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Dissecting the Signal Transduction Pathway that Directs Endothelial Differentiation Using Embryonic Stem Cell-Derived Vascular Progenitor Cells

Kyoko Kawasaki[1] and Keiji Miyazawa[2]
[1]Helen Diller Family Comprehensive Cancer Center,
University of California-San Francisco,
[2]Department of Biochemistry, Interdisciplinary Graduate School of
Medicine and Engineering, University of Yamanashi,
[1]USA
[2]Japan

1. Introduction

Blood vessels are essential for embryonic development and tissue homeostasis in adults (Coultas et al., 2005). They supply oxygen and nutrients and remove metabolic waste from tissues. Blood vessels also participate in intercellular communication. Secretion of growth/differentiation factors by blood vessels is essential for liver and pancreas organogenesis (Matsumoto et al., 2001; Lammert et al., 2001). Additionally, developing sympathetic neurons are guided by endothelins secreted by blood vessels (Makita et al., 2008), and liver regeneration in adults is triggered by factors secreted by liver sinusoidal endothelial cells (Ding et al., 2010).

In mice, vascular progenitor cells first develop in the posterior primitive streak in response to fibroblast growth factor-2 (FGF-2) and bone morphogenetic protein 4 (BMP4), and they are marked as vascular endothelial growth factor receptor 2 (VEGFR2)–positive mesodermal cells (Fig. 1; Park et al., 2004; Flamme et al., 1995). These precursor cells are committed for development into the hematopoietic and/or vascular lineage (hemangioblasts or angioblasts), and they migrate into extra-embryonic sites including the yolk sac and allantois, as well as into intra-embryonic sites (Huber et al., 2004; Hiratsuka et al., 2005). In the yolk sac, these progenitors aggregate and form clusters known as blood islands. The outer cells of the blood islands differentiate into endothelial cells, whereas the inner cells give rise to hematopoietic progenitor cells. These cells subsequently form primary capillary plexuses (vasculogenesis). In contrast, intra-embryonic angioblasts do not form a plexus intermediately and directly assembles into the dorsal aorta or cardinal vein. The extra-embryonic primary capillary plexuses then fuse with the intra-embryonic vessels to form a complete vascular network (angiogenesis). Angiopoietins and Tie receptors, along with vascular endothelial growth factor-A (VEGF-A) and Notch, are involved in this process (Thurston et al., 2003). Finally, mural cells that have differentiated in response to

transforming growth factor-β (TGF-β) are recruited to nascent vessels by platelet-derived growth factor (PDGF) secreted by endothelial cells (Betsholtz et al., 2005; Lebrin et al., 2005). This entire process ultimately leads to mature blood vessel formation.
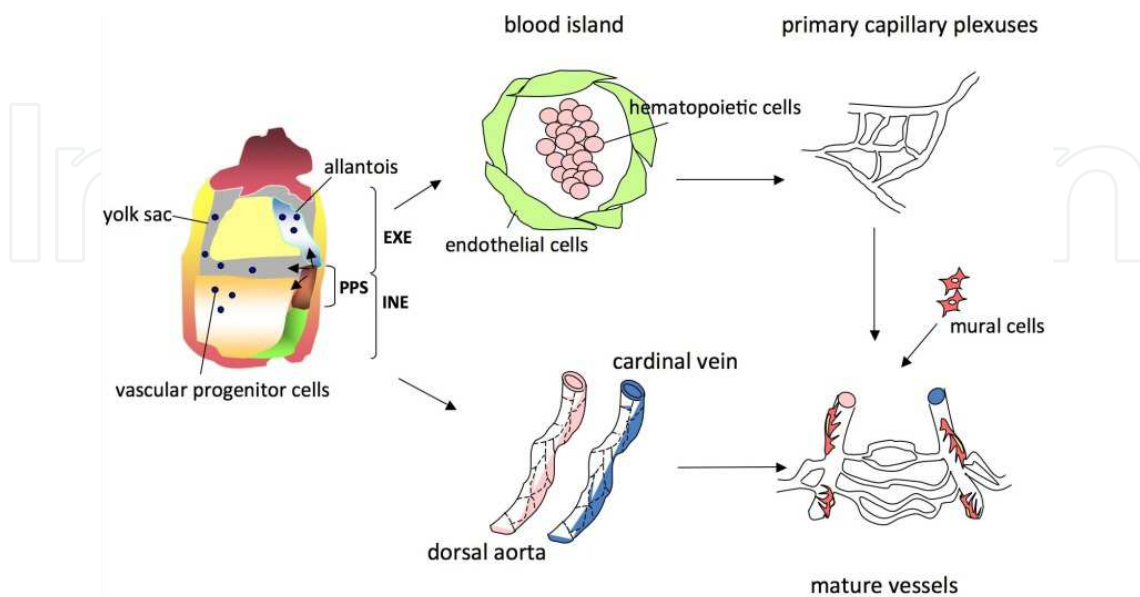

Fig. 1. Blood vessel formation during mouse development. EXE, extra-embryonic ectoderm; INE, intra-embryonic ectoderm; PPS, posterior primitive streak

A number of extracellular signaling molecules are involved in vascular development, some of which, including FGF-2, angiopoietins, PDGF, and VEGF-A, transmit their signals through receptor tyrosine kinases (RTKs). Signal transduction pathways downstream of RTKs have been extensively studied, and most share downstream effector components such as the Ras-mitogen–activated kinase (Ras-MAPK) and the phosphatidylinositol-3' kinase (PI3K) pathways. Intriguingly, these RTKs still transmit distinct signals and play unique roles that drive vascular development. The molecular basis for the observed signaling specificity remains unclear. To better understand this important process, we focused on signaling downstream of VEGFR2, a functional receptor for VEGF-A, using embryonic stem cell–derived VEGFR2+ vascular progenitor cells (Suzuki et al., 2005; Kawasaki et al., 2008; Sase et al., 2009). VEGFR2-null mice demonstrate severe defects in vasculogenesis, and VEGFR2 signaling is thought to play crucial roles in embryonic blood vessel formation (Shalaby et al., 1995; Shalaby et al., 1997; see below for details).

2. Use of differentiating embryonic stem cells for the study of lineage specification

To elucidate the signaling pathways important for the endothelial differentiation of vascular progenitor cells, we employed an *in vitro* vascular differentiation system using mouse embryonic stem cells (ESC) (Hirashima et al., 1999; Yamashita et al., 2000). *In vivo*, vascular progenitor cells must migrate to the correct microenvironment where they receive a cue for endothelial specification and further proliferate. It is challenging to identify the contributions of distinct signaling pathways to each *in vivo* event that occurs during vascular development, but the use of the *in vitro* system has allowed us to focus on the signaling

events required for endothelial specification by providing well-defined supplements to the culture medium.

