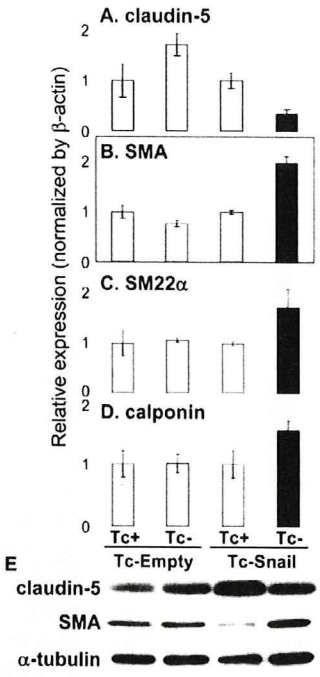

Fig. 5. Effect of Snail on expression of endothelial and mural markers in MESECs. (A-D) Levels of expression of claudin 5 (A), SMA (B), SM22α (C) and calponin (D) in MESECs derived from Tc-Empty or Tc-Snail ESCs cultured in the absence (–) or presence (+) of Tc were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (E) Protein levels of claudin 5 (top), SMA (middle) and α-tubulin (bottom) were examined by immunoblotting of total lysates of the MESECs described in A-D.

Smad4-independent pathways are partly involved in the TGFβ2-induced EndMT of MESECs

Upon ligand binding, TGFβ receptor complexes activate both Smad and non-Smad signaling pathways. In order to examine whether these non-Smad pathways are involved in the TGFβ-induced EndMT, we knocked down the expression of *Smad4*, the only co-Smad that is necessary for both the Smad2/3 and Smad1/5/8 pathways (Fig. 8A). In the MESECs in which *Smad4* expression was knocked down, TGFβ2 failed to induce the expression of PAI1 (SERPINE1) (Fig. 8B), a target of the Smad2/3 pathway, but partially induced Snail expression (Fig. 8C), suggesting that Snail is partially induced by Smad4-independent pathways. In accordance with the results of Snail expression, knockdown of *Smad4* expression failed to fully abrogate the TGFβ2-mediated EndMT (Fig. 8D), the suppression of claudin 5 (Fig. 8E) or the induction of SMA expression (Fig. 8F). These results suggest that TGFβ activates Smad4-dependent and -independent pathways, both of which play important roles in the induction of Snail expression that leads to EndMT.

We also examined whether the induction of EndMT by Snail transgene expression requires Smad4. The expression of the Snail transgene by removal of Tc from the culture of the MESECs derived from Tc-Snail ESCs was able to induce EndMT, both in the absence and presence of Smad4 expression (supplementary material Fig. S5A). These results were confirmed by quantitative RT-PCR (supplementary material Fig. S5B,C), and suggest that Snail induces the EndMT of MESECs as a downstream target of Smad4-mediated signals.

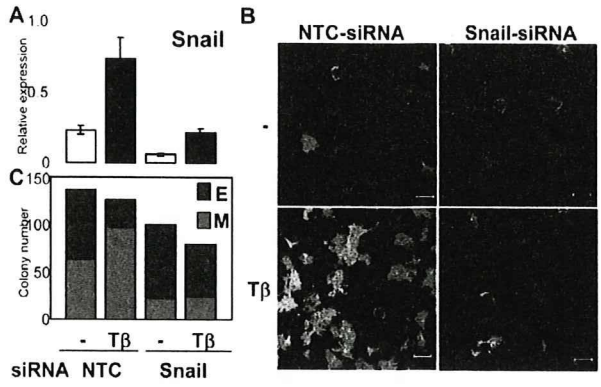

Fig. 6. Effect of *Snail* knockdown on MESECs. MESECs were sorted from the vascular cells derived from ESCs, transfected with Snail siRNA or with scrambled sequence as a negative control (NTC), and cultured in the absence (–) or presence of TGFβ2 (Tβ). (A) The levels of endogenous expression of Snail in the MESECs were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. Black and gray bars, represent +TGFβ2 and –TGFβ2, respectively. (B) The MESECs were subjected to immunofluorescence staining for PECAM1 (red) and SMA (green). (C) Quantitative analysis of the effects of Snail on colony formation from single MESECs, performed as described in Fig. 1F. Briefly, MESECs transfected with Snail siRNA or scrambled sequence were cultured at low density with 10% FCS in the absence (–) or presence of TGFβ2 (Tβ) for 4 days, followed by staining of colonies for PECAM1 and SMA. E, pure endothelial colony; M, mural-containing colony. Scale bars: 100 μm.

Discussion

In the present study, we showed that TGFβ2 induces the differentiation of endothelial cells into mural cells, with an increase in expression of the mural markers, SMA, SM22α and calponin. Previous reports have shown that TGFβ induces various mural markers during the differentiation of neural crest stem cells into smooth muscle cells (Shah et al., 1996), and that TGFβ-induced δEF1 is involved in this process (Nishimura et al., 2006). Although TGFβ has been shown to induce the expression of Snail during EMT of kidney epithelial cells (Peinado et al., 2003), functional roles of Snail during TGFβ-induced EMT were not fully elucidated. The present findings directly show, for the first time, that Snail mediates TGFβ-induced upregulation of multiple mural markers and the downregulation of claudin 5 in endothelial cells.

We also found that loss of Smad4 expression decreases, but does not completely abolish, TGFβ-induced Snail expression and EndMT (Fig. 8). We previously showed that Snail expression is upregulated within 30 minutes of addition of TGFβ to NMuMG mammary epithelial cells, in which TGFβ induces EMT (Shirakihara et al., 2007), suggesting that Snail is a direct target of TGFβ signals. The molecular mechanisms by which Smad4-dependent and -independent signals activate the Snail promoter in endothelial cells remain to be studied in the future.

Although Snail has been shown to play important roles in EMT, the molecular mechanisms by which Snail regulates the transcription of EMT-related targets have not been elucidated. In order to examine whether Snail binds to the endogenous SMA promoters in intact chromatin, we have subjected cross-linked chromatin samples prepared from Tc-Snail ESC-derived endothelial cells to chromatin immunoprecipitation (ChIP) assays. Nishimura and colleagues previously identified a TGFβ-responsive SMA promoter region containing Smad3-binding sequences and an E-box to which

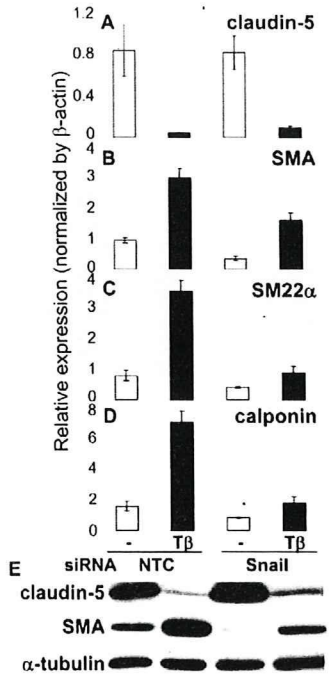

Fig. 7. Effect of Snail on expression of endothelial and mural markers in MESECs. (A-D) Levels of expression of claudin 5 (A), SMA (B), SM22α (C) and calponin (D) in MESECs transfected with Snail siRNA or scrambled sequence as a negative control (NTC), and cultured in the absence (–) or presence of TGFβ2 (Tβ) were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (E) Protein levels of claudin 5 (top), SMA (middle) and α-tubulin (bottom) were examined by immunoblotting of total lysates of the MESECs described in A-D.

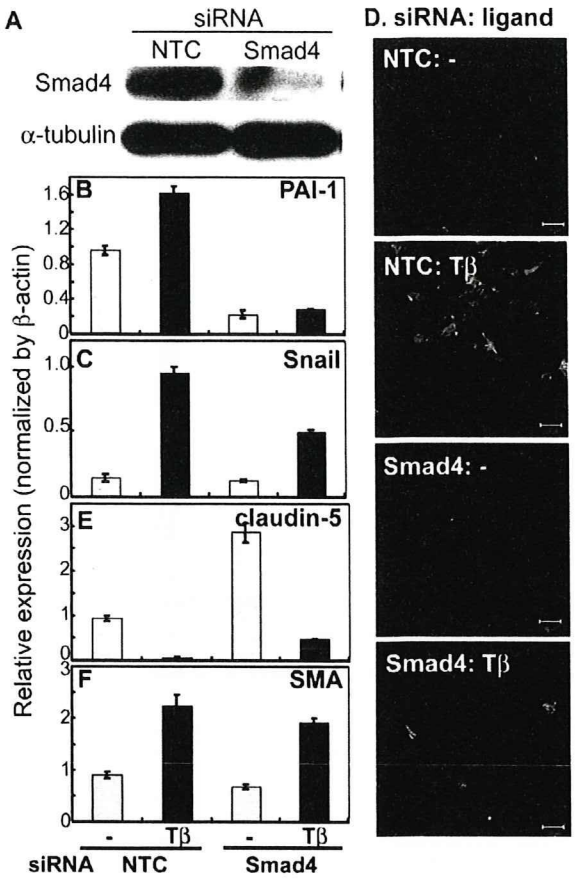

Fig. 8. Effect of *Smad4* knockdown on TGFβ-induced EndMT of MESECs. MESECs were sorted from the vascular cells derived from ESCs, transfected with Smad4 siRNA or with scrambled sequence as a negative control (NTC), and cultured in the absence (–) or presence of TGFβ2 (Tβ). (A) Levels of endogenous expression of Smad4 in the MESECs were analyzed by immunoblotting. (B,C,E,F) Levels of expression of PAI1 (B), Snail (C), claudin 5 (E) and SMA (F) in the MESECs were analyzed by quantitative real-time RT-PCR. Error bars indicate s.d. (D) The MESECs were subject to immunofluorescence staining for PECAM1 (red) and SMA (green).

