VEGFR2-PLCγ1 axis is essential for endothelial specification of VEGFR2$^+$ vascular progenitor cells

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Summary

Vascular endothelial growth factor receptor 2 (VEGFR2) plays crucial roles in vasculogenesis, a process involving cell proliferation, migration and differentiation. However, the molecular mechanism by which VEGFR2 signaling directs vascular endothelial differentiation of VEGFR2$^+$ mesodermal progenitors is not well understood. In this study, we examined the signal transduction pathway downstream of VEGFR2 for endothelial differentiation using an in vitro differentiation system of mouse embryonic stem-cell-derived VEGFR2$^+$ cells. Using chimeric receptors composed of VEGFR2 and VEGFR3, the third member of the VEGFR family, we found that signaling through tyrosine 1175 (Y1175, corresponding to mouse Y1173) of VEGFR2 is crucial for two processes of endothelial differentiation: endothelial specification of VEGFR2$^+$ progenitors, and subsequent survival of endothelial cells (ECs). Furthermore, we found that phospholipase Cγ1 (PLCγ1), which interacts with VEGFR2 through phosphorylated Y1175, is an inducer of endothelial specification. In contrast to VEGFR2, VEGFR3 does not transmit a signal for endothelial differentiation of VEGFR2$^+$ cells. We found that VEGFR3 does not activate PLCγ1, although VEGFR3 has the ability to support endothelial cell survival. Taken together, these findings indicate that VEGFR2-PLCγ1 signal relay gives rise to the unique function of VEGFR2, thus enabling endothelial differentiation from vascular progenitors.

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Key words: VEGFR2, Endothelial differentiation, PLCγ1, Embryonic stem cells

Introduction

Blood vessel formation is one of the initial events of organogenesis. Vascular progenitor cells emerge in the posterior primitive streak as vascular endothelial growth factor receptor 2 (VEGFR2)-positive mesodermal cells, and migrate into the extra-embryonic yolk sac in response to vascular endothelial growth factor (VEGF)-A (Huber et al., 2004; Hiratsuka et al., 2005). These mesodermal precursor cells, the hemangioblasts, form a cell mass referred to as the blood islands, followed by in situ differentiation into endothelial cells (ECs) and hematopoietic cells (HCs). Outer cells lining the blood islands differentiate into ECs to generate the primitive vascular plexus (vasculogenesis), whereas inner cells develop into HCs (Choi et al., 1998). Following vasculogenesis, remodeling of the vasculature (angiogenesis) occurs. New capillaries are formed from pre-existing vessels through proliferation and migration of ECs and subsequently undergo maturation, accompanied by recruitment of mural cells (MCs) such as vascular smooth muscle cells and pericytes (Yancopoulos et al., 2000; Coultas et al., 2005).

VEGFR2 plays crucial roles in vasculogenesis and hematopoiesis, as indicated by the results of analysis of VEGFR2-null mice (Shalaby et al., 1995; Shalaby et al., 1997). These mice die between embryonic days (E) 8.5 and 9.5 due to lack of organized blood vessels and reduced HCs, because of the absence of blood islands. Tyrosine-1173 (Y1173) of mouse VEGFR2 is known to play an essential role in blood vessel formation in vivo. Sakurai et al. reported that VEGFR2 Y1173F knock-in mice died between E8.5 and E9.5 because VEGFR2$^+$ cells failed to migrate and form blood islands. These phenotypes are very similar to those of VEGFR2-null mice (Sakurai et al., 2005). Although these in vivo findings indicate the importance of VEGFR2 Y1173 in the formation of blood islands, the developmental events following the formation of blood islands, including endothelial specification, have not yet been fully elucidated.

For study of signal transduction in lineage specification, it is advantageous to use differentiating embryonic stem cells (ESCs) because migration of progenitor cells to the correct microenvironment is not required. An in vitro system for analysis of ligand-dependent endothelial differentiation has been established, using mouse VEGFR2$^+$ vascular progenitor cells derived from ESC (Hirashima et al., 1999; Yamashita et al., 2000; Ema et al., 2003; Watabe et al., 2003). In this system, ESC-derived VEGFR2$^+$ cells differentiate into platelet-endothelial cell adhesion molecule-1-positive (PECAM1$^+$) ECs upon stimulation with VEGF-A, or α smooth muscle actin-positive (αSMA$^+$) MCs in the presence of serum or platelet-derived growth factor-BB. Endothelial differentiation of ESC-derived VEGFR2$^+$ cells offers a model for endothelial developmental events in the outer cells that line blood islands (Yamashita et al., 2000). VEGFR2 appears to transmit a specific signal for endothelial differentiation of vascular progenitor cells, because signaling from either VEGFR1 or VEGFR3 fails to induce endothelial differentiation (Yamashita et al., 2000; Suzuki et al., 2005). However, the VEGFR2 signaling pathways directing endothelial specification remain largely unknown, although we recently demonstrated the involvement of Ras signaling in these pathways (Kawasaki et al., 2008).

In this study, we employed an expression system of chimeric receptors in mouse ESC-derived VEGFR2$^+$ vascular progenitor cells to examine the VEGFR2 signaling involved in endothelial

differentiation of VEGFR2$^+$ progenitor cells. We found that human VEGFR2 Y1175 is essential for induction of endothelial differentiation of VEGFR2$^+$ cells, through specification of VEGFR2$^+$ cells into ECs as well as subsequent survival of ECs. We also demonstrated that PLCγ1, which binds to phosphorylated Y1175 (Y1175-*P*) of VEGFR2, is an inducer of endothelial specification.

Results

Construction of a VEGFR3-VEGFR2 chimeric receptor

We previously found that ESC-derived VEGFR2$^+$ cells differentiate into endothelial cells upon signaling from VEGFR2 but not upon signaling from VEGFR3 (Suzuki et al., 2005). Further, VEGF-C(C152S), a selective ligand for VEGFR3 (Joukov et al., 1998), failed to induce endothelial differentiation of ESC-derived VEGFR2$^+$ cells (Suzuki et al., 2005). We therefore constructed a chimeric receptor (denoted R32) containing the extracellular domain of VEGFR3 fused with the transmembrane and intracellular domains of VEGFR2 (Fig. 1). Our aim was to examine the intracellular events downstream of VEGFR2 for endothelial differentiation through analysis of ESC-derived VEGFR2$^+$ cells expressing the chimeric receptor.

We first examined phosphorylation of R32 by immunoblotting using anti-VEGFR2 Y1054-*P* antibody (Fig. 2A). Y1054 is one of the major sites of the phosphorylation required for maximal kinase activity of VEGFR2 (Dougher and Terman, 1999). We confirmed phosphorylation of the amino acid residue in R32 corresponding to Y1054 in VEGFR2, indicating that R32 is kinase-active (Fig. 2A).