We used ESC-derived vascular progenitor cells because it is practically impossible to prepare a sufficient amount of vascular progenitor cells from mouse embryos for biochemical study. In this *in vitro* system, ESCs are differentiated by culture on type IV-collagen–coated dishes in the absence of leukemia inhibitory factor (LIF) for four days, and during this time, mesodermal cells expressing VEGFR2 are induced. VEGFR2+ cells (comprising 5–10% of the total cell population) are then sorted by magnetic-activated cell sorting (MACS) and used as vascular progenitors. These cells differentiate into α-smooth muscle actin-positive (αSMA+) mural cells resembling vascular smooth muscle cells in the presence of PDGF-BB or serum, whereas they differentiate into platelet-endothelial adhesion molecule 1-positive (PECAM1+) endothelial cells in response to VEGF-A (Fig. 2; Yamashita et al., 2000; Ema et al., 2003; Watabe et al., 2003). Alteration of the differentiation fate of vascular progenitor cells was also examined by limiting dilution assay. VEGFR2+ cells were seeded at low density (90–120 cells/cm²) and allowed to form single-cell–derived colonies for four days. After immunostaining, the numbers of PECAM1+ or αSMA+ colonies, which reflect the fate of differentiation, were counted. In the presence of VEGF-A, endothelial differentiation occurs at the expense of mural differentiation: stimulation with VEGF-A increases PECAM1+ colonies and decreases αSMA+ colonies, while the total number of colonies remains constant. Thus, VEGF-A stimulation alters the differentiation fate of vascular progenitor cells.
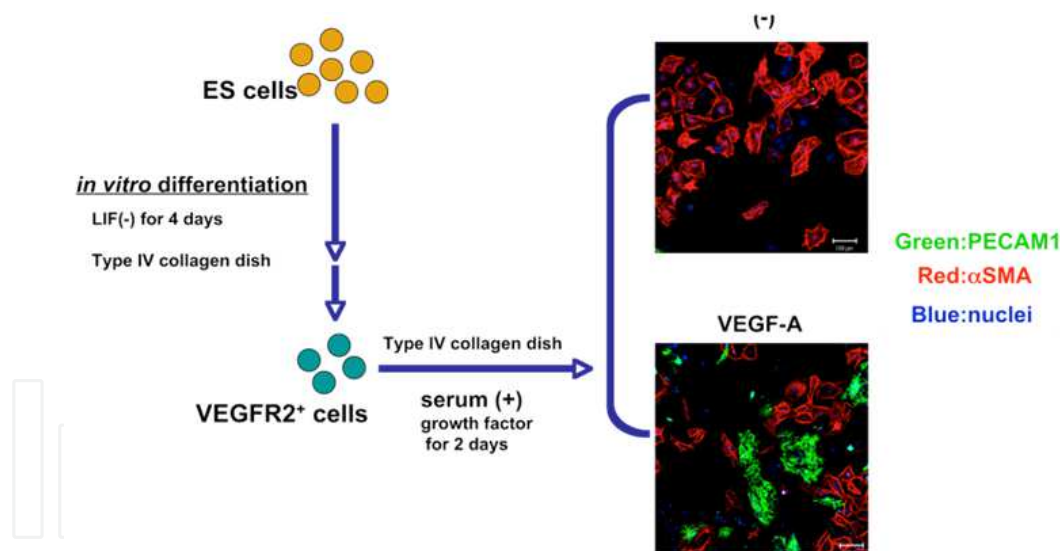

Fig. 2. *In vitro* vascular differentiation system (Yamashita et al., 2000)

This *in vitro* system was originally established using CCE embryonic stem cells. However, we used MGZ-5 cells and MGZRTcH cells because they are more amenable to transgene expression. Accordingly, we first studied MGZ-5 cells expressing genes contained in the pCAGIP vector (Fujikura et al., 2002). MGZ-5 cells express polyoma large T antigen, and this allows the efficient episomal propagation of plasmids with a polyoma origin of replication such as pCAGIP. Thus, transgenes contained within the pCAGIP vector are stably expressed in MGZ-5 cells (supertransfection method, Gassmann et al., 1996). In later experiments, we used MGZRTcH cells derived from MGZ-5 cells (Masui et al., 2005) in which a target gene

can be knocked into the *ROSA26* locus by homologous recombination using the Cre-*lox*P system, and gene expression is under the control of tetracycline (Tc) (tet-off system, Masui et al., 2005). Importantly, the *ROSA26* locus is minimally affected by epigenetic silencing during cell differentiation (Zamobrowicz et al., 1997). Therefore, expression of a transgene knocked into this locus can be regulated by Tc during nearly any stage of differentiation. Using this *in vitro* system, we have successfully pursued two different lines of investigation of vasculogenesis: examination of downstream effectors of VEGFR2 signaling (Section 3) and screening of pharmacological inhibitors (Section 4).

3. VEGFR2 transmits unique signals to induce endothelial differentiation

VEGFR2, also known as Flk1 in mice and KDR in human, is a member of the VEGFR family of RTKs, which contains three members, VEGFGR1-3 (Fig. 3; Shibuya & Claesson–Welsh, 2006). VEGFRs share characteristic structural features: they are composed of seven extracellular immunoglobulin-like domains, a transmembrane region, and an intracellular region with intrinsic tyrosine kinase activity. They have distinct expression profiles and different ligand specificities among VEGF family members.

VEGFR1, also called Flt-1 (fms-like tyrosine kinase 1) is expressed in vascular endothelial cells, hematopoietic stem cells, macrophages, and monocytes. VEGFR2 is expressed in both vascular and lymphatic endothelial cells. VEGFR3, also called Flt-4 (fms-like tyrosine kinase 4), is principally expressed in lymphatic endothelial cells.
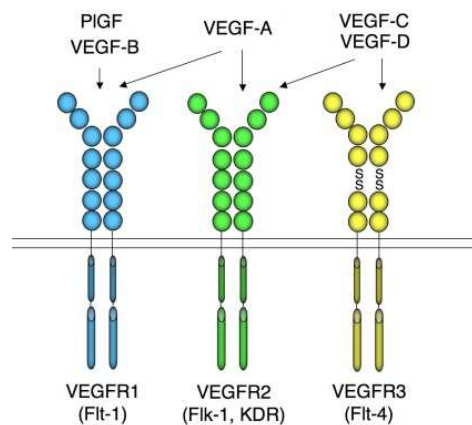

Fig. 3. VEGFR family members and their cognate ligands. The tyrosine kinase domains of VEGFRs are separated by the "kinase insert" of ~60 amino acid residues, a structural feature common to PDGF receptors and FGF receptors. The fifth immunoglobulin-like domain of VEGFR3 has an internal cleavage. The resultant N-terminal and C-terminal polypeptides are linked together by a disulfide bond.

VEGF family members, including VEGF-A, -B, -C, -D and placenta growth factor (PlGF), regulate the development, growth and function of vascular as well as lymphatic endothelial cells (Ferrara et al., 2003). VEGF-A is bound by VEGFR2 and VEGFR1, which transmit signals essential for vasculogenesis and angiogenesis. VEGF-C and VEGF-D are bound by VEGFR2 and VEGFR3 and are both involved in lymphangiogenesis (Karkkainen et al., 2004; Baldwin et al., 2005). PlGF and VEGF-B signal through VEGFR1, and they contribute to pathological angiogenesis (Carmeliet et al., 2001; Mould et al., 2003) and endothelial fatty acid uptake (Hagberg et al., 2010).

