at 37°C and then Hoechst 33342 was added for another 90 min incubation for SP staining.

Immunohistochemistry

The procedure for tissue preparation and staining was as previously reported (Takakura *et al*, 1998). For immunohistochemistry, biotin-conjugated anti-CD31 antibody (Pharmingen, BD Biosciences), Cy3-conjugated anti-α SMA antibody (Dako, Glostrup, Denmark), and anti-GFP antibody (Invitrogen) were used for staining and alkaline phosphatase (ALP)-conjugated streptavidin (Dako), biotin-conjugated polyclonal anti-rat Ig (Dako), anti-rat IgG Alexa Fluor 546 (Invitrogen) and anti-rabbit IgG Alexa Fluor 488 (Invitrogen) as the secondary antibody. Biotinylated secondary antibodies were developed using ABC kits (Vector Laboratories). 5-Bromo-4-chloro-3-indoxyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT; Boehringer Mannheim, Mannheim, Germany) was used for the ALP colour reaction. Cell nuclei were visualized with Hoechst dye (Sigma). Samples were visualized using an Olympus IX-70 equipped with UPlanFI ×4/0.13 and LCPlanFI ×20/0.04 dry objective lenses, Leica DM5500B equipped with HCX PL FLVOTAR 5/0.15 and HCX PL FLVOTAR 10 × /0.15 dry objective lenses or Leica TCS/SP5 confocal microscopy equipped with HC PLAN APO ×20/0.70 and HCXPLAPO 40/1.25–0.75 oil objective lenses. Images were acquired with a DFC 500 digital camera (Leica) and processed with the Leica application suite (Leica) and Adobe Photoshop CS3 software (Adobe systems). All images shown are representative of >6 independent experiments.

EC colony-forming assay, time-lapse analysis, limiting dilution assay, and LTC-IC assay

In all, 1000 EC-SP or MP cells were seeded onto 24-well plates and co-cultured on OP9 stromal cells in 10% FCS and 10^{-5} M 2-ME (GIBCO) containing RPMI-1640 (Sigma) and fixed for immunostaining after 10 days. Time-lapse analysis was performed using an Olympus LCV110 (Olympus) and images were processed with Metamorph software (Universal Imaging, West Chester, PA, USA). For limiting dilution assay, cells were titrated to 20, 10, 5, 3, or 1 cells in one well for SP cells and 200, 100, 50, 30, 10 cells for MP cells. Cells were cultured for 10 days and number of colonies counted after immunostaining. For the LTC-IC assay, 500 EC-SP or EC-MP cells collected from EGFP mice were cultured on OP9 cells (P0). After 12 days, GFP-positive cells were counted, and 500 EGFP cells were cultured in the same manner (P1). For EC-SP cells, the same procedure was repeated once more (P2).

In-vivo neovascularization using matrigel

Eight-week-old C57BL/6 mice were injected subcutaneously with 0.5 ml Matrigel (Becton Dickinson) and 60 units of heparin per ml (Sigma), 150 ng/ml VEGF (PeproTech), and 3000 EC-SP or MP cells from the hind limb of an EGFP mouse. Fifteen days later, Matrigel plugs were removed.

Hind limb ischaemia model and EC transplantation

The proximal portion of the right femoral artery and vein including the superficial and the deep branch as well as the distal portion of the saphenous artery and vein were occluded and resected. $CD31^+CD45^-$ ECs from hind limb were obtained 1, 2, 3, 5, 7, and 14 days after induction of ischaemia. Proportions and numbers of EC-SP cells per ischaemia-induced hind limb were analysed and calculated. Controls were the hind limbs from the other side of the animal that was sham operated. For EC-SP and EC-MP transplantation, $CD31^+CD45^-$SP and MP cells were sorted from EGFP mice. The hind limb ischaemia model was prepared and just after occlusion and removal of vessels, 3000 EC-SP cells and 3000 EC-MP cells were injected into the muscle.

Murine BM transplantation model

C57BL/6 mice underwent BM transplantation from EGFP mice. Mice were myeloablated using two different regimens as previously described (Bruscia *et al*, 2006). Regimen 1: For the adult BM transplantation model, BM cells were obtained by flushing the tibias and femurs of age-matched donor EGFP mouse. The transplantation was performed to C57BL/6 mice lethally irradiated with 10.0 Gy, by intravenous infusion of $\sim 1 \times 10^7$ donor whole BM cells. Four weeks after transplantation, by which time BM of recipient mice was reconstituted, the mice were used for analysis. Regimen 2: Mother mice were myeloablated with busulfan (15 mg/kg, Sigma) on days 17 and 18 of pregnancy. Approximately 12 h after birth, 1×10^7 GFP-positive donor whole BM cells were injected into the livers of the pups. BM chimeric mice were analysed 8 weeks after BM transplantation.

Laser Doppler blood flow analysis

Hind limb blood flow was measured using a laser Doppler blood flow meter (LDBF; MoorLDI, Moor Instrument), as described previously (Kidoya *et al*, 2010). LDBF analyses over the legs and paws were performed on postoperative days 3, 7, and 14. After scanning, stored images were analysed to quantify blood flow, and mean LDBF values of the ischaemic and non-ischemic limbs were calculated. To avoid data variations because of ambient light and temperature, hind limb blood flow was expressed as the ratio of the left (ischaemic) to right (non-ischemic) hind limb LDBF.

Capillary density analysis

Tissue samples were obtained from the ischaemic skeletal muscles on postoperative day 14. Sections were examined for the presence of capillary ECs, and capillary to muscle fibre ratios were expressed as the ratio of the number of capillaries to the number of myofibres per high-power field (×400).

In-situ hybridization

Total RNA from mammary glands was isolated and used for cDNA synthesis. The Glycam 1 primers for PCR amplification were forward primer 5′-GTGCCACCATGAAATTCTTC-3′ and reverse primer 5′-TCTTCATGACTTCGTGATAC-3′. A 467-bp PCR fragment of Glycam 1 was subcloned into pGEM-T Easy Vector (Promega). The digoxigenin-labelled RNA probes were made using DIG RNA labeling kits (Roche, Indianapolis, IN). Hind limb muscle sections were processed and hybridization was performed as previously reported (Hou *et al*, 2000). Hybridized DIG-RNA probes were detected with anti-digoxigenin-rhodamine, Fab fragments (Roche). After *in-situ* hybridization, sections were stained with polyclonal anti-type IV collagen (Cosmo Bio).

Microarray analysis

Microarray analysis was performed as previously described (Nagahama *et al*, 2010). Labelled cRNA probes were hybridized to Affymetrix Mouse Genome 430 2.0 array (Affymetrix). Raw data are available for download from Gene Expression Omnibus (GSE28240). Microarray analysis was performed in duplicate from independent RNA preparations and analysed using GeneSpring GX 11.0 (Agilent Technologies).

Statistical analysis

All data are presented as mean ± standard error of mean (s.e.m.). For statistical analysis, the statcel 2 software package (OMS) was used with analysis of variance performed on all data followed by Tukey–Kramer multiple comparison testing. When only two groups were compared, a two-sided Student's *t*-test was used. A probability value of <0.05 was considered statistically significant.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Dr RH Adams (Max Planck Institute for Molecular Biomedicine, Munster, Germany) and Dr T Suda (Keio University, Tokyo, Japan) for providing us with VE-Cadherin-Cre-ERT mice and Flox-CAT-EGFP mice, respectively. We thank K Fukuhara, C Takeshita, and N Fujimoto for technical assistance. This work was partly supported by a grant from the Ministry of Education, Science, Sports, and Culture of Japan.

Author contributions: HN, HK, SS, and TW conducted *in-vivo* and *in-vitro* experiments. HN and NT planed the experiments. HN and NT wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118:** 149–161

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM (1997) Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275:** 964–967

Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM (1999) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* **18:** 3964–3972

Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E, Robin C (2010) *In vivo* imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* **464:** 116–120

Bruscia EM, Ziegler EC, Price JE, Weiner S, Egan ME, Krause DS (2006) Engraftment of donor-derived epithelial cells in multiple organs following bone marrow transplantation into newborn mice. *Stem Cells* **24:** 2299–2308

Bunting KD, Zhou S, Lu T, Sorrentino BP (2000) Enforced P-glycoprotein pump function in murine bone marrow cells results in expansion of side population stem cells *in vitro* and repopulating cells *in vivo*. *Blood* **96:** 902–909

Cao G, Savani RC, Fehrenbach M, Lyons C, Zhang L, Coukos G, Delisser HM (2006) Involvement of endothelial CD44 during *in vivo* angiogenesis. *Am J Pathol* **169:** 325–336

Challen GA, Little MH (2006) A side order of stem cells: the SP phenotype. *Stem Cells* **24:** 3–12

Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G (1998) A common precursor for hematopoietic and endothelial cells. *Development* **125:** 725–732

Cotsarelis G, Sun TT, Lavker RM (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* **61:** 1329–1337

De Bock K, De Smet F, Leite De Oliveira R, Anthonis K, Carmeliet P (2009) Endothelial oxygen sensors regulate tumor vessel abnormalization by instructing phalanx endothelial cells. *J Mol Med* **87:** 561–569

Feinberg RN, Beebe DC (1983) Hyaluronate in vasculogenesis. *Science* **220:** 1177–1179

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C (2003) VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* **161:** 1163–1177

Golebiewska A, Brons NH, Bjerkvig R, Niclou SP (2011) Critical appraisal of the side population assay in stem cell and cancer stem cell research. *Cell Stem Cell* **8:** 136–147

Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J Exp Med* **183:** 1797–1806

Gothert JR, Gustin SE, van Eekelen JA, Schmidt U, Hall MA, Jane SM, Green AR, Gottgens B, Izon DJ, Begley CG (2004) Genetically tagging endothelial cells *in vivo*: bone marrow-derived cells do not contribute to tumor endothelium. *Blood* **104:** 1769–1777

Gothot A, Pyatt R, McMahel J, Rice S, Srour EF (1997) Functional heterogeneity of human CD34(+) cells isolated in subcompartments of the G0/G1 phase of the cell cycle. *Blood* **90:** 4384–4393

Hooper AT, Butler JM, Nolan DJ, Kranz A, Iida K, Kobayashi M, Kopp HG, Shido K, Petit I, Yanger K, James D, Witte L, Zhu Z, Wu Y, Pytowski B, Rosenwaks Z, Mittal V, Sato TN, Rafii S (2009) Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell Stem Cell* **4:** 263–274

Hou Z, Bailey JP, Vomachka AJ, Matsuda M, Lockefeer JA, Horseman ND (2000) Glycosylation-dependent cell adhesion molecule 1 (GlyCAM 1) is induced by prolactin and suppressed by progesterone in mammary epithelium. *Endocrinology* **141:** 4278–4283

Hu Y, Zhang Z, Torsney E, Afzal AR, Davison F, Metzler B, Xu Q (2004) Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. *J Clin Invest* **113:** 1258–1265

Kalabis J, Oyama K, Okawa T, Nakagawa H, Michaylira CZ, Stairs DB, Figueiredo JL, Mahmood U, Diehl JA, Herlyn M, Rustgi AK (2008) A subpopulation of mouse esophageal basal cells has properties of stem cells with the capacity for self-renewal and lineage specification. *J Clin Invest* **118:** 3860–3869

Kidoya H, Naito H, Takakura N (2010) Apelin induces enlarged and nonleaky blood vessels for functional recovery from ischaemia. *Blood* **115:** 3166–3174

Mahmoud M, Allinson KR, Zhai Z, Oakenfull R, Ghandi P, Adams RH, Fruttiger M, Arthur HM (2010) Pathogenesis of arteriovenous malformations in the absence of endoglin. *Circ Res* **106:** 1425–1433

Mazzone M, Dettori D, Leite de Oliveira R, Loges S, Schmidt T, Jonckx B, Tian YM, Lanahan AA, Pollard P, Ruiz de Almodovar C, De Smet F, Vinckier S, Aragones J, Debackere K, Luttun A, Wyns S, Jordan B, Pisacane A, Gallez B, Lampugnani MG *et al* (2009) Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. *Cell* **136:** 839–851

Medici D, Shore EM, Lounev VY, Kaplan FS, Kalluri R, Olsen BR (2010) Conversion of vascular endothelial cells into multipotent stem-like cells. *Nat Med* **16:** 1400–1406

Miller DS (2010) Regulation of P-glycoprotein and other ABC drug transporters at the blood-brain barrier. *Trends Pharmacol Sci* **31:** 246–254

Mizrak D, Brittan M, Alison MR (2008) CD133: molecule of the moment. *J Pathol* **214:** 3–9

Morita Y, Ema H, Yamazaki S, Nakauchi H (2006) Non-side-population hematopoietic stem cells in mouse bone marrow. *Blood* **108:** 2850–2856

Nagahama Y, Ueno M, Miyamoto S, Morii E, Minami T, Mochizuki N, Saya H, Takakura N (2010) PSF1, a DNA replication factor expressed widely in stem and progenitor cells, drives tumorigenic and metastatic properties. *Cancer Res* **70:** 1215–1224

Nishikawa SI, Nishikawa S, Kawamoto H, Yoshida H, Kizumoto M, Kataoka H, Katsura Y (1998) *In vitro* generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunity* **8:** 761–769

Nolan DJ, Ciarrocchi A, Mellick AS, Jaggi JS, Bambino K, Gupta S, Heikamp E, McDevitt MR, Scheinberg DA, Benezra R, Mittal V (2007) Bone marrow-derived endothelial progenitor cells are a major determinant of nascent tumor neovascularization. *Genes Dev* **21:** 1546–1558

Okuno Y, Nakamura-Ishizu A, Kishi K, Suda T, Kubota Y (2011) Bone marrow-derived cells serve as proangiogenic macrophages but not endothelial cells in wound healing. *Blood* **117:** 5264–5272

Onrust SV, Hartl PM, Rosen SD, Hanahan D (1996) Modulation of L-selectin ligand expression during an immune response accompanying tumorigenesis in transgenic mice. *J Clin Invest* **97:** 54–64

Osawa M, Hanada K, Hamada H, Nakauchi H (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273:** 242–245

Passman JN, Dong XR, Wu SP, Maguire CT, Hogan KA, Bautch VL, Majesky MW (2008) A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1+ smooth muscle progenitor cells. *Proc Natl Acad Sci USA* **105:** 9349–9354

Peters BA, Diaz LA, Polyak K, Meszler L, Romans K, Guinan EC, Antin JH, Myerson D, Hamilton SR, Vogelstein B, Kinzler KW, Lengauer C (2005) Contribution of bone marrow-derived endothelial cells to human tumor vasculature. *Nat Med* **11:** 261–262

