

Drug treatments

Gravid adult hermaphrodites were placed onto slides into 8 μ l of egg salts²⁹ containing 10 μ g per ml nocodazole (Sigma) in DMSO (0.1% final concentration) and cut open with a syringe needle to release embryos. Slides were incubated in a humid chamber for 15–20 min before processing for immunostaining with anti-PAR-2 and anti- α -tubulin antibodies as described above. This treatment severely disrupted the meiotic spindle in most embryos but was not sufficient to eliminate it entirely (data not shown).

Time-lapse videomicroscopy

Young adult hermaphrodites were anaesthetized in 0.1% tricaine, 0.01% tetramisole in M9 (ref. 30), placed on a 2% agarose pad and covered with a cover slip. We recorded time-lapse movies of embryos *in utero* from fertilization to first cleavage (~45 min) for wild-type embryos, and from fertilization to 75 min after fertilization for *mat-1* embryos, using a Zeiss Axioplan 2 equipped with a DAGE video camera and Scion LG3 frame grabber. Nomarski images were acquired every 3 s using the 4D grabber software (Laboratory for Optical and Computational Instrumentation, University of Wisconsin, Madison). Resulting Quicktime movies were analysed for directed cytoplasmic flow by following the movement of yolk granules as described⁸. In two of the 4 *mat-1* embryos analysed, we also examined PAR-2–GFP fluorescence at the end of the movie and confirmed that it had become asymmetric.

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Flk1-positive cells derived from embryonic stem cells serve as vascular progenitors

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Interaction between endothelial cells and mural cells (pericytes and vascular smooth muscle) is essential for vascular development and maintenance^{1–4}. Endothelial cells arise from Flk1-expressing (Flk1⁺) mesoderm cells⁵, whereas mural cells are believed to derive from mesoderm, neural crest or epicardial cells and migrate to form the vessel wall^{6–8}. Difficulty in preparing pure populations of these lineages has hampered dissection of the mechanisms underlying vascular formation. Here we show that Flk1⁺ cells derived from embryonic stem cells can differentiate into both endothelial and mural cells and can reproduce the vascular organization process. Vascular endothelial growth factor promotes endothelial cell differentiation, whereas mural cells are induced by platelet-derived growth factor-BB. Vascular cells derived from Flk1⁺ cells can organize into vessel-like structures consisting of endothelial tubes supported by mural cells in three-dimensional culture. Injection of Flk1⁺ cells into chick embryos showed that they can incorporate as endothelial and mural cells and contribute to the developing vasculature *in vivo*. Our findings indicate that Flk1⁺ cells can act as 'vascular progenitor cells' to form mature vessels and thus offer potential for tissue engineering of the vascular system.

Flk1, one of the receptors for vascular endothelial growth factor (VEGF), is a marker for lateral plate mesoderm⁵ and the earliest differentiation marker for endothelial cells and blood cells^{9,10}. We previously established an induction and purification system for Flk1⁺ cells from embryonic stem (ES) cells and showed that Flk1⁺ cells give rise to endothelial cells as well as blood cells *in vitro*^{11–13}. To elucidate the mechanisms underlying vascular development, we

attempted here to reconstitute the pathway required for blood vessel formation.

Undifferentiated ES cells (E-cadherin⁺) were cultured for four days on collagen IV coated dishes with 10% fetal calf serum (FCS) to induce Flk1⁺ cells^{11,12}. Flk1⁺/E-cadherin⁻ cells (at a purity of greater than 95%) obtained by flowcytometry sorting (Fig. 1a) did not express other endothelial cell (vascular endothelial cadherin (VEcad), platelet-endothelial cell adhesion molecule-1 (PECAM1),

CD34)¹¹ or mural cell (α -smooth muscle actin (SMA), PDGF- β receptor) markers (data not shown). After an additional four days of culturing of Flk1⁺ cells with 10% FCS, more than 95% of cells became positive for SMA (Fig. 1b). As unsorted cells generated heterogeneity in cell appearance with few SMA⁺ cells and PECAM1⁺ cells under the same conditions (Fig. 1c), this phenomenon appears specific to purified Flk1⁺ cells.

