Ras signaling directs endothelial specification of VEGFR2⁺ vascular progenitor cells

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Introduction

Blood vessel formation is a fundamental process in organogenesis during embryonic development (Coulats et al., 2005; Ferguson et al., 2005). Vascular progenitor cells are thought to first appear in the posterior primitive streak as vascular endothelial growth factor receptor 2-positive (VEGFR2⁺) mesodermal cells. These cells are specified for the hematopoietic and/or vascular lineage (hemangioblasts or angioblasts) and then migrate into extraembryonic sites, including the yolk sac and allantois as well as intraembryonic sites, in VEGF-A-dependent fashion (Huber et al., 2004; Hiratsuka et al., 2005). These precursor cells differentiate and assemble to form primary capillary plexuses or directly aggregate into the dorsal aorta or cardinal vein, followed by a process of remodeling through sprouting/nonsprouting angiogenesis and fusion of vessels.

Finally, maturation of the nascent vasculature is accomplished by recruitment and adhesion of mural cells to endothelial cells.

VEGFR2 (also known as Flk1 and KDR), one of the receptors for the VEGF family of growth factors, plays essential roles during vascular development. VEGFR2-deficient mice die in utero between 8.5 and 9.5 days postcoitum because of lack of endothelial cells and hematopoietic cells (Shalaby et al., 1995). Subsequent analysis suggested that the role of VEGFR2 signaling in vascular development in vivo includes proliferation, migration, and differentiation of progenitor cells (Shalaby et al., 1997). Because VEGFR2⁺ mesodermal cells can give rise to multiple lineages other than endothelial and hematopoietic cells, including vascular mural cells, skeletal muscle cells, and cardiomyocytes (Motoike et al., 2003; Ema et al., 2006), differentiation of VEGFR2⁺ cells should be appropriately specified. However, the signal transduction pathways leading to endothelial specification downstream of VEGFR2 are poorly understood, although those for cell proliferation and migration have been well explored in mature endothelial cells (Shibuya and Claesson-Welsh, 2006).
Use of differentiating embryonic stem cells (ESCs) is advantageous for the study of signaling for lineage specification because migration of progenitor cells to the correct microenvironment is unnecessary. Using mouse ESC-derived VEGFR2+ cells, an in vitro system for analysis of ligand-dependent endothelial specification has recently been established (Hirashima et al., 1999; Yamashita et al., 2000). In this system, ESC-derived VEGFR2+ cells differentiate into endothelial cells in response to VEGF-A, whereas they differentiate into α-smooth muscle actin–positive (αSMA+) mural cells resembling vascular smooth muscle cells in the presence of PDGF-BB or serum (Yamashita et al., 2000; Ema et al., 2003; Watabe et al., 2003). VEGFR2 appears to transmit a specific signal for induction of endothelial differentiation of VEGFR2+ progenitor cells because signaling from either VEGFR1 or 3 fails to induce it (Yamashita et al., 2000; Suzuki et al., 2005).

In the present study, we investigated the signaling pathway downstream of VEGFR2 for specification of endothelial lineage. Using pharmacological inhibitors, a gene silencing approach, and a gain-of-function approach, we concluded that Ras signaling is involved in endothelial specification induced by VEGF-A. Although PDGF-BB fails to induce endothelial differentiation, it also activates Ras in VEGFR2+ progenitor cells. We found that VEGF-A activates the Ras pathway at periods distinct from PDGF-BB, thus directing endothelial differentiation from VEGFR2+ vascular progenitor cells. These findings also provide mechanistic insights into signaling for cell specification through widely shared effector molecules.

Results

A farnesyltransferase inhibitor, FTI-277, inhibits VEGF-A–induced endothelial specification of ESC-derived VEGFR2+ cells

To determine the signaling components required for VEGF–A–induced endothelial differentiation from vascular progenitor cells, we used in vitro vascular differentiation systems (Yamashita et al., 2000). VEGFR2+ cells derived from CCE mouse ESCs were cultured in medium containing serum with or without VEGF-A. In the absence of VEGF-A, most cells differentiated into αSMA+ mural cells, whereas in the presence of VEGF-A, platelet-endothelial cell adhesion molecule-1–positive (PECAM1+) endothelial cells emerged (Fig. 1 A; Yamashita et al., 2000).