We next used a luciferase reporter assay to examine whether R32 transmits signals downstream. Elk1 is a transcriptional factor that is activated by extracellular signal-regulated kinase (ERK) (also known as mitogen-activated protein kinase; MAP kinase). Activation of Elk1 was monitored by luciferase activity under control of the promoter containing the 5X GAL4 binding site, which is activated by the GAL4 DNA-binding-domain–Elk-1 fusion protein. To stimulate the chimeric receptor, we used a supernatant of HEK 293T cells transfected with a VEGF-C(C152S) expression vector. Supernatants of HEK 293T cells transfected with empty vector or a VEGF-C(C152S) expression vector were denoted sMock and sVEGF-C(C152S), respectively. Luciferase activity was upregulated in cells expressing R32 or wild-type (wt) VEGFR3 upon treatment with sVEGF-C(C152S), but not sMock, indicating that R32 and VEGFR3 activated Elk1 upon ligand stimulation (Fig. 2B). Luciferase activity was increased in wt VEGFR2-expressing cells by treatment with recombinant VEGF-A but not with sVEGF-C(C152S) (Fig. 2B). sVEGF-C(C152S) thus selectively activated VEGFR3. Importantly, R32, when stimulated with sVEGF-C(C152S), induced luciferase activity to a degree comparable with that induced by VEGFR2 stimulated with VEGF-A (Fig. 2B).

Intracellular domain of VEGFR2 is sufficient to direct endothelial differentiation of VEGFR2$^+$ vascular progenitor cells

Because the signaling activity of R32 was confirmed, we next established MGZRTcH ES stable cell lines carrying tetracycline (Tc)-regulatable R32, VEGFR3 or empty vector (denoted Tc-R32, Tc-VEGFR3, and Tc-empty, respectively). In MGZRTcH cells, a gene of interest can be introduced into the *ROSA26* locus by means of the Cre-loxP system, and expression of the gene can be silenced by treatment with Tc (Masui et al., 2005).

ES cell lines were cultured for 4.5 days with Tc (1 μg/ml) for in vitro differentiation. VEGFR2$^+$ cells were then sorted from these cells, and cultured in SFO3 (a serum-free basal medium originally developed for culture of hematopoietic stem cells) with VEGF-A, sMock or sVEGF-C(C152S) in the absence of Tc (transgene-expressing condition). VEGFR2$^+$ cells derived from Tc-empty, Tc-R32 and Tc-VEGFR3 differentiated into PECAM1$^+$ cells in response to VEGF-A, indicating that these cells retain competence for VEGF-A-dependent differentiation into ECs (Fig. 2C). VEGFR2$^+$ cells derived from Tc-empty, Tc-R32 and Tc-VEGFR3 differentiated into αSMA$^+$ cells upon treatment with sMock, whereas those derived from Tc-R32, but not Tc-empty or Tc-VEGFR3, differentiated into PECAM1$^+$ cells upon stimulation with sVEGF-C(C152S) (Fig. 2C). The appearance of PECAM1$^+$ cells was inhibited by the co-presence of VEGFR3-Fc chimera protein, confirming the effect of VEGF-C(C152S) (Fig. 2D). These PECAM1$^+$ cells were also positive for other endothelial markers, including VE-cadherin, CD34 and endoglin (supplementary material Fig. S1). We therefore concluded that these PECAM1$^+$ cells represent ECs. Comparable levels of expression of R32 and VEGFR3 in differentiated states were confirmed by immunostaining using an antibody that recognizes the extracellular domain of VEGFR3 (data not shown). Therefore, the differences in phenotypes between VEGFR2$^+$ cells derived from Tc-R32 and Tc-VEGFR3 can be attributed to intrinsic properties of the intracellular domains of VEGFR2 and VEGFR3.

We next performed a colony formation assay to quantify the endothelial differentiation induced by R32 signaling (Fig. 2E).

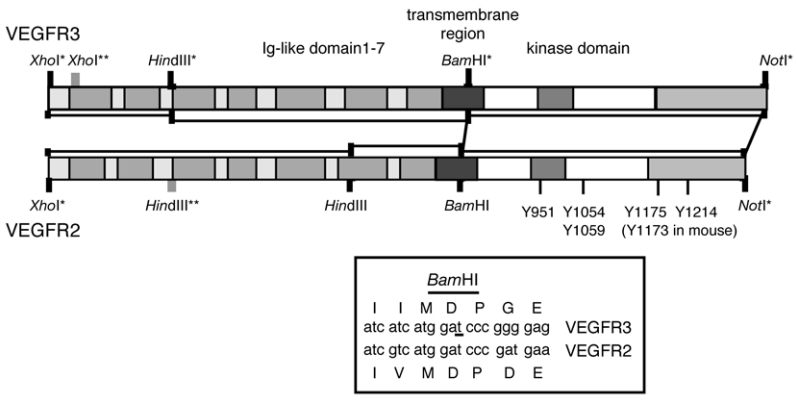

Fig. 1. Schematic illustration of R32 and its mutants. Restriction enzyme sites for *Xho*I, *Hin*dIII, *Bam*HI and *Not*I were generated or destroyed without changing the amino acid residues. Asterisks and double asterisks denote generated restriction sites and destroyed sites, respectively. Mutated tyrosine residues are also shown. Y1175 in human VEGFR2 corresponds to Y1173 in mouse VEGFR2. The nucleotide sequences as well as amino acid sequence around the *Bam*HI sites of VEGFR2 and VEGFR3 are shown in the box. The mutated base in VEGFR3 is underlined. The intracellular domain of VEGFR3 was swapped for that of VEGFR2 at the *Bam*HI sites of VEGFR2 and VEGFR3. The transmembrane region is shaded dark gray.