Snail proteins might bind (Nishimura et al., 2006). We were also able to pull down the TGFβ-responsive element with antibodies against Smad3 in the Tc-Snail ESC-derived endothelial cells treated with TGFβ (supplementary material Fig. S6A), but not with antibodies against FLAG-Snail (supplementary material Fig. S6B). These results suggest that Snail does not bind to the TGFβ-responsive element to induce SMA expression in MESECs.

During EMT, a decrease in the expression of multiple tight-junction molecules, such as ZO1 and claudins, is accompanied by an increase in the expression of mesenchymal markers. We observed a decrease in the expression of claudin 5, an endothelium-specific tight-junction molecule, induced by TGFβ in MESECs. We previously reported that expression of claudin 5 is downregulated by TGFβ during endothelial differentiation from ESC-derived vascular progenitor cells (Watabe et al., 2003). Since claudin 1 expression is also repressed by Snail and Slug during EMT of kidney epithelial cells (Martinez-Estrada et al., 2006), downregulation of claudin family members might be a crucial event during EMT and EndMT.

During EMT and EndMT, expression of E-cadherin and VE-cadherin is, respectively, also decreased. However, VE-cadherin expression was not altered by Snail in MESECs, whereas E-cadherin expression was suppressed by Snail in undifferentiated ESCs. This might suggest that repression of VE-cadherin requires other transcription factors. We recently showed that TGFβ-induced δEF1 and SIP1, but not Snail, are involved in the downregulation of E-cadherin expression in mammary epithelial cells (Shirakihara et al., 2007). However, expression of the other EMT-related transcription factors was unaffected by TGFβ in MESECs, suggesting that other EMT-related signaling pathways are involved in the repression of VE-cadherin expression. In the embryonic heart, Notch functions to promote the TGFβ-induced EMT that results in formation of the cardiac valvular primordia (Timmerman et al., 2004). Liebner and colleagues showed that TGFβ induction of EndMT during heart cushion development is strongly inhibited in mice deficient for β-catenin, suggesting that an interaction between TGFβ and Wnt signaling pathways plays important roles in this process (Liebner et al., 2004). The roles of Notch and Wnt signals in the EndMT of MESECs remain to be elucidated in the future.

The expression of Twist, another EMT-related transcription factor, has been reported to be regulated by BMP2 (Ma et al., 2005), a member of the TGFβ family that has been implicated in cardiac cushion EndMT. In the embryos that lack BMP2 or BMP type IA receptor in AV myocardium or endocardium, respectively, cardiac

cushion formation is perturbed, with loss of expression of various transcription factors, including Twist (Ma et al., 2005). However, the present study shows that BMP4 fails to induce EndMT of MESECs. In accordance with this, Snail expression in the endocardium was unaffected by the loss of BMP2 or BMP type IA receptor (Ma et al., 2005). A recent report showed that BMP7 inhibits the TGFβ-induced EndMT of cardiac endothelial cells (Zeisberg et al., 2007a). We also found that BMP7 partially inhibits the TGFβ-mediated SMA expression in MESECs (supplementary material Fig. S7). These results suggest that certain types of BMPs play roles in the EndMT in a manner independent of TGFβ.

Recently, EndMT was implicated in two pathological situations. During cardiac fibrosis, accumulated fibroblasts cause the deposition of extracellular matrix, which can cause heart failure. Furthermore, activated fibroblasts can induce the progression of cancers. Zeisberg and colleagues reported that the TGFβ-induced EndMT plays important roles in the formation of fibroblasts from endothelial cells during cardiac fibrosis (Zeisberg et al., 2007a) and cancer progression (Zeisberg et al., 2007b). Since fibroblasts are key to both situations, EndMT is expected to be a target in the therapy of cardiac dysfunction and cancer. Therefore, the present findings might lead to a greater understanding of not only normal cardiovascular development, but also of such pathological situations, and eventually to the development of strategies to manipulate these signals for therapeutic benefit.

Materials and Methods

Cells and cell culture

The maintenance, differentiation, culture and cell sorting of mouse CCE and MGZ5TcH2 ESCs (gifts from Drs M. J. Evans and H. Niwa, respectively) were as described (Yamashita et al., 2000). Differentiated ESC-derived endothelial cells were sorted using PE-conjugated anti-CD34 antibodies (Pharmingen) and a MACS separation system (Miltenyi Biotec). Establishment of Tc-inducible ESC lines from parental MGZ5TcH2 cells was as described (Masui et al., 2005; Mishima et al., 2007). HUVECs were obtained from Sanko Junyaku and cultured as described (Mishima et al., 2007). VEGF (R&D, 30 ng/ml), TGFβ1, 2 and 3 (R&D, 1 ng/ml), BMP4 (R&D, 50 ng/ml), BMP7 (R&D, 500 ng/ml), activin (R&D, 25 ng/ml), TβR-I inhibitor (Calbiochem LY364947, 1 μM) and tetracycline (Sigma-Aldrich, 1 μg/ml) were used.

RNA interference and oligonucleotides

siRNAs were introduced into cells as described (Shirakihara et al., 2007). The target sequences for mouse Snail and Smad4 siRNAs were 5′-UGCAGUUGAAGAUCU-UCCGCGACUG-3′ and 5′-UUAAUCCUGAGAGAUCAAUUCCAGGS-3′, respectively. Control siRNAs were obtained from Ambion.

Immunohistochemistry and immunoblot analysis

Immunohistochemistry of cultured cells was performed as described (Yamashita et al., 2000) using monoclonal antibodies to PECAM1 (Mec13.3, BD Pharmingen), SMA (1A4, Sigma-Aldrich) and FLAG (M2, Sigma-Aldrich). Stained cells were photographed using a confocal microscope (LSM510 META, Carl Zeiss MicroImaging) with 10× (Plan-Neofluar 10×/0.30) objectives and LSM Image Browser. All images were taken at room temperature, and imported into Adobe Photoshop as TIFs for contrast adjustment and figure assembly. Immunoblot analyses were performed as described (Kawabata et al., 1998) using antibodies to claudin 5 (Zymed), SMA (Sigma-Aldrich), α-tubulin (Sigma-Aldrich), Snail (Cell Signaling) and E-cadherin (BD Transduction Laboratories).

RNA isolation and RT-PCR

Total RNA was prepared using RNeasy Reagent (Qiagen) and reverse-transcribed by random priming and using a Superscript First-Strand Synthesis Kit (Invitrogen). Quantitative RT-PCR analysis was performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems). All expression data were normalized to those for β-actin. For primer sequences, see supplementary material Table S1.

Chromatin immunoprecipitation (ChIP) assay

Endothelial cells derived from Tc-Snail ESCs were obtained in the absence or presence of Tc, and were incubated with or without TGFβ for 3 hours. Cells were fixed by adding formaldehyde and harvested. ChIP assays were carried out as described (Nishimura et al., 2006). In order to precipitate Smad3 and FLAG-tagged Snail, anti-Smad3 antibody (Upstate Biotechnology) and anti-FLAG (M2) antibody were used. PCR of the SMA promoter around the TGFβ hypersensitivity region was performed using immunoprecipitated chromatin with primers 5′-CAGTTGTTCTGAGGGCT-TAGGATGTTTATC-3′ and 5′-ACAAGGAGCAAAGACGGGCTGAAGCTGGCC-3′.

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Development of stabilin2$^+$ endothelial cells from mouse embryonic stem cells by inhibition of TGFβ/activin signaling

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ABSTRACT

To understand the endothelial cell (EC) development, arterial, venous, and lymphatic EC (LEC) have been successfully induced from embryonic stem cells (ESC). However, tissue-specific EC, such as hepatic sinusoidal EC (HSEC), have never been generated from ESC. Based on the findings that TGFβ/activin signaling negatively regulates differentiation of both LEC and HSEC, and that HSEC and LEC are distinguishable by the expression of marker genes, we assessed the role of TGFβ/activin signaling in EC development from ESC. Here we show that the inhibition of TGFβ/activin signaling by a TGFβ receptor I (TGFβRI) kinase inhibitor increased the expression of Lyve1 and stabilin2 but not podoplanin in CD31$^+$CD34$^+$ EC derived from ESC. EC generated by the inhibition of TGFβRI signaling also exhibited stronger endocytic activity than control EC, indicating that their phenotype is similar to fetal HSEC. Our results reveal that TGFβ/activin signaling negatively regulates the early events of HSEC differentiation.