We first examined the effects of various inhibitors targeting signal molecules. Among those tested, we found that FTI-277 (Lerner et al., 1995), a farnesyltransferase inhibitor, had a selective inhibitory effect on endothelial differentiation. When FTI-277 was added, VEGF-A–induced appearance of PECAM1+ cells was suppressed, whereas that of αSMA+ cells was not markedly altered (Fig. 1 A). To determine whether the reduction in number of PECAM1+ cells by FTI-277 was caused by inhibition of differentiation, we next performed quantitative analyses using a limiting dilution assay (Fig. 1 B). When VEGFR2+ cells were seeded at low density (90–120 cells/cm2), they formed single-cell–derived colonies in 4 d. We counted the number of colonies after immunostaining for PECAM1 and αSMA, which reflects the fate of differentiation. In the absence of FTI-277, stimulation with VEGF-A increased PECAM1+ colonies and decreased αSMA+ colonies, indicating that VEGF-A directs endothelial differentiation at the expense of mural differentiation. In the presence of FTI-277, the number of PECAM1+ colonies was decreased and that of αSMA+ colonies was increased, whereas the total number of colonies was not markedly changed. These findings indicate that FTI-277 specifically inhibits endothelial differentiation of ESC-derived VEGFR2+ cells. Similar results were obtained using MGZ5 ESCs (unpublished data).

To determine when FTI-277–sensitive signal is transmitted, we added FTI-277 at different time points after VEGF-A stimulation (Fig. 1 C). When FTI-277 was added 3 h after stimulation, the appearance of PECAM1+ cells was suppressed but when FTI-277 was added 6 h after stimulation, it was not. We concluded that the FTI-277–sensitive signal for endothelial specification is transmitted later than 3 h after VEGF-A stimulation.

We also performed ex vivo whole-embryo culture assay to investigate the effects of FTI-277 on vascular development in mouse embryo. Embryonic day (E)–6.75 concepti were picked out from the uteri of pregnant mice and cultured for 3 d, during which PECAM1+ blood vessels were formed in the yolk sac. In the presence of FTI-277, however, PECAM1+ vessels were diminished, although overall development of the yolk sac was not affected (Fig. 1 D). We then examined the expression of vascular markers by quantitative RT-PCR. FTI-277 treatment resulted in decrease in the level of expression of PECAM1 and VE-cadherin compared with control, whereas expression of αSMA was unchanged (Fig. 1 E). These findings suggest that FTI-277 suppresses vascular development.

Loss of H-Ras abrogates endothelial differentiation of VEGFR2+ cells

Because the principal targets of FTI-277 include H-Ras, it appeared possible that Ras signaling could be involved in VEGF–A–induced endothelial differentiation of vascular progenitor cells. To examine the effect of H-Ras inactivation on vascular development, we investigated the vascular phenotype of H-ras knockout mice. Heterozygous H-ras+/− mice produced homozygous H-ras−/− offspring in Mendelian ratio (+/+; 17; +/−, 36; and −/−, 17), as described previously (Ise et al., 2000; Esteban et al., 2001). We therefore focused on vascular phenotypes during early development, and found vascular aberration in the periphery of the brain of 73% (8/11) of H-ras−/− embryos studied at E9.5, although they contained similar numbers of somites, as did wild-type and heterozygous littermates (Fig. 2 A). H-ras−/− embryos exhibited no clear difference from wild-type embryos. We further double stained the cephalic region for PECAM1 and VEGFR2, the earliest marker of differentiation of endothelial cells (Fig. 2 B). In H-ras−/− embryos, complex vascular networks were stained for both PECAM1 and VEGFR2, whereas in H-ras−/− embryos, vascular structures positive for either PECAM1 or VEGFR2 were strikingly reduced. Furthermore, we found that vascular structures were rare in cross sections of the head region of H-ras−/− embryos (Fig. 2 C). This vascular aberration was transient,
down condition), VEGF-A–induced PECAM1+ colonies decreased in number, whereas αSMA+ colonies increased compared with those in the presence of Tc (Fig. 2 D). In Tc-miR-NTC cells expressing negative control miRNA, PECAM1+ colonies did not decrease in number (unpublished data). These findings suggest that H-Ras plays a role in endothelial specification of VEGFR2+ progenitor cells.