Addition of 50 ng ml⁻¹ VEGF (VEGF₁₆₅) to culture resulted in the appearance of PECAM1⁺ sheets of endothelial cells (Fig. 1d, n), which were also positive for VEcad, CD34, endoglin and acetyl-LDL incorporation (Fig. 1e–h). The remaining cells surrounding these sheets were SMA⁺ (Fig. 1d, n) and expressed other mural cell markers such as CGA7, a marker for differentiated vascular smooth muscle cells (VSMC)^{14,15} (Fig. 1i–l). Analysis by polymerase chain reaction with reverse transcription confirmed that other SMC

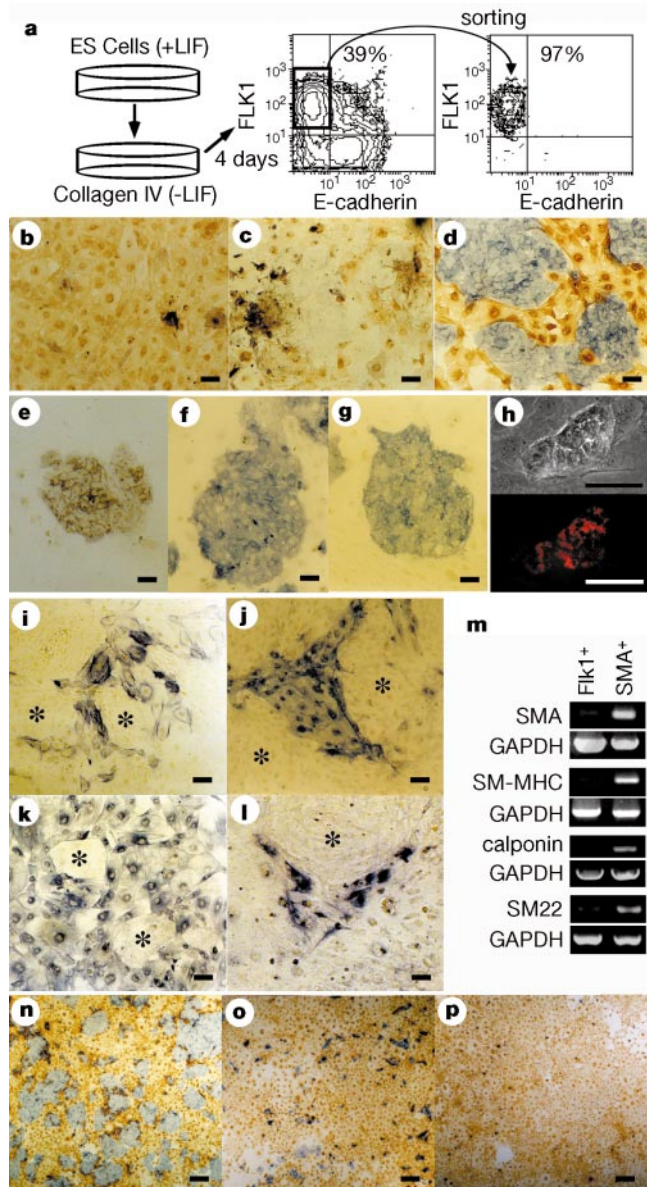


Figure 1 Differentiation of Flk1⁺ cells into endothelial and mural cells. **a**, Purification of Flk1⁺ cells from embryonic stem (ES) cells. Undifferentiated ES cells (E-cadherin⁺) cultured without LIF for four days induced Flk1 expression. Flk1⁺/E-cadherin⁻ population was purified by flowcytometry sorting. **b–d**, PECAM1 (purple) and SMA (1A4; brown) immunostaining. **b**, 10% FCS results in >95% SMA⁺ cells. **c**, Heterogenous cells are obtained in unsorted fractions. **d**, Flk1⁺ cells with 10% FCS and VEGF produce PECAM1⁺ sheets; expression of PECAM1 and SMA are mutually exclusive. PECAM1⁺ sheets were examined for VEcad (**e**), CD34 (**f**), endoglin (**g**) and acetyl-LDL incorporation (**h**). Immunostaining for mural cell markers: **i**, CGA7 (SMA in differentiated vascular SMC); **j**, PDGF- β receptor; **k**, smooth muscle tropomyosin; **l**, desmin. Asterisks, endothelial cell sheets negative for mural cell markers. **m**, RT-PCR analysis of mural cell markers in Flk1⁺ and SMA⁺ cells. **n–p**, PECAM1/SMA double immunostaining of Flk1⁺ cells with 50 ng ml⁻¹ VEGF₁₆₅ (**n**), VEGF₁₂ (**o**), PIGF (**p**). Scale bars: **b–h**, 100 μ m; **n–p**, 400 μ m.