Purhonen S, Palm J, Rossi D, Kaskenpaa N, Rajantie I, Yla-Herttuala S, Alitalo K, Weissman IL, Salven P (2008) Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. *Proc Natl Acad Sci USA* **105:** 6620–6625

Risau W (1995) Differentiation of endothelium. *FASEB J* **9:** 926–933

Risau W (1997) Mechanisms of angiogenesis. *Nature* **386:** 671–674

Sainz J, Al Haj Zen A, Caligiuri G, Demerens C, Urbain D, Lemitre M, Lafont A (2006) Isolation of 'side population' progenitor cells from healthy arteries of adult mice. *Arterioscler Thromb Vasc Biol* **26:** 281–286

Shantsila E, Watson T, Lip GY (2007) Endothelial progenitor cells in cardiovascular disorders. *J Am Coll Cardiol* **49:** 741–752

Summers YJ, Heyworth CM, de Wynter EA, Hart CA, Chang J, Testa NG (2004) AC133+ G0 cells from cord blood show a high incidence of long-term culture-initiating cells and a capacity for more than 100 million-fold amplification of colony-forming cells *in vitro*. *Stem Cells* **22:** 704–715

Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T (1999) Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* **5:** 434–438

Takakura N, Huang XL, Naruse T, Hamaguchi I, Dumont DJ, Yancopoulos GD, Suda T (1998) Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. *Immunity* **9:** 677–686

Takakura N, Watanabe T, Suenobu S, Yamada Y, Noda T, Ito Y, Satake M, Suda T (2000) A role for hematopoietic stem cells in promoting angiogenesis. *Cell* **102:** 199–209

Walter DH, Haendeler J, Reinhold J, Rochwalsky U, Seeger F, Honold J, Hoffmann J, Urbich C, Lehmann R, Arenzana-Seisdesdos F, Aicher A, Heeschen C, Fichtlscherer S, Zeiher AM, Dimmeler S (2005) Impaired CXCR4 signaling contributes to the reduced neovascularization capacity of endothelial progenitor cells from patients with coronary artery disease. *Circ Res* **97:** 1142–1151

Zengin E, Chalajour F, Gehling UM, Ito WD, Treede H, Lauke H, Weil J, Reichenspurner H, Kilic N, Ergun S (2006) Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development* **133:** 1543–1551

Zhang XQ, Takakura N, Oike Y, Inada T, Gale NW, Yancopoulos GD, Suda T (2001) Stromal cells expressing ephrin-B2 promote the growth and sprouting of ephrin-B2(+) endothelial cells. *Blood* **98:** 1028–1037

Cancer Science
The official journal of the Japanese Cancer Association

Changes in blood vessel maturation in the fibrous cap of the tumor rim

Hisamichi Naito,[1,4] Kazuhiro Takara,[1,4] Taku Wakabayashi,[1] Hiroki Kawahara,[1] Hiroyasu Kidoya[1] and Nobuyuki Takakura[1,2,3]

[1]Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, Osaka; [2]JST, CREST, Sanbancho, Tokyo, Japan

(Received August 30, 2011/Revised November 10, 2011/Accepted November 16, 2011/Accepted manuscript online November 20, 2011/Article first published online December 21, 2011)

It is widely accepted that blood vessels in the tumor microenvironment are immature because mural cell (MC) adhesion to endothelial cells (ECs) is broadly lacking. Hyperpermeability of the tumor vasculature then results in interstitial hypertension that mitigates against penetration of anticancer drugs into the depths of the tumor. It has been suggested that treatment with angiogenesis inhibitors normalizes blood vessels, resulting in restoration of normal permeability and improved drug delivery. However, recent reports suggest that cancer cell invasion is induced from the edge of the tumor into peripheral areas after treatment with angiogenesis inhibitors. Therefore, it is important to assess the status of blood vessels in the fibrous cap at the tumor rim after antiangiogenesis therapy. In the present study, we found that mature blood vessels in which ECs are covered with MCs are present in the fibrous cap. After treatment with angiogenesis inhibitors, immature blood vessels were destroyed and vascular function was significantly improved, but maturing blood vessels in which ECs were covered with MCs remained visible. These maturing blood vessels showed a less dilated character after treatment with the angiogenesis inhibitors. It is widely accepted that well-matured blood vessels are sheathed in extracellular matrix (ECM) and that cancer cells migrate along tracks made of ECM collagen fibers. Therefore, our data indicate the importance of destroying maturing blood vessels outside the tumor parenchyma to prevent cancer cell invasion. (*Cancer Sci* 2012; 103: 433–438)

Tumor growth commonly depends on newly developed blood vessels that supply oxygen and nutrients to the tumor microenvironment; indeed, antiangiogenic therapy has been clinically available for some time.[1] Vascular endothelial growth factor (VEGF) plays a fundamental role in this process by inducing the proliferation, migration, and tube formation of endothelial cells (ECs) in physiological as well as pathological angiogenesis. Accordingly, many agents have been developed targeting VEGF itself or VEGF receptors.[2] Although effective antiangiogenic activity is mediated by these agents in mouse tumor models, suppression of tumor growth by angiogenesis inhibitors alone in clinical studies has been disappointing. However, enhanced efficacy of combination therapy using anticancer drugs together with angiogenesis inhibitors for tumor suppression has been reported.[3] Therefore, a new concept has emerged that the normalization of tumor vasculature by angiogenesis inhibitors may act to attenuate tumor growth.[4]

In the tumor microenvironment, proangiogenic factors are abundantly produced by the tumor cells themselves, as well as by stromal cell components such as fibroblasts, myofibroblasts, hematopoietic cells, ECs, and other cell types, in amounts sufficient to overcome antiangiogenic factors in normal homeostasis. This can result in the disorganized growth of immature blood vessels lacking mural cell (MC) coverage. Moreover, the EC–EC junctions are loose; therefore, blood vessels in the tumor exhibit hyperpermeability and both spatially and temporally non-uniform blood flow, which may result in interstitial hypertension. This contributes to an inability of anticancer drugs to penetrate deeply into the tumor mass. Angiogenesis inhibitors seem to cause maturation and normalization of the tumor vasculature by rebalancing the dysregulated production of pro- and antiangiogenic factors. This results in normalized vascular permeability and enhanced ability of anticancer drugs and oxygen to exit from the intravascular lumen and penetrate into the parenchyma of the tumor mass. Therefore, normalization of blood vessels may increase the efficacy of standard drug therapy and radiation therapy.

Although this benefit of angiogenesis inhibitors has been advocated, it has also been reported that cancer cells tend to acquire invasive potential when treatment with angiogenesis inhibitors is terminated.[5] Moreover, one line of evidence suggests that cancer recurrence is induced from the edge of the tumor after treatment with vascular disrupting agents.[6] It is hypothesized that responsiveness to angiogenesis inhibitors can differ depending on the structure or localization (i.e. center, periphery, or fibrous cap) of the tumor. The sequential changes to the vasculature inside the tumor after antiangiogenic therapy have been studied extensively;[7] however, changes outside the tumor mass have not. Therefore, in the present study we investigated how tumor blood vessels respond to angiogenesis inhibitors depending on their characteristics, with a particular focus on the MC coverage of the tumor vasculature in the fibrous cap of the tumor rim because cancer cell invasion or recurrence occurs from these peripheral areas.

Materials and Methods

Mice, cell lines, and tumors. All experiments were performed in accordance with the guidelines of the Osaka University Committee for Animal and Recombinant DNA Experiments. Mice were handled and maintained according to the Osaka University guidelines for animal experimentation. KSN nude mice (7–10 weeks of age) were purchased from Japan SLC (Shizuoka, Japan). The HT29 (human colorectal adenocarcinoma) and Colo320DM (human sigmoid colon cancer) cell lines were cultured in RPMI-1640 (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Subcutaneous xenografts were established by injecting 1.0 or 2.0×10^6 cells into the flanks of mice and tumor size was measured with calipers. Tumor volume (V) was calculated according to the formula $V = ½ \times \text{length} \times \text{width} \times \text{height}$.[8] Bevacizumab (Avastin; Genentec, San Francisco, CA, USA) and AG-013736 (Axitinib; Selleck Chemicals, Houston, TX, USA) were used as angiogenesis inhibitors. Bevacizumab, a VEGF neutralizing antibody, was dissolved in saline and injected twice a week at a dose of 5 mg/kg, i.p. Axitinib, which inhibits

[3]To whom correspondence should be addressed.
E-mail: ntakaku@biken.osaka-u.ac.jp
[4]These authors contributed equally to this work.

doi: 10.1111/j.1349-7006.2011.02157.x

multiple kinases, including the VEGF receptor (VEGFR), was dissolved in a solution of PEG-400 (Sigma)–acidified (pH 2–3) water (3:7) and injected at a dose of 25 mg/kg, i.p., for 7 days.[9]

Immunohistological analysis. The procedure for tissue preparation and staining was as reported previously.[10] Briefly, fixed specimens were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and sectioned at 8 or 12 μm. For immunofluorescence analyses, anti-CD31 mAb (clone MEC 13.3; Pharmingen, BD Biosciences, San Diego, CA, USA) was used to stain ECs and Cy3-conjugated mouse anti-α-smooth muscle actin (α-SMA) mAb (Sigma) was used to stain MCs. Anti-rat IgG Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) was used as the secondary Ab. To measure hypoxia in tumor tissues, HypoxyProbe-1 (60 mg/kg, i.v.; Hypoxyprobe, Burlington, MA, USA) was injected 2 h before tissues were harvested. Tumor sections were stained using an anti-HypoxyProbe antibody.[11] To evaluate macromolecule infiltration, mice were injected intravenously with 0.5 mg of FITC-conjugated dextran (MW 40 000; 0.5 mg/body; MP Biomedicals, Solon, OH, USA) and the dextran was allowed to circulate for 2 h. Samples were visualized using a Leica DM5500B or Leica TCS/SP5 confocal microscope (Leica Microsystems, Nussloch, Germany) and processed with the Leica application suite (Leica Microsystems) and Adobe Photoshop CS3 software (Adobe Systems, San Jose, CA, USA). All images shown are representative of more than five independent experiments. The ratio of matured vessels ($CD31^+$ SMA^+ vessels/$CD31^+$ vessels) and the number of $CD31^+$ vessels were counted in more than seven different fields at a magnification of ×100.

Statistical analysis. All data are presented as the mean ± SEM. Statistical analyses were performed using statcel 2 (OMS, Tokorozawa, Japan). Data were analyzed by analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison tests. When only two groups were compared, two-sided Student's *t*-test was used. $P < 0.01$ was considered significant.

Results

Histological evaluation of sequential changes to the vasculature in the fibrous cap after tumor inoculation. We first evaluated vascularity in the fibrous cap during tumor growth. Five days after inoculation of HT29 colon tumor cells, areas with abnormally high numbers of blood vessels appeared outside the tumor mass (Fig. 1a,b). On Day 7, the vasculature in the fibrous cap did not differ from that observed on Day 5, but the density of the vasculature in the parenchyma of the tumor mass was greater (Fig. 1c,d). On Day 10, blood vessels in which ECs were covered with α-SMA-positive MCs gradually appeared in the fibrous cap (Fig. 1e,f). On Day 14, mature blood vessels covered with α-SMA-positive MCs had increased in number; these were irregular in size, with both small and large diameter vessels seen (Fig. 1g,h). These changes continued after Day 14. Compared with the maturation of blood vessels in the fibrous cap, in the tumor parenchyma the ECs were rarely covered with MCs, despite the presence of α-SMA-positive cells.

Mature blood vessels in the fibrous cap selectively survive treatment with angiogenesis inhibitors. Next we treated HT29 tumor-bearing mice with bevacizumab from Day 14 after tumor cell inoculation. As reported previously,[12] tumor growth was significantly slowed by three injections of bevacizumab (Fig. 2a,b). There was a significant decrease in the number of microvessels inside the tumor parenchyma that were not covered with α-SMA-positive MCs (Fig. 3a,b). To determine the effect of VEGF inhibition on the vasculature in the fibrous cap surrounding the tumor, we used anti-CD31 and anti-α-SMA mAbs. The results of these experiments revealed that there was a decrease in the number of immature vessels in which ECs were not covered with MCs and that the ratio of mature:total blood vessels was increased from 22.2 ± 3.9% to 59.1 ± 3.5% after treatment with bevacizumab (Fig. 2c,d). Moreover, compared with untreated controls, most of the mature blood vessels covered with MCs were shrunken and looked squashed

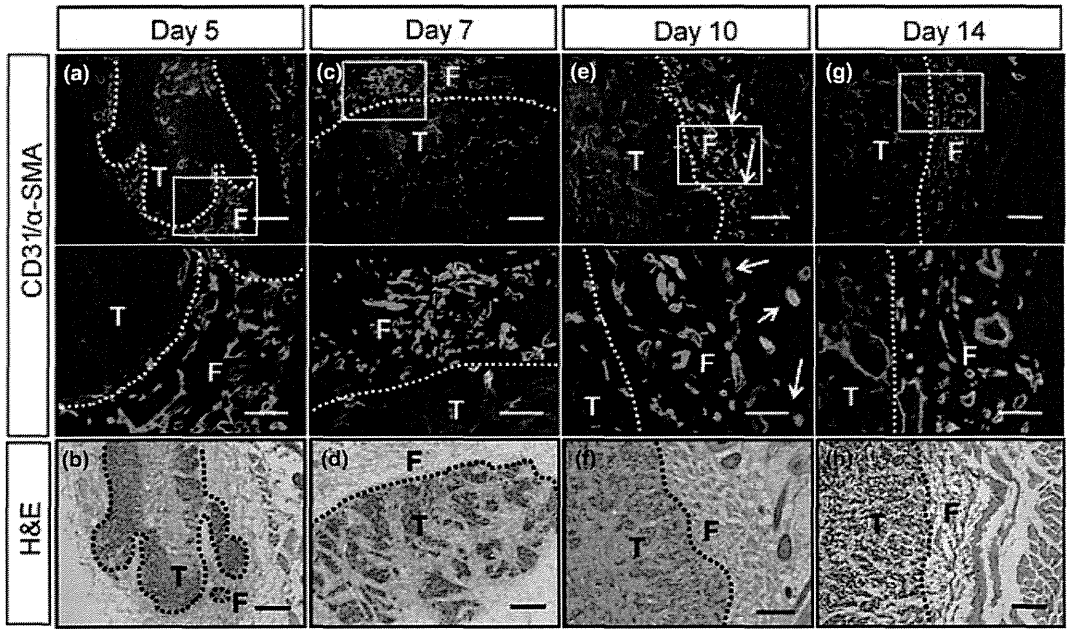

Fig. 1. Sequence of histological changes in the vasculature of the fibrous cap after inoculation of tumor cells. Sections of HT29 tumor tissues 5 (a,b), 7 (c,d), 10 (e,f), and 14 days (g,h) after inoculation of tumor cells were stained with (a,c,e,g) anti-CD31 mAb (green) and anti-α-smooth muscle actin (α-SMA) Ab (red) or (b,d,f,h) H&E. (a,c,e,g) High-power views of the areas indicated by the white boxes in the top panels are shown in the bottom panels. Dotted lines show the border between the fibrous cap surrounding the tumor mass (F) and the tumor mass (T). Arrows in (e) show mature vessels covered with α-SMA^+ mural cells. Scale lines: 200 μm.