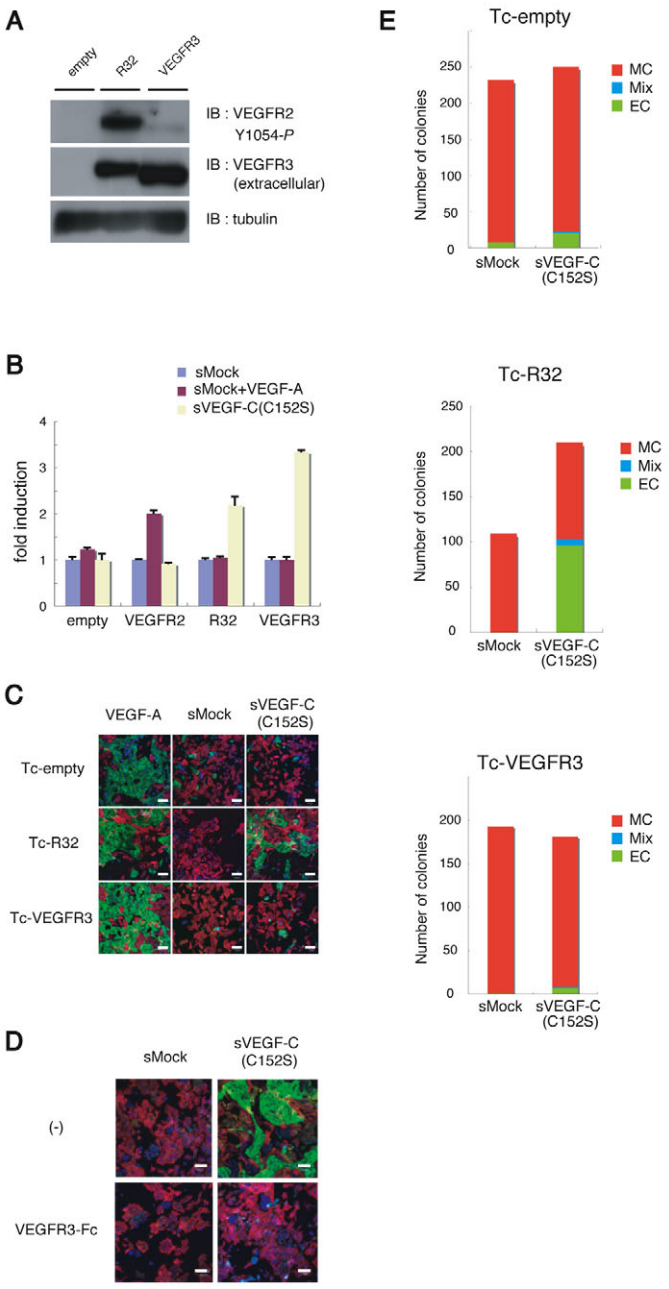

Fig. 2. Induction of endothelial cells from VEGFR2$^+$ cells by R32 signaling. (A) Phosphorylation of the R32 chimeric receptor. HEK 293T cells were transfected with the indicated plasmids and subjected to immunoblotting using anti-VEGFR2 Y1054-*P* antibody (top panel). The lower two panels show the expression of each protein as indicated. (B) Luciferase reporter assay to detect activation of Elk1 by VEGF receptors that were stimulated as indicated. HepG2 cells were used to achieve modest expression levels, at which ligand-independent activation of the receptors is avoided. sMock treatment did not enhance luciferase activity compared with that of non-treated cells. Flt1-Fc was not added to 'sMock + VEGF-A'. Error bars represent s.d. (C) Endothelial differentiation assay of VEGFR2$^+$ cells derived from Tc-empty, Tc-R32 and Tc-VEGFR3. Cells were immunostained for PECAM1 (green), αSMA (red) and nuclei (blue). Scale bars: 100 μm. (D) Endothelial differentiation assay of VEGFR2$^+$ cells derived from Tc-R32 in the absence or presence of VEGFR3-Fc (5 μg/ml). Cells were immunostained for PECAM1 (green), αSMA (red) and nuclei (blue). Scale bars: 100 μm. (E) Colony formation assay of VEGFR2$^+$ cells derived from Tc-empty, Tc-R32 or Tc-VEGFR3. The numbers of colonies of ECs, MCs or mixture of both were quantified. Representative data from two independent experiments are shown.

When treated with sMock, more than 95% of VEGFR2$^+$ cells derived from Tc-empty, Tc-R32 and Tc-VEGFR3 formed MC colonies. When treated with sVEGF-C(C152S), about 45% of VEGFR2$^+$ cells derived from Tc-R32 differentiated to form EC colonies. By contrast, EC colonies were only minimally induced from VEGFR2$^+$ cells derived from Tc-empty and Tc-VEGFR3. These findings indicate that the intracellular domain of VEGFR2 is sufficient to direct endothelial differentiation of ESC-derived VEGFR2$^+$ cells.

Construction of R32 mutants in which phosphorylated tyrosine residues were mutated to phenylalanine

To identify which tyrosine residue(s) in VEGFR2 are crucial for the induction of endothelial differentiation, five R32 mutants (R32Y951F, R32Y1054F/Y1059F, R32Y1175F, R32Y1214F and R32Y951F/Y1214F), in which tyrosine residues were mutated to phenylalanine, were constructed. Y951, Y1175 and Y1214 are major sites of phosphorylation of VEGFR2 (Matsumoto et al., 2005). Y951 and Y1175 are unique tyrosine residues in VEGFR2, whereas Y1214 is conserved in VEGFR2 and VEGFR3. Autophosphorylation of these mutants was confirmed, except for Y1054F/Y1059F, which was used as a negative control (Fig. 3A). We next examined signal transduction of these mutants by monitoring activation of Elk1 (Fig. 3B). R32Y951F, R32Y1214F and R32Y951F/Y1214F transactivated Elk1 reporter activities after stimulation with sVEGF-C(C152S), whereas R32Y1175F did not. This was consistent with the previous report that signaling from Y1175 leads to activation of ERK (Takahashi et al., 2001). However, because autophosphorylation of R32Y1175F was detected (Fig. 3A), we regarded R32Y1175F as a kinase-active receptor and proceeded with subsequent experiments.

Signaling through Y1175 is indispensable for endothelial differentiation of VEGFR2$^+$ vascular progenitor cells

We next established stable ES cell lines carrying a Tc-regulatable R32Y951F, R32Y1175F or R32Y1214F (Tc-Y951F, Tc-Y1175F and Tc-Y1214F). VEGFR2$^+$ cells derived from these cell lines were sorted and cultured in SFO3 with VEGF-A, sMock, or sVEGF-C(C152S). These cells also exhibited competence for endothelial differentiation in response to VEGF-A (Fig. 3C). Upon stimulation with sVEGF-C(C152S), VEGFR2$^+$ cells derived from Tc-Y951F and Tc-Y1214F differentiated into ECs, whereas those from Y1175F failed to do so (Fig. 3C). To exclude the possibility that signals from Y951 and Y1214 compensate for each other in endothelial differentiation, we also established a stable cell line carrying Tc-regulatable R32Y951F/Y1214F (Tc-Y951F/Y1214F). VEGFR2$^+$ cells derived from this cell line did not lose the ability to differentiate into ECs upon stimulation with sVEGF-C(C152S) (Fig. 3C). Comparable levels of expression of these chimeric receptors were confirmed by immunostaining in differentiated states (data not shown). These findings indicate that signals from Y951 and Y1214 are not required for endothelial differentiation but that signaling from Y1175 are required for it. We performed a colony formation assay using these cells (Fig. 3D). When treated with sMock, more than 98% of VEGFR2$^+$ cells derived from the four mutant cell lines formed MC colonies. However, when treated with sVEGF-C(C152S), 25-55% of VEGFR2$^+$ cells derived from Tc-Y951F, Tc-Y1214F or Tc-Y951F/Y1214F differentiated to form EC colonies. In contrast, EC colonies were rarely formed from VEGFR2$^+$ cells derived from Tc-Y1175F, indicating that signaling through Y1175 is essential for endothelial differentiation.

