cells was confirmed under an Eclipse TE300 fluorescence microscope (Nikon Co., Tokyo, Japan) (Tsuji-Tamura et al., 2011).

An expression vector that carries the DsRed.T4 reporter (Bevis and Glick, 2002) under the control of the SMC-specific promoter of the *SM22* gene (Akyurek et al., 2000) was constructed as follows. A 440-bp DNA fragment encoding the *SM22* promoter was isolated by PCR from C57BL/6 mouse genomic DNA. A full-length DsRed.T4 cDNA was isolated from the pDsRed.T4 plasmid. The *SM22* promoter was ligated to the DsRed.T4 cDNA and the SV40 poly(A) signal sequence by subcloning into pBlueScript. This SM22/DsRed.T4 expression vector was co-transfected with pPyCAGIRESzeocinpA into F10-EGFP/ESCs by electroporation. Zeocin-resistant clones (F10-EGFP/SM22-DsRed.T4 ESCs) were induced to differentiate as described later. Expression of EGFP and DsRed.T4 in endothelial cells and SMCs, respectively, was confirmed under an Eclipse TE300 fluorescence microscope.

### Induction of ESC differentiation

Flk-1-positive VPCs were induced from ESCs by culturing on an OP9 cell layer as previously described (Park et al., 2009). VPCs were isolated by fluorescence-activated cell sorting (FACS) using a Special Order FACSAria II cell sorter (BD Biosciences, San Jose, CA, USA).

### Screening system for modulators of endothelial cell morphology

The formation of endothelial cell colonies from ESC-derived Flk-1-positive VPCs on the OP9 feeder layer was originally described by Hirashima et al. (1999). We adapted the original method to a new screening system for the detection of chemicals that modulate endothelial cell morphology. Flk1-positive VPCs derived from F10-EGFP/SM22-DsRed.T4 ESCs were seeded (2000 cells per well) onto an OP9 cell layer, which was prepared in a 96-well glass-bottom culture plate (IWAKI, Tokyo, Japan), and cultured in induction medium (α-MEM, 10% FCS, 50 mM 2-mercaptoethanol) for

24 h. Chemical inhibitors (5 µM) were then added to the culture, and cells were further incubated for 3 days in the presence of the inhibitors. Fluorescence images of live endothelial cells (EGFP) and SMCs (DsRed. T4) were captured using an IN Cell Analyzer 6000 laser confocal imaging system (GE Healthcare, Buckinghamshire, UK). Developer Toolbox 1.9 was used to stitch the images that were separately captured from different fields of the same well. When necessary, to make a detailed observation of colonies, adjustments of contrast and threshold, and tone reversal were applied uniformly to the entire image of the original data. Endothelial cell colonies were classified into two types, the sheet-like colony and the thread-like colony. The sheet-like colony is a round colony composed of flat polygonal shaped endothelial cells. The thread-like colony is a scattered colony composed of long spindle shaped endothelial cells. The length of each branch of thread-like colonies was determined by converting the images into eight-bit images by Adobe Photoshop software (Adobe Systems, San Jose, CA, USA), followed by manual tracing of the branches using the line tool of the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The average length of branches in each group is presented as a ratio relative to that of DMSO control.

For detailed analysis of the cell biological effects of chemical inhibitors, Flk-1-positive VPCs were induced from several types of ESCs and cultured as described above. Recombinant murine VEGF (10 ng/ml) (Peprotech, Rocky Hill, NJ, USA) or recombinant mouse VEGFR1–Flt1-Fc chimera (0.2 µg/ml) (R&D Systems, Minneapolis, MN, USA) was added to some wells to explore the effects of VEGF signaling.

### Formation of vessel-like structures in 3D culture

Flk-1-positive VPCs were induced to form a vessel-like structure in a 3D culture as previously described (Park et al., 2009; Yamashita et al., 2000). In brief, Flk-1-positive VPCs were incubated at a density of $1.5\times10^5$ cells per well in a 96-well round-bottomed Sumilon cell-tight spheroid plate (Sumitomo Bakelite, Tokyo, Japan) with the induction medium containing 10 ng/ml VEGF. This step allowed cells to aggregate effectively. At 1 day after seeding, the aggregated cells were mounted in induction medium containing 1.5 mg/ml type I collagen (Nitta Gelatin, Osaka, Japan) and 10 ng/ml VEGF. The cell suspension was poured onto a plastic disc (Cell Desk LF; Sumitomo Bakelite), followed by the polymerization of collagen at 37°C. The collagen gel was cultured for 4 days in induction medium containing 10 ng/ml VEGF in the presence or absence of a chemical inhibitor (0.5 µM). For immunofluorescence staining of the vessel-like structures, the thickness of the collagen gel was reduced by absorbing medium using a filter paper.

### Immunofluorescence microscopy

Immunofluorescence staining of cell cultures was performed as previously described (Matsukawa et al., 2009; Park et al., 2009). Microscopy images were taken using an IN Cell Analyzer 6000 or an FV1000D confocal laser scanning microscope (Olympus, Tokyo, Japan). When necessary to make a detailed observation of cells, adjustments of the gamma setting were applied uniformly to the entire image of the original data.

### DNA microarray

*Foxo1*$^{+/+}$ ESCs or *Foxo1*$^{-/-}$ ESCs were seeded on an OP9 cell layer and cultured for 6.5 days in the induction medium containing 10 ng/ml VEGF. After culture, VE-cadherin- and CD31-positive endothelial cells were isolated by FACS as previously described (Matsukawa et al., 2009). Total RNA was extracted from the endothelial cells by using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Genome-wide gene expression profiles of the endothelial cells were obtained by Toray Industries (Tokyo, Japan) using the 3D-Gene Mouse Oligo chip 24K. The heat map images of the expression levels of each gene were generated using the R software (R Project, http://www.r-project.org/). Microarray data are deposited in the NCBI GEO database under accession number GSE76366.

### Statistical analysis

The results were first evaluated by a one-way analysis of variance (ANOVA) for significant differences among the means of samples, and then analyzed by Dunnett's multiple comparison tests using MEPHAS (http://www.gen-info.osaka-u.ac.jp/testdocs/tomocom/dunnett-e.html). Values of $P<0.05$ were considered statistically significant. All data are reported as mean±s.d.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

K.T-T. developed the research plans and the original hypothesis, performed all the experiments and analysis of data, and prepared the manuscript. M.O. prepared the manuscript, directed and supervised the project.

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#### Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.178434/-/DC1

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