doi: 10.1111/j.1349-7006.2011.02157.x

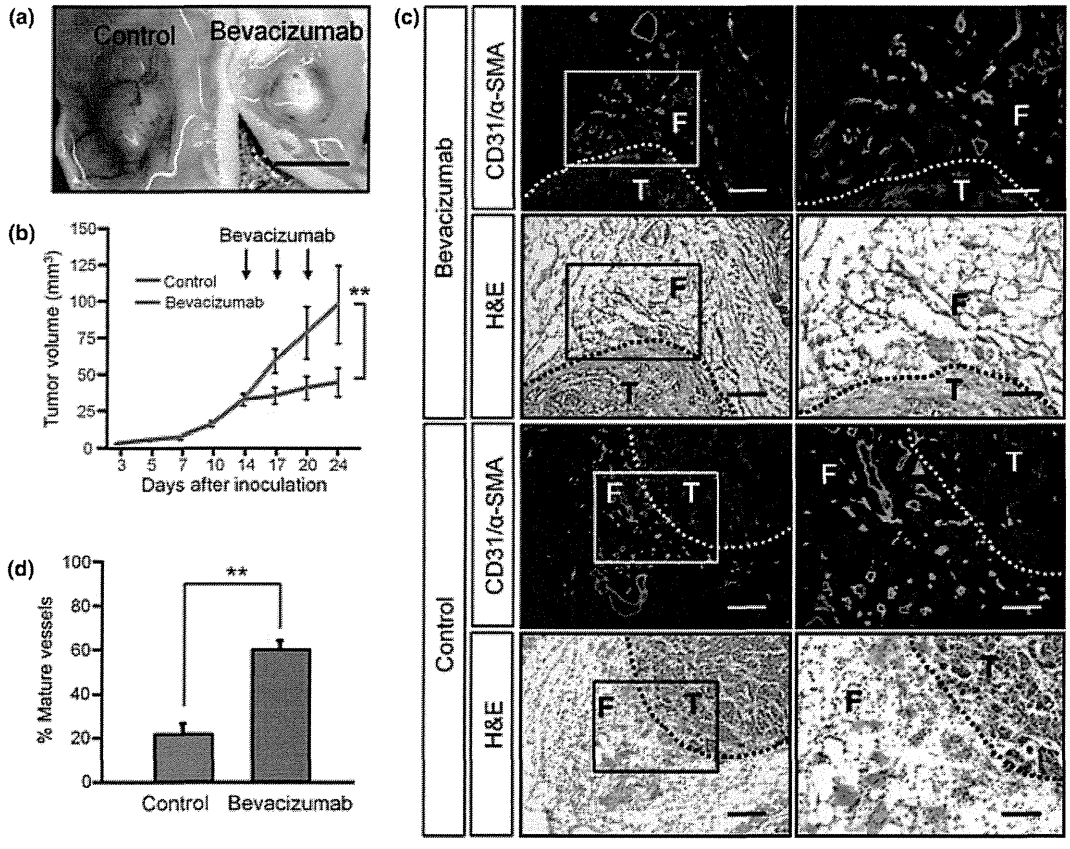

Fig. 2. Resistance of mature blood vessels in the fibrous cap to antiangiogenic therapy with anti-vascular endothelial growth factor (VEGF) neutralizing antibody. HT29 tumor cells were inoculated subcutaneously into mice, which were commenced on bevacizumab treatment 2 weeks later. (a) Gross appearance of the tumor after bevacizumab treatment. Scale lines: 5 mm. (b) Tumor volumes in untreated mice or mice treated with bevacizumab. Bevacizumab was injected when indicated (black arrows). Data are the mean ± SEM tumor volume ($n > 7$). $^{**}P < 0.01$. (c) Tumor sections, including the fibrous cap surrounding the tumor, 24 days after tumor cell inoculation were stained with anti-CD31 mAb (green) and anti-α-smooth muscle actin (α-SMA) Ab (red) or H&E. Higher-power views of the areas indicated by the boxes in the left-hand panels are shown in the right-hand panels. Dotted lines show the border between the fibrous cap surrounding the tumor mass (F) and the tumor mass (T). Scale lines: 200 μm (left-hand panels); 100 μm (right-hand panels). (d) Quantitative evaluation of the percentage of mature blood vessels, in which endothelial cells were covered with mural cells, of total blood vessels in the fibrous cap. More than six random fields of view were evaluated. Data are the mean ± SEM. $^{**}P < 0.01$.

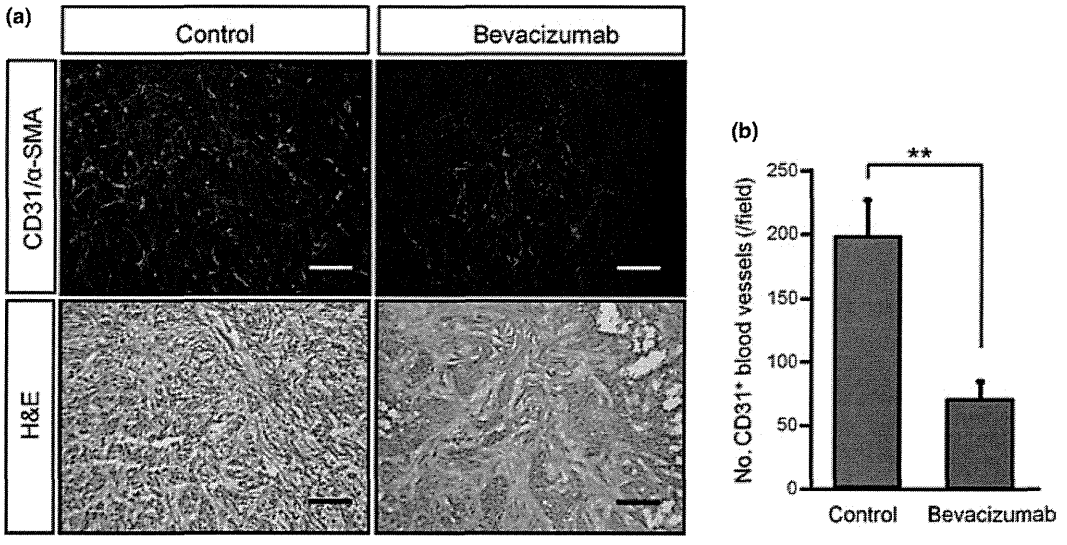

Fig. 3. Vascular density in the tumor parenchyma after bevacizumab treatment. (a) Tissue sections from the HT29 tumor mass in untreated mice or mice treated with bevacizumab were stained with anti-CD31 mAb (green) and anti-α-smooth muscle actin (α-SMA) Ab (red) or H&E. Scale lines: 200 μm. (b) Quantitative evaluation of the number of blood vessels in the tumor parenchyma. More than seven random fields of view were evaluated. Data are the mean ± SEM. $^{**}P < 0.01$.

(Fig. 2c). These dynamic phenotypic changes were not observed routinely in the tumor parenchyma (Fig. 3).

Because bevacizumab is specific inhibitor of human VEGF and does not neutralize murine VEGF[13] and, moreover, cells composing the fibrous cap are of murine origin, it is likely that human tumor cell-derived VEGF is involved in this blood vessel formation in mouse tissue (fibrous cap). Therefore, it is important to determine the degree to which tumor cell-derived VEGF drives vascular formation in the fibrous cap. To assess this, we used axitinib, a potent small molecule inhibitor for both human and mouse VEGFR and related receptor tyrosine kinases.

After 1 week treatment with axitinib, HT29 tumor growth was significantly reduced and, even more importantly, the volume of the tumor shrank such that is was less that the volume at the beginning of treatment (Fig. 4a,b). As observed in HT29 tumors treated with bevacizumab, immunohistological analysis of the fibrous cap after axitinib treatment revealed a decrease in immature vessels and an increase in the fraction of mature vessels:total blood vessels, from 28.6 ± 2.4% to 60.3 ± 5.0% after treatment (Fig. 4c,d). Shrunken vessels with reduced diameters were observed after axitinib treatment, similar to the observations after bevacizumab treatment (Fig. 4c). These data suggest that mature blood vessels in the fibrous cap are resistant to treatment with bevacizumab and other common angiogenesis inhibitors. Moreover, it is the tumor-derived VEGF that affects blood vessel formation in the fibrous cap, because attenuation of immature blood vessels was not significantly different following treatment with inhibitors specific for human, but not mouse, VEGF or inhibitors blocking both human and murine VEGFR.

Next, to confirm that these changes in vascularity in the fibrous cap are not specific to this one tumor cell line, we inoculated Colo320DM tumor cells into mice and treated them with bevacizumab. Three injections of bevacizumab suppressed tumor growth, as was observed with HT29 cells (Fig. 5a). On Day 24 after tumor cell inoculation, the vasculature of the fibrous cap was evaluated. As expected, the number of immature vessels was decreased and the ratio of mature vessels to total blood vessels increased significantly from 17.6 ± 2.0% to 53.0 ± 5.6% (Fig. 5b,c). The mature blood vessels also tended to be less dilated. Together, these results suggest that treatment with antiangiogenic agents not only affects the vasculature in the tumor parenchyma, but also the abnormal vasculature surrounding the tumor mass (i.e. causing a reduction of immature vessels and an induction of the mature squashed vascular phenotype).

Improvement in blood vessel function in the fibrous cap following treatment with bevacizumab. We next evaluated whether the increased numbers of blood vessels covered by MCs in the fibrous cap resulted in any functional changes. First, we performed vascular permeability assays with dextran. Although the fibrous cap is not composed of tumor tissue, the permeability of the blood vessels in it was significantly increased (Fig. 6a). After treatment with bevacizumab, this

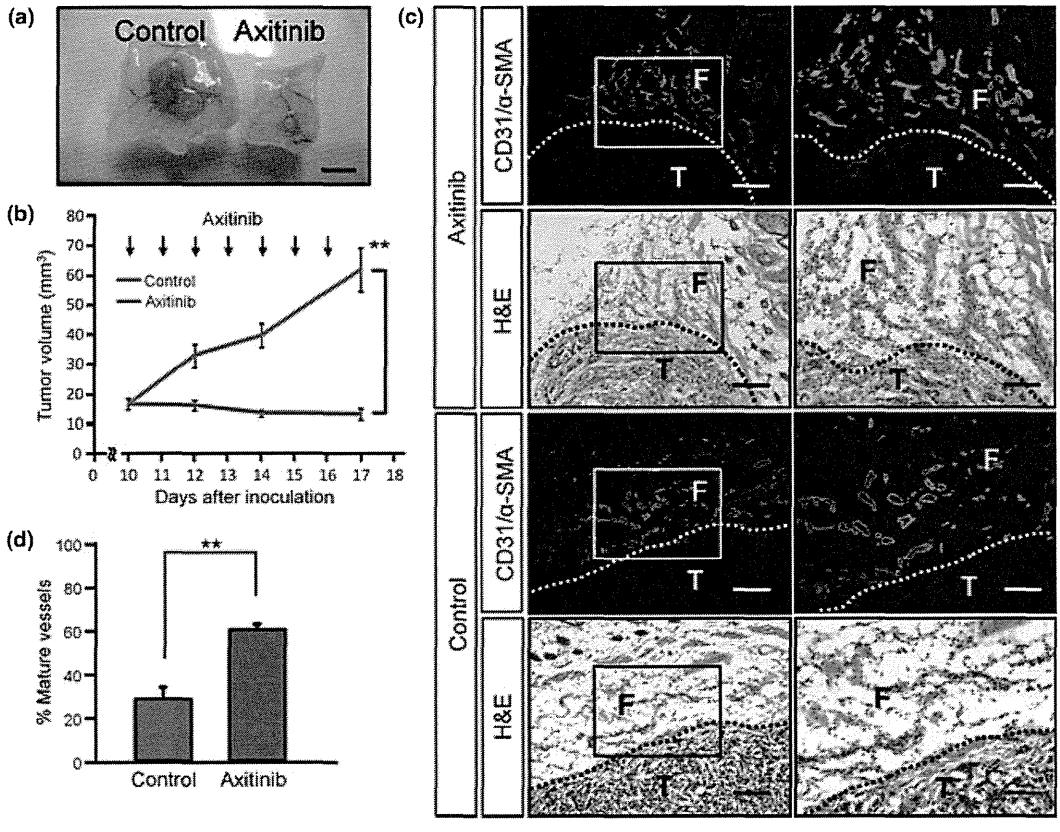

Fig. 4. Resistance of mature blood vessels in the fibrous cap after treatment with the potent, small molecule vascular endothelial growth factor receptor inhibitor, axitinib. HT29 tumor cells were inoculated subcutaneously into mice, which were commenced on axitinib treatment 10 days later. (a) Gross appearance of the tumors after treatment. Scale line: 5 mm. (b) Axitinib was injected as indicated (black arrows). Data are the mean ± SEM tumor volume ($n > 9$). $^{**}P < 0.01$. (c) Tumor sections, including the fibrous cap surrounding the tumor, 17 days after tumor cell inoculation were stained with anti-CD31 mAb (green) and anti-α-smooth muscle actin (α-SMA) Ab (red) or H&E. Higher-power views of the areas indicated by the boxes in the left-hand panels are shown in the right-hand panels. Dotted lines show the border between the fibrous cap surrounding the tumor mass (F) and the tumor mass (T). Scale lines: 200 μm (left-hand panels); 100 μm (right-hand panels). (d) Quantitative evaluation of the percentage of mature blood vessels, in which endothelial cells were covered with mural cells, of total blood vessels in the fibrous cap. More than seven random fields of view were evaluated. Data are the mean ± SEM. $^{**}P < 0.01$.