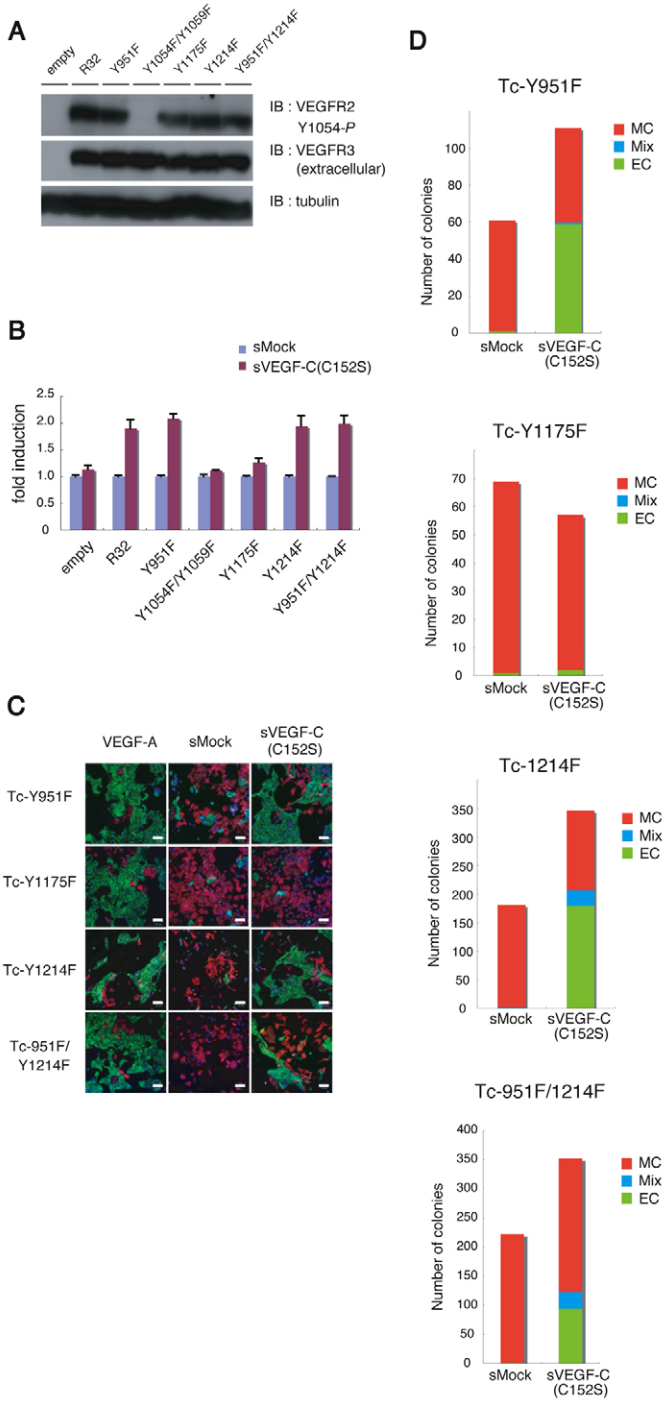

Fig. 3. Y1175F mutation results in failure of R32 to induce endothelial differentiation of VEGFR2$^+$ cells. (A) Phosphorylation of R32 chimeric receptor mutants. HEK 293T cells were transfected with the indicated plasmids and subjected to immunoblotting using anti-VEGFR2 Y1054-*P* antibody (top panel) and anti-VEGFR3 extracellular domain (middle panel). α-tubulin was used as a loading control (bottom panel). R32Y1054F/Y1059F was used as a negative control. (B) Luciferase reporter assay to detect activation of Elk1 by R32 mutants. Error bars represent s.d. (C) Endothelial differentiation assay of VEGFR2$^+$ cells derived from Tc-Y951F, Tc-Y1175F, Tc-Y1214F and Tc-Y951F/Y1214F. Cells were immunostained for PECAM1 (green), αSMA (red) and nuclei (blue). Scale bars: 100 μm. (D) Colony formation assay of VEGFR2$^+$ cells derived from Tc-R32 mutants. The numbers of colonies of ECs, MCs or mixture of both were quantified. Representative data from two independent experiments are shown.

Signaling from Y1175 plays crucial roles in the survival of ECs

Endothelial differentiation requires two processes: specification from VEGFR2$^+$ progenitor cells into ECs, and subsequent survival/proliferation of ECs. We hypothesized that signaling from Y1175 plays roles in either or both processes. To test this hypothesis, endothelial survival assay was performed (Fig. 4).

We knocked down endogenous mouse VEGFR3 in order to exclude effects of signaling from endogenous VEGFR3, which is expressed in ESC-derived ECs (Suzuki et al., 2005). When ESC-derived ECs were cultured in the absence of sVEGF-C(C152S), cell number was markedly decreased within 12 hours (Fig. 4A-C). Cell number was restored in the case of ECs expressing R32 by stimulation with sVEGF-C(C152S) irrespective to the expression of endogenous mouse VEGFR3 (Fig. 4A). In contrast, cell number was not restored in ECs expressing R32Y1175F by stimulation with sVEGF-C(C152S) when endogenous mouse VEGFR3 was knocked down (Fig. 4B). These findings indicate that Y1175 is involved in the transmission of survival signals for ECs. We also examined the effect of VEGFR3 signaling on the survival of ECs (Fig. 4C). We observed increase in survival of ECs expressing transgenic human VEGFR3 by sVEGF-C(C152S), indicating that VEGFR3 also transmits survival signals for ECs. Survival of ESC-derived ECs

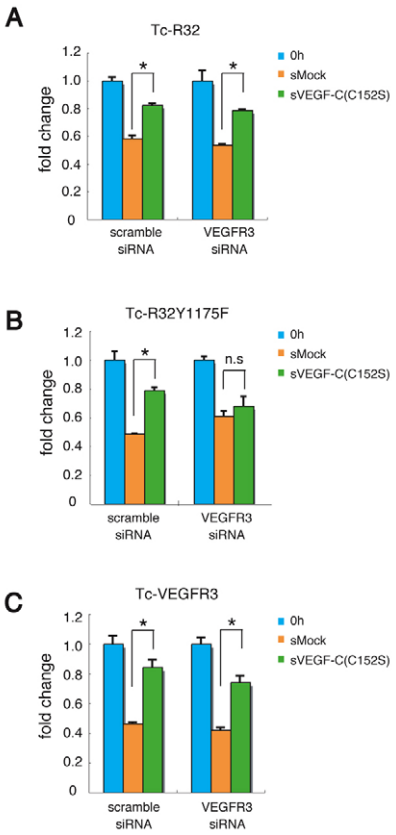

Fig. 4. Signaling through VEGFR2 Y1175 supports survival of ECs. Survival assay for ECs expressing R32 (A), R32Y1175F (B) or human VEGFR3 (C). Cells were treated with sMock or sVEGF-C(C152S). Cell numbers are shown as fold-changes relative to those at the point of medium change (0h). Values are the means ± s.d. **P*<0.05 (Student's *t*-test); n.s., not significant. Endogenous mouse VEGFR3 was knocked down to exclude effects of endogenous signaling. Efficiency of knockdown was measured by quantitative RT-PCR as follows: ECs expressing R32, 60%; ECs expressing R32Y1175F, 40%; ECs expressing VEGFR3, 40%.

by sVEGF-C(C152S) was abrogated in the presence of LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K) (supplementary material Fig. S2), suggesting that the PI3K pathway is involved in endothelial survival by VEGFR3.

Neither R32Y1175F nor VEGFR3 is able to transmit signal sufficient for endothelial differentiation. VEGFR3, however, transmits survival signal for ECs, in contrast to R32Y1175F. The present in vitro system is thus suitable for analysis of each of the individual processes involved in endothelial differentiation.

Signaling from Y1175 plays crucial roles in endothelial specification of VEGFR2$^+$ vascular progenitor cells

We next investigated the involvement of signaling from Y1175 in endothelial specification of ESC-derived VEGFR2$^+$ cells. To do so, we examined differentiation of VEGFR2$^+$ cells derived from Tc-Y1175F into ECs under culture conditions that support the survival of ECs. Fibroblast growth factor 2 (FGF-2) was used to support the survival of ECs. We first examined survival of ECs in the presence of FGF-2, and found that 0.5 ng/ml was sufficient to maintain the survival of ECs (data not shown). We next examined endothelial differentiation of VEGFR2$^+$ cells in the presence of FGF-2 (0.5 ng/ml). The majority of VEGFR2$^+$ cells derived from both Tc-R32 and Tc-Y1175F differentiated into MCs upon treatment with sMock (Fig. 5). Upon stimulation with sVEGF-C(C152S), significant endothelial differentiation was observed for Tc-R32-derived VEGFR2$^+$ cells, but not for Tc-Y1175F-derived VEGFR2$^+$ cells (Fig. 5). Signaling from Y1175 thus appears to direct endothelial specification of ESC-derived VEGFR2$^+$ cells.