Endothelial cells (EC) are actively involved in various biological processes in different tissues, such as organ development, homeostasis, regeneration and disease [1], and their characteristics are differentially regulated in each vascular bed [2]. Progress has been made to distinguish various types of EC by expression of molecular markers, allowing us to address the development of EC heterogeneity [3]. Lymphatic endothelial cells (LEC) are EC that line the lymphatic vasculature. Because lymphatic vessels are important not only for the maintenance of fluid balance, but also for the pathogenesis of diseases, such as cancer [4], much attention has been paid to lymphatic development [5]. Hepatic sinusoidal EC (HSEC) are EC that line the hepatic microvasculature, sinusoids. It has been well known that HSEC have unique structural and functional characteristics, such as fenestrae and the scavenging activity [6], but the mechanism of their development remains largely unknown. Interestingly, it has been noted that there are some similarities between LEC and HSEC [7]. For example, both LEC and HSEC have minimal basement membranes and loose cell–cell junctions, express Lyve1, but lack CD34 [7]. Moreover, both EC are originated from veins or mesenchyme [8–10]. These observations suggest that the development of LEC and HSEC is regulated by the same signaling pathway to some extent.

We have recently described development of mouse HSEC by immunohistochemical studies [11]. Together with studies on rat and human HSEC [12–14], we have proposed that development of HSEC can be divided into at least three stages [11]. First, at the beginning of the liver specification, from embryonic day (E) E8.0 to E9.5 in mouse, EC in the emerging liver bud do not express an HSEC specific marker. At this stage, HSEC cannot be distinguished from other EC. At the second stage from E9.5 to perinatal period, HSEC acquire some of their specific features, such as the expression of stabilin2 (Stab2) and Lyve1 and high endocytic activity. At this stage, HSEC can be clearly distinguished from other EC based on such characteristics. However, their characteristics are different from adult HSEC. E14.5 HSEC express CD34 but lack the receptors for the Fc fragment of IgG (FcgRs) and fenestrae, whereas adult mature HSEC are CD34$^-$FcgRs$^+$ and possess fenestrae. It should be noted that the earliest marker of HSEC is Stab2 expression.

A recent report revealed that TGFβ/activin signaling inhibits the proliferation, migration and differentiation of LEC [15]. It has also been shown that TGFβ/activin signaling inhibits maturation of HSEC from the immature stage [16]. However, it still remains unknown what signals regulate the early stage of HSEC development.

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Embryonic stem cells (ESC) have been shown to differentiate into Flk1$^+$ vascular progenitors that give rise to both endothelial and mural cells [17]. This system has been considered to mimic the EC differentiation in vivo, and has been used to study the development of EC heterogeneity [18]. By using this differentiation system, we previously demonstrated that TGFβ/activin signaling inhibits proliferation and sheet formation of EC [19]. However, it remains unknown whether TGFβ/activin signaling also inhibits differentiation of LEC and/or HSEC in this system. In this study, we addressed this possibility by using the markers that distinguish LEC and HSEC from other EC [3,7,11]. Our results show that the inhibition of TGFβ/activin signaling in EC differentiated from ESC increases expression of Lyve1, a common marker for LEC and HSEC, but not podoplanin (Pdpn) and chemokine (C–C motif) ligand 21 (Ccl21), LEC markers. It also increases the expression of Stab2 and coagulation factor VIII (F8), and induces stronger endocytic activity. Our results suggest that TGFβ/activin signaling negatively regulates the early step of HSEC differentiation.

Materials and methods

Cell culture. Maintenance, differentiation and culture of CCE ESC (a gift from Sir Martin J. Evans, Cardiff University, UK) were performed as described previously [17,20]. Briefly, CCE ESC were maintained on gelatin-coated tissue culture dishes in KnockOut D-MEM (Invitrogen, California, USA) supplemented with 15% fetal bovine serum (FBS, SAFC Biosciences, Tokyo, Japan), 2000 U/mL LIF (Millipore, Billerica, USA), 5×10^{-5} M 2-mercaptoethanol (2-ME), L-glutamine and non-essential amino acids (Invitrogen). For differentiation, ESC were cultured 4 days on type-IV collagen-coated dishes (AGC TECHNO GLASS, Chiba, Japan) in differentiation medium (alpha minimal essential medium (Invitrogen) supplemented with 10% FBS (SAFC Biosciences) and 5×10^{-5} M 2-ME). Flk1$^+$ vascular progenitor cells were cultured in differentiation medium supplemented with 30 ng/mL recombinant human VEGF (R&D systems, Minneapolis, USA). To block TGFβ/activin signaling, 0.3 μM TGFβ Type I Receptor Kinase

Table 1
Oligonucleotides used in RT-PCR

Gene symbol	Description	GeneBank		Sequence
Actb	Actin, beta, cytoplasmic	NM_007393.1	Sense	5′-GAT ATC GCT GCG CTG GTC GTC-3′
			Antisense	5′-ACG CAG CTC ATT GTA GAA GGT GTG G-3′
Ccl21	Chemokine (C–C motif) ligand 21	NM_023052.1	Sense	5′-GAT GAT GAC TCT GAG CCT CC-3′
			Antisense	5′-CTC TTG AGG GCT GTG TCT GT-3′
F8	Coagulation factor VIII	NM_007977.1	Sense	5′-AAA GAA GGC AGT CTC TCC AAA-3′
			Antisense	5′-GGA ACT GCC CAA GAT CTA TCA-3′
Fcgr2b	Fc receptor, IgG, low affinity IIb	NM_001077189.1	Sense	5′-TGT GGA CAG CCG TGC TAA AT-3′
			Antisense	5′-CAG CAG CCA GTC AGA AAT CA-3′
Mrc1	Mannose receptor, C type 1	NM_008625	Sense	5′-ACC CTG TAT GCC TGT GAT TCG-3′
			Antisense	5′-AGG TGC AGT CTG CAT ACC ACT TGT-3′
Stab2	Stabilin-2	NM_138673.1	Sense	5′-GCA CCA CCT CAC TAA TGT CAA-3′
			Antisense	5′-CCC AAG AGG GTC ACT GTT CT-3′

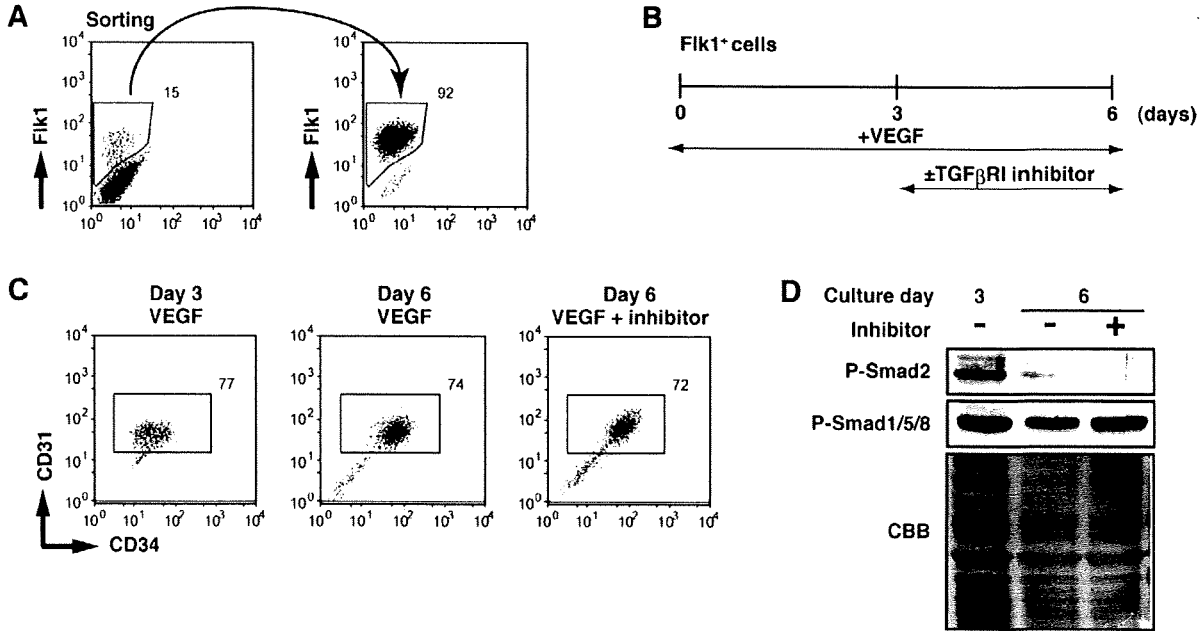

Fig. 1. Inhibition of TGFβRI signaling does not alter the differentiation of CD34$^+$CD31$^+$ cells from Flk1$^+$ vascular progenitors. (A) Isolation of Flk1$^+$ cells from ES-derived differentiated cells. ESC were cultured for 4 days. Flk1$^+$ cells were purified by using the autoMACS. The enclosures indicate Flk-1$^+$ cells. (B) Two protocols to culture the Flk1$^+$ vascular progenitors. (C) Expression of molecular markers for endothelial lineage, CD31 and CD34. Cells were harvested on culture day 3 and 6, and were subjected to flow cytometry. The enclosures indicate CD34$^+$CD31$^+$ cells. (D) Phosphorylation of Smad2 and Smad1/5/8 after cultivation of Flk1$^+$ cells. Cells were harvested on culture day 3 and 6. Cell lysates were directly subjected to immunoblotting using phospho-Smad2 and phosphor-Smad1/5/8 antibodies. Coomassie blue staining (CBB) was used as loading controls. The numbers in (A) and (C) indicate percentages of cells in each box. All results are representative of at least three independent experiments.