doi: 10.1111/j.1349-7006.2011.02157.x

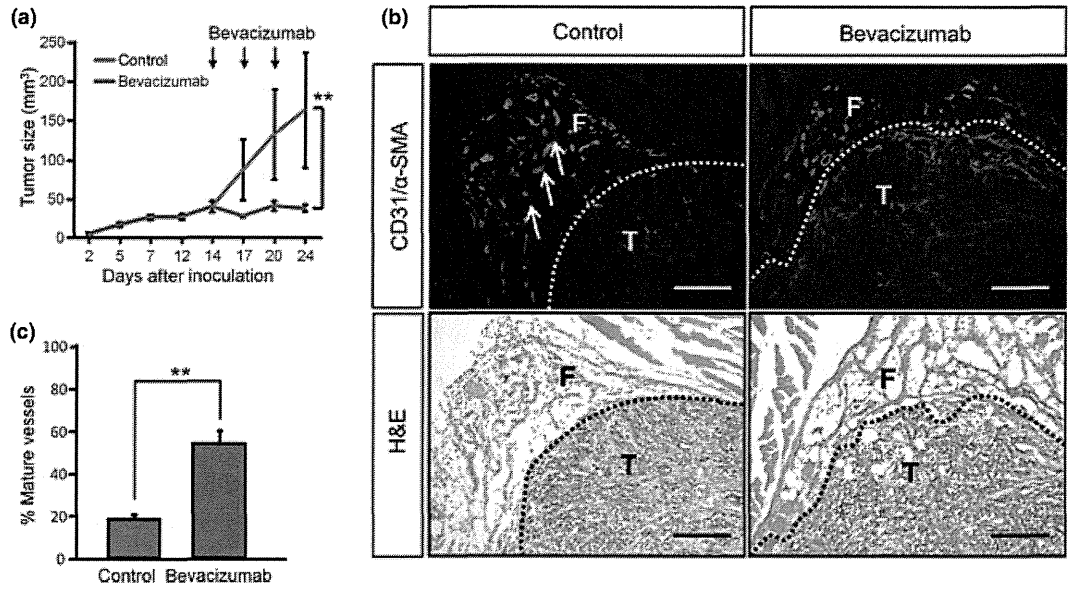

Fig. 5. Mature blood vessels in the fibrous cap of Colo320DM tumors show resistance to bevacizumab. Colo320DM tumor cells were inoculated subcutaneously into mice, which were commenced on bevacizumab treatment 2 weeks later. (a) Bevacizumab was injected as indicated (black arrows). Data are the mean ± SEM tumor volume ($n > 5$). $^{**}P < 0.01$. (b) Tumor sections, including the fibrous cap surrounding the tumor, 24 days after tumor cell inoculation were stained with anti-CD31 mAb (green) and anti-α-smooth muscle actin (α-SMA) Ab (red) or H&E. Dotted lines show the border between the fibrous cap surrounding the tumor mass (F) and the tumor mass (T). Scale lines: 200 μm (c) Quantitative evaluation of percentage of mature blood vessels, in which endothelial cells were covered with mural cells, of total blood vessels in the fibrous cap. More than seven random fields of view were evaluated. Data are the mean ± SEM. $^{**}P < 0.01$.

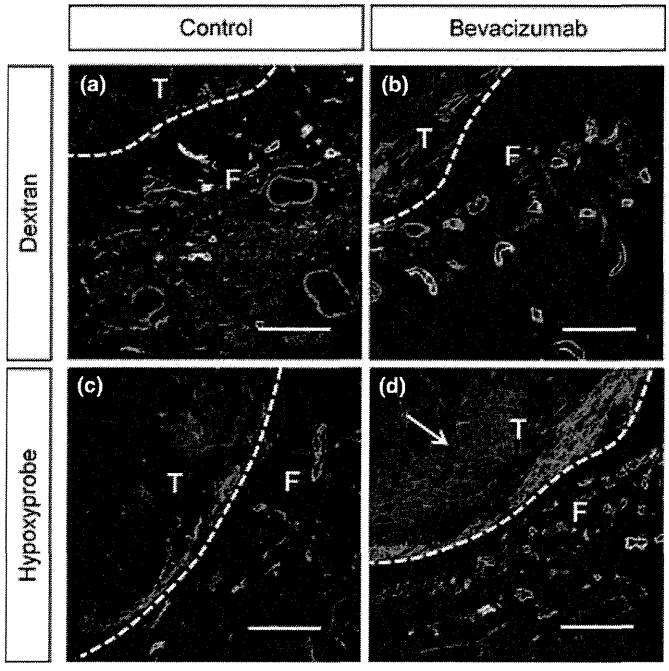

Fig. 6. Improvement in blood vessel function following bevacizumab treatment. (a,b) Microscopic images of FITC-dextran (blue) angiography and the vasculature (CD31, green; α-smooth muscle actin (α-SMA), red) of the fibrous cap from the control (a) and bevacizumab-treated (b) groups. (c,d) Hypoxia in the fibrous cap was detected by immunofluorescence staining of pimonidazole (blue), triple-stained with anti-CD31 mAb (green) and anti-α-SMA mAb (red). The arrow indicates a hypoxic area observed in the tumor parenchyma. Scale lines: 200 μm.

dextran extravasation decreased to a level comparable to that in normal tissue (Fig. 6b). These results indicate that the vasculature in the fibrous cap is influenced by VEGF secreted from the tumor cells and is functionally abnormal. Therefore, administration of bevacizumab not only normalizes blood vessels in the tumor parenchyma, but also in the fibrous cap. Moreover, it has been reported that treatment with bevacizumab induces hypoxia in the tumor parenchyma;[14] therefore, we evaluated the oxygenation level in the fibrous cap. Without bevacizumab treatment, the fibrous cap was not hypoxic (Fig. 6c) but, unlike the tumor parenchyma, which became hypoxic after treatment, the oxygenation level of the fibrous cap did not change after treatment (Fig. 6d).

Discussion

In the present study, we found that most blood vessels in the fibrous cap that formed at the tumor rim were immature, lacking MC coverage of the ECs. Upon treatment with angiogenesis inhibitors, these immature blood vessels were destroyed and only blood vessels covered with MCs remained. Moreover, despite MC coverage, dilated blood vessels were characteristically less dilated after treatment with angiogenesis inhibitors. These responses of blood vessels to angiogenesis inhibitors were quite similar to those observed in the parenchyma of the tumor itself. Therefore, these observations suggest that the fibrous cap is an environment mimicking the tumor microenvironment. Recently, it was reported that treatment with angiogenesis inhibitors induces normalization of tumor vascular function.[15] Indeed, in the present study the permeability of the blood vessels in the fibrous cap was significantly increased, as in the tumor parenchyma. After bevacizumab treatment, we found that the permeability of the vasculature in the fibrous cap was decreased. This functional change to the vasculature in the fibrous cap seems to be caused by the destruction of immature blood vessels not covered by MCs. However, when a hypoxic situation was established, hypoxia was not induced in the fibrous cap, suggesting a different mechanism underlying oxygenation in the fibrous cap compared with that in the tumor parenchyma. At present, the reasons underlying these differences in leakiness and hypoxia between the fibrous cap and the tumor parenchyma are

not known and further analysis of oxygenation in the fibrous cap is required.

It is interesting to know how angiogenesis inhibitors affect vascular maturation in the fibrous cap. Previously, we reported that hematopoietic stem cells (HSCs) promote angiogenesis,[16] that c-Kit$^+$ hematopoietic stem/progenitor cells accumulate around the edge of the tumor,[17] and that HSCs differentiate into MCs through a CD45$^+$ CD11b$^+$ stage.[18] Moreover, another group has reported that bone marrow-derived α-SMA-positive fibroblasts promote tumor growth.[19] Therefore, it is possible that bone marrow-derived cells more effectively contribute to the maturation and coverage of blood vessels in the fibrous cap after treatment with angiogenesis inhibitors.

Previous studies have suggested that well-matured blood vessels are sheathed in ECM and that cancer cells migrate along tracks made of ECM collagen fibers.[20] In terms of tumor invasion, it has been suggested that antiangiogenic therapy may be involved in this process.[21] Vascular remodeling induced by angiogenesis inhibitors leads to a more hypoxic tumor microenvironment, which results in enhanced tumor cell invasion into normal tissue.[22] Mature vessels are fully covered by ECM and it is therefore possible that tumor cells invade into the normal tissues along these ECM-rich vessels that are induced by angiogenesis inhibitors. In addition, regarding lymphatic metastasis, development of lymphatic vessels in the fibrous cap must be involved in the process. Further analysis of lymphangiogenesis in the fibrous cap is required.

How to destroy mature vessels may be the next task for treating cancer after the induction of vascular maturation by angiogenesis inhibitors. Moreover, the relationship between cancer stem cells and the fibrous cap after antiangiogenic therapy remains of great interest. We have reported that malignant cancer cells exhibiting high tumorigenic and metastatic ability locate in the vascular region at the edge of the tumor.[23] Therefore, a drug delivery system that targets mature vessels only in the tumor rim, and not in normal tissue, will be required in future to destroy vascular niches for malignant cancer cells.

Acknowledgments

The authors thank Ms N. Fujimoto and Ms C. Takeshita for technical assistance. This work was supported, in part, by a Grant-in-Aid from The Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Disclosure Statement

The authors have no conflict of interest.

References

1 Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov* 2004; **3**: 391–400.
2 Crawford Y, Ferrara N. VEGF inhibition: insights from preclinical and clinical studies. *Cell Tissue Res* 2009; **335**: 261–9.
3 Ellis LM, Hicklin DJ. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nat Rev Cancer* 2008; **8**: 579–91.
4 Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005; **307**: 58–62.
5 Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell* 2009; **15**: 232–9.
6 Tozer GM, Kanthou C, Baguley BC. Disrupting tumour blood vessels. *Nat Rev Cancer* 2005; **5**: 423–35.
7 Mancuso MR, Davis R, Norberg SM *et al.* Rapid vascular regrowth in tumors after reversal of VEGF inhibition. *J Clin Invest* 2006; **116**: 2610–21.
8 Tomayko MM, Reynolds CP. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* 1989; **24**: 148–54.
9 Inai T, Mancuso M, Hashizume H *et al.* Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. *Am J Pathol* 2004; **165**: 35–52.
10 Takakura N, Huang XL, Naruse T *et al.* Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. *Immunity* 1998; **9**: 677–86.
11 Kidoya H, Kunii N, Naito H *et al.* The apelin/APJ system induces maturation of the tumor vasculature and improves the efficiency of immune therapy. *Oncogene* 2011; doi: 10.1038/onc.2011.489. [Epub ahead of print].
12 Kim KJ, Li B, Winer J *et al.* Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* 1993; **362**: 841–4.
13 Yu L, Wu X, Cheng Z *et al.* Interaction between bevacizumab and murine VEGF-A: a reassessment. *Invest Ophthalmol Vis Sci* 2008; **49**: 522–7.
14 Paez-Ribes M, Allen E, Hudock J *et al.* Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 2009; **15**: 220–31.
15 Dickson PV, Hamner JB, Sims TL *et al.* Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenografts results in improved delivery and efficacy of systemically administered chemotherapy. *Clin Cancer Res* 2007; **13**: 3942–50.
16 Takakura N, Watanabe T, Suenobu S *et al.* A role for hematopoietic stem cells in promoting angiogenesis. *Cell* 2000; **102**: 199–209.
17 Okamoto R, Ueno M, Yamada Y *et al.* Hematopoietic cells regulate the angiogenic switch during tumorigenesis. *Blood* 2005; **105**: 2757–63.
18 Yamada Y, Takakura N. Physiological pathway of differentiation of hematopoietic stem cell population into mural cells. *J Exp Med* 2006; **203**: 1055–65.
19 Quante M, Tu SP, Tomita H *et al.* Bone marrow-derived myofibroblasts contribute to the mesenchymal stem cell niche and promote tumor growth. *Cancer Cell* 2011; **19**: 257–72.
20 Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. *Nat Rev Mol Cell Biol* 2009; **10**: 445–57.
21 Sato Y. Persistent vascular normalization as an alternative goal of anti-angiogenic cancer therapy. *Cancer Sci* 2011; **102**: 1253–6.
22 Keunen O, Johansson M, Oudin A *et al.* Anti-VEGF treatment reduces blood supply and increases tumor cell invasion in glioblastoma. *Proc Natl Acad Sci USA* 2011; **108**: 3749–54.
23 Nagahama Y, Ueno M, Miyamoto S *et al.* PSF1, a DNA replication factor expressed widely in stem and progenitor cells, drives tumorigenic and metastatic properties. *Cancer Res* 2010; **70**: 1215–24.

doi: 10.1111/j.1349-7006.2011.02157.x

Journal of Thrombosis and Haemostasis, **9** (Suppl. 1): 144–150 DOI: 10.1111/j.1538-7836.2011.04275.x

INVITED REVIEW

Role of intimate interactions between endothelial cells and the surrounding accessory cells in the maturation of blood vessels

N. TAKAKURA

Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

To cite this article: Takakura N. Role of intimate interactions between endothelial cells and the surrounding accessory cells in the maturation of blood vessels. *J Thromb Haemost* 2011; **9** (Suppl. 1): 144–150.

Summary. While blood vessels clearly have a fundamental role in supplying oxygen and nutrients, and transporting inflammatory cells throughout the body, they are also involved in organogenesis and maintenance. Blood vessels provide a niche that supports self-renewal of stem cell populations in normal organs. This role unfortunately also extends to the field of cancer biology; it has been suggested that cancer stem cells are located in perivascular regions supporting their proliferation. Through cell-to-cell interactions, arteries also have the important function of guiding appropriate migration of neurites. Therefore, analysis of the molecular mechanisms responsible for blood vessel formation and maintenance is important for developing strategies to regulate tissue regeneration. According to the usual concept of angiogenesis, it is widely accepted that homogeneous endothelial cells from preexisting vessels sprout and proliferate during angiogenesis. Recently, however, at least three different endothelial cell types designated tip, stalk and phalanx cells have been suggested to be involved in new blood vessel formation in sprouting angiogenesis. Given this endothelial cell heterogeneity, the involvement of a stem cell system in preexisting blood vessels is proposed. In addition, endothelial cells possess the capacity to differentiate into mesenchymal stem cells upon stimulation with growth factors, and pericytes show stem cell behaviour in their ability to differentiate into a variety of different histotypes. Moreover, under normal physiological conditions, haematopoietic stem cells differentiate into mural cells to provide blood vessel stability. These findings make it necessary to reconsider issues concerning the regulation of blood vessels by accessory cells situated around those vessels.