PLCγ1, which interacts with phosphorylated Y1175, is an inducer of endothelial specification

PLCγ1 has been reported to play roles in cell proliferation through interaction with Y1175-*P* in VEGFR2 (Takahashi et al., 2001; Takahashi and Shibuya, 1997). PLCγ1 deficiency in mice is lethal because vasculogenesis and erythrogenesis do not occur, though hemangioblasts appear to be present (Liao et al., 2002). We hypothesized that R32Y1175F fails to induce endothelial differentiation from ESC-derived VEGFR2$^+$ cells because PLCγ1 is not activated. To elucidate the function of PLCγ1 in endothelial differentiation, we constructed a constitutively active form of PLCγ1, PalmPLCγ1, which has an additional sequence for myristoylation and palmitoylation in its N-terminus (Veri et al., 2001). We established a stable ES cell line carrying Tc-regulatable PalmPLCγ1 (Tc-PalmPLCγ1). These cells exhibited competence for endothelial differentiation in response to VEGF-A (Fig. 6A).

Expression of PalmPLCγ1 resulted in the appearance of PECAM1$^+$ cells from ESC-derived VEGFR2$^+$ cells in the presence, but not the absence, of FGF-2 (Fig. 6A). These PECAM1$^+$ cells induced by PalmPLCγ1 and FGF-2 were also positive for other endothelial markers, such as VE-cadherin, CD34 and endoglin (supplementary material Fig. S3), indicating that they represent ECs. We performed a colony formation assay for quantification of endothelial differentiation induced by PalmPLCγ1 and FGF-2. In the absence of survival signals, expression of PalmPLCγ1 did not affect induction of EC colonies, whereas in the presence of such signals PalmPLCγ1 drastically induced formation of EC colonies (Fig. 6B). Stimulation with PalmPLCγ1 and FGF-2 thus reconstituted signaling for endothelial differentiation of ESC-derived VEGFR2$^+$ progenitor cells. Because induction of endothelial differentiation by PalmPLCγ1 was observed only in the presence of survival signal for ECs, PalmPLCγ1 appears to direct endothelial specification but not to promote the survival of ECs. We also performed a cell survival assay (Fig. 6C). Numbers of ECs expressing PalmPLCγ1 were decreased to levels comparable to those of control ECs in the absence of VEGF-A in culture (Fig. 6C). These findings indicate that PLCγ1 signaling is involved in endothelial specification of VEGFR2$^+$ cells but not in the survival of ECs.

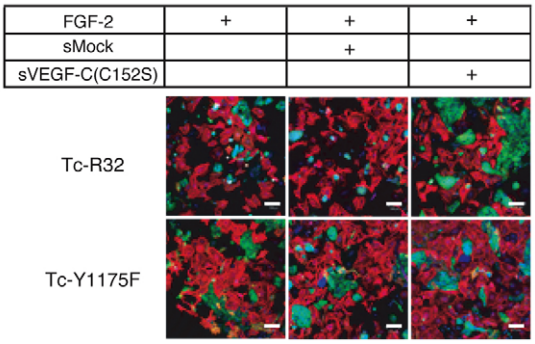

Fig. 5. Signaling through VEGFR2 Y1175 is indispensable for endothelial specification of VEGFR2$^+$ cells. Endothelial differentiation assay of VEGFR2$^+$ cells derived from Tc-R32 and Tc-Y1175F in αMEM supplemented with 10% FBS in the presence FGF-2, and sMock or sVEGF-C(C152S). Cells were then fixed and immunostained for PECAM1 (green), αSMA (red) and nuclei (blue). Scale bars: 100 μm.

We next established a stable ES cell line in which expression of PLCγ1 can be knocked down by microRNA (miRNA) under the control of Tc (Tc-miRNA-PLCγ1). Expression of miRNA targeting PLCγ1 resulted in modest decrease of PLCγ1 expression (Fig. 6D, right) and decreased appearance of ECs (Fig. 6D, left), thus confirming the important role of PLCγ1 in endothelial differentiation.

We previously reported that a farnesyltransferase inhibitor, FTI-277, suppressed VEGF-A-induced endothelial specification of ESC-derived VEGFR2$^+$ vascular progenitor cells (Kawasaki et al., 2008). In this study, we found that FTI-277 also suppressed the endothelial differentiation induced by PalmPLCγ1 plus FGF-2 (Fig. 6E). Because FTI-277 did not inhibit FGF-2-induced survival of ECs (data not shown), the FTI-277-sensitive process is probably located downstream of PLCγ1.

Signaling from VEGFR3 fails to induce endothelial differentiation due to lack of PLCγ1 activation

As described above, VEGFR3 signaling supports the survival of ECs (Fig. 4C) but does fail to induce endothelial differentiation (Fig. 2C). We therefore anticipated that VEGFR3 would not activate PLCγ1. We found that activation of R32 by VEGF-C induced phosphorylation of PLCγ1, whereas activation of VEGFR3 by VEGF-C did not induce phosphorylation of PLCγ1 (Fig. 6F). Collectively, these findings indicate that the differences in ability to induce endothelial differentiation between VEGFR2 and VEGFR3 can be attributed to the differences between them in activation of PLCγ1. These findings further accentuate the crucial role of PLCγ1 in endothelial specification.

Discussion

The development of blood vessels requires orchestrated behavior of cells, including proliferation, migration and differentiation. These complex dynamics are mediated through a variety of receptor-dependent signaling pathways. Of the signaling pathways that

regulate the formation and maturation of blood vessels, VEGFR2 signaling is one of the most important (Olsson et al., 2006). VEGFR2 plays essential roles in the migration of VEGFR2$^+$ cells from the primitive streak to the extra-embryonic yolk sac (Huber et al., 2004; Hiratsuka et al., 2005), in situ differentiation into both HCs and ECs (Choi et al., 1998), and subsequent vascular remodeling processes (Yancopoulos et al., 2000; Coultas et al., 2005). In this study, we used ESC-derived VEGFR2$^+$ cells to examine the molecular relay of VEGFR2 signaling that leads to endothelial differentiation. With this experimental system, we found that, among the major sites of phosphorylation of VEGFR2, Y1175 is indispensable for endothelial differentiation, whereas Y951 and Y1214 are not. We also found that signaling from Y1175 is required in two processes of endothelial differentiation: that which specifies the endothelial fate of VEGFR2$^+$ cells, and that which maintains the survival of ECs. Sakurai et al. previously reported that migration of VEGFR2$^+$ cells from the primitive streak to the yolk sac is impaired in knock-in mice, in which Y1173 of VEGFR2 has been substituted with phenylalanine (Y1173 of mouse VEGFR2 corresponds to Y1175 of human VEGFR2) (Sakurai et al., 2005). This defect results in accumulation of these cells in the allantois and amnion, and lack of blood island formation in the yolk sac (Sakurai et al., 2005). This report and the findings of the present study indicate that VEGFR2 Y1175 is involved in the formation of blood islands through effects on the migration, subsequent endothelial specification and survival of ECs. These findings indicate that signaling from Y1175 plays diverse and important roles in VEGFR2-dependent endothelial differentiation.