Inhibitor II (Tbr1ki2) (EMD Chemicals, Darmstadt, Germany) was added.

Cell preparation. Flk1$^+$ vascular progenitors were isolated from differentiated ESC after a 4-day culture as described previously [20]. Briefly, cells were trypsinized and suspended in staining buffer (phosphate-buffered saline (PBS) containing 0.5% BSA and 2 mM EDTA). Cells were stained with phycoerythrin (PE)-conjugated anti-mouse Flk1 monoclonal antibody (mAb) (eBioscience, San Diego, USA) followed by anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells labeled with the microbeads were separated by using an autoMACS Separator (Miltenyi).

Flow cytometry. MAb against mouse CD16/32, CD31, CD34, and CD45 were purchased from BD Biosciences (San Jose, USA). MAb against mouse Stab2 and Lyve1 were established in our laboratory [11]. MAb against mouse Pdpn was kindly provided by Drs. Fujita and Tsuruo [21]. Cells were blocked with an anti-mouse CD16/32 mAb, co-stained with fluorescein- and biotin-conjugated antibodies, washed, and incubated with allophycocyanin-conjugated streptavidin (Invitrogen). Dead cells were stained with propidium iodide. Labeled cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences) and with FlowJo software (Tree Star, Inc., Ashland, USA).

Immunostaining, western blot analysis, and cellular uptake of scavenger ligands. Staining of cultured cells was performed as described previously [11]. Western blot analysis was performed as described previously [22]. Antibodies against phospho-Smad1/5/8 and phopho-Smad2 were purchased from Cell Signaling Technology (Danvers, USA). For cellular uptake of acetylated low density lipoprotein (Ac-LDL) and hyaluronan, cells were incubated with 5 μg/mL Ac-LDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine (DiI-Ac-LDL, Biomedical Technologies, Stoughton, USA) or 25 μg/mL fluoresceinamine labeled sodium hyaluronate (FITC-HA, PG Research, Tokyo, Japan) at 37 °C for 4 h, and counterstained with Hoechst dye. Images were captured and fluorescence intensity was quantified as described previously [11].

RT-PCR analysis. RNA was extracted using a High Pure RNA Isolation Kit (F. Hoffmann-La Roche, Basel, Switzerland). First-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen) and random hexamer primers. The thermal cycle (denaturation at 94 °C for 15 s, annealing at an appropriate temperature for each pair of primers for 15 s and extension at 72 °C for 15 s) was repeated 35 times. The primers used are shown in Table 1.

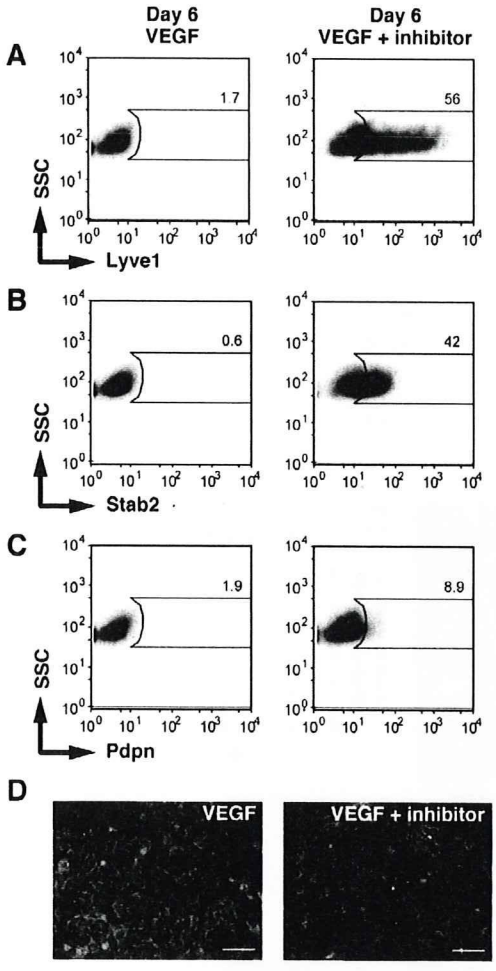

Fig. 2. Inhibition of TGFβRI signaling upregulates the expression of Lyve1 and Stab2, but not Pdpn. (A–C) Expression of molecular marker for HSEC and LEC. Flk1$^+$ cells were cultured for 6 days with or without TGFβRI inhibitor. On day 6, cells were harvested and were stained with antibodies against cell surface proteins as indicated. Shown are density plots of expression of Lyve1 (A), Stab2 (B) and Pdpn (C) in CD31$^+$CD34$^+$ cells. The numbers are percentages of the cells in each box. (D) Expression of Stab2 in the CD31$^+$ endothelial sheet. Flk1$^+$ cells were cultured for 6 days with or without the inhibitor and were stained with anti-CD31 (green), anti-Stab2 (magenta) antibodies and Hoechst dye (blue). All results are representative of at least three independent experiments. The bars represent 100 μm.

Results

Induction of endothelial cells from ESC

To examine whether TGFβ/activin signaling inhibits differentiation of LEC and/or HSEC in the ESC differentiation system, EC were induced from Flk1$^+$ vascular progenitors derived from ESC (Fig. 1A–C) [17]. Flk1$^+$ vascular progenitors were isolated (Fig. 1A), and further cultured for 6 days in the presence of VEGF to induce EC differentiation (Fig. 1B). EC differentiation was assessed by the expression of pan-EC markers, CD31 and CD34. At the beginning of the culture, Flk1$^+$ cells did not express the pan-EC markers [17], however, on culture day 3, over 70% of cultured Flk1$^+$ cells expressed both CD31 and CD34, indicating that differentiation of EC had been induced at this period (Fig. 1C). To inhibit endogenous TGFβ/activin signaling in CD31$^+$CD34$^+$ EC, TGFβRI inhibitor (Tbr1ki2) was added to the cells on culture day 3 (Fig. 1B). On culture day 6, about 70% of the cells were CD31$^+$CD34$^+$ in the presence or absence of the Tbr1ki2 (Fig. 1C). Of note, both the percent-

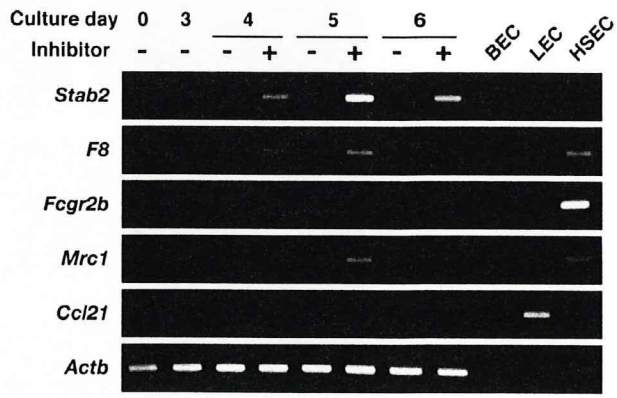

Fig. 3. Inhibition of TGFβRI signaling upregulates the expression of HSEC but not LEC markers. PCR analyses of genes for HSEC and LEC markers. Flk1$^+$ cells were cultured for 6 days with or without TGFβRI inhibitor (last 3 days). Cells were harvested on day 3, 4, 5 and 6, and were subjected to RT-PCR analyses. Positive and negative control templates were from blood vessels EC (BEC), LEC, and HSEC. All results are representative of at least three independent experiments.

ages and total numbers of the EC were not significantly different between VEGF alone and VEGF plus the inhibitor (Fig. 1C, Nonaka et al. unpublished observation).

To confirm the effect of Tbr1ki2, we examined phosphorylation of Smad2 and Smad1/5/8, which function downstream of the TGFβ/activins and bone morphogenetic proteins signaling pathways, respectively. On day 3, both Smad2 and Smad1/5/8 were phosphorylated by endogenous ligands (Fig. 1D), as we previously reported [19]. On day 6, the phosphorylation level of Smad2 was decreased in the absence of the inhibitor, and was hardly detectable in the presence of the inhibitor. However, the phosphorylation levels of Smad1/5/8 did not change between these conditions (Fig. 1D). These results indicate that TGFβ/activin signaling was actually blocked by Tbr1ki2 during the 3- to 6-day culture period.