3.1 Essential role of VEGFR2 during vasculogenesis

Individual knock-out mice lacking VEGFR1, VEGFR2, or VEGFR3 all demonstrate vascular defects. VEGFR1-deficient mice exhibit disorganized vasculature secondary to endothelial cell overgrowth (Fong et al., 1995). VEGFR3 knockout mice have defects in vascular remodeling and develop fluid accumulations in the pericardial sac (Dumont et al., 1998). Blood vessel formation occurs in VEGFR1$^{-/-}$ and VEGFR3$^{-/-}$ mice. VEGFR1 and VEGFR3 are thus indispensable for angiogenesis but not vasculogenesis. In contrast, VEGFR2 plays essential roles during vasculogenesis. It is first expressed in the vascular progenitor cells at the posterior primitive streak. VEGFR2-deficient mice die *in utero* between 8.5 and 9.5 d.p.c. due to lack of organized blood vessels and hematopoietic cells. VEGFR2 signaling is required for the formation of blood islands at extraembryonic sites (Shalaby et al., 1995), where vascular endothelial cells and hematopoietic cells differentiate to form primary plexuses. In the absence of VEGFR2 signaling, vascular progenitor cells fail to migrate to extraembryonic sites from the posterior primitive streak (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 located at the correct anatomical niche, but they fail to complete the differentiation pathway. The identified roles of VEGFR2 signaling in vascular development *in vivo* include promoting the proliferation, migration, and differentiation of progenitor cells. Additionally, VEGF-A$^{+/-}$ mice have defects in vascular development (Carmeliet te a., 1996; Ferrara et al., 1996), but VEGF-C$^{-/-}$ mice and VEGF-D$^{-/-}$ mice exhibit lymphatic defects (Karkkainen et al., 2004; Baldwin et al., 2005). Therefore, the VEGF-A/VEGFR2 axis appears to be essential for vasculogenesis.

Because VEGFR2^{+} mesodermal cells can give rise to 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. We examined the nature of this unique and specific signaling.

3.2 VEGFR2, but not VEGFR3, directs endothelial differentiation

Although VEGFRs differ in their ligand-binding properties, their intracellular domains share structural similarities. However, the signaling properties of VEGFRs remain incompletely understood.

VEGF-A, which initiates signaling from both VEGFR1 and VEGFR2, promotes the differentiation of endothelial cells from ESC-derived VEGFR2^{+} cells, whereas PlGF, a specific ligand for VEGFR1, fails to induce endothelial differentiation despite VEGFR1 expression by precursor cells (Yamashita et al., 2000). Thus, VEGFR1 signaling does not direct endothelial differentiation. We then examined the ability of VEGFR3 to induce endothelial differentiation (Suzuki et al., 2005). While VEGFR3 is not expressed by VEGFR2^{+} vascular progenitor cells, we ectopically expressed VEGFR3 to examine whether VEGFR3 signaling induces endothelial cell differentiation.

3.2.1 VEGFR3 fails to induce endothelial differentiation

VEGFR3 cDNA was introduced into MGZ-5 ES cells using the supertransfection technique followed by drug selection (Gassmann et al., 1996). VEGFR2^{+} vascular progenitor cells were then collected from the transfected cells after *in vitro* differentiation (Suzuki et al., 2005).

These cells differentiated into PECAM1^{+} endothelial cells in response to VEGF-C stimulation. Although VEGF-C is bound by both VEGFR2 and VEGFR3, it does not promote endothelial differentiation through endogenous VEGFR2 signaling, because it failed to induce endothelial differentiation of mock-transfected cells. We next examined the effect of VEGF-C(C152S), a mutant that selectively stimulates VEGFR3 (Kirkin et al., 2001). This mutant did not induce endothelial differentiation, but it caused vascular progenitor cell differentiation into mural cells (Suzuki et al., 2005). Thus, VEGFR3 signaling alone is not sufficient to induce endothelial differentiation. It remains possible that VEGF-C induces endothelial differentiation through heterodimer formation between ectopic VEGFR3 and endogenous VEGFR2 in these experimental conditions. Collectively, these findings indicate that VEGFR2 has unique properties among VEGFR family members even in this *in vitro* system.

3.2.2 The intracellular domain of VEGFR2 can transmit signals for endothelial differentiation

We next examined the intracellular events downstream of VEGFR2 that are required for endothelial differentiation. For this purpose, we constructed a chimeric receptor (denoted R32) that is composed of the extracellular and the transmembrane domains of VEGFR3 fused with the intracellular domain of VEGFR2 (Fig. 4; Sase et al., 2009). We then established ES cell lines expressing either R32 or VEGFR3 under the control of the Tc-regulated promoter in MGZRTcH cells (denoted Tc-R32 and Tc-VEGFR3, respectively).
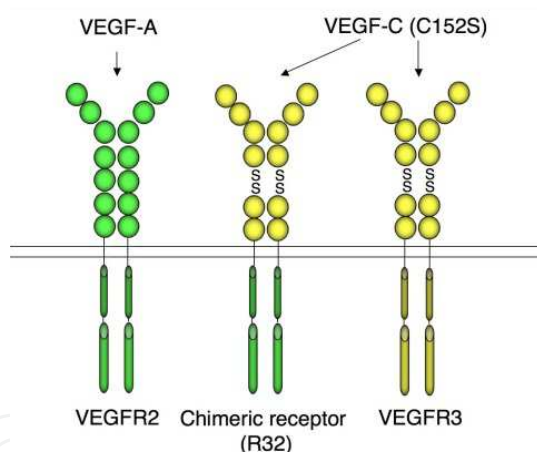

Fig. 4. Schematic structure of the chimeric receptor R32

VEGFR2[+] vascular progenitor cells prepared from these cell lines differentiated into PECAM1^{+} cells following VEGF-A stimulation, indicating that these cell lines retain their ability to differentiate into endothelial cells. However, VEGFR2^{+} cells derived from Tc-R32, but not Tc-VEGFR3, differentiated into PECAM1^{+} cells upon stimulation with VEGF-C(C152S). The PECAM1^{+} cells were also positive for other endothelial markers (VE-cadherin, CD34, and endoglin) and appeared to be endothelial cells. Therefore, the differences in phenotypes between VEGFR2^{+} cells derived from Tc-R32 and Tc-VEGFR3 cells are due to intrinsic properties of the intracellular domains of VEGFR2 and VEGFR3. The intracellular domain of VEGFR2 is sufficient to direct endothelial differentiation of ESC-derived vascular progenitor cells.