The roles of the major sites of phosphorylation in VEGFR2 have been investigated in 'mature' ECs. Y951 is involved in actin stress fiber organization and migration through interaction with an adaptor molecule, TSAd (T-cell-specific adaptor) (Matsumoto et al., 2005; Wu et al., 2000; Zeng et al., 2001). TSAd-deficient mice exhibited decreased tumor growth rate due to reduced vascularization in an in vivo pathological angiogenesis model (Matsumoto et al., 2005). Y1214 is implicated in actin polymerization and reorganization through activation of Cdc42 and p38 (Lamalice et al., 2004; Lamalice et al., 2006). However, Y1212F knock-in mice are viable and fertile (Sakurai et al., 2005), consistent with our finding that Y1214 does not play a role in endothelial differentiation. Phosphorylation of Y1175 is known to result in recruitment and activation of PLCγ, followed by Ras-independent activation of ERK via protein kinase C (PKC), leading to enhancement of cell

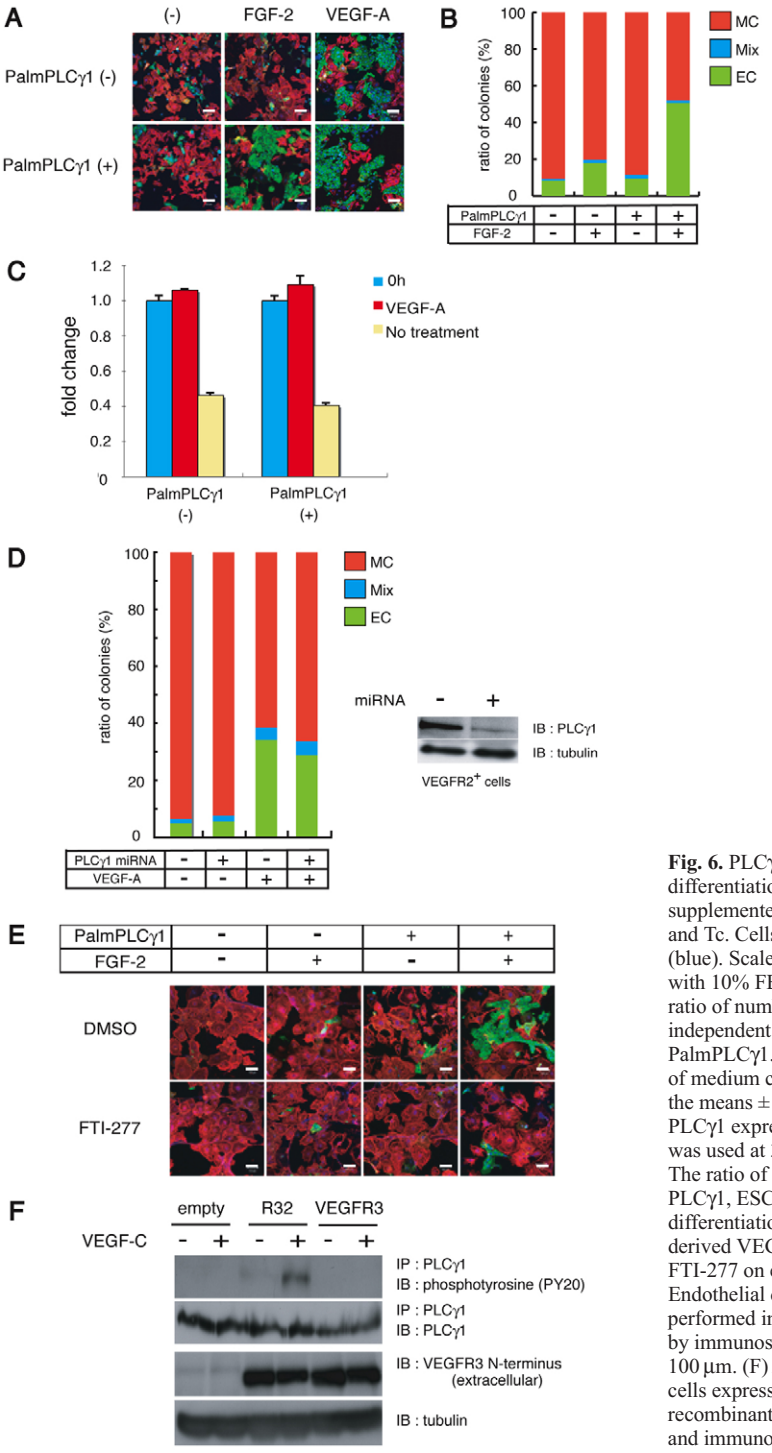

Fig. 6. PLCγ1 is an inducer of endothelial specification. (A) Endothelial differentiation assay of VEGFR2$^+$ cells derived from Tc-PalmPLCγ1 in αMEM supplemented with 10% FBS in the presence (+) or absence (-) of FGF-2, VEGF-A and Tc. Cells were immunostained for PECAM1 (green), αSMA (red) and nuclei (blue). Scale bars: 100 μm. (B) Colony formation assay in αMEM supplemented with 10% FBS. Colonies of ECs, MCs and mixtures of both were quantified. The ratio of number of colonies is displayed in the graph. Representative data from three independent experiments are shown. (C) Survival assay for ECs expressing PalmPLCγ1. Cell numbers are shown as fold-changes relative to those at the point of medium change (0h). Cells were treated with VEGF-A or not treated. Values are the means ± s.d. (D) Colony formation assay of Tc-miR-PLCγ1 cells in which PLCγ1 expression was knocked down by miRNA in the absence of Tc. VEGF-A was used at 30 ng/ml. Colonies of ECs, MCs and mixtures of both were quantified. The ratio of number of colonies is displayed in the graph (left). For knockdown of PLCγ1, ESCs were cultured in the absence of Tc for the last 2 days of in vitro differentiation to induce expression of miRNA. Knockdown efficiencies in ESC-derived VEGFR2$^+$ cells were examined by immunoblotting (right). (E) Effect of FTI-277 on endothelial differentiation induced by PalmPLCγ1 and FGF-2. Endothelial differentiation assay in αMEM supplemented with 10% FBS was performed in the presence or absence of FGF-2, Tc and FTI-277 (3 μM), followed by immunostaining for PECAM1 (green), αSMA (red) and nuclei (blue). Scale bars: 100 μm. (F) Activation of PLCγ1 by ligand-stimulated R32 or VEGFR3. HEK 293T cells expressing R32 or VEGFR3 were starved for 14 hours and stimulated with recombinant VEGF-C (400 ng/ml) for 5 minutes, followed by immunoprecipitation and immunoblotting to detect phosphorylation of PLCγ1.

proliferation (Takahashi et al., 1999; Takahashi et al., 2001). Phosphorylation of Y1175 has also been reported to trigger activation of PI3K to promote cell survival, as well as activation of focal adhesion kinase (FAK) to regulate formation of stress fibers and focal adhesions via the adaptor protein Shb (Dayanir et al., 2001; Holmqvist et al., 2004). Y1054 and Y1059 have been found to be required for maximal kinase activity of VEGFR2 (Dougher and Terman, 1999). Although VEGFR2 signaling pathways mediating cell proliferation and migration have been well explored, those directing endothelial differentiation in vascular progenitor cells have remained largely unknown.