Cell surface phenotype in EC developed with the TGFβRI inhibitor from ESC

To reveal the phenotype of EC derived from ESC, the expression of cell surface markers was examined in $CD31^+CD34^+$ cells (boxes in Fig. 1C) by flow cytometry. Lyve1 expression was expected to increase, because the previous study showed that the inhibition of TGFβ/activin signaling induced it in human dermal LEC [15]. As expected, treatment of ESC-derived EC with Tbr1ki2 for 3 days upregulated Lyve1 expression in $CD31^+CD34^+$ EC. 56% of them were $Lyve1^+$ in the presence of the inhibitor, but only 1.7% in its absence (Fig. 2A). To reveal whether the increased expression of Lyve1 represented LEC and/or HSEC differentiation, we examined the expression of Pdpn and Stab2, an LEC and an HSEC specific marker, respectively [11]. In the absence of Tbr1ki2, $CD31^+CD34^+$ EC did not express both markers on culture day 6 (Fig. 2B and C, left). In the presence of the inhibitor, Pdpn expression remained unchanged (Fig. 2C), however, Stab2 expression was increased in over 40% of $CD31^+CD34^+$ cells (Fig. 2B). The expression of Stab2 in EC was further confirmed by immunocytochemistry. As shown in Fig. 2D, Stab2 expression was detected on the $CD31^+$ EC sheet formed in the presence of Tbr1ki2, but not in the absence of it. These results indicate that the inhibition of TGFβRI signaling promotes differentiation of HSEC rather than LEC.

Gene expression profile of EC generated from ESC by the TGFβRI inhibitor

To further characterize the nature of the EC developed in the presence or absence of Tbr1ki2, we examined the expression of other LEC/HSEC markers by RT-PCR. As shown in Fig. 3, transcripts for HSEC markers, Stab2, F8, Fcgr2b and mannose receptor, C type 1 (Mrc1), were upregulated by the addition of Tbr1ki2. On the other hand, the expression of Ccl21, an LEC marker, was not detected regardless of the culture conditions. The upregulation of HSEC markers was observed on one day after the addition of Tbr1ki2, though the number of $Stab2^+$ EC and the expression level of Stab2 protein were more prominent on three days after the addition of the inhibitor (Nonaka et al. unpublished observation). These results further confirm differentiation of HSEC by the inhibition of TGFβRI signaling, and indicate that the induction begins just after the addition of Tbr1ki2.

Functional properties of EC generated from ESC by the TGFβRI inhibitor

To evaluate the functional properties of EC developed with Tbr1ki2, we examined the cellular uptake of fluorescently labeled Ac-LDL and hyaluronan. The incorporation of Ac-LDL is a common characteristic of EC. We previously showed that mouse E14.5 HSEC exhibited 2–3 times higher endocytic activity for Ac-LDL than E14.5 $Stab2^-Lyve1^-$ EC [11]. Furthermore, uptake of hyaluronan is a characteristic feature of HSEC [23]. As shown in Fig. 4A and B, EC developed with or without Tbr1ki2 incorporated Ac-LDL, while quantitative analysis revealed that those developed with Tbr1ki2 exhibited a higher (>3-fold) endocytic activity than those without the inhibitor (Fig. 4C). Moreover, EC developed with the inhibitor incorporated hyaluronan, while those without the inhibitor failed to do so (Fig. 4D and E). These results indicate that EC developed with Tbr1ki2 exhibit functional similarity with HSEC.

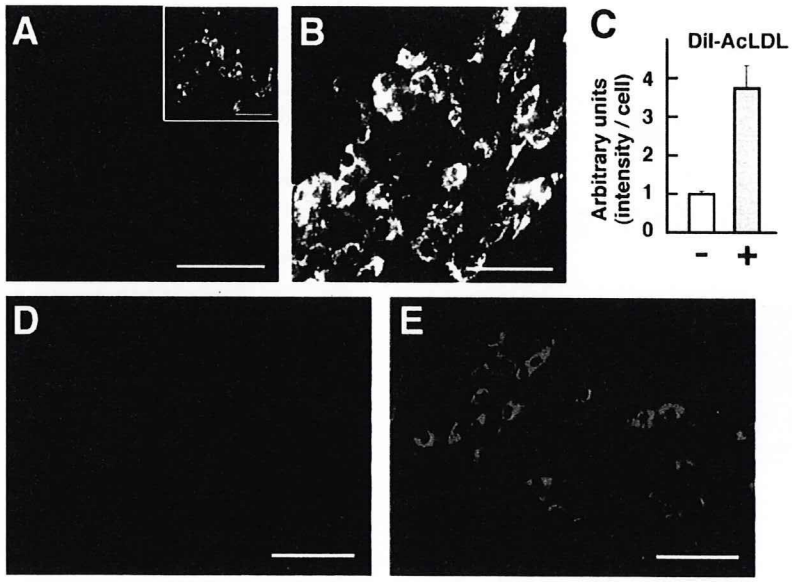

Fig. 4. EC with TGFβRI inhibitor exhibit higher endocytic activities. Endocytic activities in cultured EC differentiated from ESC. $Flk1^+$ cells were cultured for 6 days with (B,E) or without (A,D) the inhibitor, and were incubated with 5 μg/mL DiI-AcLDL (A,B) or 25 μg/mL FITC-hyaluronan (D,E) at 37 °C for 4 h. Cells were counterstained with Hoechst dye and were observed by fluorescence microscopy. The DiI fluorescence intensity was measured to quantify DiI-Ac-LDL uptake (C). Bars express means ± SD from three different microscopic fields. All results are representative of at least three independent experiments. The inset in A is an image of the same field taken by longer exposure (over 4 times long). The bars in (A,B) and (D,E) represent 80 and 100 μm, respectively.

Discussion

HSEC are characterized by their unique morphological feature and functions [24]. As the blood flows from the gut into the liver, one important function of the liver is to eliminate various foreign substances absorbed in the gut. HSEC play a key role as a filter by their strong endocytic activity [6]. Lyve1 and Stab2 are such scavenger receptors, implicated in the uptake of hyaluronan as well as other scavenger receptor-ligands [25–27]. We used Lyve1 and Stab2 to analyze EC differentiation, because they are functional receptors involved in the scavenger function of HSEC, and their expression is the earliest sign of HSEC differentiation [7,11].

The features of EC developed with Tbr1ki2 were similar to those of fetal immature HSEC, because (i) they expressed Stab2, (ii) they also expressed CD34, (iii) they exhibited a higher endocytic activity than control EC, and (iv) expression of FcgR proteins was undetectable by flow cytometric analysis (Nonaka et al. unpublished observation), though the transcript was detected in EC developed with Tbr1ki2 on culture day 6 (Fig. 3), and (v) fenestrae was not observed (Nonaka et al. unpublished observation). Further maturation of HSEC seems to require an additional factor. Yoshida et al. previously demonstrated that EC with fenestrae could be induced from rat E13.5 HSEC by the inhibition of TGFβ/activin signaling [16]. Since their culture system contains hepatoblasts, stellate cells, and others besides HSEC, paracrine factors from hepatic cells may promote HSEC maturation cooperatively with the TGFβRI inhibition.

The negative impact of TGFβ signaling on Lyve1 expression was also observed in human dermal LEC and EC induced from ESC in 3-dimensional culture [15]. We show in this paper that the TGFβRI inhibitor also upregulated HSEC markers, but not LEC markers such as Pdpn and Ccl21, while the previous study showed that the inhibition of TGFβ/activin signaling upregulated another LEC marker, Prox1 [15]. Since LEC and HSEC share some common characteristic features, it is possible they use the same signaling cascade for regulating their differentiation. An intriguing question is how the same signaling cascade, TGFβ/activin signaling, elicits different biological outputs, LEC/HSEC differentiation.

Since differentiation of ESC often mirrors a normal developmental process [28], to establish a tissue-specific EC differentiation system from ESC will also contribute to a better understanding of the development of EC heterogeneity. Interestingly, a recent study demonstrated that transplantation of healthy HSEC might be useful for cell therapy of relevant disorders [29]. Because a large number of homogenous cells can be generated from ESC, our successful induction of Stab2$^+$ EC from ESC raises an intriguing possibility for their use in future clinical applications.

Acknowledgments

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ARTICLE

Ras signaling directs endothelial specification of VEGFR2$^+$ vascular progenitor cells

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Vascular endothelial growth factor receptor 2 (VEGFR2) transmits signals of crucial importance to vasculogenesis, including proliferation, migration, and differentiation of vascular progenitor cells. Embryonic stem cell–derived VEGFR2$^+$ mesodermal cells differentiate into mural lineage in the presence of platelet derived growth factor (PDGF)–BB or serum but into endothelial lineage in response to VEGF-A. We found that inhibition of H-Ras function by a farnesyltransferase inhibitor or a knockdown technique results in selective suppression of VEGF-A–induced endothelial specification. Experiments with ex vivo whole-embryo culture as well as analysis of H-*ras*$^{-/-}$ mice also supported this conclusion. Furthermore, expression of a constitutively active H-Ras[G12V] in VEGFR2$^+$ progenitor cells resulted in endothelial differentiation through the extracellular signal-related kinase (Erk) pathway. Both VEGF-A and PDGF-BB activated Ras in VEGFR2$^+$ progenitor cells 5 min after treatment. However, VEGF-A, but not PDGF-BB, activated Ras 6–9 h after treatment, preceding the induction of endothelial markers. VEGF-A thus activates temporally distinct Ras–Erk signaling to direct endothelial specification of VEGFR2$^+$ vascular progenitor cells.

Introduction

Blood vessel formation is a fundamental process in organogenesis during embryonic development (Coultas 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 d 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).