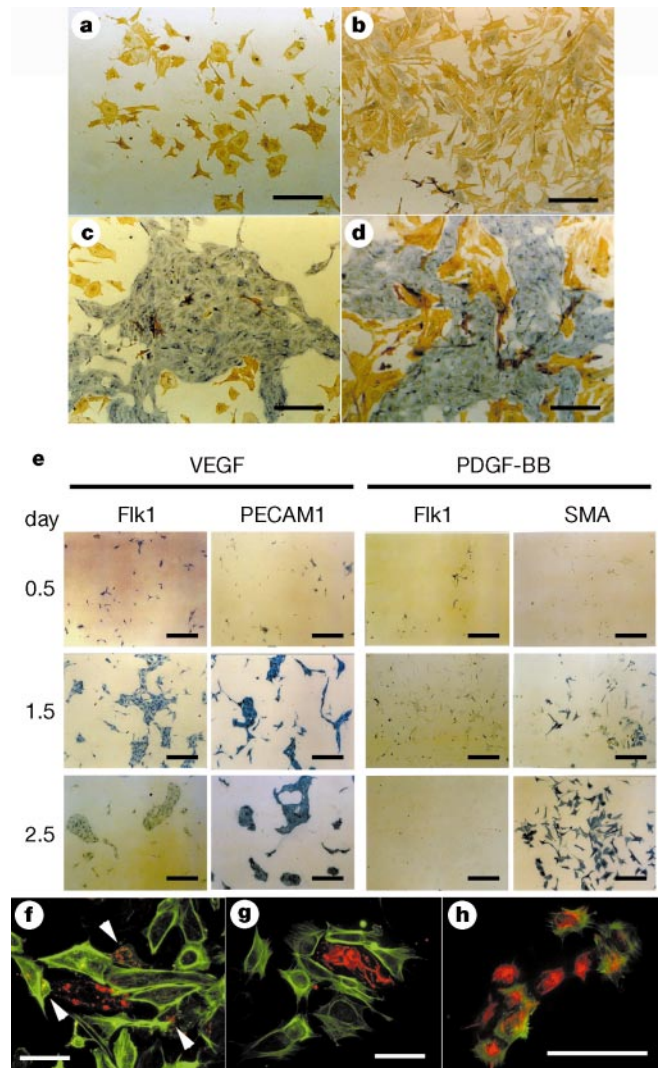


Figure 2 Flk1⁺ cell differentiation in serum-free culture. **a–d**, PECAM1 (purple)/SMA (1A4; brown) immunostaining (day 3). **a**, Vehicle-treated cells. **b**, PDGF-BB. **c**, VEGF. **d**, Simultaneous administration of VEGF and PDGF-BB. **e**, Time course of Flk1⁺ cell differentiation. VEGF maintains expression of Flk1 and PECAM1⁺ sheets from day 1.5. PDGF-BB-treated cells gradually lose Flk1 expression, SMA⁺ mural cells appear and dominate from day 1.5 to 2.5. **f**, Fluorescent immunostaining for Flk1 (red) and SMA (green) in 1.5-day PDGF-BB treatment. Arrowheads, Flk1⁺/SMA⁺ cells. **g**, VEcad (red)/SMA (green) double staining in 1.5-day PDGF-BB treatment. **h**, Flk1 (red)/SMA (green) double staining in 2.5-day VEGF and 10% FCS treatment. Flk1⁺/SMA⁺ cells are present. VEGF (50 ng ml⁻¹), PDGF-BB (10 ng ml⁻¹). Scale bars: **a–e**, 200 μ m; **f–h**, 100 μ m.

markers such as smooth muscle myosin heavy chain, calponin and SM22 α were upregulated in SMA⁺ cells (Fig. 1m), indicating that Flk1⁺ cells predominantly differentiate into either endothelial cells or mural cells under these culture conditions. As VEGF₁₆₅ was more efficient than VEGF₁₂₁ in endothelial cell induction (Fig. 1n, o) and placental growth factor (PIGF) had no activity (Fig. 1p), Flk1 and neuropilin, but not Flt1, are involved in this process¹⁶.