Constitutively active G12V mutant of H-Ras induces PECAM1+ cells from VEGFR2+ progenitor cells

We next established ESC lines carrying a Tc-regulatable active form of H-Ras (Tc-H-Ras[G12V]) or no transgene (Tc-empty). In Tc-H-Ras[G12V] cells, Ras is expressed at high levels in the absence of Tc (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). We then examined differentiation of VEGFR2+
were cultured in type I collagen gel for 7 d. When active Ras was inducibly expressed, cells formed tube-like structures even in the absence of VEGF-A (Fig. 3D). Furthermore, we performed in vivo vascular formation assay. Tc-empty and Tc-H-Ras[G12V] cells were labeled with retrovirus carrying YFP. These cells were differentiated in vitro and subcutaneously injected, together with Matrigel, into the abdominal region of mice. After 10 d, Matrigel was harvested, frozen sectioned, and immunostained for PECAM1 and αSMA. When Tc-H-Ras[G12V] cells were injected, PECAM1+ blood vessels surrounded by αSMA+ cells were observed. These PECAM1+ cells were also positive for other endothelial markers, including VE-cadherin (Fig. 3B), CD34, and endoglin (not depicted), and they incorporated acetylated low-density lipoprotein (AcLDL; Fig. 3C) and expressed mRNA for endothelial nitric oxide synthase and claudin-5 (not depicted). We next examined the ability of VEGFR2+ cells to form vascular structures in three-dimensional culture upon expression of H-Ras[G12V]. Aggregated VEGFR2+ cells derived from Tc-H-Ras[G12V] cells were cultured in type I collagen gel for 7 d. When active Ras was inducibly expressed, cells formed tube-like structures even in the absence of VEGF-A (Fig. 3D). Furthermore, we performed in vivo vascular formation assay. Tc-empty and Tc-H-Ras[G12V] cells were labeled with retrovirus carrying YFP. These cells were differentiated in vitro and subcutaneously injected, together with Matrigel, into the abdominal region of mice. After 10 d, Matrigel was harvested, frozen sectioned, and immunostained for PECAM1 and αSMA. When Tc-H-Ras[G12V] cells were injected, PECAM1+ blood vessels surrounded by αSMA+ cells were observed. These PECAM1+ cells were also positive for YFP, indicating that they originated from Tc-H-Ras[G12V] cells (Fig. 3E). In contrast, PECAM1+ vessels were not observed when Tc-H-Ras[G12V] cells were injected, but H-Ras[G12V] expression was suppressed by treatment with Tc or when Tc-empty cells were injected. These findings suggest that active Ras induces differentiation of cells with characteristics of endothelial cells from VEGFR2+ progenitor cells.
Signaling for endothelial specification is mediated through the Ras–Erk pathway

To investigate whether Ras signaling is involved in cell fate determination, we next performed a limiting dilution assay (Fig. 4 A). When H-Ras[G12V] was expressed, the total number of colonies increased. PECAM1+ colonies dramatically increased in number. Notably, αSMA+ colonies decreased in number. These findings suggest that expression of active Ras leads to endothelial differentiation at the expense of mural differentiation. To confirm the causal relationship between Ras expression and endothelial differentiation, cells were immunocytochemically examined for Ras expression (Fig. 4 B). Cells that successfully expressed Ras at high levels were positive for PECAM1, whereas those that failed to express Ras were positive for αSMA. These findings suggest that expression of constitutively active Ras directs endothelial specification of VEGFR2+ cells.

Ras signaling is known to induce the expression of VEGF-A (Rak et al., 1995; Grugel et al., 1995; Arbiser et al., 1997). It thus appeared possible that VEGF-A induced by signaling from H-Ras[G12V] directed differentiation of VEGFR2+ progenitor cells to PECAM1+ cells in the present experimental system. To exclude this possibility, we examined Ras-induced endothelial differentiation in the presence of SU5614 (Spiekermann et al., 2002), an inhibitor of VEGFR2 kinase, as well as VEGFR1 (Flt1)-Fc chimera protein, which competes with VEGFR2 for binding with VEGF-A. Ras-induced endothelial differentiation was not inhibited under these conditions (Fig. S3 B and not depicted).

Furthermore, Ras-induced endothelial cells formed tube-like structure in the presence of SU5614 (Fig. S3 C). These findings suggest that differentiation depends primarily on intracellular signal transduction from Ras protein.