3.2.3 Tyrosine 1175 of human VEGFR2 is required for endothelial specification of vascular progenitor cells and endothelial cell survival

The role of signal transduction pathways downstream of VEGFR2 for cell proliferation, migration and survival have been well explored in mature endothelial cells. Five tyrosine residues (Y951, Y1054, Y1059, Y1175, and Y1214) have been identified in the intracellular domain of VEGFR2 as major phosphorylation sites (Matsumoto et al., 2005). Y1054 and Y1059 are located in the activation loop of the kinase domain and are required for the activation of intrinsic kinase activity. The remaining tyrosine residues are located outside of the kinase domain and are required for recruitment of downstream effectors. Phosphorylation of Y1175 leads to phospholipase Cγ-activation, followed by protein kinase C (PKC) β-mediated Raf activation to induce cell proliferation (Takahashi et al., 2000). Y1175 is also involved in the activation of the PI3 kinase pathway through the adaptor protein Shb (Welch et al., 1994). In contrast, phosphorylation of Y951 leads to cell migration and actin stress fiber organization through interactions with T cell specific adaptor (TSAd) (Matsumoto et al., 2005). Phosphorylation of Y1214 is also implicated in actin stress fiber remodeling through the p38 mitogen activated kinase pathway (Lamalice et al., 2004). Y951 and Y1175 are unique tyrosine residues in VEGFR2, whereas Y1214 is conserved in VEGFR2 and VEGFR3.
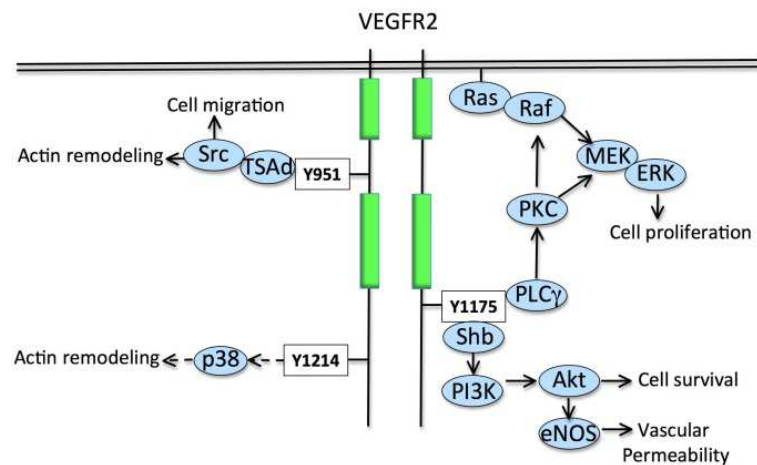

Fig. 5. Signal transduction downstream of VEGFR2

Y1173 in mouse VEGFR2 (corresponding to Y1175 in humans) is indispensable for blood vessel formation *in vivo* (Sakurai et al., 2005). VEGFR2 Y1173F knock-in mice died between E8.5 and E9.5, similar to VEGFR2-null mice. In Y1173F knock-in mice, VEGFR2+ vascular progenitor cells failed to migrate into the yolk sac to form blood islands, and, because vasculogenesis was aborted and progenitor cells did not receive specification signals, it remained unclear whether Y1173 is also important for endothelial specification of vascular progenitor cells. Therefore, we addressed this question using the *in vitro* differentiation system.

3.2.3.1 Tyrosine 1175 of human VEGFR2 is required for endothelial differentiation

To examine the roles of individual tyrosine residues in the induction of endothelial differentiation, we constructed three mutants of the R32 chimeric receptor (R32Y951F, R32Y1175F, and R32Y1214F) in which the indicated tyrosine residues were mutated to phenylalanine. After confirming the ability of these mutants to activate effective signals, we

established MGZRTcH ES cell lines expressing these mutant receptors (Tc-R32Y951F, Tc-R32Y1175F, and Tc-R32Y1214F). VEGFR2+ vascular progenitor cells were prepared from these cell lines and examined for endothelial differentiation in the presence of VEGF-C(C152S) (Sase et al., 2009).

VEGFR2+ cells derived from Tc-R32Y951F and Tc-R32Y1214F differentiated into endothelial cells following treatment with VEGF-C(C152S) whereas those from Tc-R32Y1175F failed to do so. To exclude the possibility that signals associated with residues Y951 and Y1214 could compensate for one another to promote endothelial differentiation, we also established a cell line expressing a double mutant, Tc-R32Y951/1214F. VEGFR2+ cells derived from this cell line retained their ability to undergo endothelial differentiation. These findings indicate that signaling from Y1175 plays a central role in the endothelial differentiation of vascular progenitor cells, but residues Y951 and Y1214 are dispensable for this function.

In this *in vitro* differentiation system, endothelial cells appear only when vascular progenitor cells are successfully specified into endothelial cells and when the differentiated endothelial cells are able to survive and proliferate (Fig. 6, top panel). Endothelial cells are unable to survive in the presence of serum alone, and they typically require growth factors such as VEGF-A or FGFs for survival and/or proliferation. We investigated whether signaling from Y1175 plays a role in specification, survival, or both processes.
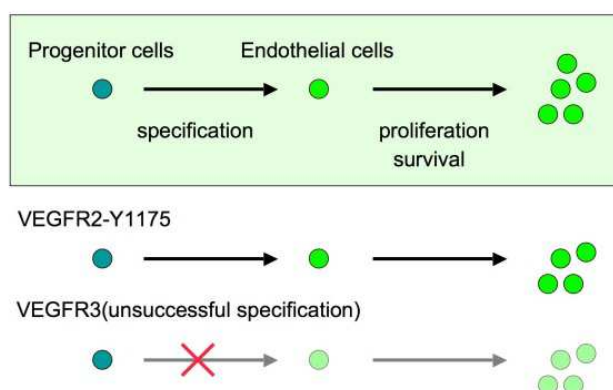

Fig. 6. VEGFR signaling and endothelial differentiation

3.2.3.2 Tyrosine 1175 of human VEGFR2 is required for endothelial survival

We first performed an endothelial survival assay (Fig. 7) with Tc-R32, Tc-R32Y1175F, and Tc-VEGFR3. ESC-derived VEGFR2+ cells were cultured with VEGF-A in serum-free medium for two days to induce mature endothelial cells, and the endothelial cells were then cultured in serum-free medium with or without VEGF-C(C152S) to activate the chimeric receptors.

The number of endothelial cells cultured in the absence of VEGF-C(C152S) was considerably decreased within 12 h. VEGF-C(C152S)–induced signaling from R32 led to a significant recovery in the number of cells, but VEGF-C(C152S) treatment of cells expressing R32Y1175 failed to do so. Thus, Y1175 is involved in the transmission of survival signals to endothelial cells. As described in 3.2.1, VEGFR3 signaling is not sufficient for inducing endothelial differentiation from VEGFR2+ vascular progenitor cells. However, VEGFR3 signaling increased the survival of ESC-derived endothelial cells. We also found that LY294002, an inhibitor of PI3K, abrogated the effects of VEGFR3 on endothelial survival. These findings suggest that VEGFR3 signaling promotes endothelial survival despite its inability to direct endothelial specification (Fig. 6).
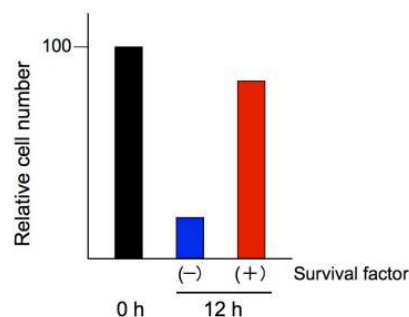
Fig. 7. Endothelial survival assay. When ESC-derived endothelial cells are cultured in serum-free medium, the cell number largely decreases within 12 h, while in the presence of an appropriate survival factor, the cell number is maintained.