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Abbreviations used in this paper: AcLDL, acetylated low-density lipoprotein; αSMA$^+$, α–smooth muscle actin positive; E, embryonic day; ESC, embryonic stem cells; Erk, extracellular signal-related kinase; HMEC, human microvascular endothelial cell; miRNA, microRNA; PECAM1$^+$, platelet-endothelial cell adhesion molecule-1 positive; VEGFR2$^+$, vascular endothelial growth factor receptor 2 positive.

The online version of this paper contains supplemental material.

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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/cm^2), 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,

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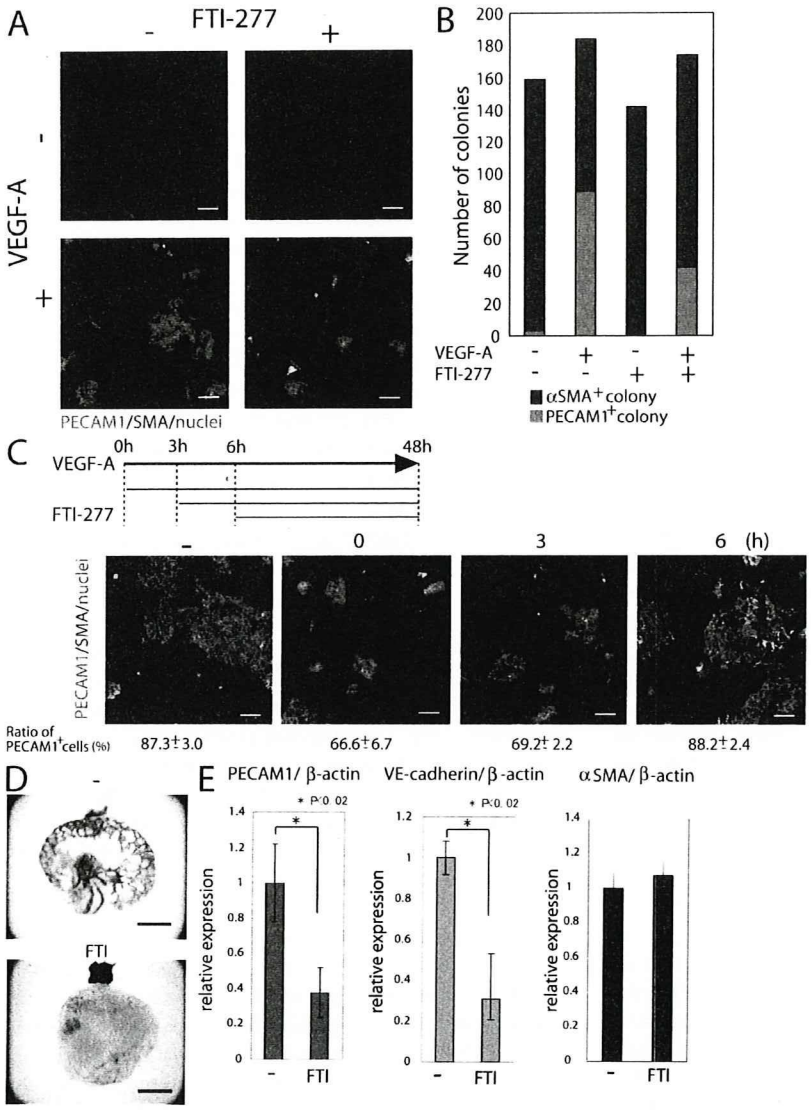

Figure 1. **Inhibitory effect of FTI-277 on endothelial differentiation.** (A) ESC-derived VEGFR2$^+$ cells were cultured for 2 d with 1 μM FTI-277 and/or 30 ng/ml VEGF-A, followed by immunostaining for PECAM1 (green), αSMA (red), and nuclei (blue). Bars, 100 μm. (B) Quantification of colony formation. FTI-277 was used at 3 μM. Representative results for three independent experiments are shown. (C) Time course of changes in FTI-277 sensitivity of VEGF-A-dependent endothelial differentiation. 1 μM FTI-277 was added at 0, 3, and 6 h after stimulation with VEGF-A, and cultured until 48 h. Cells were immunostained for PECAM1 (green), αSMA (red), and nuclei (blue). Bars, 100 μm. Quantification of appearance of PECAM1$^+$ cells is indicated below the panels (% of PECAM1$^+$ cells; means ± SD from three independent fields). (D) Ex vivo culture of mouse embryo E6.75 concepti were picked out and cultured with or without 10 μM FTI-277 for 3 d. Vasculature in yolk sacs of concepti were immunostained for PECAM1 (blue). Bars, 1 mm. (E) Quantitative RT-PCR analysis of PECAM1, VE-cadherin, and αSMA of ex vivo–cultured whole concepti. Each value is normalized to expression of β-actin. Values are the means ± SD of triplicate measurements. *, $P < 0.02$ (Student's *t* test).

and no obvious abnormality was observed in E10.5 H-*ras*$^{-/-}$ embryos (unpublished data).

To determine whether H-Ras regulates endothelial differentiation from vascular progenitor cells in vitro, we established stable ESC lines in which expression of H-Ras can be knocked down by microRNA (miRNA) under the control of tetracycline (Tc) because siRNA duplex was only minimally incorporated into ESC-derived VEGFR2$^+$ cells. A premiRNA sequence targeting H-Ras was knocked into the *ROSA26* locus in MGZ-RTcH cells (Tc-miR-H-Ras; Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). In MGZRTcH cells, expression of transgene at the *ROSA26* locus was induced by removal of Tc (Masui et al., 2005). In Tc-miR-H-Ras cells, expression of endogenous H-Ras was knocked down in the absence of Tc (Fig. S1 B). Limiting dilution assay was then performed for Tc-miR-H-Ras–derived VEGFR2$^+$ cells in the presence or absence of Tc. In the absence of Tc (Ras–knocked 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 but is not expressed in the presence of Tc (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). We then examined differentiation of VEGFR2$^+$

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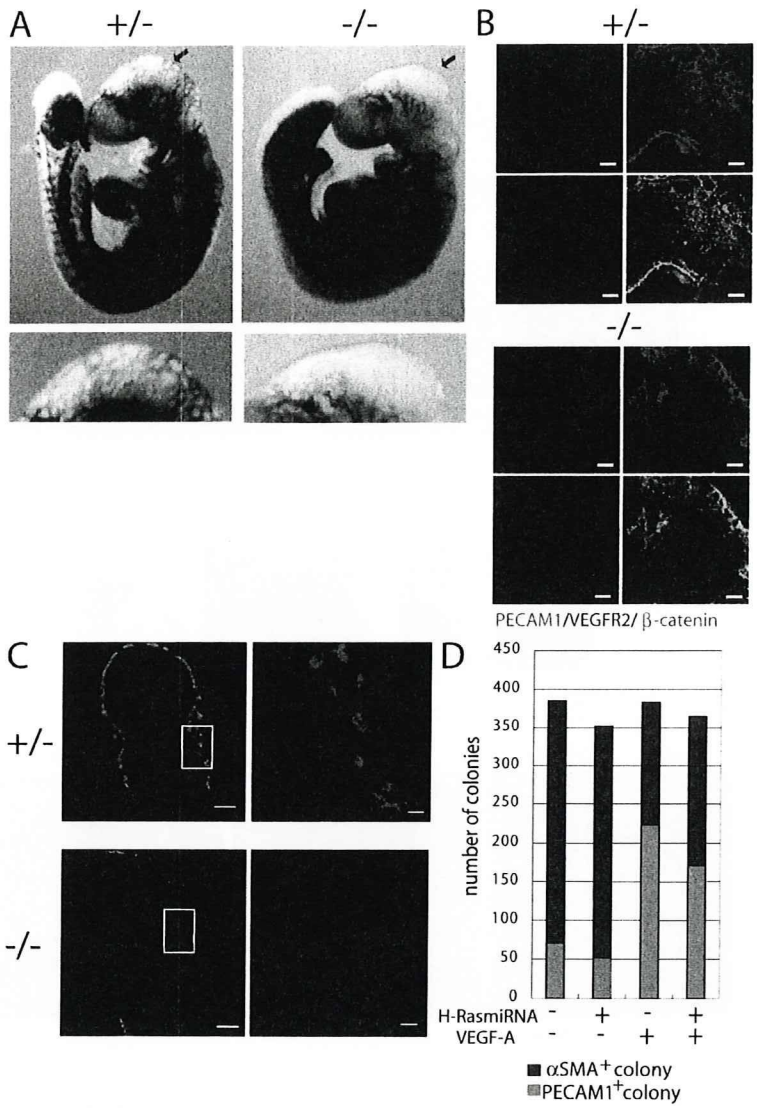

Figure 2. **Loss of H-Ras impairs vascular development.** (A) Whole-mount PECAM1 staining of E9.5 H-*ras*$^{+/-}$ and H-*ras*$^{-/-}$ mice. Magnifications of the areas marked with arrows in the top are shown in the bottom. (B) Immunostaining for PECAM1 (green), VEGFR2 (red), and β-catenin (blue) of cephalic region of E9.5 H-*ras*$^{+/-}$ and H-*ras*$^{-/-}$ mice. Bars, 100 μm. (C) Immunostaining for PECAM1 (green) of cross sections of cephalic region of E9.5 H-*ras*$^{+/-}$ and H-*ras*$^{-/-}$ mice. Magnifications of the boxed areas in the left are shown in the right. Bars: (left) 100 μm; (right) 20 μm. (D) Quantification of colony formation of Tc-miR-H-Ras cells, in which H-Ras has been knocked down by miRNA in the absence of Tc. Representative results of three independent experiments are shown.

cells derived from these cell lines. When H-Ras[G12V] was not expressed in the presence of Tc, appearance of PECAM1$^+$ cells was VEGF-A–dependent (Fig. 3 A, H-Ras[G12V](-)). Upon expression of H-Ras[G12V] by removal of Tc, PECAM1$^+$ cells appeared even in the absence of VEGF-A (Fig. 3 A, H-Ras[G12V](+)). Among Tc-empty cells, PECAM1$^+$ cells were not induced by removal of Tc (unpublished data). We further confirmed that the appearance of PECAM1$^+$ cells induced by H-Ras[G12V] was inhibited by FTI-277 (Fig. S3 A).