We next established ESC lines in which H-Ras effector mutants H-Ras[G12V, T35S] or H-Ras[G12V, Y40C] can be inducibly expressed to examine the signaling pathway mediating Ras-induced endothelial specification. H-Ras[G12V, T35S] and H-Ras[G12V, Y40C] preferentially activate the Raf–MEK–Erk and PI3K–Akt pathways, respectively (Joneson et al., 1996; Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1).
appears to be different from that by PDGF receptors. In this respect, it is notable that FTI-277 was still effective in inhibiting endothelial differentiation when added 3 h after VEGF-A stimulation (Fig. 1C). The specificity of Ras signaling induced by VEGFR2 can be attributed to the timing of Ras activation. We therefore investigated the window of time within which Ras protein is specifically activated by VEGF-A, focusing on the period more than 3 h after stimulation with VEGF-A. We first examined levels of phosphorylation of Erk, a downstream effector of Ras, 3–12 h after stimulation with VEGF-A (Fig. 5A). Erk phosphorylation peaked at 6 and 9 h after stimulation, suggesting that Ras may be activated with a similar time course. We next examined activation of Ras in cells stimulated with VEGF-A or PDGF-BB for 6 h (Fig. 5B). Activated Ras was detected by pulldown assay using the Raf-Ras binding domain. We found that Ras activation in response to VEGF-A or PDGF-BB was markedly different at 6 h after stimulation. VEGF-A caused intense activation of Ras and Erk, whereas PDGF-BB failed to activate both Ras and Erk. At 5 min after stimulation with VEGF-A, when VEGF-A efficiently activates Erk (Takahashi et al., 1999; Yashima et al., 2001), the levels of activation of Ras and

Figure 4. Induction of endothelial differentiation by Ras–Erk signaling. (A) Quantification of colony formation of Tc-H-Ras[G12V] cells. Representative results of three independent experiments are shown. (B) Cells overexpressing Ras were positive for PECAM1. ESC-derived VEGFR2+ cells were cultured for 2 d in the absence of Tc and VEGF-A, followed by immunostaining for PECAM1 (green), αSMA (red), and Ras (blue). Bars, 100 μm. (C and D) Quantification of colony formation of Tc-H-Ras[G12V, T35S] (C) and Tc-H-Ras[G12V, Y40C] (D) cells. Representative results of three independent experiments are shown.
Erk were not notably different from those induced by PDGF-BB (Fig. 5B). Activation of Ras and Erk by VEGF-A was also observed at 9 h but not at 3 h (unpublished data).

We next compared phosphorylation of Erk 3–12 h after stimulation with VEGF-A, PDGF-BB, FGF-2, and PlGF (Fig. S5A, available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). Two ligands, PDGF-BB and PlGF, which lack ability to induce endothelial differentiation of VEGFR2+ progenitor cells (Yamashita et al., 2000), failed to activate Erk during the period. FGF-2 that modestly supports endothelial progenitor cells (Yamashita et al., 2000), failed to activate Erk pathway 6–9 h after stimulation with VEGF-A stimuli (Fig. 5D). These findings suggest that activation of the Ras–Erk pathway 6–9 h after stimulation with VEGF-A directs endothelial specification of VEGFR2+ progenitor cells.

We further examined the farnesylation status of H-Ras after treatment with FTI-277 (Fig. 5C). Nonfarnesylated Ras began to increase 3 h after stimulation but not at later time points. These findings indicate that activation of Erk at late time points is specific for ligands that induce endothelial differentiation of VEGFR2+ vascular progenitor cells. We also examined time course of phosphorylation of Erk after stimulation of human microvascular endothelial cells (HMECs) with VEGF-A (Fig. S5B, available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). Erk was intensively activated 5–15 min after stimulation but not at later time points. These findings indicate that activation of Erk at later time points is not a common feature of VEGFR2 signaling.

We further examined the farnesylation status of H-Ras after treatment with FTI-277 (Fig. 5C). Nonfarnesylated Ras began to increase 3 h after treatment and was constant after 6 h. Consistent with this finding, FTI-277 inhibited phosphorylation of Ras 6 h after VEGF-A stimulation (Fig. 5D). We thus confirmed that activation of Ras around 6 h after VEGF-A stimulation is sensitive to FTI-277. These findings suggest that activation of the Ras–Erk pathway 6–9 h after stimulation with VEGF-A directs endothelial specification of VEGFR2+ progenitor cells.

**VEGF-A-induced Ras activation precedes the expression of endothelial markers**

We next examined the expression of vascular markers over time during in vitro differentiation of ESC-derived VEGFR2+ cells by VEGF-A (Fig. 6A). The expression of the endothelial markers PECAM1 and VE-cadherin began to increase from 12 h after stimulation with VEGF-A. Interestingly, the level of expression of VEGFR2 in VEGF-A–stimulated cells was similar to that in unstimulated cells up to 6 h after stimulation. During the period beyond 12 h after stimulation, VEGFR2 expression increased in VEGF-A–stimulated cells, whereas it decreased in nonstimulated cells. These findings suggest that endothelial specification occurs between 6 and 12 h after stimulation with VEGF-A, which is preceded by VEGF-A–induced Ras activation. Consistent with these findings, the level of expression of αSMA, a mural cell marker, began to increase later than 24 h. Genes up-regulated at 48 h after VEGF-A stimulation were analyzed by oligonucleotide microarray (Affymetrix) and listed in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). We observed induction of PECAM1 and VE-cadherin, as well as VEGFR2, e-NOS, Tie1, and other genes expressed in endothelial cells by treatment with VEGF-A.