3.2.3.3 Tyrosine 1175 of human VEGFR2 is required for endothelial specification

We next examined the involvement of signaling from Y1175 in the endothelial specification of vascular progenitor cells. Because R32Y1175F failed to transmit appropriate survival signals, we supplemented the cell cultures with a low dose of FGF-2 (0.5 ng/ml) to promote endothelial cell survival. VEGFR2$^+$ cells derived from Tc-R32Y1175F failed to differentiate into endothelial cells upon stimulation with VEGF-C(C152S) although those derived from Tc-R32 successfully underwent differentiation. Thus, human VEGFR2 Y1175 is essential for the induction of endothelial cells from vascular progenitor cells through both specification of VEGFR2$^+$ cells and their subsequent survival (Fig. 6).

3.2.4 The VEGFR2-Y1175-PLCγ1 pathway is indispensable for endothelial specification

In mature endothelial cells, Y1175 of human VEGFR2 primarily transmits cell proliferation signals through the recruitment of PLCγ and cell survival signals through the recruitment of Shb (Fig. 5). Intriguingly, PLCγ1 deficient mice are embryonically lethal secondary to a lack of vasculogenesis and erythrogenesis despite the presence of hemangioblasts (Liao et al., 2002). Thus, PLCγ1 may be involved in the specification of vascular progenitor cells and/or subsequent survival of endothelial cells. We hypothesized that R32Y1175F failed to induce endothelial differentiation because it does not activate PLCγ1.

3.2.4.1 Knockdown of PLCγ1 attenuated endothelial differentiation

To elucidate the function of PLCγ1 in endothelial differentiation, we established a stable ES cell line in which expression of PLCγ1 can be knocked down by expression of pre-miRNA under the control of Tc (Tc-miRNA-PLCγ1) (Sase et al., 2009). We used this system to perform gene silencing studies because we had difficulty introducing siRNA oligonucleotides to VEGFR2$^+$ vascular progenitor cells; we were able to easily introduce them into ES cells or differentiated endothelial cells, however (Kawasaki et al., 2008). It is likely that differentiating ES cells may be resistant to transfection at certain stages of development.

Expression of the miRNA targeting PLCγ1 resulted in a modest decrease in PLCγ1 protein expression and decreased appearance of endothelial cells, indicating that PLCγ1 signaling is required for endothelial differentiation.

3.2.4.2 Constitutively active PLCγ1 induces endothelial specification

We next constructed a constitutively active form of PLCγ1, PalmPLCγ1, in which a sequence for myristoylation and palmitoylation was added to its N-terminus. This mutant protein is

constitutively localized at the plasma membrane. We then established a stable ES cell line harboring Tc-regulatable PalmPLCγ1 (Tc-PalmPLCγ1) or a negative control cell line (Tc-empty) and examined the differentiation of VEGFR2^{+} cells derived from these cell lines.

The induced expression of PalmPLCγ1 did not lead to the appearance of PECAM1^{+} cells from VEGFR2^{+} progenitor cells, and, after performing an endothelial survival assay, we found that PalmPLCγ1 did not transmit signals for cell survival in ESC-derived endothelial cells. We next examined endothelial differentiation of VEGFR2^{+} cells in the presence of a low dose of FGF-2 to support survival of endothelial cells. Stimulation with PalmPLCγ1 plus FGF-2 reconstituted signaling for endothelial differentiation although stimulation with only FGF-2 did not affect the number of differentiated endothelial cells. PalmPLCγ1-expressing progenitor cells treated with FGF-2 became positive for PECAM1, VE-cadherin, CD34, and endoglin, and these findings indicate that PLCγ1 signaling is involved in the endothelial specification of vascular progenitor cells but not in the survival of differentiated endothelial cells (Fig. 8). Consistent with this, VEGFR3, which transmits signals for endothelial survival but not endothelial specification, failed to activate PLCγ1 (Sase et al., 2009).

At present, the signaling pathways downstream of Y1175 that mediate survival of endothelial cells remain to be elucidated. However, Shb may be a good candidate for a signaling effector because it activates the PI3K pathway.
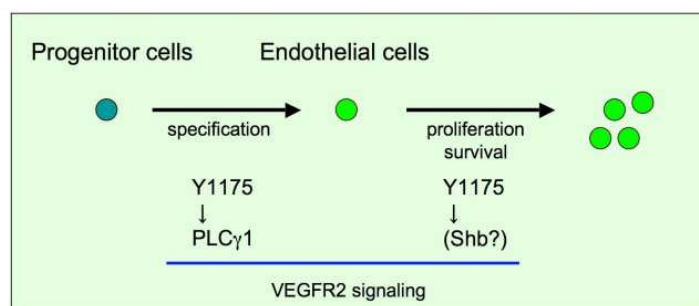

Fig. 8. Signaling from VEGFR2-Y1175 for induction of endothelial cells *in vitro*

4. Ras signaling specifies endothelial lineage

In addition to the chimeric receptor approach described above, we also used various pharmacological inhibitors to study endothelial differentiation (Kawasaki et al., 2008), and we found that temporally regulated Ras signaling plays a crucial role in endothelial specification downstream of PLCγ1.

4.1 A farnesyltransferase inhibitor, FTI-277, inhibits VEGF-A–induced endothelial differentiation of ESC-derived VEGFR2^{+} cells

To determine the signaling components involved in VEGF-A–induced endothelial differentiation from vascular progenitor cells, we screened various low molecular weight compounds targeting signaling molecules for their ability to inhibit endothelial differentiation *in vitro*. Among the compounds tested, we found that the farnesyltransferase inhibitor FTI-277 (Lerner et al., 1995) selectively affects endothelial differentiation. FTI-277 interferes with the farnesylation of small G proteins and their subsequent association with the plasma membrane, thus abrogating their signaling functions. FTI-277 inhibited the appearance of VEGF-A–induced PECAM1^{+} cells, but that of αSMA[+] was not. The effect of

FTI-277 was also confirmed in a limiting dilution assay. In the presence of FTI-277, the number of PECAM1+ colonies was decreased while αSMA+ colonies were increased. Importantly, the total number of derived colonies was not significantly changed. These findings indicate that FTI-277 specifically inhibits the endothelial differentiation of ESC-derived VEGFR2+ cells.

We next performed an *ex vivo* whole-embryo culture assay to confirm the effects of FTI-277 on vascular development in a mouse embryo (Fig. 9; Kawasaki et al., 2008). Concepti at embryonic day 6.75 (E 6.75) were picked out from the uteri of pregnant mice and cultured *in vitro* for three days, and, in the absence of further manipulation, PECAM1+ blood vessels formed in the yolk sac during this time. However, FTI-277 treatment led to a reduced number of PECAM1+ vessels, but the overall development of the yolk sac was not affected. Quantitative RT-PCR analysis also indicated that PECAM1 expression was decreased in the presence of FTI-277, while the expression of αSMA was not affected. These findings suggest that FTI-277 selectively suppresses vascular development. Importantly, endothelial differentiation induced by constitutively active PalmPLCγ1 plus FGF-2 was inhibited by FTI-277, indicating that FTI-277 targets signaling pathway(s) downstream of PLCγ1.
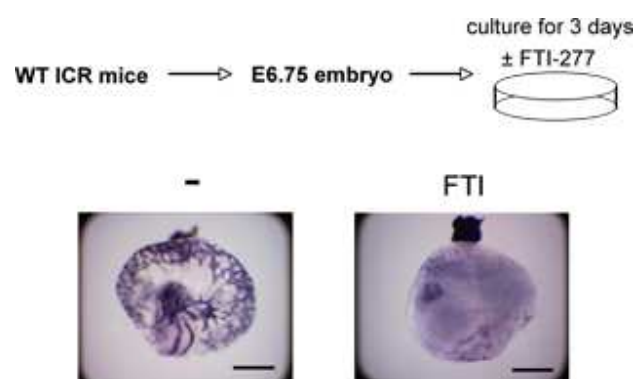

Fig. 9. FTI-277 inhibited *ex vivo* yolk sac vascularization (Kawasaki et al., 2008. Originally published *in Journal of Cell Biology*. doi: 10.1083/jcb.200709127). E6.75 concepti were picked out and cultured with or without FTI-277 (10 μM) for three days and whole-mount stained for PECAM1.