In this study, we have demonstrated that signaling through VEGFR2 Y1175 is involved in endothelial specification of VEGFR2$^+$ cells and subsequent survival of ECs. We further examined the role of PLCγ1, an effector that interacts with Y1175-*P*, in VEGFR2-mediated endothelial differentiation. We found that PLCγ1 enhances endothelial differentiation in the presence of survival signal, suggesting that the VEGFR2-PLCγ1 axis mediates endothelial specification but not the survival of ECs (Fig. 6A-C). Importantly, our finding that PLCγ1 plays an essential role in endothelial specification is consistent with a previous report on PLCγ1-deficient mice. Liao et al. reported that vasculogenesis and erythrogenesis were not observed in PLCγ1-null mice, though non-erythroid granulocytes and macrophages were present (Liao et al., 2002). These observations suggest that hemangioblasts are present, but that the subsequent differentiation into ECs or cells of the erythroid lineage is impaired in the absence of PLCγ1. They also suggested the possibility that impaired vasculogenesis in PLCγ1-null mice might be due to failure of survival of endothelial progenitors, because PLCγ1 also plays roles in cell survival (Lee et al., 1999; Wang et al., 2001). However, the findings of our colony formation assay (Fig. 6B) and endothelial survival assay (Fig. 6C) revealed that the principal role of PLCγ1 during endothelial differentiation of VEGFR2$^+$ progenitor cells is to direct endothelial specification and not to support cell survival. PLCγ2 is also expressed in VEGFR2$^+$ cells and ECs, as well as PLCγ1 (data not shown). PLCγ2-deficient mice were viable, although they were obtained at approximately two-thirds the expected frequency and were often smaller than wild-type mice, presumably because of occasional hemorrhage (Wang et al., 2000). Given the difference in phenotype between PLCγ1-null mice and PLCγ2-null mice, PLCγ1 appears to play more crucial roles than PLCγ2 in the process of development of the vasculature.

We recently reported that Ras signaling plays an important role in VEGF-A-dependent endothelial specification of VEGFR2$^+$ cells (Kawasaki et al., 2008). VEGF-A induces delayed Ras activation 6-9 hours after stimulation in VEGFR2$^+$ vascular progenitor cells, which specifies endothelial differentiation. In this study, our findings suggested that the VEGFR2-PLCγ1 axis is upstream of Ras signaling because the endothelial specification induced by PLCγ1 signaling was abrogated by FTI-277, a farnesyltransferase inhibitor that inhibits H-Ras (Fig. 6E). It will be important to determine the link between activation of PLCγ1 and the delayed Ras activation in the transmission of signaling for endothelial specification. Activation of PLCγ results in formation of diacylglycerol and inositol-1,4,5-triphosphate [Ins(1,4,5)P_3]. The former activates the C1-domain-containing molecules including PKCs and Ras guanine-releasing proteins (RasGRPs) (Kazanietz, 2000), whereas the latter triggers Ca^{2+} signaling pathways. VEGFR2-induced PLCγ signaling activates Erk through PKC (Takahashi et al., 1999). PKC is also reported to activate Ras (Marais et al., 1998) although the detailed mechanism still remains to be determined. These two pathways, however, do not appear to be involved in the delayed Ras activation by VEGF-A in VEGFR2$^+$ cells, because U0126 (a MEK inhibitor) and Go6983 (a PKC inhibitor) did not affect Ras activation although they inhibited endothelial differentiation induced by VEGF-A (supplementary material Figs S4 and S5). Thus, other pathways downstream of PLCγ1 appear to induce the delayed Ras activation. Notably, we did not observe the delayed activation of Ras after VEGF-A stimulation in human microvascular endothelial cells (Kawasaki et al., 2008), suggesting that Ras might be activated through a mechanism unique to VEGFR2$^+$ vascular progenitor cells. It is possible that the delayed Ras activation is mediated through transcriptional induction of certain signaling molecules.

Akt has been reported to play an essential role in VEGF-A-induced endothelial survival (Fujio and Walsh, 1999). By contrast, the PI3K-Akt pathway is not involved in endothelial specification (Kawasaki et al., 2008). Thus, it appears likely that the PI3K-Akt pathway contributes to endothelial differentiation through supporting cell survival. We also found that the PI3K-Akt pathway is involved in endothelial survival by VEGFR3 signaling. The PI3K-Akt pathway can be activated by Ras, but it appears to be activated independently of Ras in VEGF-A-stimulated VEGFR2$^+$ vascular progenitor cells, because FTI-277 inhibited phosphorylation of Erk but not Akt at 6 hours after VEGF-A-stimulation (supplementary material Fig. S6).

Our findings are schematically summarized in Fig. 7. In conclusion, we found that signaling through VEGFR2 Y1175 is indispensable for endothelial specification and subsequent survival of ECs, which are two elementary processes in endothelial differentiation. We also demonstrated that signaling for endothelial specification, a function of VEGFR2 characteristic among the VEGFR family members, is mediated by VEGFR2-PLCγ1 signal relay via VEGFR2 Y1175.

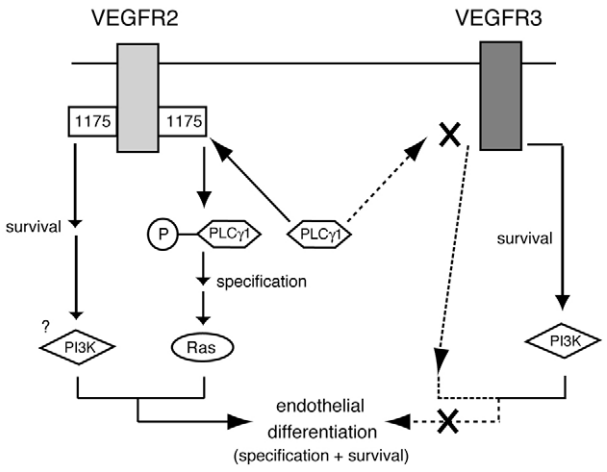

Fig. 7. Hypothetical model of endothelial differentiation of VEGFR2$^+$ cells. PLCγ1 is phosphorylated through interaction with Y1175-*P* of VEGFR2 in response to VEGF-A stimulation. The VEGFR2-PLCγ1 signaling axis might lead to activation of Ras, one of the pivotal regulators of endothelial specification. PI3K might also be activated via VEGFR2 Y1175-*P* to maintain survival of ECs. VEGFR3 does not induce endothelial specification of VEGFR2$^+$ cells, presumably because VEGFR3 fails to activate PLCγ1, although VEGFR3 has the potential to maintain the survival of ECs via PI3K.

Materials and Methods

Construction of a chimeric receptor and its mutants

cDNAs coding for human VEGFR2 and VEGFR3 were described previously (Suzuki et al., 2005). All chimeric receptors were cloned into the pPthC vector. The chimeric receptor denoted R32 contains the extracellular domain of VEGFR3 fused with the transmembrane and the intracellular domain of VEGFR2. R32 was constructed by a PCR-based method as follows. Restriction sites were generated or destroyed with amino acid residues being unaffected, as shown in Fig. 1. A *Bam*HI site was generated in the intracellular domain of VEGFR3 at 2451-2456 bp from the translational start site, which corresponds to the *Bam*HI site (2418-2423 bp) in VEGFR2. The intracellular domain of VEGFR3 was swapped with that of VEGFR2 at the *Bam*HI sites of VEGFR2 and VEGFR3. R32Y951F, R32Y1054F/1059F, R32Y1175F, R32Y1214F and R32Y951F/Y1214F, in which tyrosine residues were mutated to phenylalanine, were also generated from R32 by a PCR-based method.