We further determined expression of vascular markers after induction of H-Ras[G12V] (Fig. 6B). mRNA for H-Ras[G12V] was detected at 3–6 h, followed by induction of PECAM1 and VE-cadherin later than 12 h. Earlier induction of these endothelial markers may be caused by the earlier onset of Ras signaling through expression of the constitutively active form. Up-regulation of VEGFR2 was, however, delayed. The reason for this delay remains to be elucidated.

We conclude that VEGF-A stimulation of VEGFR2+ vascular progenitor cells specifically induces Ras–Erk activation around 6–9 h after stimulation, which in turn specifies endothelial differentiation.

**Discussion**

The development of multicellular organisms requires the orchestrated growth, migration, and differentiation of numerous cells. Various extracellular factors, as well as intracellular signaling molecules, are involved in the robust regulation of the behaviors...
endothelial cells, though with low efficiency (Schuh et al., 1999). The endothelial differentiation observed in vitro may be caused by an effect of FGF-2, which was included in the culture medium (Schuh et al., 1999), because we previously found that FGF-2 supports endothelial differentiation of ESC-derived VEGFR2+ cells to a modest extent (Kano et al., 2005). VEGFR2 signaling thus appears to be a pathway for endothelial specification of biological importance and high efficiency.

The roles of specific pathways downstream of VEGFR2 in mediating cell proliferation and migration have been elucidated. Phosphorylation of Y1175 of VEGFR2 leads to phospholipase C-γ activation, followed by PKC-γ-mediated Raf activation to induce cell proliferation (Takahashi et al., 2001). In contrast, phosphorylation of Y951 mediates signaling for cell migration and actin stress fiber organization through interaction with T cell–specific adaptor (Matsumoto et al., 2005). Phosphorylation of Y1214 is also implicated in actin stress fiber remodeling through the p38 pathway (Lamalice et al., 2004). However, which

of cells during development. VEGFR2 signaling plays a central role in de novo blood vessel formation (vasculogenesis). In extraembryonic sites, VEGFR2 signaling is primarily required for the formation of blood islands (Shalaby et al., 1995), where vascular endothelial cells and hematopoietic cells differentiate to form primary plexuses. In the absence of VEGFR2 signaling, VEGFR2+ progenitor cells fail to migrate to the extraembryonic sites (Shalaby et al., 1997). In the embryo proper, VEGFR2 signaling is required for endothelial specification of the vascular progenitor cells (Shalaby et al., 1997). Potential endothelial precursor cells are observed in the correct location where they would develop into embryonic blood vessels but fail to complete the pathway of differentiation. Recently, shear stress has been shown to induce differentiation of endothelial cells from progenitor cells (Yamamoto et al., 2003; Yamamoto et al., 2005), which is mediated through ligand-independent activation of VEGFR2 (Yamamoto et al., 2005). In embryoid body culture system in vitro, however, VEGFR2+ ESCs still give rise to endothelial cells, though with low efficiency (Schuh et al., 1999). The endothelial differentiation observed in vitro may be caused by an effect of FGF-2, which was included in the culture medium (Schuh et al., 1999), because we previously found that FGF-2 supports endothelial differentiation of ESC-derived VEGFR2+ cells to a modest extent (Kano et al., 2005). VEGFR2 signaling thus appears to be a pathway for endothelial specification of biological importance and high efficiency.

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signaling pathway downstream of VEGFR2 is involved in endothelial specification has not been elucidated.

VEGF-A promotes the differentiation of endothelial cells from ESC-derived VEGFR2 \(^2\) cells, whereas PGF, a specific ligand for VEGFR1, fails to induce endothelial differentiation (Yamashita et al., 2000). We have also reported that ectopically expressed VEGFR3 fails to transmit signal for endothelial differentiation of VEGFR2 \(^2\) progenitor cells (Suzuki et al., 2005). These findings suggest that VEGFR2 has unique features of signal transduction among VEGF receptor family members. In the present study, we unexpectedly found that Ras signaling downstream of VEGFR2 is involved in specifying endothelial differentiation of VEGFR2 \(^2\) vascular progenitor cells. We also found that the Raf–Erk pathway plays an important role downstream of Ras in endothelial specification. Interestingly, activation of Erk has been reported in blood islands of the E7.5 mouse embryo (Corson et al., 2003).