4.2 Loss of H-Ras attenuates endothelial differentiation of VEGFR2+ cells *in vitro*

Because the principal targets of FTI-277 include H-Ras, we hypothesized that H-Ras signaling could be involved in the VEGF-A–induced endothelial differentiation of vascular progenitor cells. To examine this possibility, we established an ES cell line based on MGZRTcH cells in which a pre-miRNA sequence targeting H-Ras is expressed under the control of Tc (Tc-miR-H-Ras). In Tc-miR-H-Ras cells, expression of endogenous H-Ras was efficiently knocked down in the absence of Tc (Tet-off system). A limiting dilution assay was performed using Tc-miR-H-Ras–derived VEGFR2+ cells in the presence or absence of Tc. In the absence of Tc (H-Ras knocked-down condition), the number of VEGF-A–induced PECAM1+ colonies was decreased while the number of αSMA+ colonies was increased compared to cells grown in the presence of Tc (PECAM1:αSMA=51.6%:48.4% to 36.8%:63.2%). These findings suggest that H-Ras plays a role in the endothelial differentiation of VEGFR2+ progenitor cells.

4.3 Loss of H-Ras attenuates endothelial differentiation in mouse embryos

To examine the importance of H-Ras signaling during vascular development *in vivo*, we investigated the vascular phenotype of H-*ras* knockout mice. Because heterozygous H-*ras*+/- mice produced homozygous H-*ras*−/− offspring in the expected Mendelian ratio as previously reported (Ise et al., 2000), we focused on the vascular phenotype during early development. There were no clear differences in the vascular phenotypes of wild-type (WT) and H-*ras*+/- embryos. Vascular anomalies were found in the brain of 73% of H-*ras*-/- embryos examined at E9.5 although they contained similar numbers of somites as their WT littermates (Fig. 10; Kawasaki et al., 2008).

We next double-stained the cephalic region of the embryos for PECAM1 and VEGFR2, the earliest marker of endothelial cell differentiation. In H-*ras*+/- embryos, numerous complex vascular networks were stained for both PECAM1 and VEGFR2, but vascular structures positive for either PECAM1 or VEGFR2 were strikingly reduced in H-*ras*-/- embryos. However, these defects were transient because no abnormalities were apparent in E10.5 H-*ras*-/- embryos. Collectively, these data suggest that H-Ras signaling is involved in the *in vivo* differentiation of endothelial cells although it is not indispensable.
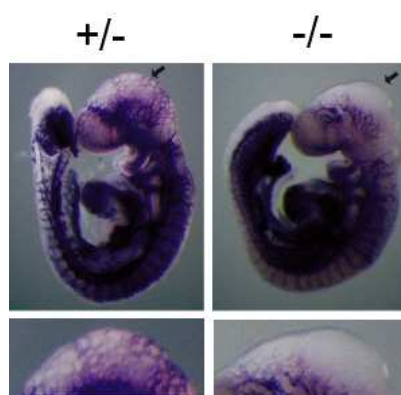

Fig. 10. Whole-mount PECAM1 staining of E9.5 H-*ras*+/- and H-*ras*-/- mice (Kawasaki et al., 2008. Originally published *in Journal of Cell Biology*. doi: 10.1083/jcb.200709127). Magnifications of the area marked with arrows in the top are shown in the bottom.

4.4 A constitutively active G12V mutant of H-Ras induces PECAM1^{+} cells from VEGFR2^{+} progenitor cells

We next established an ES cell line carrying a constitutively active form of H-Ras (Tc-H-Ras[G12V]) under the control of Tc, and we examined the differentiation of VEGFR2+ cells derived from this cell line. When H-Ras[G12V] expression was suppressed by Tc treatment, the appearance of PECAM1+ cells was VEGF-A–dependent. However, expression of H-Ras[G12V] led to the VEGF-A–independent induction of PECAM1+ cells. These PECAM1+ cells were also positive for other endothelial markers including CD34 and endoglin, and they incorporated AcLDL. Additionally, the aggregated VEGFR2+ cells derived from Tc-H-Ras[G12V] cells formed tube-like structures even in the absence of VEGF-A when cultured in type I collagen gel for seven days, indicating that vascular formation by VEGFR2+ cells is dependent on H-Ras signaling. Furthermore, Tc-H-Ras[G12V] cells formed vascular structures when subcutaneously injected with Matrigel into the abdominal region of mice, dependent upon the expression of H-Ras[G12V]. These findings suggest that active Ras induces the differentiation of cells with characteristics of endothelial cells from VEGFR2+

progenitor cells (Kawasaki et al., 2008). Additionally, data from a limiting dilution assay showed that expression of H-Ras[G12V] led to endothelial differentiation at the expense of mural differentiation. Thus, the fate of cell differentiation was altered by Ras signaling.

Although Ras signaling induces the expression of VEGF-A (Rak et al., 1995; Gruger et al., 1995), Ras-induced endothelial differentiation was not dependent on autocrine stimulation by induced VEGF-A. Endothelial differentiation by H-Ras[G12V] proceeded normally in the presence of SU5614, an inhibitor of VEGFR2 kinase (Spiekermann et al., 2002), or VEGFR1 (Flt1)-Fc chimeric protein which competes with VEGFR2 for binding to VEGF-A. Collectively, these findings suggest that differentiation depends on intracellular Ras signaling.

4.5 Signaling for endothelial differentiation is mediated through the Ras-Erk pathway

Ras proteins activate both the Raf/MEK/Erk and the PI3K/Akt pathways. To determine the signaling pathway(s) that mediate Ras-induced endothelial differentiation, we established ES cell lines in which H-Ras effector mutants H-Ras[G12V, T35S] or H-Ras[G12V, Y40C] can be inducibly expressed. The effector mutants H-Ras[G12V, T35S] and H-Ras[G12V, Y40C] preferentially activate the Raf/MEK/Erk and PI3K/Akt pathways, respectively (Joneson et al., 1996). When H-Ras[G12V, T35S] was expressed, PECAM1$^+$ colonies increased in number, while αSMA$^+$ colonies were decreased as determined by limiting dilution assay. In contrast, when H-Ras[G12V, Y40C] was expressed, the numbers of PECAM1$^+$ colonies and αSMA$^+$ colonies were unchanged. These findings suggest that the Ras-PI3K pathway does not affect the determination of cell fate, and we concluded that the Ras-Erk pathway specifies the endothelial fate of VEGFR2$^+$ progenitor cells.