These PECAM1$^+$ cells were also positive for other endothelial markers, including VE-cadherin (Fig. 3 B), CD34, and endoglin (not depicted), and they incorporated acetylated low-density lipoprotein (AcLDL; Fig. 3 C) 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. 3 D). 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. 3 E). 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.

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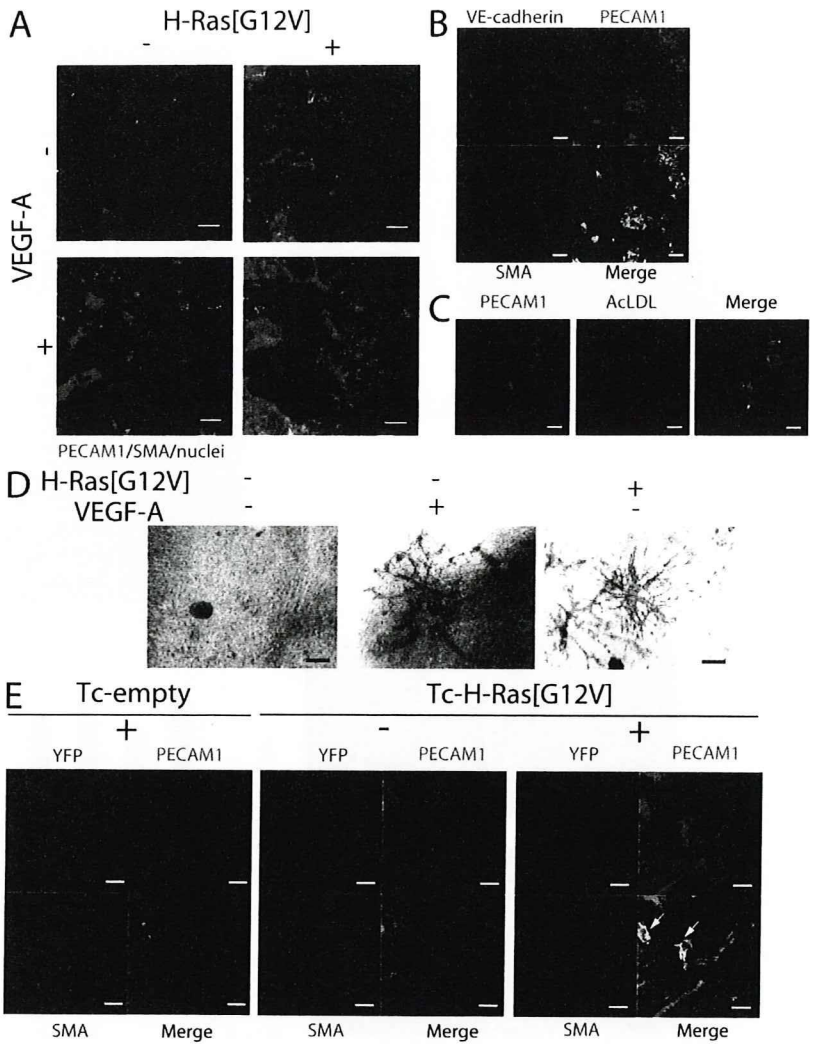

Figure 3. **Induction of PECAM1$^+$ cells by H-Ras[G12V].** (A) Differentiation of VEGFR2$^+$ Tc-H-Ras[G12V] cells in which constitutively active form of Ras is induced. Tc-H-Ras[G12V] ESCs were cultured on type IV collagen-coated dishes in the absence of LIF for 4 d during which expression of H-Ras[G12V] was suppressed by addition of Tc. ESC-derived VEGFR2$^+$ cells were then sorted and further cultured for 2 d with or without 1 μg/ml Tc and/or 30 ng/ml VEGF-A, followed by immunostaining for PECAM1 (green), αSMA (red), and nuclei (blue). Bars, 100 μm. (B) Immunostaining of H-Ras[G12V]–induced PECAM1$^+$ cells for VE-cadherin. PECAM1, green; αSMA, blue; and VE-cadherin, red. Bars, 100 μm. (C) PECAM1 staining (green) and AcLDL uptake (red) of PECAM1$^+$ cells induced by H-Ras[G12V]. Bars, 50 μm. (D) Three-dimensional culture of Tc-H-Ras[G12V] cells. 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. Bars, 200 μm. (E) In vivo vascular formation assay. Tc-empty and Tc-H-Ras[G12V] cells were labeled with retrovirus encoding YFP. After in vitro differentiation, Tc-H-Ras[G12V] or Tc-empty cells were mixed with Matrigel and subcutaneously injected into 129svJ 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. After 10 d, Matrigels containing ES-derived cells were picked out and frozen sectioned, followed by immunostaining for PECAM1 (green) and αSMA (red). Fluorescence of YFP is also shown (blue). +, transgene-induced condition (-Tc); -, transgene-suppressed condition (+Tc). Arrows indicate PECAM1$^+$ vessels covered with αSMA$^+$ cells. Bars, 20 μm.

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).

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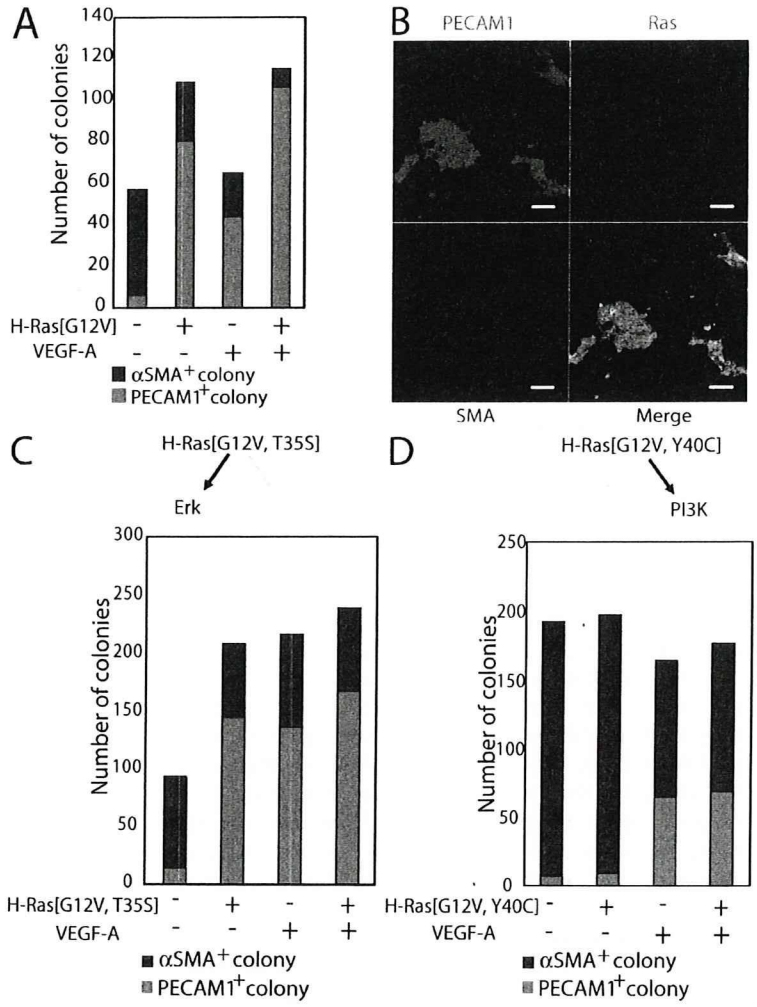

Figure 4. **Induction of endothelial differentiation by Ras–Erk signaling.** (A) Quantification of colony formation of Tc-H-Ras[G12V] cells. Representative results of three independent experiments are shown. (B) Cells overexpressing Ras were positive for PECAM1. ESC-derived VEGFR2$^+$ cells were cultured 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.