Ras signaling is known to act as a switch that determines cell fate in vulval formation in Caenorhabditis elegans (Sternberg and Han, 1998) and in photoreceptor development in Drosophila melanogaster (Wasserman et al., 1995). Ras is, however, activated by various extracellular stimuli in mammalian cells. ESC-derived VEGFR2 \(^2\) cells are differentiated into endothelial cells by VEGF-A, but not by PDGF-BB, although both ligands activate Ras in the cells. It will thus be important to determine how VEGFR2 transmits specific signals using an effector that is widely shared among different signaling pathways like Ras. In PC12 cells, EGF stimulation results in transient activation of Erk to induce cell proliferation, whereas NGF stimulation results in sustained activation of Erk to cause growth arrest and outgrowth of neurites (Marshall, 1995). Similarly, unique utilization of Ras by the VEGFR2 system likely accounts for the specific signaling to induce endothelial differentiation. In the present study, we found that Ras is specifically activated by VEGF-A around 6–9 h after stimulation. This delayed activation of Ras appears to transmit specific signaling for endothelial differentiation, which is consistent with the time course of FTI-277 sensitivity.

Usage of Ras by the VEGFR2 system differs in cells of various origins. In human aortic and umbilical vein endothelial cells as well as rat sinusoidal endothelial cells, activation of Ras by VEGF-A is modest. The PKC-dependent pathway, but not Ras, principally transmits the signal for Erk activation (Doanes et al., 1999; Takahashi et al., 1999; Yashima et al., 2001). In contrast, VEGF-A induces intense activation of Ras and Ras-mediated activation of Erk in HMECs (Yashima et al., 2001). These differential signaling properties may reflect the unique profiles of expression of signaling molecules in each type of cell. In our experiments using ESC-derived VEGFR2 \(^2\) progenitor cells, the PKC-dependent pathway appeared to be activated in the early phase because phosphorylation of Erk was notably increased but activation of Ras was modest 5 min after VEGF-A stimulation. In contrast, the Ras pathway was strongly activated to induce phosphorylation of Erk in the delayed phase (6–9 h after stimulation), a finding supported by the inhibition of Erk phosphorylation by FTI-277 (Fig. 5 D). The mechanism of this delayed activation of Ras remains to be elucidated in detail. It is possible that the activation is not direct and instead is mediated through transcriptional induction of certain signaling molecules. Notably, the delayed activation of Ras was not observed in mature endothelial cells, suggesting that it is not a common feature of VEGFR2 signaling.

In mature endothelial cells, Ras signaling appears to be involved in cell proliferation, tubule formation, and cell survival downstream of FGF receptor or integrin \(\alpha\)v \(\alpha\) (Klint et al., 1999; Hooi et al., 2003). However, the role of Ras downstream of VEGFR2 has been regarded as marginal (Shibuya and Claesson-Welsh, 2006). The present study is the first to suggest the crucial role of Ras–Erk signaling downstream of VEGFR2 in endothelial specification of vascular progenitor cells.

We examined vasculogenesis in allantoic explants obtained from E8.5 embryos and found reduced vascular formation in those from H-\(ras^{+/−}\) (2 out of 12 embryos), whereas those from H-\(ras^{+/+}\) \((n = 16)\) or H-\(ras^{−/−}\) \((n = 26)\) embryos exhibited no such phenotype (unpublished data). We also examined vascular formation in H-\(ras^{−/−}\) mice and found vascular aberration in the periphery of the brain of 73% of E9.5 H-\(ras^{−/−}\) embryos. However, there was no obvious abnormality of E10.5 H-\(ras^{−/−}\) embryos, which is consistent with the previous results that suggest H-ras knockout mice are born and grow normally (Ise et al., 2000; Esteban et al., 2001). These findings suggest that H-\(ras^{−/−}\) embryos catch up for the delay in vascular formation in cephalic region until E10.5. One possibility is that expression of other members of the Ras family, N-Ras and K-Ras, is up-regulated and compensates for the loss of H-Ras as reported previously (Ise et al., 2000). Alternatively, reduction of endothelial differentiation in the absence of H-Ras may be permissive for embryonic development although quantitative assay in vitro exhibits substantial reduction (Fig. 1 B and Fig. 2 D). Compensatory growth of differentiated endothelial cells may offset reduction in endothelial differentiation. It appears likely that N-Ras and K-Ras are also involved in endothelial specification because treatment with FTI-277 that principally targets H-Ras or knockdown of H-Ras failed to completely inhibit endothelial specification induced by VEGF-A in the in vitro vascular differentiation assay.