4.6 Kinetics of Ras activation by VEGF-A

Ras proteins are activated by extracellular stimuli including hormones, cytokines and growth factors. ESC-derived VEGFR2$^+$ cells differentiate into endothelial cells upon stimulation with VEGF-A, but not upon stimulation with PDGF-BB, a known activator of Ras signaling. Thus, the activation statuses of Ras are different between that downsteam of VEGFR2 and that downstream of PDGF receptors.

We examined the effects of FTI-277 treatment at different time points after VEGF-A stimulation. FTI-277 inhibited endothelial differentiation even when added 3 h after VEGF-A stimulation, indicating that Ras activation later than 3 h after stimulation plays a role in endothelial differentiation. We hypothesized that the specificity of Ras signaling induced by VEGFR2 may be attributed to the timing of Ras activation, and we investigated the window within which Ras is specifically activated by VEGF-A, focusing on the period later than 3 h after VEGF-A stimulation.

We examined the levels of phosphorylation of Erk, a downstream effector of Ras, 3–12 h after stimulation with VEGF-A. Erk phosphorylation peaked at 6 h and 9 h after stimulation, suggesting that Ras could be activated with a similar time course. We next examined the activation of Ras in cells stimulated with VEGF-A or PDGF-BB for 6 h, and the patterns of Ras activation in response to VEGF-A or PDGF-BB were 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, both VEGF-A and PDGF-BB induced activation of Ras and Erk although to different extents. Activation of Ras and Erk by VEGF-A was also observed at 9 h, but not at 3 h after stimulation (Fig. 11). Importantly,

phosphorylation of Erk around 6 h after VEGF-A stimulation was sensitive to FTI-277. Activation of the Ras-Erk pathway 6–9 h after stimulation with VEGF-A thus appears to direct endothelial differentiation of VEGFR2+ progenitor cells. These findings provide mechanistic insights into signaling events required for cell specification through widely-shared effector molecules.

4.7 VEGF-A–specific Ras activation precedes the expression of endothelial markers

We finally examined whether the expression of vascular markers is induced after the delayed activation of the Ras-Erk pathway during *in vitro* differentiation of vascular progenitor cells. The mRNA expression of the endothelial markers PECAM1 and VE-cadherin increased from 12 h after VEGF-A stimulation. Intriguingly, the expression of VEGFR2 in VEGF-A–stimulated cells was similar to that seen in non-stimulated cells up to 6 h after treatment. However, at time points later than 12 h after stimulation, VEGFR2 expression was up-regulated in VEGF-A–stimulated cells, but it was down-regulated in non-stimulated cells. These observations suggest that specification to endothelial lineage occurs between 6–12 h after stimulation with VEGF-A, and this is preceded by the delayed activation of Ras (Fig. 11). We conclude that VEGF-A stimulation of VEGFR2+ vascular progenitor cells specifically induces Ras-Erk activation around 6–9 h after stimulation, and this event in turn specifies endothelial differentiation.
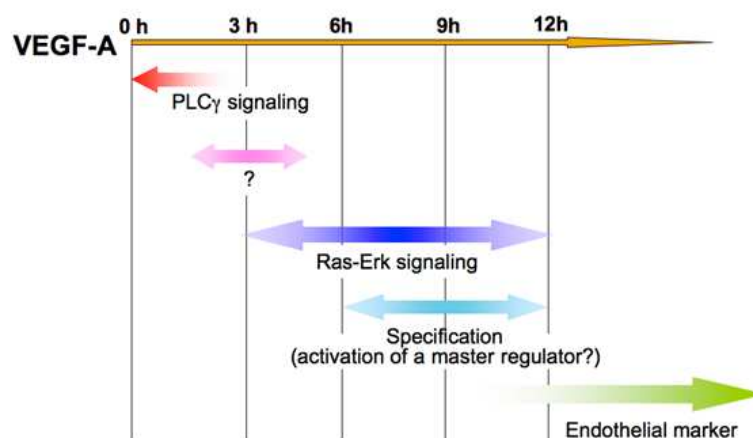

Fig. 11. Kinetics of endothelial differentiation of vascular progenitor cells after VEGF-A stimulation. The link between PLCγ and Ras-Erk (the second double-headed arrow) remains to be elucidated.

5. Unsolved questions in signaling for endothelial differentiation

The development of multicellular organisms requires the orchestrated interactions of a wide variety of cells, including growth, migration, and differentiation. Extracellular factors as well as intracellular signaling molecules are involved in the regulation of cellular processes during development. Each signaling molecule appears to have defined functions that are dependent upon the appropriate cellular context.

VEGFR2 signaling plays a central role in *de novo* blood vessel formation. In an *in vitro* embryoid body culture system, VEGFR2-/- ES cells still give rise to endothelial cells with low efficiency (Schuh et al., 1999). This may be due to an effects of FGF-2 included in the culture

medium (Schuh et al., 1999), because FGF-2 induces endothelial differentiation of ESC-derived VEGFR2+ cells to a modest extent (Kano et al., 2005). VEGFR2 signaling appears to be the primary pathway for controlling endothelial specification with high efficiency, although it is not the sole pathway capable of mediating these effects. We found that activation of the Ras-Erk pathway 6–9 h after stimulation with VEGF-A plays a critical role in endothelial specification of vascular progenitor cells. The mechanism(s) regulating the delayed activation of Ras and the downstream signaling of the Ras-Erk pathway are the next questions to be solved.

5.1 Upstream and downstream of the delayed Ras activation induced by VEGF-A

In ESC-derived VEGFR2+ progenitor cells, the PKC-dependent pathway (Fig. 5) appears to be activated early, since phosphorylation of Erk was notably increased while activation of Ras was modest 5 min after VEGF-A stimulation (Kawasaki et al., 2008). In contrast, the Ras pathway strongly induced Erk phosphorylation 6–9 h after stimulation, a finding supported by the inhibition of Erk phosphorylation by FTI-277. The mechanism(s) controlling the delayed activation of Ras remain unclear. At present, we only know that PLCγ1 lies upstream of the delayed Ras activation. It is possible that Ras activation is mediated through the transcriptional induction of some signaling molecules and/or activation of other signaling receptor(s). It is also important to identify transcription factors that are phosphorylated by Erk to better understand the signaling specificity of early and late Erk activation. VEGFR2+ cells develop into mural cells in the absence of growth factor, and mural differentiation may be the default fate of VEGFR2+ cells. During the differentiation of vascular progenitor cells, induction of mural markers is delayed compared to the induction of endothelial markers by VEGF-A: expression of αSMA was increased at 24 h while PECAM1 became expressed from 12 h after stimulation. It appears likely that VEGFR2+ cells in which the delayed Ras-Erk signaling is activated before the determination of cell fate to mural cells are differentiated into endothelial cells. However, it remains possible that VEGF-A signaling merely suppresses mural differentiation, thus altering the default differentiation fate. Alternatively, it is possible that VEGF-A signaling not only induces endothelial differentiation but also suppresses mural differentiation.