We performed a limiting dilution assay using these cells. When H-Ras[G12V, T35S] was expressed, PECAM1$^+$ colonies increased in number, whereas αSMA$^+$ colonies decreased (Fig. 4 C). In contrast, when H-Ras[G12V, Y40C] was expressed, numbers of PECAM1$^+$ colonies and αSMA$^+$ colonies were unchanged (Fig. 4 D). Additionally, among H-Ras[G12V, T35S] cells, those expressing Ras at high levels all differentiated into PECAM1$^+$ cells, whereas among H-Ras[G12V, Y40C] cells, those expressing Ras differentiated into either PECAM1$^+$ cells or αSMA$^+$ cells (unpublished data). These findings suggest that the Ras–PI3K pathway does not affect determination of cell fate. We concluded that the Ras–Erk pathway transmits signals required to specify endothelial differentiation of VEGFR2$^+$ progenitor cells.

The window of time within which Ras is specifically activated by VEGF-A

Ras proteins are known to be activated by various extracellular stimuli including cytokines and growth factors. In the present experimental system, ESC-derived VEGFR2$^+$ cells differentiate into endothelial cells upon stimulation with VEGF-A but not upon stimulation with PDGF-BB. Utilization of Ras by VEGFR2 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. 1 C). 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. 5 A). 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. 5 B). 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

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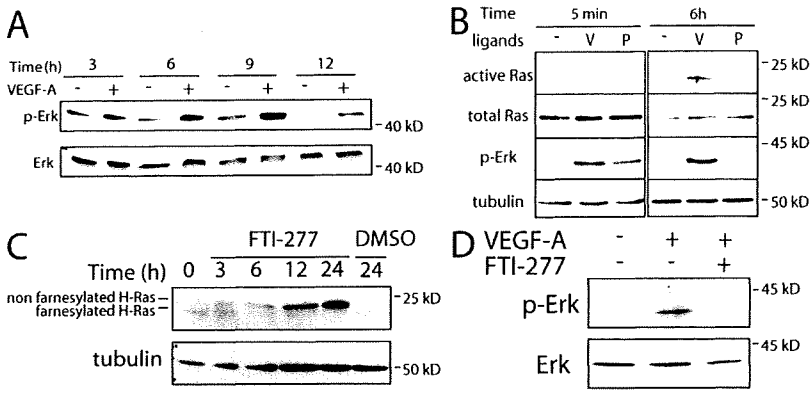

Figure 5. **Window of time within which Ras is specifically activated by VEGF-A.** (A) Time course of phosphorylation of Erk 3–12 h after stimulation with VEGF-A. VEGFR2$^+$ cells were stimulated with 30 ng/ml VEGF-A and lysed at the indicated time. Protein lysates were subjected to immunoblot analysis using anti–p-44/42 antibody and anti-Erk antibody. (B) Ras activation at 5 min and 6 h after stimulation with VEGF-A or PDGF-BB. VEGFR2$^+$ cells were stimulated with 30 ng/ml VEGF-A or 15 ng/ml PDGF-BB. At 5 min and 6 h after stimulation, cells were lysed and 1 mg of lysates was subjected to pulldown assay using a Ras activation assay kit (top). Residual lysates were subjected to immunoblot analysis (bottom three panels) using anti-Ras antibody, anti-p44/42 antibody, and anti-tubulin antibody (loading control). –, no ligand control cells; V, VEGF-A–stimulated cells; P, PDGF-BB–stimulated cells. (C) Time course of change in ratios of farnesylated and nonfarnesylated H-Ras after addition of FTI-277. 1 μM FTI-277 and 30 ng/ml VEGF-A were added to ESC-derived VEGFR2$^+$ cells. At 0, 3, 6, 12, and 24 h after addition, cells were lysed and lysates were subjected to immunoblot analysis using anti–H-Ras antibody. The top bands correspond to nonfarnesylated H-Ras and the bottom bands to farnesylated H-Ras. DMSO was used as vehicle control. Tubulin expression is shown as loading control. (D) Inhibitory effect of FTI-277 on phosphorylation of Erk. At 6 h after the addition of 1 μM FTI-277 and 30 ng/ml VEGF-A, cells were lysed and lysates were subjected to immunoblot analysis using anti-p44/42 and anti-Erk antibodies.

Erk were not notably different from those induced by PDGF-BB (Fig. 5 B). 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. S5 A, 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 differentiation of VEGFR2$^+$ progenitor cells (Kano et al., 2005), however, resulted in strong and sustained activation of Erk from 3 to 9 h after stimulation. 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. S5 B, 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. 5 C). Nonfarnesylated Ras began to increase 3 h after treatment and was constant after 6 h. Consistent with this finding, FTI-277 inhibited phosphorylation of Erk 6 h after VEGF-A-stimulation (Fig. 5 D). 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. 6 A). 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. 6 B). 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

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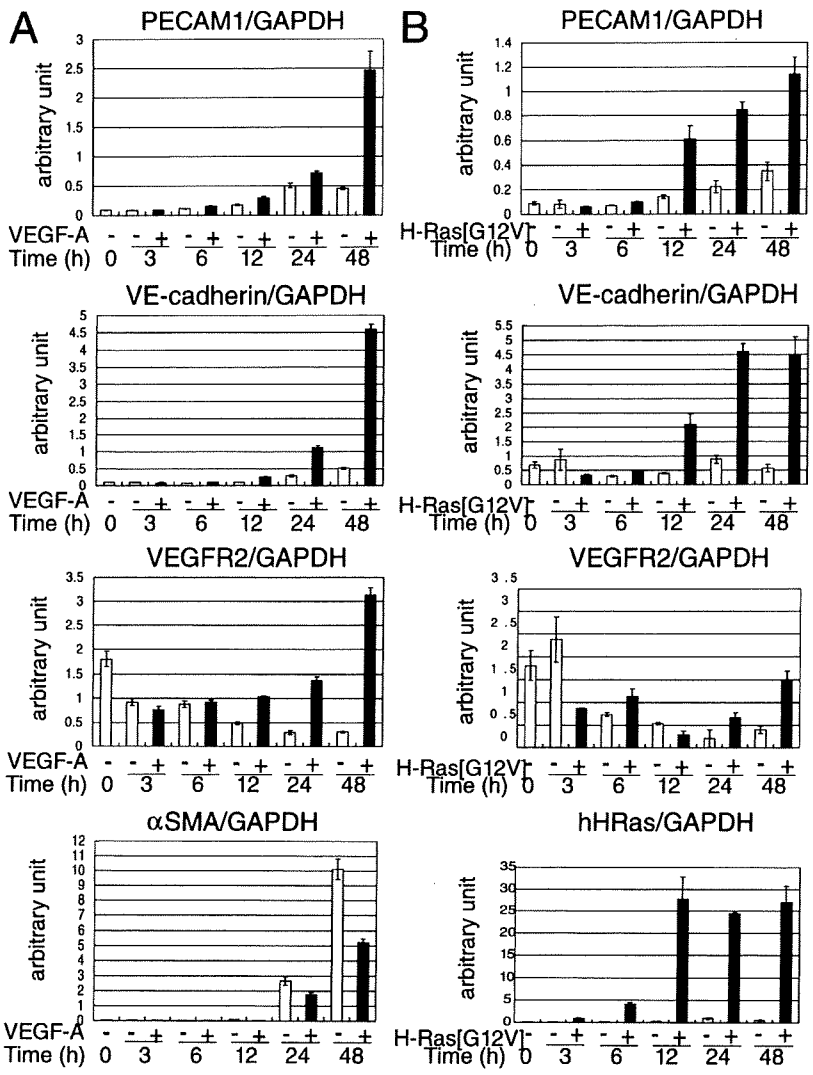

Figure 6. **Expression of vascular markers over time after in vitro differentiation of VEGFR2$^+$ cells.** Quantitative RT-PCR analysis of vascular markers of cells stimulated with 30 ng/ml VEGF-A (A) or H-Ras[G12V] (B) in the presence of serum. Each value is normalized to the expression of GAPDH. Error bars represent SD.

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.

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 β–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

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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$^+$ cells, whereas PlGF, 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$^+$ 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$^+$ 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* (Wassarman et al., 1995). Ras is, however, activated by various extracellular stimuli in mammalian cells. ESC-derived VEGFR2$^+$ 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$^+$ 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 αv (Klint et al., 1999; Hood 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 CCE 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 × 10^4 ESC-derived VEGFR2$^+$ cells per well on type IV collagen-coated 8-well CultureSlides (IWAKI) for immunostaining or 0.6–1 × 10^3 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),

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FGF-2 (R&D Systems), PlGF (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 (11D4.1; 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), p-44/42 (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 (MZ6; 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-Neofluar 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 27.5 mM 2-mercaptoethanol, preequilibrated at 37°C with 5% CO_2. Embryos were cultured at 37°C with 5% CO_2 and analyzed. FTI-277 (dissolved in DMSO) was used at 10 µM. The concentration of DMSO 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 genotyped 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-iT Pol 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 pPthC-EmGFP-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-empty cells were established as previously described (Masui et al., 2005; Mishima et al., 2007). cDNAs for H-Ras [G12V], H-Ras [G12V, T35S], and 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 10^7 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 129svJ 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-Neofluar; 1.3). All images were taken at room temperature.

Ras activation assay and immunoblot analysis

ESC-derived VEGFR2$^+$ cells (6×10^6) 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% FBS in the absence or presence 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 tubule 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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