In summary, we have demonstrated the involvement of Ras signaling in VEGFR2-mediated endothelial specification of vascular progenitor cells and provided novel insights into temporal aspects of signaling for cell lineage specification through widely shared effector molecules.

### Materials and methods

**Cells and cell culture**

The C57Bl/6 ESC line was obtained from M.J. Evans (University of Cambridge, Cambridge, UK) and MGZ5 and MGZRTcH ESC cells were obtained from H. Niwa (RIKEN Center for Developmental Biology, Kobe, Japan). Maintenance, differentiation, culture, and cell sorting of CCE, MGZ5, and MGZRTcH ESCs were performed as previously described (Yamashita et al., 2000). For in vitro differentiation, mouse ESCs were cultured on type IV collagen-coated dishes in the absence of leukemia inhibitory factor for 4 d. VEGFR2 vascular progenitor cells were then sorted and used for analysis of endothelial differentiation. We plated 2 x 10^5 ESC-derived VEGFR2 cells per well on type IV collagen-coated 8-well CultureSlides (IWAKI) for immunostaining or 0.6–1 x 10^5 cells per well on 1-well CultureSlides for limiting dilution assay. The cells were cultured for 2–4 d in α-minimum essential medium (Invitrogen) supplemented with 10% FBS in the presence of the following various ligands or inhibitors: VEGF-A (VEGF165, R&D Systems), FLT1-Fc chimera proteins (R&D Systems), PDGF-BB (Peprotech),...
FGF-2 (R&D Systems), PIGF (R&D Systems), FTI-277 (EMD), and SU5614 (EMD). HMEC, an immortalized human dermal microvascular endothelial cell line, was obtained from T. Lawley (Emory University, Atlanta, GA) and was cultured in EGM-2 (Cambrex) containing 2% FBS and endothelial cell growth supplements (Clonetics).

Antibodies

For immunohistochemistry, monoclonal antibodies to murine VEGFR2 (AVAS12; BD Biosciences), PECAM1 (Mec13.3 [eBioscience] or 2H8 [Millipore]), VE-cadherin (11D4; BD Biosciences), CD34 (RAM34; BD Biosciences), endoglin (M17/18; BD Biosciences), αSMA (1A4; Sigma-Aldrich), and Ras (clone RAS10; Millipore) were used. Anti–β-catenin antibody was obtained from Santa Cruz Biotechnology, Inc. Secondary antibodies conjugated with Alexa 488, 594, or 647 anti-murine/rat IgG were obtained from Invitrogen, and HRP-conjugated anti–rat IgG and HRP-conjugated anti–Armenian hamster IgG were obtained from Invitrogen and Jackson ImmunoResearch Laboratories, respectively. TOTO3 iodide for nuclear staining was obtained from Invitrogen. For immunoblot analysis, antibodies to Ras (clone RAS10; Millipore), H-Ras (EPITOMICS), p44/42 (Cell Signaling Technology), Erk (Millipore), p-Akt (Cell Signaling Technology), Akt (Cell Signaling Technology), and tubulin (Sigma-Aldrich) were used.

Immunohistochemistry

Whole-mount staining of embryos and yolk sacs was performed as described previously (Hogan et al., 1994), and microscopy was performed using a microscope (M2B; Leica) with 5× objectives (Leica 10411589). Staining of culture cells was performed as described previously (Kano et al. 2005). For staining of AcLDL in endothelium, we used Alexa Fluor 594-conjugated AcLDL (Invitrogen) in accordance with the manufacturer's protocol. Stained cells were photographed using a confocal microscope (LSM510 META; Carl Zeiss, Inc.) with 10× objectives (Plan-Neofluor 0.3 NA) and LSM Image Browser (Carl Zeiss, Inc.). All images were taken at room temperature.

Ex vivo whole-embryo culture

Embryos were dissected out of the deciduum and placed in 500 μL DME containing 50% Rat IC serum (Charles River Laboratories), 5 mM of non-essential amino acids, 50 mM sodium pyruvate, and 2.75 mM 2-mercaptoethanol, preequilibrated at 37°C with 5% CO2. Embryos were cultured at 37°C with 5% CO2 and analyzed. FTI-277 (dissolved in DMSO) was used at 1 μM. The concentration of DMEM was set at 0.1% in all cultures.

Mice

H-Ras+/− mice (Ise et al., 2000) backcrossed into the C57BL/6J background were used. Mice were allowed to mate naturally at night. E0.5 was considered to be noon on the day the vaginal plug was observed. Embryos were generated by PCR analysis using yolk sacs as a DNA source as previously described (Ise et al., 2000). All animal experimental protocols were performed in accordance with the policies of the Animal Ethics Committee of the University of Tokyo.