Although Ras appears to be activated downstream of PLCγ1 in vascular progenitor cells after stimulation with VEGF-A, constitutively active Ras transmits sufficient signals for full endothelial differentiation while constitutively active PLCγ1 transmits signals only for endothelial specification. This discrepancy remains unexplored. It is likely that constitutively active effector molecules may transmit artificial signals. In particular, G12V mutants of Ras proteins are resistant to intrinsic negative regulation through hydrolysis of GTP. H-Ras[G12V] may constitutively send strong signals that are sufficient to fully induce endothelial differentiation.

5.2 Phenotypes of H-Ras knockout mice

We examined vascular formation in H-ras-/- mice, and vascular anomalies were found in the brain of 73% of E9.5 H-ras-/- embryos. However, there were no obvious abnormalities in E10.5 H-ras-/- embryos, consistent with the previous report that H-ras knockout mice are born and grow normally (Ise et al., 2000). These findings suggest that H-ras-/- embryos catch up for the delay in vascular formation in the cephalic region until E10.5. It is possible that expression of other Ras family members, N-Ras and K-Ras, is upregulated, and these

proteins may compensate for the loss of H-Ras (Ise et al., 2000). Alternatively, a reduction of endothelial differentiation in the absence of H-Ras may be permissive for embryonic development. Compensatory growth of differentiated endothelial cells may offset the reduction in endothelial differentiation. It appears likely that N-Ras and K-Ras are also involved in endothelial specification because treatment with FTI-277 or knockdown of H-Ras failed to completely inhibit endothelial specification induced by VEGF-A in the *in vitro* vascular differentiation system.

5.3 Towards more efficient differentiation of endothelial cells

In recent years, revascularization therapy using endothelial progenitor cells (EPCs) has been studied for the treatment of ischemic disorders and arteriosclerosis. In this therapy, EPCs implanted at sites of disease form new vascular structures. Identification of the signaling pathways required for endothelial differentiation could provide valuable information for establishing a highly efficient endothelial differentiation system. This would be a boon to the field of regenerative medicine.

The efficiency of endothelial differentiation of vascular progenitor cells *in vitro* remains ~50%. Differentiating ES cells actively proliferate, indicating that they are going through cell cycles. Recent work revealed that Ras can efficiently transmit signals during G1 phase but not during G1/S or S/G2 (Sakaue–Sawano et al., 2008). Signals for endothelial specification are likely successfully transmitted in vascular progenitor cells at G1 phase (Fig. 12). Manipulations that control the cell cycle status may improve the efficiency of specification of vascular progenitor cells to endothelial cells, which is transmitted principally through Ras proteins.
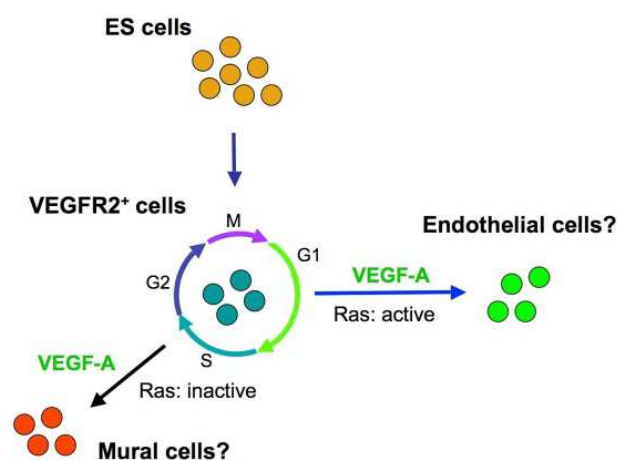

Fig. 12. Possible cell cycle–dependent signaling for endothelial differentiation through Ras activation

Modulation of the VEGFR2 signaling pathway at the level of receptor–ligand interaction may also improve the efficiency of endothelial differentiation *in vitro*. Recently, protein kinase A was found to induce expression of VEGFR2 and its co-receptor neuropilin-1, thus enhancing vascular progenitor potential (Yamamizu et al., 2009).

6. Conclusions

Vascular endothelial growth factor 2 (VEGFR2) is a critical signaling component controlling many aspects of vasculogenesis during embryonic development, including proliferation,

migration, and endothelial differentiation of vascular progenitor cells. However, the signaling pathways specifying endothelial differentiation downstream of VEGFR2 are poorly understood. We investigated these signaling pathways using an *in vitro* endothelial differentiation system (Kawasaki et al., 2008; Sase et al., 2009). Using a variety of different approaches including chimeric receptors, gene silencing, gain-of-function mutants, and pharmacological inhibitors, we dissected the signal transduction pathway that directs endothelial differentiation (Fig. 13). In response to VEGF-A stimulation, VEGFR2 transmits signals for specification of vascular progenitor cells and survival of endothelial cells through Y1175. PLCγ1, a downstream effector that is recruited to phosphorylated Y1175, relays endothelial specification signals. Activation of H-Ras that occurs at a delayed phase after VEGF-A stimulation triggers endothelial specification. The unique activation of signaling molecules downstream of VEGFR2/PLCγ/Ras is essential for transmitting signals controlling endothelial specification. At the present time, the link between PLCγ1 and H-Ras remains to be identified. Shb, another downstream effector recruited to phosphorylated Y1175, may be involved in the survival of endothelial cells through activation of the PI3K pathway. VEGFR3 lacks the ability to induce endothelial specification, and this may be due to its inability to activate PLCγ1, although it activates the PI3K pathway to support endothelial cell survival. It has previously been shown that Y1175 signaling is indispensable for migration of vascular progenitor cells to the site of their differentiation (Sakurai et al., 2005). Therefore, Y1175 of VEGFR2 appears to be a central node transmitting signals required for endothelial differentiation including migration, specification, and subsequent cell survival.
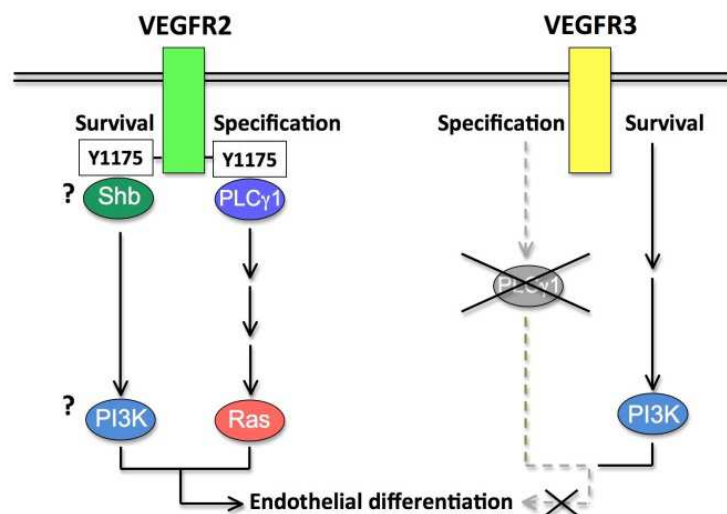

Fig. 13. Signal transduction pathways for endothelial differentiation from VEGFR2

7. Acknowledgement

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Embryonic Stem Cells - Differentiation and Pluripotent Alternatives

Edited by Prof. Michael S. Kallos

The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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