To define precisely the factors required for this differentiation, we tested the effects of various growth factors on Flk1⁺ cells cultured in

serum-free conditions. Some SMA⁺ cells were observed without addition of factors (Fig. 2a), indicating that de novo differentiation of mural cells could occur independently of exogenous growth factors. Administration of PDGF-BB but not PDGF-AA resulted in selective induction of SMA⁺ cells with spindle-like shapes resembling VSMC (Fig. 2b). This is consistent with previous reports indicating that PDGF-B/PDGF- β R signalling is required for vascular integrity through involvement in pericyte proliferation and recruitment to the vascular wall^{15,17–19}. In contrast, VEGF (50 ng ml⁻¹) administration induced predominantly PECAM1⁺ sheets (Fig. 2c). Both types of cell were increased by the simultaneous administration of VEGF and PDGF-BB (Fig. 2d), indicating that the induction of vascular cells from Flk1⁺ cells is probably regulated by a balanced combination of growth factors.

With VEGF treatment, Flk1 expression was maintained and Flk1⁺ cells formed PECAM1⁺ endothelial cell sheets after 1.5 days of incubation (Fig. 2e). In contrast, PDGF-BB-treated cells gradually lost Flk1 expression from 1.5 days and SMA⁺ mural cells appeared and became dominant from day 1.5 to 2.5 (Fig. 2e). Double fluorescent immunostaining of Flk1 and SMA in 1.5-day cultures of PDGF-BB-treated cells showed Flk1⁺/SMA⁺ cells as well as Flk1 and SMA single-positive cells (Fig. 2f). VECad⁺ endothelial cells were distinct from SMA⁺ cells (Fig. 2g). Flk1⁺/SMA⁺ cells were also detected in the cultures with serum (Fig. 2h), unlike VECad⁺/SMA⁺ cells. Although transdifferentiation of endothelial cells to SMA⁺ cells in chick embryo has been reported²⁰, VECad⁺/SMA⁺ cells rarely appeared from Flk1⁺ cells under our culture conditions with or without serum. Thus, transdifferentiation of VECad⁺ cells to SMA⁺ cells may rarely occur in our experimental system. These results indicate that VEGF is required for maintenance of Flk1 expression and differentiation to endothelial cells. In the absence of VEGF, Flk1 expression is lost and cells proliferate and differentiate into mural

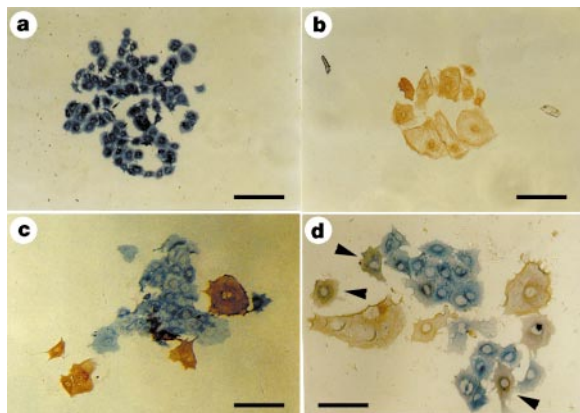


Figure 3 Single-cell deposition of Flk1⁺ cells cultivated with 10% FCS and 50 ng ml⁻¹ VEGF for four days and stained for PECAM1 (purple) and SMA (1A4; brown). Three colony types were observed. **a**, Pure endothelial cells (PECAM1⁺). **b**, Pure mural cells (SMA⁺). **c, d**, Mixed colonies consisting of PECAM1⁺ and SMA⁺ cells with some cells expressing both PECAM1 and SMA (arrowheads). Scale bars: 50 μ m.