Establishment and differentiation of ESC lines in which H-Ras is knocked down with inducible miRNA

We used the Block-T Pal II mir RNAi expression system (Invitrogen) in MGZRTcH ESCs (Fig. S1 A; Masui et al., 2005). Stable ESC clones (Tc-miR-H-Ras) were established by transfecting pRhCEmGFP-miRNA-H-Ras into MGZRTcH ESCs as described previously (Masui et al., 2005). Negative control cells (Tc-mir-NTC) were also established. For endothelial differentiation assay, ESCs were cultured in the absence of Tc for the last 2 d of in vitro differentiation. To induce expression of miRNA, VEGFR2+ cells were then sorted and used for limiting dilution assay. Results were confirmed in at least two independent cell lines.

Establishment of ESC lines inducibly expressing H-Ras (G12V), H-Ras [G12V, T35S], or H-Ras (G12V, Y40C).

Tc-H-Ras[G12V], Tc-H-Ras[G12V, T35S], Tc-H-Ras[G12V, Y40C], and Tc-H-Ras[G12V, Y40C] mutants were described previously (Yoshida-Koide et al., 2004). Results were confirmed in at least two independent cell lines.

Three-dimensional culture

ESC-derived VEGFR2+ cells were cultured for 12 h on Petri dishes with 1 μg/ml Tc and/or 30 ng/ml VEGF-A. Aggregates formed were suspended in type I collagen gel and cultured for 7 d in medium containing 1 μg/ml Tc and/or 30 ng/ml VEGF-A, followed by microscopic observation. In some of the samples, SU5614 was added. Collagen gels were photographed using microscopy (IX70; OLYMPUS) with 10× objectives (UPlanFl; 0.3 NA), at room temperature.

In vivo vascular formation assay

All ESCs were labeled with YFP retrovirus before in vivo vascular formation assay to distinguish cells of ESC origin and host origin. ESCs were cultured on type IV collagen-coated dishes in the absence of leukemia inhibitory factor for 4 d. Then 107 cells were pelleted and mixed with 100 μL PBS and 100 μL Matrigel and injected subcutaneously into the abdominal region of 4-wk-old male 129sv mice. In vivo suppression of transgene was maintained by adding 1 μg/ml Tc in Matrigel and supplementing drinking water with 2 mg/ml doxycycline. The mice were killed on day 10, and the plaques were harvested and fixed with formalin. They were then frozen sectioned and stained with anti–PECAM1 and αSMA antibodies. Stained sections were photographed using a confocal microscope (LSM510 META) with 40× oil objectives (Plan-Neofluor; 1.3). All images were taken at room temperature.

Ras activation assay and immunoblot analysis

ESC-derived VEGFR2+ cells (6 × 104) were stimulated with 30 ng/ml VEGF-A or 15 ng/ml PDGF-BB. Cells were harvested at the indicated time points and lysed. The cell lysates were subjected to pulldown assay using Raf-RBD (Ras activation assay kit; Millipore). The precipitated GTP-bound Ras was detected by anti-Ras antibody. Immunoblot analysis was performed as described previously (Suzuki et al., 2005). Image processing and storage (TIFF format) was performed using Photoshop software (Adobe).

RNA isolation, quantitative RT-PCR, and oligonucleotide microarray analysis

Culture of VEGFR2+ cells with 10% PBS in the absence of VEGF-A cells was used as a source of RNA. Total RNA was prepared with RNeasy (QIAGEN), according to the manufacturer's instructions, and reverse-transcribed with the SuperScript III first-strand synthesis system (Invitrogen). Expression of various markers of differentiation was compared by quantitative RT-PCR analysis. Primer sequences are listed in Table S2 (available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). For oligonucleotide microarray analysis, GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix) were used according to the manufacturer's instruction.

Online supplemental material

Fig. S1 shows Tc-regulated inducible expression of premiRNA in ESCs. Fig. S2 shows Tc-regulated inducible expression of H-Ras[G12V] in ESCs. Fig. S3 shows effects of pharmacological inhibitors on the induction of PECAM1+ cells and tube formation by H-Ras[G12V]. Fig. S4 shows Tc-regulated inducible expression of Ras effector mutants in ESCs. Fig. S5 shows time course of phosphorylation of Erk in ESC-derived VEGFR2+ cells and HMECs after ligand stimulation. Table S1 shows genes induced by VEGF-A treatment of ESC-derived VEGFR2+ cells for 48 h. Table S2 shows primers used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1.

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