Keywords: angiogenesis, endothelial cell, hematopoietic stem cell, stem cell, vascular niche.

Correspondence: Nobuyuki Takakura, Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan.
Tel.: +81 6 6879 8316; fax: +81 6 6879 8314.
E-mail: ntakaku@biken.osaka-u.ac.jp

Complex differentiation pathways of mesenchymal cells and mural cells supporting endothelial cells

Vascular development is regulated by two different processes – vasculogenesis and angiogenesis [1]. In the former, angioblasts derived from mesoderm differentiate into mature endothelial cells (ECs). These then form a tube and a primitive vascular plexus which initially possesses a homogeneous dilated structure, but in later stages, generate highly hierarchical architecture ranging from smaller to larger calibre vessels. This process, termed remodelling of blood vessels, is known to be regulated at several different levels. For example, interactions of ECs and mural cells (such as pericytes or vascular smooth muscle cells) recruited near the blood vessels induce vessel maturation [2]. This process promotes vessel enlargement and structural stabilisation for the maintenance of the lumen by direct mural cell adhesion to ECs and production of extracellular matrices from the basal part of blood vessels, imbedding them in the matrix. Fusion of smaller blood vessels can result in enlargement, and reciprocally, intussusception produces smaller blood vessels with an increased density. During blood vessel formation, superfluous vessels regress, but upon tissue hypoxia, new blood vessels are generated mainly by sprouting angiogenesis [1].

Growth factors involved in the processes of vasculogenesis and angiogenesis have been identified. Development, proliferation, and migration of vascular cells, interactions between them, and arterio-venous patterning, have been characterised based on the analysis of such growth factors [2–5]. Of the many growth factors and their cognate receptors, two receptor tyrosine kinase subfamilies are characterised by their largely EC-specific expression. One comprises the vascular endothelial growth factor (VEGF) family and its cognate receptors (Flt-1/VEGFR1, Flk-1/KDR/VEGFR2, and Flt-4/VEGFR3). Experiments on genetically engineered mice with mutations in genes of this receptor family showed that these receptors play crucial roles in the development, tube formation and proliferation of ECs, and for other aspects of vessel formation [6–9]. The second family includes Tie1 and Tie2. The onset of expression of these receptors in the embryo seems to follow VEGFR expression [10]. Gene knockout mice provide evidence that these receptors also play a crucial role in embryonic

vascular formation [11–13]. Embryos lacking Tie2 or Tie1 fail to develop a structurally stable vasculature, resulting in haemorrhage at E9.5 and 13.5, respectively. Compared with the early defect in vasculogenesis seen in *VEGF*- or *VEGFR*-mutant embryos, mice lacking *Tie1* or *Tie2* exhibit later defects in angiogenesis and vascular remodelling, as well as in vascular integrity.

For both vasculogenesis and angiogenesis, mural cells need to adhere to ECs to promote the maturation of blood vessels, as described above. It has been suggested that adhesion and dissociation of ECs and mural cells are regulated by angiopoietin-1 (Ang1) and Ang2, ligands for Tie2 on ECs [13–17]. To trigger adhesion, ECs produce and release platelet-derived growth factors (PDGFs) for the recruitment of nearby mural cells. This results in ligation of the PDGFRβ expressed on mural cells [18,19]. Under normoxic conditions, Ang1 is continuously produced by mural cells and stimulates Tie2 to induce cell adhesion between ECs and mural cells, resulting in the maintenance of stability of the blood vessels. However, under hypoxic conditions, Ang2, an antagonist of Ang1, is produced and released from ECs [20]. In this situation, Tie2 is inactivated, resulting in dissociation of mural cells from ECs. Although unequivocal evidence is still lacking, it has been shown that new vessels may sprout from those areas of the blood vessel where mural cells are not adhering. In consideration of regenerative as well as anti-angiogenic therapy, not only regulation of ECs but also mural cells is thus likely to be very important for modulating angiogenesis.

Regarding the origin of mural cells, several sources have been identified *in vivo* on the basis of developmental biological analyses. Mesenchymal cells surrounding newly-developed blood vessels are thought to be the main source of mural cells. However, diverse origins of smooth muscle cells have also been reported. Thus, neural crest cells or cells in the secondary heart field can give rise to smooth muscle cells in the aorta and pulmonary artery and those of the coronary artery can be derived from the epicardium. Moreover, several reports in the literature argue for complex differentiation pathways of mesenchymal cells and mural cells supporting ECs.

In models of post-angioplasty restenosis, graft vasculopathy and hyperlipidaemia-induced atherosclerosis, bone-marrow (BM) cells, especially haematopoietic stem cells (HSCs), give rise to most of the vascular smooth muscle cells that contribute to arterial remodelling [21]. However, such trans-differentiation of HSCs into smooth muscle cells has not been fully investigated in the context either of physiological or pathological vascular growth in humans. On the other hand, consistent with such trans-differentiation of HSCs into mesenchymal cells, evidence for the plasticity of HSCs is accumulating [22]. These findings indicate that adult BM HSCs can differentiate into mature, non-haematopoietic cells of multiple histotypes, including epithelial cells of the liver, kidney, lung, skin, gastrointestinal (GI) tract, and myocytes of heart and skeletal muscle. Although this indicates that HSCs might indeed be developmentally plastic and capable of producing both blood cells and other tissues, the mechanism underlying this phenomenon remains unclear. Although two reports have raised the issue of whether fusion between donor haematopoietic cells and host cells could explain this apparent stem cell plasticity [23,24] and some investigators are sceptical concerning the multi-lineage plasticity of HSCs, any final judgment on this issue is currently very difficult.

Despite these discrepancies, we have demonstrated that HSCs do have the physiological ability to differentiate into mesenchymal cells or into the mural cell lineage and thus promote maturation/stabilisation of blood vessels [25], although many reports, including our own, suggest that ECs can differentiate from adult BM cells. We found that a haematopoietic lineage marker (Lin)-negative, haematopoietic marker CD45-positive and c-Kit-positive HSC population sorted from brains of E10.5 mouse embryos could differentiate into mural cells *in vitro*. In culture, cells weakly double-positive for CD31 and smooth muscle actin are observed. These proliferating cells are predominantly PDGFRβ and smooth muscle actin double-positive mural cells [25]. Moreover, we discovered that HSCs from adult BM as well as embryonic HSCs could trans-differentiate into mural cell populations via a myeloid progenitor cell pathway. HSCs in the neural tube readily differentiate into mural cells without requiring any factors for trans-differentiation. However, TGFβ is necessary for the *in vitro* differentiation of adult BM HSC into mural cells. Recently, Medici *et al.* [26] reported conversion of ECs into mesenchymal stem cells with the potential to differentiate into osteoblasts, chondrocytes, and adipocytes. They reported that TGFβ2 or BMP4 induces such conversion of ECs into mesenchymal stem cells. Therefore, it is possible that ECs derived from HSCs differentiate into mesenchymal stem cells and subsequently into mural cells. Moreover, it has been shown that pericytes expressing CD146, NG2, and PDGFRβ are identical to mesenchymal stem cells showing osteogenic, chondrogenic, and myogenic activity [27]. Taken together, these data strongly suggest that differentiation of HSCs into pericytes occurs physiologically in the perivascular region to provide stable blood vessel support. However, the physiological role of HSCs in terms of their contribution to mural cells should be further clarified *in vivo*.

Currently, so-called haemogenic endothelium derived from mesoderm is thought to be the origin of haematopoietic cells [28]. However, we and other groups have suggested the differentiation of HSCs into mural or mesenchymal stem cells. Therefore, development of ECs, mural cells and haematopoietic cells is complex (Fig. 1) and differentiation of HSCs into mural cells may be one of the physiologically occurring events of the de-differentiation pathway.

Function of haematopoietic cells as accessory cells for blood vessel formation

Angiogenesis occurs under physiological conditions in adult organs as well as in the embryo and is also very closely associated with different pathological states [29]. Abnormally rapid proliferation of blood vessels is implicated in many

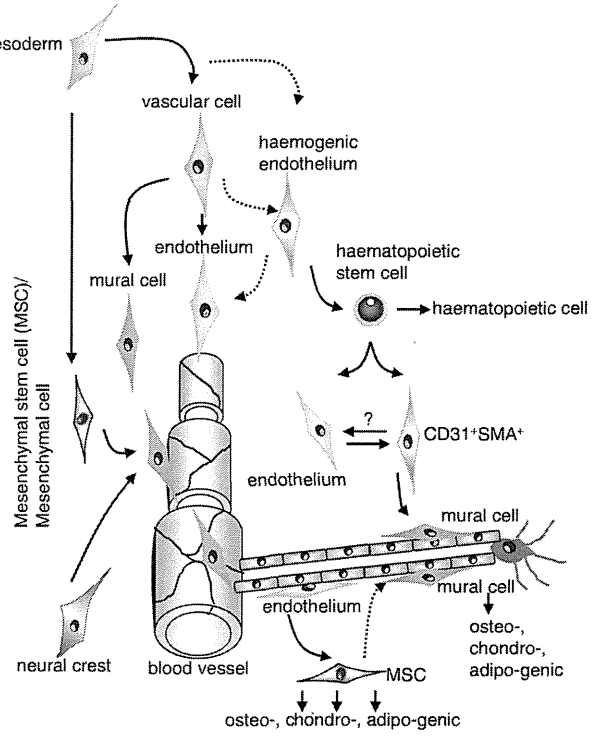

Fig. 1. Differentiation pathways of vascular cells. Complex pathways for differentiation to vascular cells, such as endothelium and mural cells, have been reported. In the early embryo, haematopoietic cells develop from haemogenic endothelium already committed to ECs; however, in the later stage of embryonic development and after birth, haematopoietic cells conversely differentiate into mural cells as well as ECs. Moreover, ECs can differentiate into mesenchymal stem cells by endothelial-mesenchymal transition and pericytes posses a multipotential for differentiation. This suggests that blood vessels have their own stem cell system for long-term maintenance. Arrows indicate the direction of differentiation. Broken line arrows indicate possible differentiation pathways. For details, see text.

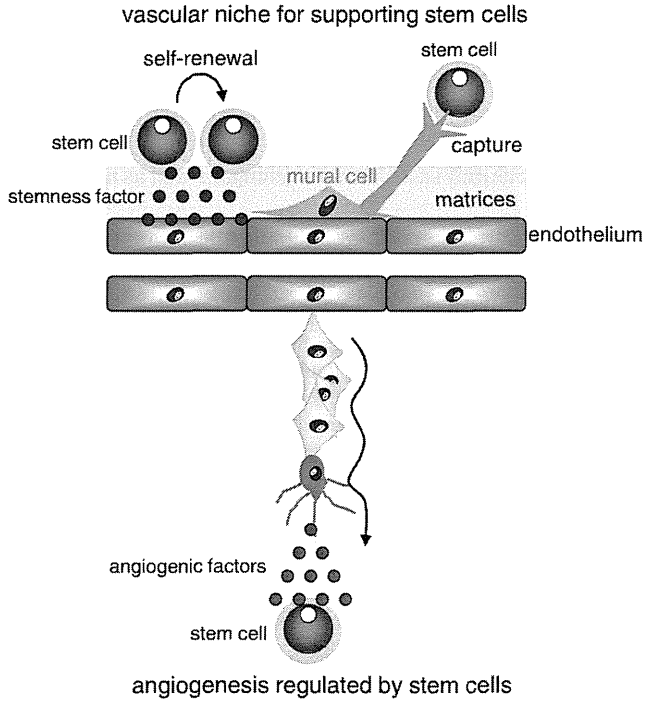

Fig. 2. Mutual interactions between angiogenesis and haematopoiesis. HSCs play a role in angiogenesis by inducing migration of ECs into avascular areas and therefore act as a guide for determining the direction of migration of ECs. Moreover, haematopoietic cells can differentiate into mural cells to provide stability for newly-developed blood vessels. Conversely, ECs form a niche for maintenance of stem cell populations including haematopoietic stem cells, neural stem cells, and perhaps stem cells in other organs. In vascular niches, angiocrine factors produced by ECs regulate stem cell division. Moreover, the importance of the extracellular matrix and mural cells in the perivascular region for adhesion and capture of stem cells has been suggested.

diseases, such as cancer, psoriasis, inflammatory diseases, and diabetic retinopathy. It is widely accepted that ECs derived from preexisting vessels are responsible for neovascularisation during angiogenesis. However, cells derived from the BM may also contribute to postnatal angiogenesis [30]. Most studies have focused initially on the contribution of endothelial progenitor cells (EPCs) to neovascularisation. However, several studies have not supported a direct contribution of BM-derived cells to vascular ECs [31], and argued that their contribution may not be a major one in newly-developed blood vessels. Further precise analysis will be required to clarify the contribution of EPCs both physiologically and pathologically.

On the other hand, in contrast to the concept that BM-derived cells contribute to vascular cells directly, many reports suggest that cells of the haematopoietic lineage are mobilised and then entrapped in lesions where they may function as accessory cells promoting sprouting angiogenesis by releasing angiogenic signals such as VEGF, angiopoietins and matrix metalloproteinases (MMPs). In cancer, it is widely believed that tumour cells are the major producers of proangiogenic factors [32]. However, not only cancer cells but also haematopoietic cells produce proangiogenic factors and function as accessory cell components to induce angiogenesis.

Of course, the major function of HSCs is to generate all the mature hematopoietic cells. However, HSCs are present in peripheral blood and organs in adults as well as embryos. The function of such motile HSCs is completely unclear. *AML1*-deficient mice provide a tool to analyse interactions between haematopoiesis and angiogenesis because haematopoiesis is fully impaired. Inspection of blood vessels in AML1 mutant embryos revealed no major defects for those generated by vasculogenesis, but sprouting of capillaries generated during angiogenesis was retarded in several regions such as the neural tube and pericardium. Observation of HSC localisation in the neural tube during angiogenesis reveals that HSCs migrate into the parenchyma and then ECs migrate toward them. Based on this analysis, we hypothesised that HSCs induce migration of ECs into avascular regions. Accordingly, we found that Angl produced by HSCs stimulates Tie2 expressed on ECs, resulting in their attraction near HSCs and promoting sprouting angiogenesis (Fig. 2) [33]. Based on the evidence that HSCs play a role in promoting angiogenesis, accompanied by the direct differentiation capacity of the HSC fraction into vascular cells, transplantation of HSCs into ischaemic patients has

already been used in the clinic and shown to be effective for repair in ischaemic diseases.