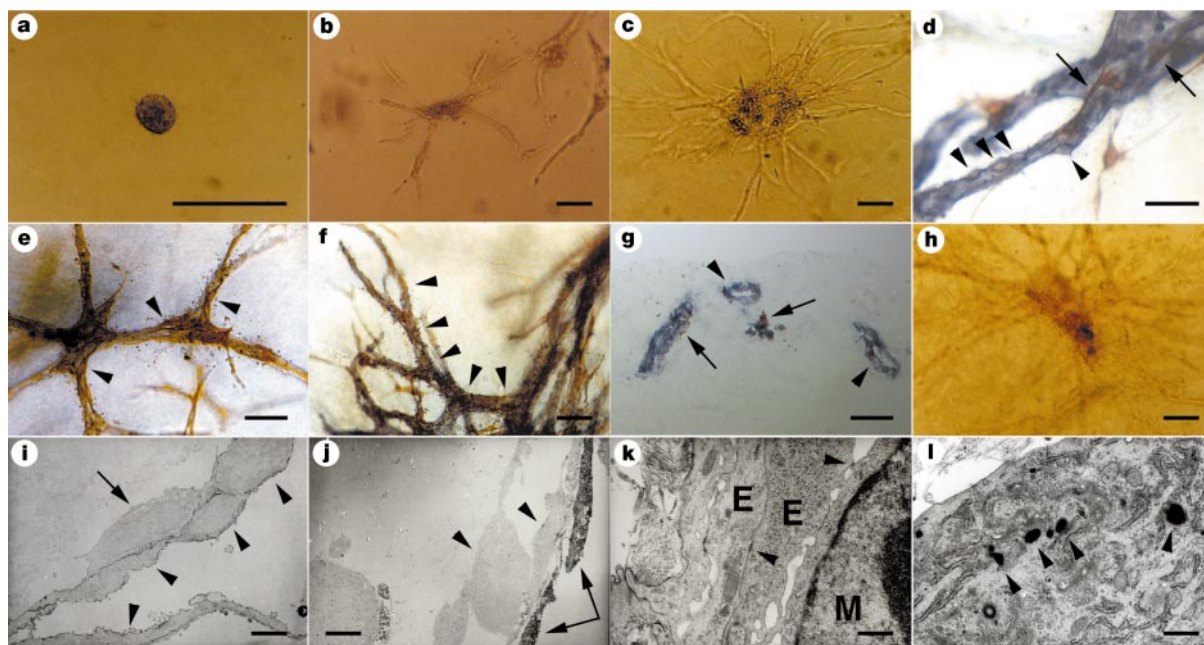


Figure 4 Vascular formation of Flk1⁺ cell aggregates in three-dimensional culture with 10% serum and 50 ng ml⁻¹ VEGF. **a–c**, Time course of tube formation. **a**, Flk1⁺ cell aggregate, day 0; **b**, day 3; **c**, day 5. **d–f**, In-gel double staining of PECAM1 (purple) and SMA (brown). **d**, PECAM1⁺ tube structure; endothelial cell nuclei (arrowheads) and attached SMA⁺ cells (arrows). **e**, Elongated SMA⁺ cells attached to tube with stellate processes (arrowheads). **f**, Tree-like structure with SMA⁺ cells (arrowheads). **g**, Cross-section reveals lumen with PECAM1⁺ endothelial cells (arrowheads) and attached SMA⁺ cells (arrows). **h**, Round cells in centre of aggregates are positive for blood cell markers;

antibody mixture for CD45 and Ter119 (brown). Immunoelectron microscopy for PECAM1 (**i**) and SMA (**j**). PECAM1⁺/SMA⁻ endothelial tube structure (arrowheads) with adhering PECAM1⁺/SMA⁺ mural cells (arrows). **k**, Junction formation in Flk1⁺ cell-derived structure. Desmosome-like structures (arrowheads) were observed in endothelial–endothelial and endothelial–mural cell junctions. E, endothelial cell; M, mural cell. **l**, Ultrastructure of endothelial cell revealing Weibel–Palade bodies (arrowheads). Scale bars: **a–c**, 100 μ m; **d–h**, 25 μ m; **i, j**, 5 μ m; **k, l**, 1 μ m.

cells through mainly PDGF-BB signalling. Although transforming growth factor- β (TGF- β) was reported to promote differentiation of mesenchymal cells to pericytes²¹, TGF- β inhibited growth of Flk1⁺ cells in our system (data not shown).