Reagents and antibodies

VEGF-A (VEGF-A165), FGF-2 and Flt1-Fc were purchased from R&D Systems (Flanders, NJ). VEGF-C, FTI-277, U0126, LY294002 and Go6983 were from Calbiochem (La Jolla, CA). VEGFR3-Fc was from Sigma. The following antibodies were used: anti-VEGFR2 Y1054-*P* (44-1046, Invitrogen, Carlsbad, CA), anti-VEGFR3 (sc-28297, Santa Cruz Biotechnology, Santa Cruz, CA), anti-tubulin (Sigma-Aldrich), anti-PLCγl (sc-81, Santa Cruz Biotechnology), anti-phosphotyrosine (PY20, BD Pharmingen, San Diego, CA), anti-VEGFR2 (Avas12, BD Pharmingen), anti-PECAM1 (Mec13.3, BD Pharmingen), anti-αSMA (1A4, Sigma-Aldrich), anti-VE-cadherin (11D4.1, BD Pharmingen), anti-CD34 (RAM34, BD Pharmingen), anti-endoglin (MJ7/18, BD Pharmingen), anti-phospho-p44/42 (#9101S, Cell Signaling Technology), anti-phospho-Akt (#9271S, Cell Signaling Technology), anti-Erk (1B3B9, Millipore), secondary antibodies conjugated with Alexa Fluor 488, 594 or 647 anti-rat/mouse IgG (Invitrogen Molecular Probes), and anti-PE conjugated with magnetic beads (Miltenyi Biotec, Auburn, CA). TOTO3 iodide for nuclear staining was from Invitrogen Molecular Probes.

Cells and cell culture

HEK 293T and HepG2 cells, obtained from American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS). MGZRTcH ESCs were obtained from Hitoshi Niwa (RIKEN Center for Developmental Biology, Kobe, Japan). The CCE ESC line was obtained from Martin J. Evans (University of Cambridge, Cambridge, UK). Maintenance, differentiation, culture and magnetic-activated cell sorting (MACS) of ESCs were performed as described previously (Yamashita et al., 2000). Stable ES cell lines carrying Tc-regulatable R32 mutants, PalmPLCγl and miRNA targeting PLCγl were established and cultured as described previously (Kawasaki et al., 2008). The pre-miRNA sequence targeting mouse PLCγl was selected as 5′-TCAAGAAGAACTTAGGAGTCCGTTTTGGCCACT-GACTGACGGACTCCTGTTCTTCTTGA-3′ and used as 16 tandem repeats (Kawasaki et al., 2008). For endothelial differentiation assay, mouse ESC-derived VEGFR2$^+$ cells were plated on type IV collagen-coated eight-well CultureSlides (IWAKI, Chiba, Japan) at 2.5×10^4 cells per well and cultured for 2 days in SFO3 (Sanko Junyaku, Tokyo, Japan), or at 1.5×10^4 cells per well and cultured for 2-4 days in αMEM (Gibco, Grand Island, NY) supplemented with 10% FBS, in the presence or absence of VEGF-A (30 ng/ml), FGF-2 (0.5 ng/ml), sMock or sVEGF-C(C152S). For colony formation assay, ESC-derived VEGFR2$^+$ cells were plated at $1.0\text{-}4.0\times10^3$ cells per well on type IV collagen-coated one-well CultureSlides and further cultured for 2-4 days in SFO3 medium or αMEM supplemented with 10% FBS, in the presence of various ligands, reagents and supernatants. ESC-derived VEGFR2$^+$ cells were cultured in SFO3 medium except as noted otherwise.

Preparation of sVEGF-C(C152S)

cDNA encoding mouse VEGF-C(C152S) was described previously (Suzuki et al., 2005). The coding region was subcloned into pcDEF vector (Goldman et al., 1996). For preparation of cell culture supernatants, HEK 293T cells were transfected with pcDNA3 or a VEGF-C(C152S) expression vector by FuGene6 (Roche Diagnostics, Indianapolis, IN). After 24 hours, cells were washed twice with serum-free medium and further cultured in DMEM containing 5 mM glutamax (Gibco) for an additional 72 hours. Supernatants of HEK 293T cells transfected with empty vector or VEGF-C(C152S) expression vector were denoted sMock and sVEGF-C(C152S), respectively. sVEGF-C(C152S) had a VEGFR3-stimulating activity that was equivalent to 1.5 μg/ml of recombinant VEGF-C, as determined by luciferase reporter assay. Flt1-Fc chimera (300 ng/ml) was added to sMock or sVEGF-C(C152S) for removal of endogenous VEGF-A produced by HEK 293T cells, but was not added to the 'sMock plus VEGF-A' used in the luciferase assay. Flt1-Fc (300 ng/ml) was sufficient for neutralizing recombinant VEGF-A up to 60 ng/ml.

Luciferase assay

Activation of Elk1 was measured by the GAL4 DNA-binding-domain (DB)–Elk1 fusion system (PathDetect in vivo signal transduction pathway trans-reporting system, Stratagene, La Jolla, CA). HepG2 cells were transfected with plasmids as follows: Renilla expression vector under control of thymidine kinase promoter, luciferase expression vector under control of 5X GAL4 binding site, DB–Elk1 fusion protein expression vector, chimeric receptor expression vectors, and tetracycline-sensitive transactivator expression vector. At 24 hours after transfection, cells were treated with sMock, sMock plus VEGF-A (100 ng/ml) or sVEGF-C(C152S). After 6 hours, cells were harvested and luciferase assay was performed according to the manufacturer's protocol (Promega, Madison, WI).

Immunocytochemistry

Cells were fixed in 1:1 acetone-methanol solution, followed by incubation with primary antibodies and then with secondary antibodies, as described previously (Kano et al., 2005).

siRNA

Three siRNAs against mouse VEGFR3 (Flt4) were purchased from Invitrogen. Equal amounts of Flt4-MSS204362, Flt4-MSS204363 and Flt4-MSS204364 were mixed and used to knock down mouse VEGFR3.

Survival assay for ECs

Mouse ESC-derived VEGFR2$^+$ cells were plated at 2.0×10^5 to 4.0×10^5 cells per well on type IV collagen-coated six-well plates and cultured in SFO3 to differentiate into mature ECs by stimulation with VEGF-A (30 ng/ml). To exclude effects of endogenous mouse VEGFR3 expressed in ECs, ESC-derived VEGFR2$^+$ cells were reverse-transfected with negative control siRNA or VEGFR3 siRNA (5 nM) by HiPerfect (Qiagen, Chatsworth, CA). After 36-42 hours of culture of the ESC-derived VEGFR2$^+$ cells in SFO3 with VEGF-A, the medium was changed to one containing sMock or sVEGF-C(C152S). Cells were counted at the point of medium exchange and 12 hours after medium exchange. RNA was also prepared to examine the efficiency of knockdown of endogenous VEGFR3 at the point of medium exchange.

RNA isolation and quantitative RT-PCR

Total RNA was prepared using RNeasy (Qiagen) and reverse-transcribed with the SuperScript III first-strand synthesis system (Invitrogen). Expression of mouse VEGFR3 was measured by quantitative RT-PCR. The primer sequences used for VEGFR3 and GAPDH were as follows: VEGFR3: 5′-TCTCCA-ACTTCTTGCGTGTCAA-3′ and 5′-GCTTTGGCGCCTTCTACCAT-3′. GAPDH: 5′-TGCAGTGGCAAAGTGGAGATT-3′ and 5′-TGCCGTTGAATTTGCCGT-3′. All expression data were normalized to those for GAPDH.

Ras activation assay

Determination of activated Ras was performed as described previously (Kawasaki et al., 2008).

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