As described above, Ang1 produced by mural cells induces cell-to-cell adhesion between these cells and ECs. This results in finalization of angiogenesis for structural stabilization of blood vessels. However, Ang1 from HSCs induces mobility of ECs for chemotaxis and acts as a proangiogenic factor in this case. The paradoxical function of Ang1 on ECs has not been clearly explained. We recently found that the Akt signalling pathway downstream of Tie2 mainly induces mural cell adhesion to ECs when EC-to-EC cell contact is already established, but that Tie2 activation of the ERK pathway induces migration of ECs when the latter are not tightly adherent [34].

Other than HSCs, the function of several haematopoietic lineage cells in the context of angiogenesis has been extensively documented, especially in tumours. In tissue repair accompanied by inflammation, granulation tissue formation is initiated by proliferation of fibroblasts and remodelling of the extracellular matrix, together with infiltration of inflammatory cells and angiogenesis. Once inflammation is attenuated, the newly-developed blood vessels usually regress, with restoration of the remodelled tissue. However, the tumour environment continues to contain many inflammatory cells such as macrophages, granulocytes, eosinophils, dendritic cells and mast cells, as well as lymphocytes. Under these conditions, there is no subsidence of angiogenic activity and tissue remodelling with extracellular matrices and myofibroblasts also continues. For this reason, cancer has been called an 'unresolved inflammation'. From the lesson of angiogenesis learned in inflammatory tissue, especially in the cancer environment, the function of haematopoietic cells in angiogenesis has been clarified.

In acute inflammatory responses, neutrophils are recruited to and accumulate in the lesion. Neutrophils are abundantly present in most tumour types and a correlation between the number of these cells and clinical prognosis as well as microvessel density in tumours has been reported [35]. Neutrophils infiltrating tumours produce MMP9, which degrades extracellular matrices and induces angiogenesis [36]. Additionally, they produce the chemokines CXCL8 and CXCL1 [37]. CXCL8 is suggested to act on neutrophils via an autocrine loop, resulting in release of MMP9. When they are stimulated with tumour necrosis factor (TNF)α, neutrophils release VEGF, resulting in induction of angiogenesis [38]. When neutrophils are in contact with cancer cells, they may secrete oncostatin M, which in turn induces VEGF production from the tumour cells [39].

Subsequent to neutrophil infiltration, monocytes, which finally differentiate into macrophages, enter the inflammatory foci. Activated macrophages produce different growth factors, which promote proliferation and activation of ECs and fibroblasts in the inflammatory foci. Macrophages infiltrating into tumours are usually termed tumour-associated macrophages (TAMs). It has been commonly accepted that activated macrophages are effector cells that phagocytose microorganisms and kill tumour cells. However, many reports have highlighted a correlation between the degree of macrophage infiltration in tumours and a poor prognosis with increased blood vessel density [40]. TAMs express a variety of proangiogenic and matrix-remodelling factors such as VEGF, basic fibroblast growth factor (bFGF), TNFα, interleukin 1β (IL-1b) CXCL8, PDGF, MMPs and urokinase-type plasminogen activator (uPA) [41]. As described above, BM myeloid cells can differentiate into ECs and mural cells, and when exposed to sustained stimulation by angiogenic growth factors, TAM may also trans-differentiate into vascular cells. However, those ECs derived from TAM may not survive long-term and may only transiently contribute to producing a neovasculature. It has recently been reported that Tie2-expressing monocytes (TEM) have a specific function for angiogenesis. These TEM may represent a sub-population of TAMs, possibly overlapping with M2 macrophages [42]. At present, how TEM regulate tumour angiogenesis is not clear, but secretion of the proangiogenic cytokine bFGF from TEMs has been suggested as one possible mechanism [43].

Mast cells (MC) are also important regulators for tissue remodelling by releasing inflammatory mediators such as cytokines, histamine, and proteases. MC are found in the tumour environment from premalignant stages through to malignant tumour progression, and especially at the periphery of invasive tumours, suggesting a host-tumour barrier activity as their original function for host defence. The density of MC in tumours correlates with microvessel density [44]. It has been reported that MC produce a variety of proangiogenic cytokines and growth factors, including VEGF, bFGF, IL-8, MMP9, TNFα, TGFβ, CCL2, CXCL8, heparin and histamine and that they also contain angiogenic proteases in their secretory granules [45]. In addition, MC indirectly stimulate angiogenesis by secreting MC-specific serine proteases that activate pro-MMPs and stimulate fibroblasts to synthesise collagen for tissue remodelling [44]. It is suggested that these factors affect proliferation, migration, and tube formation of ECs [44]. MCs are observed at the edge of the tumour before the formation of new blood vessels and can be seen to adhere to blood vessel walls in tumours. From this intimate co-localisation of MCs and ECs, trans-differentiation of MCs to ECs has even been suggested; however, such trans-differentiation capacity is controversial.

Myeloid-derived suppressor cells (MDSC), a population of $CD11b^+/Gr1^+$ cells phenotypically quite similar to immature myeloid cells, are associated with suppression of immune function. Indeed, a possible role of MDSC in suppressing the anti-tumour functions of T and natural killer (NK) cells has been suggested [46]. However, MDSC also induce tumour angiogenesis. Co-injection of MDSC and cancer cells enhanced angiogenesis in the tumour environment [47]. MDSC in the tumour environment produce several MMPs for matrix remodelling. It has been reported that some MDSC change their morphology from a round to a bipolar shape and also express endothelial markers such as CD31 and VEGFR2 [47], suggesting that some have the potential to differentiate into ECs.

Although their contribution to physiological and pathological angiogenesis is uncertain, eosinophils and dendritic cells

(DCs) are also associated with angiogenesis. In the case of eosinophils, it has been suggested that they produce a variety of proangiogenic factors, such as VEGF, bFGF, IL-6, CXCL8, GM-CSF, PDGF, TGFβ, and MMP9 [48]. In the case of DCs, it has similarly been suggested that immature DCs (iDC) produce proangiogenic factors and induce angiogenesis directly and indirectly. TNFα and CXCL8 produced by iDC in cancer ascites have been shown to promote angiogenesis *in vivo* [49]. In addition, chemokines such as CXCL1, CXCL2, CXCL3 and CXCL5 produced by DC have been suggested to recruit other proangiogenic myeloid cells [50]. Interestingly, it was recently reported that a population of $CD45^+CD11c^+MHC\text{-}II^+$ DC precursors that massively infiltrate human ovarian carcinomas also express the EC-specific marker VE-cadherin. Those cells could assemble into blood vessels *in vivo* following implantation of tumour-derived $CD45^+$VE-cadherin$^+$ cells into the flanks of immunodeficient mice [51]. Moreover, differentiation of tumour-derived $CD11c^+$ DC into endothelial-like cells can be induced by culture with VEGF [52]. Therefore, it is possible that such iDC directly contribute to blood vessel formation in tumours; hence they are designated 'vascular DC'.

Vascular niches for maintenance of stem cells

In relation to tissue generation, the importance of analysing self-renewal and maintenance of stem cells in a specific microenvironment, the so-called niche, has been suggested, and the localisation of organ-specific stem cells and supporting niche cells has been extensively investigated [53]. In the case of HSCs, we found that self-renewal of HSCs is induced on ECs of the omphalomesenteric artery at E9.5 in mouse embryos and that Tie2 on HSCs is critical for adhesion of these cells to ECs through activation of integrin [16]. Other groups have also found that proliferation of HSCs is induced within the vascular labyrinth region of E10.5–11.5 mouse placenta [54]. However, in the adult BM, there are two different niches for HSCs. One comprises osteoblasts at the endosteum of BM [55,56]. Proliferation of N-cadherin-positive osteoblasts by the ablation of BMP type 1A receptor on these cells correlates with an increase of HSCs; however, in the osteoblast niche, HSCs are dormant (caused by tight adhesion to osteoblasts due to Tie2 activation of HSCs by the Ang1 produced from osteoblasts). Therefore, the osteoblast niche may regulate HSC pool size by inhibiting their disorganised expansion. On the other hand, as observed in the embryo, HSCs are also found in a vascular region termed the sinusoid [57]. At present, whether HSCs are in direct contact with the sinusoidal endothelium is not clear; other investigators have suggested that reticular cells near the sinusoidal endothelium directly attach to HSCs [58]. Nonetheless, control of HSC self-renewal must be regulated in such vascular regions, whatever the exact mechanism (Fig. 2).

In addition to the identification of HSC niches, mechanisms of self-renewal or expansion of HSCs induced by ECs has been gradually clarified. The potential support by ECs of HSC proliferation has been documented by coculturing HSCs with ECs *in vitro* [59]. However, experiments using primary ECs in the analysis of HSCs have some serious drawbacks. Addition of serum and growth factors is critical for the maintenance of primary ECs but such supplements also affect proliferation or differentiation of HSCs. Rafii's group established a coculture system of HSCs with primary ECs transduced with the adenovirus gene early region 4-encoded open reading frame-1, which leads to constitutive activation of Akt in transfected cells. In their system, primary ECs are maintained without serum- and in growth factor-free medium, thus allowing direct effects of ECs on HSCs to be observed [60]. Using this coculture system, they showed that Akt activation in ECs induces expansion of HSCs through upregulation of specific factors for HSCs, such as FGF2, BMP4, Ang1, and DHH and concomitant downregulation of inhibitory factors, such as Ang2 and DKK1. Conversely, when ECs are activated with MAPK by constitutively active c-raf expression, production of differentiation factors such as Ang2 or IL6, was upregulated. Along the same lines as this experiment, we also previously reported that osteoblast cells alter the differentiation or proliferation of HSCs, via factors produced following their stimulation [61]. In our experiment, we used OP9 stromal cells as osteoblastic cells and stimulated them with bFGF or EGF in a chemically-defined serum-free medium. When BM HSCs were cocultured with OP9 stimulated with bFGF, they immediately differentiated, but in the case of EGF, immature haematopoietic progenitors as well as HSCs proliferated long-term. To exclude direct effects of EGF on HSCs, we transduced constitutively active EGFR (v-erbB2) into OP9 cells and confirmed the expansion of HSCs without exogenous EGF. Taken together, these data indicate that self-renewal/differentiation of HSCs is regulated by exogenous molecular cues affecting niche cells, and raise the possibility that differentiation or self-renewal of HSCs occurs in both the osteoblastic and vascular niches. However, the identity of the molecules altering cell growth in both vascular and osteoblast niches *in vivo* requires clarification, as does the role of basement membrane proteins [62].

Vascular niches for stem cells have been reported in other normal organs and the concept also extended to cancer tissues. It has been suggested that neural progenitor/stem cells proliferate within an angiogenic microenvironment [63] in the brain and that a similar microenvironment for brain tumour stem cells is observed in the microvascular proliferating zone (also the so-called perivascular niche) [64]. In our work, we also reported the localisation of cancer stem-like cells in the vascular region, especially at the tumour rim [65]. Importantly, these putative cancer stem cells were located beside mature blood vessels in which ECs are fully covered with mural cells. At present, the maximum dose of angiogenesis inhibitors that can be used in cancer patients is restricted by the need to prevent vascular damage in normal organs. The immature blood vessels frequently observed in the core region of tumours may be responsive to low doses of angiogenesis inhibitors. However, blood vessels in the niches used by cancer stem cells are mature, implying that such vascular niches cannot be destroyed by low-dose drug, and hence the rim of the tumour containing the cancer stem cells would not be affected.

It has been suggested that various angiocrine factors produced by ECs may support stemness in the vascular niche, that is, BMP2, BMP4, FGF2, TGFβ etc. [66]. Most such factors may be used as stemness factors in both normal organs and tumours. In the context of tissue regeneration, enhancement of stemness factor availability may be beneficial for effective tissue repair. However, blocking these factors for the suppression of tumour growth may affect maintenance of normal organs. When considering tissue damage to stem cell systems of normal organs, it will be important to destroy the vascular niche composed of mature blood vessels in the tumour to inhibit proliferation of cancer stem cells, but to leave normal organs intact. For this purpose, the identification of specific antigens expressed on mature blood vessels in the tumour but not in normal organs is essential. By targeting such antigens, destruction of mature blood vessels in the tumour but not in normal organs can be achieved by the development of novel drug delivery systems.

Acknowledgements

We thank K. Fukuhara and N. Fujimoto for technical assistance. This work was supported by a grant from the Ministry of Education, Science, Sports, and Culture of Japan.

Disclosure of Conflict of Interests

The author states that he has no conflict of interests.

References

1 Risau W. Mechanisms of angiogenesis. *Nature* 1997; **386**: 671–4.
2 Folkman J, D'Amore PA. Blood vessel formation: what is its molecular basis? *Cell* 1996; **87**: 1153–5.
3 Hanahan D. Signaling vascular morphogenesis and maintenance. *Science* 1997; **277**: 48–50.
4 Wang HU, Chen Z-F, Anderson DJ. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* 1998; **93**: 741–53.
5 Gale NW, Yancopoulos GD. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev* 1999; **13**: 1055–66.
6 Shalaby F, Rossant J, Tamaguchi TP, Gertsenstein M, Wu X-F, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 1995; **376**: 62–6.
7 Fong G-H, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 1995; **376**: 66–70.
8 Dumont DJ, Jussila L, Taipale J, Lymboussaki T, Mustonen T, Pajusola K, Breitman M, Alitalo K. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* 1998; **282**: 946–9.
9 Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996; **380**: 439–42.
10 Dumont DJ, Fong G-H, Puri MC, Gradwohl G, Alitalo K, Breitman ML. Vascularization of the mouse embryo: a study of *flk-1*, *tek*, *tie*, and vascular endothelial growth factor expression during development. *Dev Dyn* 1995; **203**: 80–92.
11 Dumont DJ, Gradwohl G, Fong G-H, Puri MC, Gerstenstein M, Auerbach A, Breitman ML. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev* 1994; **8**: 1897–909.
12 Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J. The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO J* 1995; **14**: 5884–91.
13 Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 1995; **376**: 70–4.
14 Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* 1996; **87**: 1161–9.
15 Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD. Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. *Science* 1997; **277**: 55–61.
16 Takakura N, Huang X-L, Naruse T, Hamaguchi I, Dumont DJ, Yancopoulos GD, Suda T. Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. *Immunity* 1998; **9**: 677–86.
17 Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD. Requisite role of angiopoietin-1, a ligand for the Tie-2 receptor, during embryonic angiogenesis. *Cell* 1996; **87**: 1171–80.
18 Hellström M, Kalén M, Lindahl P, Abramsson A, Betsholtz C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* 1999; **126**: 3047–55.
19 Lindblom P, Gerhardt H, Liebner S, Abramsson A, Enge M, Hellstrom M, Backstrom G, Fredriksson S, Landegren U, Nystrom HC, Bergstrom G, Dejana E, Ostman A, Lindahl P, Betsholtz C. Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. *Genes Dev* 2003; **17**: 1835–40.
20 Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y. Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. *J Biol Chem* 1999; **274**: 15732–9.
21 Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makuuchi M, Hirata Y, Nagai R. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med* 2002; **8**: 403–9.
22 Orkin SH, Zon LI. Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. *Nat Immunol* 2002; **3**: 323–8.
23 Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 2003; **422**: 897–901.
24 Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 2003; **425**: 968–73.
25 Yamada Y, Takakura N. Physiological pathway of differentiation of hematopoietic stem cell population into mural cells. *J Exp Med* 2006; **203**: 1055–65.
26 Medici D, Shore EM, Lounev VY, Kaplan FS, Kalluri R, Olsen BR. Conversion of vascular endothelial cells into multipotent stem-like cells. *Nat Med* 2010; **16**: 1400–6.
27 Crisan M, Yap S, Casteilla L, Chen C, Corselli M, Park TS, Andriolo G, Sun B, Zheng B, Zhang L, Norotte C, Teng P, Traas J, Schugar R, Deasy BM, Badylak S, Buhring H, Giacobino J, Lazzari L, Huard J, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008; **3**: 301–13.