To determine whether Flk1⁺ cells contain common progenitors for endothelial and mural cells, we performed single cell deposition. Three types of colony emerged from single Flk1⁺ cells: pure endothelial cells (PECAM1⁺), pure mural cells (SMA⁺) and mixtures of both (Fig. 3a–d). Mixed colonies also contained PECAM1⁺/SMA⁺ cells (Fig. 3d, arrowheads). The frequencies of endothelial cell, mural cell and mixed colonies in the limiting dilution assay were around 27%, 43% and 30%, respectively. Plating efficiency was 14%. Similarly, Flk1⁺ cells (Flk1⁺/VEcad⁻) obtained from embryonic day (E) 8.5 embryos (85–90% purity) gave rise to all three colony types: endothelial cells (11%), mural cells (80%) and mixed (9%) (data not shown), indicating that Flk1⁺ cells from ES cells as well as embryos possess the potential to serve as common ‘vascular progenitor cells’ (VPC).

We next tested whether Flk1⁺ cells can form a vascular structure *in vitro*. Aggregates of around several hundred Flk1⁺ cells from ES cells were cultivated in collagen I gels with 10% FCS and 50 ng ml⁻¹ VEGF. Cells migrated out from the aggregates and formed tube structures within 3–5 days (Fig. 4a–c; see Supplementary Information). Double immunostaining revealed that these structures consisted of PECAM1⁺ endothelial tubes associated with SMA⁺ cells, which attached to endothelial tubes with stellate processes (Fig. 4d, e), a feature compatible with that of pericytes in capillary vessels. Occasional formation of vascular tree-like structures with massive investment of SMA⁺ cells was observed (Fig. 4f). Cross-sections showed a true lumen with attached SMA⁺ cells (Fig. 4g). In addition, spherical cells within the lumen were positive for the blood cell markers CD45 and Ter119, indicating generation of blood cells (Fig. 4h). This *in vitro* culture system of Flk1⁺ cell aggregates appears to mimic vascular development from yolk sac blood islands.

Immunoelectron microscopy suggested that the tube formation occurred by direct contact of PECAM1⁺ and SMA⁺ cells (Fig. 4i, j). Desmosome-like structures were formed in both endothelial–endothelial and endothelial–mural cell junctions (Fig. 4k), indicating direct interaction of these vascular cells. Relatively long adhesive structures were formed between endothelial cells, whereas endothelial and mural cells exhibited patchy formation of desmosome-like junctions²². Endothelial cells contained rough endoplasmic reticulum (RER), Golgi apparatus, microfilaments and sometimes round or rod-shaped electron-dense Weibel–Palade bodies (Fig. 4l)²³. Pericytes possessed thin stellate processes, abundant microfilaments, microtubules, RER and Golgi apparatus, but not Weibel–Palade bodies. There were a few collagen fibres adjacent to the pericytes, but we could not confirm clear basement membranes or external laminae. These results indicate that endothelial and mural cells that differentiate from Flk1⁺ cells interact with each other to form mature vessel-like structures *in vitro*.

To analyse the *in vivo* differentiation potential of Flk1⁺ cells, we induced LacZ-expressing Flk1⁺ cells from CCE/nLacZ (see Methods), injected them intracardially into stage 16–17 chick embryos and traced them by whole-mount staining. Most LacZ signals appeared along vessels in the head, yolk sac, heart and intersomitic region, in some cases forming a network structure 2–3 days after injection (Fig. 5a, b). Immunohistochemical staining of sections demonstrated incorporation of donor cells as both endothelial cells (PECAM1⁺/nLacZ⁺) (Fig. 5c) and mural cells (SMA⁺/nLacZ⁺) (Fig. 5d). This indicates that Flk1⁺ cells differentiate to both types of cell *in vivo* and contribute to the vascular component.