28 Eilken HM, Nishikawa S, Schroeder T. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature* 2009; **457**: 896–900.

29 Red-Horse K, Crawford Y, Shojaei F, Ferrara N. Endothelium–microenvironment interactions in the developing embryo and in the adult. *Dev Cell* 2007; **12**: 181–94.

30 Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275**: 964–7.

31 Purhonen S, Palm J, Rossi D, Kaskenpää N, Rajantie I, Ylä-Herttuala S, Alitalo K, Weissman IL, Salven P. Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. *Proc Natl Acad Sci U S A* 2008; **105**: 6620–5.

32 Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004; **25**: 581–611.

33 Takakura N, Watanabe T, Suenobu S, Yamada Y, Noda T, Ito Y, Satake M, Suda T. A role for hematopoietic stem cells in promoting angiogenesis. *Cell* 2000; **102**: 199–209.

34 Fukuhara S, Sako K, Minami T, Noda K, Kim HZ, Kodama T, Shibuya M, Takakura N, Koh GY, Mochizuki N. Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1. *Nat Cell Biol* 2008; **10**: 513–26.

35 Bellocq A, Antoine M, Flahault A, Philippe C, Crestani B, Bernaudin JF, Mayaud C, Milleron B, Baud L, Cadranel J. Neutrophil alveolitis in bronchioloalveolar carcinoma: induction by tumor derived interleukin-8 and relation to clinical outcome. *Am J Pathol* 1998; **152**: 83–92.

36 Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 2000; **103**: 481–90.

37 Cassatella MA. Neutrophil-derived proteins: selling cytokines by the pound. *Adv Immunol* 1999; **73**: 369–509.

38 McCourt M, Wang JH, Sookhai S, Redmond HP. Proinflammatory mediators stimulate neutrophildirected angiogenesis. *Arch Surg* 1999; **134**: 1325–31.

39 Queen MM, Ryan RE, Holzer RG, Keller-Peck CR, Jorcyk CL. Breast cancer cells stimulate neutrophils to produce oncostatin M: potential implications for tumor progression. *Cancer Res* 2005; **65**: 8896–904.

40 Lin EY, Pollard JW. Role of infiltrated leucocytes in tumour growth and spread. *Br J Cancer* 2004; **90**: 2053–8.

41 Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 2003; **3**: 401–10.

42 Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 2005; **7**: 211–7.

43 De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M, Naldini L. Tie-2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* 2005; **8**: 211–26.

44 Ribatti D, Vacca A, Nico B, Crivellato E, Roncali L, Dammacco F. The role of mast cells in tumour angiogenesis. *Br J Haematol* 2001; **115**: 514–21.

45 Theoharides TC, Kempuraj D, Tagen M, Conti P, Kalogeromitros D. Differential release of mast cell mediators and the pathogenesis of inflammation. *Immunol Rev* 2007; **217**: 65–78.

46 Gabrilovich DI, Bronte V, Chen SH, Colombo MP, Ochoa A, Ostrand-Rosenberg S, Schreiber H. The terminology issue for myeloid-derived suppressor cells. *Cancer Res* 2007; **67**: 425.

47 Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, Carbone DP, Lin PC. Expansion of myeloid immune suppressor Gr^+CD11b^+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer Cell* 2004; **6**: 409–21.

48 Puxeddu I, Alian A, Piliponsky AM, Ribatti D, Panet A, Levi-Schaffer F. Human peripheral blood eosinophils induce angiogenesis. *Int J Biochem Cell Biol* 2005; **37**: 628–36.

49 Curiel TJ, Cheng P, Mottram P, Alvarez X, Moons L, Evdemon-Hogan M, Wei S, Zou L, Kryczek I, Hoyle G, Lackner A, Carmeliet P, Zou W. Dendritic cell subsets differentially regulate angiogenesis in human ovarian cancer. *Cancer Res* 2004; **64**: 5535–8.

50 Scimone ML, Lutzky VP, Zittermann SI, Maffia P, Jancic C, Buzzola F, Issekutz AC, Chuluyan HE. Migration of polymorphonuclear leucocytes is influenced by dendritic cells. *Immunology* 2005; **114**: 375–85.

51 Conejo-Garcia JR, Buckanovich RJ, Benencia F, Courreges MC, Rubin SC, Carroll RG, Coukos G. Vascular leukocytes contribute to tumor vascularization. *Blood* 2005; **105**: 679–81.

52 Conejo-Garcia JR, Benencia F, Courreges MC, Kang E, Mohamed-Hadley A, Buckanovich RJ, Holtz DO, Jenkins A, Na H, Zhang L, Wagner DS, Katsaros D, Caroll R, Coukos G. Tumor-infiltrating dendritic cell precursors recruited by a b-defensin contribute to vasculogenesis under the influence of VEGF-A. *Nat Med* 2004; **10**: 950–8.

53 Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: stem cells and their niche. *Cell* 2004; **116**: 769–78.

54 Ottersbach K, Dzierzak E. The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev Cell* 2005; **8**: 377–87.

55 Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li L. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 2003; **425**: 836–41.

56 Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, Ito K, Koh GY, Suda T. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* 2004; **118**: 149–61.

57 Kiel MJ, Morrison SJ. Maintaining hematopoietic stem cells in the vascular niche. *Immunity* 2006; **25**: 862–4.

58 Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 2006; **25**: 977–88.

59 Li W, Johnson SA, Shelley WC, Ferkowicz M, Morrison P, Li Y, Yoder MC. Primary endothelial cells isolated from the yolk sac and para-aortic splanchnopleura support the expansion of adult marrow stem cells *in vitro*. *Blood* 2003; **102**: 4345–53.

60 Kobayashi H, Butler JM, O'Donnell R, Kobayashi M, Ding BS, Bonner B, Chiu VK, Nolan DJ, Shido K, Benjamin L, Rafii S. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol* 2010; **12**: 1046–56.

61 Takakura N, Kodama H, Nishikawa S, Nishikawa S. Preferential proliferation of murine colony-forming units in culture in a chemically defined condition with a macrophage colony-stimulating factor-negative stromal cell clone. *J Exp Med* 1996; **184**: 2301–9.

62 Nikolova G, Strilic B, Lammert E. The vascular niche and its basement membrane. *Trends Cell Biol* 2007; **17**: 19–25.

63 Palmer TD, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol* 2000; **425**: 479–94.

64 Calabrese C, Poppleton H, Kocak M, Hogg TL, Fuller C, Hamner B, Oh EY, Gaber MW, Finklestein D, Allen M, Frank A, Bayazitov IT, Zakharenko SS, Gajjar A, Davidoff A, Gilbertson RJ. A perivascular niche for brain tumor stem cells. *Cancer Cell* 2007; **11**: 69–82.

65 Nagahama Y, Ueno M, Miyamoto S, Morii E, Minami T, Mochizuki N, Saya H, Takakura N. PSF1, a DNA replication factor expressed widely in stem and progenitor cells, drives tumorigenic and metastatic properties. *Cancer Res* 2010; **70**: 1215–24.

66 Butler JM, Kobayashi H, Rafii S. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nat Rev Cancer* 2010; **10**: 138–46.

The American Journal of Pathology, Vol. 179, No. 6, December 2011

DOI: 10.1016/j.ajpath.2011.08.024

Short Communication

Apelin Attenuates UVB-Induced Edema and Inflammation by Promoting Vessel Function

Mika Sawane,* Hiroyasu Kidoya,† Fumitaka Muramatsu,† Nobuyuki Takakura,† and Kentaro Kajiya*

From the Shiseido Innovative Science Research Center, Yokohama; and the Department of Signal Transduction,† Research Institute of Microbial Diseases, Osaka University, Suita, Osaka, Japan*

Apelin, the ligand of the G protein–coupled receptor APJ, is involved in the regulation of cardiovascular functions, fluid homeostasis, and vessel formation. Recent reports indicate that apelin secreted from endothelial cells mediates APJ regulation of blood vessel caliber size; however, the function of apelin in lymphatic vessels is unclear. Here we report that APJ was expressed by human lymphatic endothelial cells and that apelin induced migration and cord formation of lymphatic endothelial cells dose-dependently *in vitro*. Furthermore, permeability assays demonstrated that apelin stabilizes lymphatic endothelial cells. *In vivo*, transgenic mice harboring apelin under the control of keratin 14 (K14-apelin) exhibited attenuated UVB-induced edema and a decreased number of CD11b-positive macrophages. Moreover, activation of apelin/APJ signaling inhibited UVB-induced enlargement of lymphatic and blood vessels. Finally, K14-apelin mice blocked the hyperpermeability of lymphatic vessels in inflamed skin. These results indicate that apelin plays a functional role in the stabilization of lymphatic vessels in inflamed tissues and that apelin might be a suitable target for prevention of UVB-induced inflammation. *(Am J Pathol 2011, 179:2691–2697; DOI: 10.1016/j.ajpath.2011.08.024)*

The lymphatic vascular system is composed of a dense network of thin-walled capillaries that drain protein-rich lymph from the extracellular space; its function is important for homeostasis of the circulatory and immune systems, maintenance of interstitial fluid composition and volume, and immune cell trafficking in health and in disease.[1,2] Chronic skin inflammation in mice has been associated with lymphatic endothelial cell (LEC) proliferation, and the skin disease psoriasis exhibited pronounced cutaneous lymphatic hyperplasia,[3] indicating that the lymphatic vascular system participates in both acute and chronic inflammation.

Acute exposure of skin to UVB irradiation (290 to 320 nm) leads to inflammation associated with epidermal hyperplasia, erythema, vascular hyperpermeability, and edema formation.[4,5] Previous studies have demonstrated that acute UVB irradiation of both human and mouse skin promotes marked angiogenesis.[6,7] Several angiogenesis factors, including vascular endothelial growth factor-A (VEGF-A), basic fibroblast growth factor, and interleukin-8, were up-regulated in skin after UVB-irradiation.[7–9] Thrombospondin-1, a potent endogenous angiogenesis inhibitor, was up-regulated.[7] Moreover, targeted overexpression of VEGF-A enhanced sensitivity to UVB-induced cutaneous photodamage,[10] but transgenic overexpression of thrombospondin-1 in the epidermis completely prevented UVB-induced photodamage.[11] Taken together, these findings indicate that the cutaneous blood vasculature plays an important role in the mediation of photodamage. A previous study from our research group demonstrated that UVB irradiation caused enlargement of lymphatic vessels with leaky and hyperpermeable function.[12] More recently, we found that activation of the VEGF-C/VEGFR-3 pathway attenuates UVB-induced inflammation by promoting lymphangiogenesis.[13] These studies point to a crucial role of lymphatic vessels in UVB-induced inflammation.

Apelin is an endogenous ligand for the previously orphan G protein–coupled receptor, APJ. The apelin gene (*APLN*), which is located on the long arm of the human X chromosome, encodes a 77-amino-acid preproprotein that is then cleaved to shorter active peptides.[14,15] The full-length mature peptide, which was originally isolated from bovine stomach extracts, comprises 36 amino acids and is known as apelin-36; the short-length peptide is known as apelin-13. Both peptides activate APJ.[16] APJ

Accepted for publication August 31, 2011.

M.S. and H.K. contributed equally to the present work.

Address reprint requests to Kentaro Kajiya, Ph.D., Shiseido Innovative Science Research Center, 2-12-1 Fukuura Kanazawa-ku, Yokohama 236-8643, Japan. E-mail: kentaro.kajiya@to.shiseido.co.jp.

expression has been reported in the cardiovascular system and in the central nervous system.[17,18] In the brain, the apelin/APJ system plays a role in maintaining body fluid homeostasis and regulating release of vasopressin from the hypothalamus.[19] In the cardiovascular system, APJ is expressed in endothelial cells, vascular smooth muscle cells, and cardiomyocytes.[20,21] Apelin/APJ in cells of endothelial lineage promotes hypotensive activity[22]; the activation of APJ leads to nitric oxide (NO) production by the endothelial cells,[23] and this possibly plays a role in the relaxation of smooth muscle cells.

Apelin is also essential for blood vessel formation. The apelin/APJ system plays a role in the cardiovascular system of *Xenopus laevis*[24] and of zebrafish.[25] *Xenopus* apelin (Xapelin) is expressed in the region around the presumptive blood vessels during early embryogenesis as *Xenopus* APJ (Xmsr). Knockdown of Xapelin or Xmsr resulted in a defect of blood vessel formation in the posterior cardinal vein, intersomitic vessels, and vitelline vessels. The regulation of blood vessel formation by apelin in mammals has been described recently. The Apelin/APJ system was shown to be involved in downstream signaling of Ang1/Tie2 in endothelial cells and in regulation of blood vessel diameter during angiogenesis.[26] However, the function of apelin in lymphatic vessels and its role in inflammation is not completely clear.

In the present study, we found that the APJ receptor is expressed in lymphatic endothelial cells *in vitro* and *in vivo*, and that apelin/APJ signaling promotes stabilization of lymphatic vessels. Moreover, using apelin transgenic mice, we demonstrated that apelin attenuates UVB-induced inflammation by promoting stabilization of lymphatic and blood vessels. These results suggest that apelin might be a suitable target for prevention of UVB-induced skin inflammation and photodamage.