We thus showed that ES-derived Flk1⁺ cells give rise to two major vascular cell types (endothelial cells and mural cells) *in vitro* and *in vivo*. We started from a purified population of Flk1⁺ cells that allowed the specific differentiation of vascular cells and facilitated

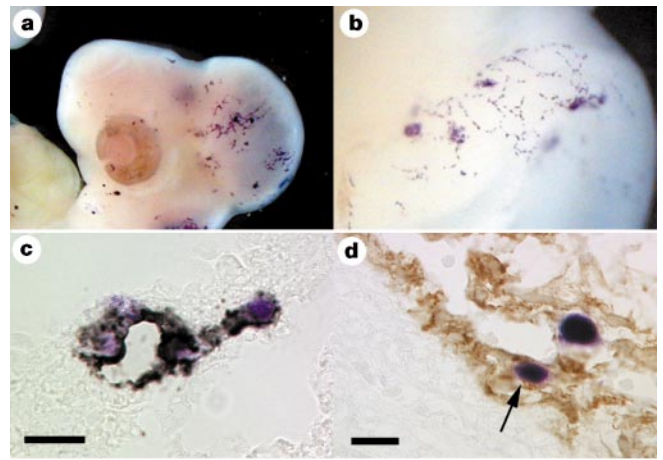


Figure 5 Contribution of Flk1⁺ cells to vascular formation *in vivo*. **a, b**, Whole-mount β-gal staining of chick embryos. **a**, 2 days; **b**, 3 days after injection of Flk1⁺ cells (CCE/nLacZ). LacZ⁺ nuclei formed network structure along vasculature. **c, d**, Immunostaining of sectioned LacZ-stained embryos for PECAM1 (grey) and SMA (1A4; brown). Anti-PECAM1 antibody is specific to murine endothelial cells. 1A4 reacts with both murine and chick SMA. **c**, Incorporation of PECAM1⁺/nLacZ⁺ cells into vasculature in yolk sac. Donor cells formed a true lumen. **d**, Incorporation of SMA⁺/nLacZ⁺ cells in head vascular wall (arrow). Blood cells are observed in the lumen. Scale bars: 10 μm.

analyses of vascular development. Our study gives new insight into the vascular developmental pathway that originates in Flk1⁺ cells (see Supplementary Information). Observations that other growth factors such as basic fibroblast growth factor induced cells which were negative for both PECAM1 and SMA (data not shown) indicate that Flk1⁺ cells can also differentiate into lineages other than vascular. Further roles of Flk1⁺ cells in embryogenesis should be explored and might provide additional potential of Flk1⁺ cells for tissue engineering beyond the vascular system. □

Methods

Cell culture and sorting

Maintenance, differentiation, culture and cell sorting of CCE ES cells (gift from M. J. Evans) were as described¹². We plated 2 × 10⁴ Flk1⁺ cells per well on collagen IV (Col. IV)-coated 24-well dishes (Becton Dickinson) in differentiation medium¹². For serum-free culture, cells were incubated in SFO3 (Sanko Junyaku) as described²⁴. Single sorted cells were plated into individual wells of Col IV-coated 96-well dishes¹². In limiting dilution assay, 200–500 Flk1⁺ cells were plated per plate. Before three-dimensional culture, cells (4 × 10⁵ cells per ml) were incubated in differentiation medium¹² containing 50 ng ml⁻¹ VEGF on uncoated petri dishes for 12 h to induce aggregation. Aggregates were resuspended in 2× differentiation medium and mixed with an isovolume of collagen I-A gel (3 mg ml⁻¹) (Nitta Gelatin). We plated 250 μl of this mixture onto a lucent insert disc, Cell Disk (Sumitomo Bakelite), in 24-well dishes. After 15 min at 37 °C to allow polymerization, we added 500 μl differentiation medium with VEGF (final 50 ng ml⁻¹). Human VEGF₁₆₅, VEGF₁₂₁ and PlGF were purchased from R&D Systems Inc., and human PDGF-AA and PDGF-BB from GIBCO BRL.

Monoclonal antibodies

Monoclonal antibodies for murine E-cadherin (ECCD2), Flk1 (AVAS12) and VEcad (VECD1) as described^{25–27} and murine PDGF- β R (APB5) were prepared and labelled in our laboratory. Monoclonal antibodies for murine PECAM1 (Mec13.3), VEcad (11D4.1) for immunohistochemistry, Ter119 and CD45 were purchased from Pharmingen; those for SMA (1A4, CGA7) from NeoMarkers and ENZO Diagnostics, respectively, and those for desmin, tropomyosin and endoglin from DAKO, Sigma and Southern Biotechnology Associates, respectively.

Immunohistochemistry

Single staining of culture cells on dishes was as described¹¹. For double staining, cells were incubated with a mixture of Mec13.3 and 1A4 followed by ALP-conjugated anti-rat IgG (H+L) (Jackson ImmunoResearch Lab. Inc.) and HRP-conjugated anti-mouse IgG (BIO SOURCE International). For three-dimensional cultures, gel blocks on discs were fixed in 4% paraformaldehyde for 5 min at room temperature, then processed for whole-mount immunohistochemistry²⁸. Stained blocks were frozen in OCT compound (Miles Inc.) and sectioned by Cryostat (MICROM) at 5–7 μm thickness.

Electron microscopy

Electron microscopy and immunoelectron microscopy were performed as described^{29,30}. For electron microscopy, cell samples were fixed in 1.5% glutaraldehyde for 2 h. For immunoelectron microscopy, cells were fixed with 0.1% glutaraldehyde for 10 min.

Reverse transcription-polymerase chain reaction (RT-PCR)

After four days culture of Flk1⁺ cells with 10% FCS, cells (Fig. 1b) were used as a source of SMA⁺ RNA. Total RNA was prepared with TRIzol reagent (Life Technologies, Inc.), reverse transcribed by oligo (dT) priming and PowerScript-Reverse Transcriptase (CLONTECH Laboratories), and PCR was performed with the following primers: SMA forward: 5'-ACGGCCCTCCTCTTCTC-3', reverse 5'-GCCAGCTTCGTCGATTCC-3', smooth muscle myosin heavy chain forward: 5'-GACAACTCTCTCGCTTTGG-3', reverse: 5'-GCTCTCCAAAAGCAGGTAC-3', h1-calponin forward: 5'-GATACGAATTCAGAGG TGCAGACGGAGGCTC-3', reverse: 5'-GATACAAGCTTTCAATCCACTCTCTCAG CTCC-3', SM22 α forward: 5'-GCAGTCCAAAATTGAGAAGA-3', reverse: 5'-CTGTTGCTGCCCATTTGAAG-3'.

Chick/mouse chimaeric assay

CCE/nLacZ cells were generated by cotransfection of elongation factor 1 promoter-driven LacZ gene with nuclear localization signal (T. Kunisada) and murine phosphoglycerate kinase 1 promoter-driven puromycin resistant gene constructs and selection by 2 μ g ml⁻¹ puromycin. We injected 1–2 \times 10⁶ Flk1⁺ cells in 2–4 μ l of phosphate buffered saline (PBS) into hearts of stage 16–17 chick embryos with glass needles. Embryos were killed 2–3 days after injection and fixed with 2% paraformaldehyde at 4 °C for 10 min. A X-gal analogue, magenta gal (Nakarai), was used as substrate for whole-mount staining; stained embryos were frozen in OCT, cryosectioned at 6–7 μ m and stained for PECAM1 or SMA. PECAM1 staining was performed using TSA Indirect, tyramide signal amplification reagent (NEN Life Science Products).

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Bidirectional control of airway responsiveness by endogenous cannabinoids

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Smoking marijuana or administration of its main active constituent, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), may exert potent dilating effects on human airways^{1–4}. But the physiological significance of this observation and its potential therapeutic value are obscured by the fact that some asthmatic patients respond to these compounds with a paradoxical bronchospasm^{3,5}. The mechanisms underlying these contrasting responses remain unresolved. Here we show that the endogenous cannabinoid anandamide exerts dual effects on bronchial responsiveness in rodents: it strongly inhibits bronchospasm and cough evoked by the chemical irritant, capsaicin, but causes bronchospasm when the constricting tone exerted by the vagus nerve is removed. Both effects are mediated through peripheral CB1 cannabinoid receptors found on axon terminals of airway nerves. Biochemical