Materials and Methods

Cells

Human dermal LECs were isolated from neonatal human foreskins by immunomagnetic purification, as described previously.[27] The lineage-specific differentiation was confirmed by real-time RT-PCR for the lymphatic vascular markers Prox1, LYVE-1, and podoplanin, as well as by immunostaining for Prox1 and podoplanin, as described previously.[28] Human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell (Heidelberg, Germany). Cells were cultured in endothelial basal medium (Lonza, Verviers, Belgium) supplemented with supplements provided by the suppliers for up to 11 passages.

Immunoblotting

For Western blot analyses of APJ, Akt, and p-Akt, confluent LECs and HUVECs were homogenized in lysis buffer, and protein concentrations were determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of lysates (10 μg protein) were immunoblotted with a rabbit polyclonal antibody against APJ, as described previously.[26] LECs were also cultured with apelin-36 (1000 ng/mL; Peptide Institute, Osaka, Japan) for 2 minutes, followed by homogenization in lysis buffer. Untreated cells were prepared as controls in the same manner. Cell lysates (100 μg total protein each) were immunoprecipitated with antibodies against p-Akt and Akt (Cell Signaling Technology, Danvers, MA). Equal loading was confirmed with an antibody against β-actin (Sigma-Aldrich, St. Louis, MO).

Migration and Cord Formation Assays

The LEC migration assay was performed as described previously,[29] using 24-well FluoroBlock inserts of 8-μm pore size (Falcon; BD Biosciences, Franklin Lakes, NJ). The bottom sides of the inserts were coated with 10 μg/mL fibronectin (BD Biosciences, Bedford, MA) for 1 hour, followed by incubation with 100 μg/mL of bovine serum albumin. Cells (10^5 cells in 100 μL) were seeded in serum-free endothelial basal medium into the upper chambers, and were incubated for 5 hours at 37°C in the presence or absence of human recombinant apelin-13 (500 to 1000 ng/mL) or apelin-36 (500 to 1000 ng/mL). Cells on the underside of inserts were stained with Hoechst dye 33342 (Molecular Probes; Invitrogen, Carlsbad, CA). Five different digital images were captured per well, and the number of migrated cells was counted. Cord formation assays were performed as described previously.[30] LECs were grown on fibronectin-coated 24-well plates until confluence. In all, 0.5 mL of neutralized isotonic bovine dermal collagen type I (Vitrogen; Celtrix Laboratories, Palo Alto, CA) in the presence or absence of apelin-13 (50 to 1000 ng/mL) or apelin-36 (50 to 1000 ng/mL) was added to the cells. After incubation at 37°C for 24 hours, cells were fixed with 4% paraformaldehyde for 30 minutes at 4°C. Before lymphatic endothelial cells form tubes in collagen gels, endothelial cells connect with each other to make cords *in vitro*. Representative images were captured, and the total length of cordlike structures per area was measured using IP-LAB software version 4.0. All studies were performed in triplicate. Statistical analyses were performed using the unpaired Student's *t*-test.

Permeability Assay

LECs were grown into confluence on the fibronectin-coated surface of tissue culture inserts of 0.4-μm pore size (Transwell; Corning, Lowell, MA) and then in serum-free endothelial basal medium for 24 hours. Apelin-13 (500 to 1000 ng/mL) was placed into the upper and lower chambers for 6 hours. Fluorescein isothiocyanate-dextran was added to the upper chambers, and the apparatus was then placed in a CO_2 incubator at 37°C. After incubation for 15 minutes, a 100-μL sample was taken from the lower chamber, and the absorbance of fluorescein isothiocyanate-dextran was determined at 492 nm using a spectrophotometer (Fluoroskan Ascent; Thermo Fisher Scientific, Waltham, MA).

UVB Irradiation Regimen

Transgenic mice harboring apelin under the control of keratin 14 (K14-apelin) were generated on a C57BL/6 background, as described previously.[31] A total of 10 K14-apelin mice and wild-type (WT) mice, 12 weeks old (n = 5/group) were exposed to a single dose of 200 mJ/cm^2 of UVB irradiation using 10 Toshiba FL-20 SD fluorescent lamps that deliver energy in the UVB wavelength range (280 to 340 nm) with maximum energy at a wavelength of 305 nm. On day 3 or 4 after UVB irradiation, mouse ears were collected and were processed for histological analysis of frozen sections. Control mice without UVB irradiation were also analyzed. All procedures including UVB irradiation were performed under anesthesia. The study was approved by the ethics committee of Shiseido Research Center in accordance with guidelines of the U.S. National Institutes of Health (7th edition).

Intravital Lymphangiography and Plasma Extravasation

WT and K14-apelin mice (n = 5/group) were anesthetized with avertin (0.4 g/kg; Sigma-Aldrich), and 1 mL of a 1% solution of Evans Blue dye in 0.9% NaCl was injected intradermally at the inner surface of the rim of the ear, using a 10-mL Hamilton syringe, to visualize the lymphatic vessels. Mouse ears were photographed at 1 and 5 minutes after dye injection. To determine blood vascular permeability, a Miles assay was performed as described previously.[31] Briefly, mice were anesthetized and intravenously injected with 100 μL of a 1% solution of Evans Blue dye in 0.9% NaCl. At 60 minutes after dye injection, ears were photographed and then removed. The dye was eluted from the dissected samples with formamide at 56°C, and the optical density was measured by spectrophotometry (Biotrak II; GE Healthcare, Piscataway, NJ) at 620 nm.

Immunostaining and Computer-Assisted Morphometric Vessel Analysis

Immunofluorescence analysis was performed on cryostat sections (6 μm thick) of mouse ears using antibodies against the macrophage monocyte marker CD11b (Pharmingen; BD Biosciences, San Diego, CA), the blood vessel-specific marker Meca-32 (BD Biosciences), and the lymphatic-specific marker podoplanin (Acris Antibodies, Hiddenhausen, Germany) and using corresponding secondary antibodies labeled with Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes; Invitrogen). Routine H&E staining was also performed. Sections were examined with an Olympus AX80T microscope (Olympus, Tokyo, Japan), and images were captured with a DP controller digital camera (DP71; Olympus). Morphometric analyses were performed using IP-LAB software version 4.0, as described previously.[28] Three different fields of each section were examined, and ear thickness and the number of macrophages and average vessel size in the dermis were determined. Statistical analyses were performed using the unpaired Student's t-test.

Results

Apelin Is Expressed by Lymphatic Vessels both in Vitro *and* in Vivo *and Promotes Lymphatic Function*

To investigate whether apelin functions in lymphatic endothelial cells, we analyzed the expression of apelin receptor APJ in LECs. Western blot analyses demonstrated that APJ was expressed by both LECs and HUVECs (Figure 1A). Moreover, immunofluorescence analysis of mouse ear skin using antibodies against APJ and the blood vessel marker Meca-32 or the lymphatic marker podoplanin revealed that APJ was expressed by both lymphatic vessels (Figure 1C) and blood vessels (Figure 1B) *in vivo*. Apelin is known to activate the phosphorylation of Akt in HUVECs.[32] Treatment of LECs with 1000 ng/mL apelin-36 resulted in the increased phosphorylation of Akt, compared with untreated cells (Figure 1D). Migration assays performed to further characterize the effects of apelin on LEC revealed that both apelin-13 and apelin-36 induced LEC migration in a dose-dependent manner (Figure 1, E and F). To investigate whether apelin stimulation might promote cord formation of lymphatic endothelial cells *in vitro*, confluent LECs were overlaid with type I collagen. Cord formation of LECs was clearly enhanced in the presence of apelin-13 and apelin-36, compared with control cells (Figure 1, G–I). Morphometric analyses confirmed that both apelin-13 and apelin-36 induced cord formation of LECs dose-dependently ($P <$ 0.01) (Figure 1, J and K). A permeability assay was performed to determine whether apelin contributes to the stabilization of LECs *in vitro*. LECs were cultured on Transwell culture inserts into confluence, and the concentration of fluorescein isothiocyanate-dextran that permeated across the culture inserts was measured with or without apelin-13. The addition of 500 or 1000 ng/mL of apelin-13 in LECs decreased the fluorescence intensity of permeated fluorescein isothiocyanate-dextran, indicating that apelin promoted the stabilization of LECs ($P <$ 0.01) (Figure 1L).

Apelin Enhances Recovery from UVB-Induced Edema Formation and Inflammation

To determine the functional role of apelin in the cutaneous vasculature *in vivo*, transgenic mice harboring apelin under the control of keratin-14 (K14-apelin) and WT control mice were exposed to 200 mJ cm^{-2} of UVB irradiation. H&E staining of skin sections at 3 days after UVB irradiation revealed characteristic features of acute photodamage in the ear skin of WT mice, including epidermal hyperplasia and edema formation in the dermis (Figure 2C). Of note, K14-apelin mouse ears irradiated with UVB were closely similar to those of non-UVB-irradiated skin (Figure 2, A, B, and D). In a physiological condition, by contrast, no obvious difference was found between WT

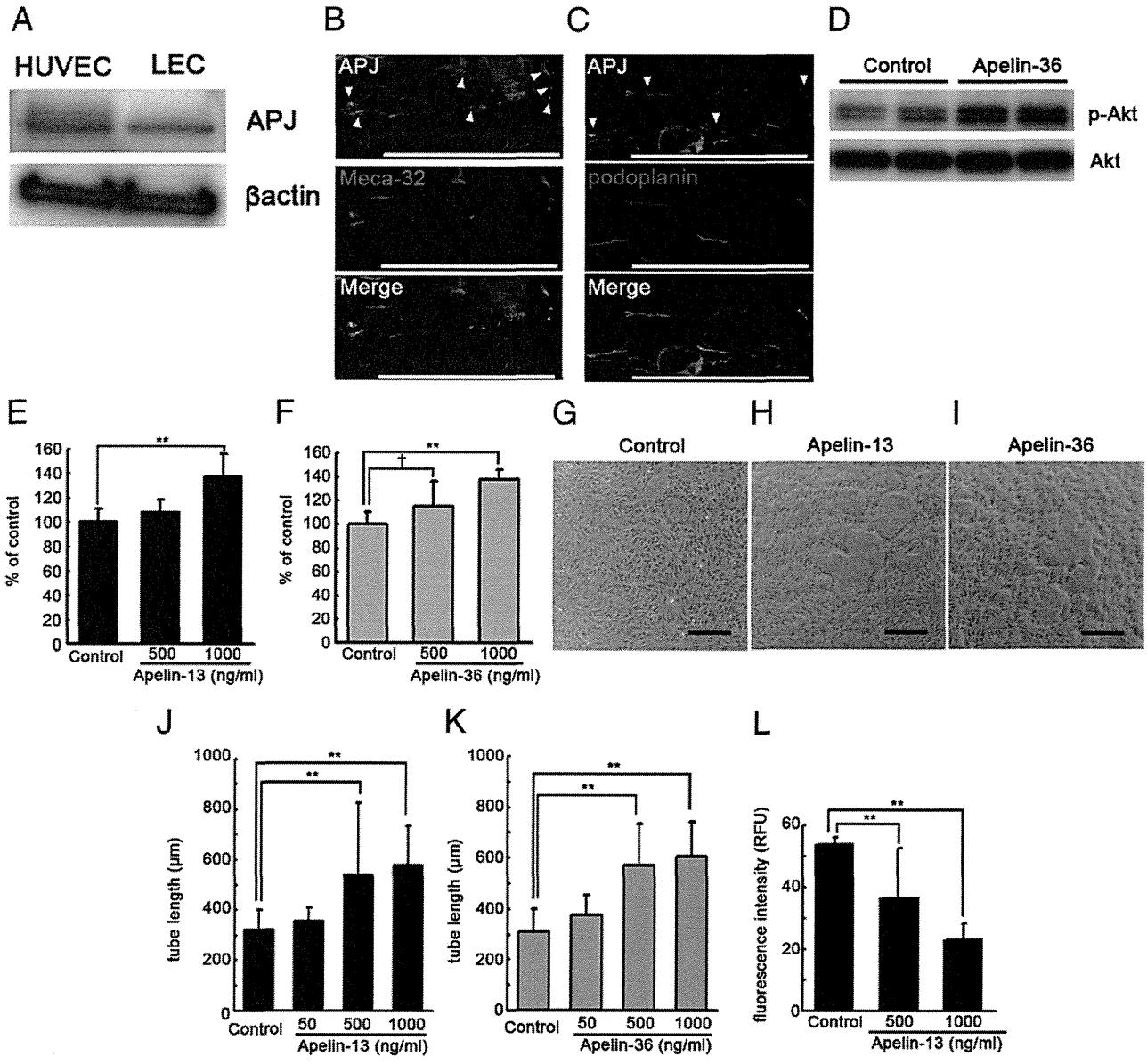

Figure 1. Apelin promotes formation and stabilization of lymphatic endothelial cells (LECs) *in vitro*. **A:** Immunoblot analyses confirmed that the apelin receptor APJ was expressed by both LECs and HUVECs. **B** and **C:** Double immunofluorescence analyses for APJ (green) and blood vessel marker Meca-32 (red, **B**) or lymphatic vessel marker podoplanin (red, **C**) revealed that APJ was expressed by both blood vessels and lymphatic vessels *in vivo*. **Arrowheads** show APJ expression in blood vessels (**B**) and lymphatic vessels (**C**). **D:** Treatment of LECs with 1000 ng/mL apelin-36 resulted in increased phosphorylation of Akt, compared with untreated cells. **E** and **F:** Apelin-13 (**E**) and apelin-36 (**F**) induced migration of LECs in a dose-dependent manner, compared with untreated control cells. **G–I:** Incubation of LECs with 500 ng/mL or 1000 ng/mL apelin-13 (**H** and **J**) and apelin-36 (**I** and **K**) enhanced cord formation in a dose-dependent manner after overlay with a type 1 collagen gel, compared with controls (**G**). Scale bars: 100 μm. **L:** The addition of 500 or 1000 ng/mL of apelin-13 decreased the fluorescence intensity of permeated fluorescein isothiocyanate-dextran from LEC, compared with controls. Data are expressed as means ± SD. $^{**}P < 0.01$; $^{\dagger}P < 0.1$.

and K14-apelin mice. The measurement of skin thickness confirmed that ear swelling was decreased in K14-apelin mouse ears, compared with WT mouse ears, after UVB irradiation ($P < 0.05$) (Figure 2I). Immunohistochemical staining for a monocyte macrophage marker, CD11b, demonstrated an increased number of infiltrating macrophages in the dermis of WT ears after UVB irradiation, compared with non-UVB-irradiated skin (Figure 2, E–G); however, the ear skin of UVB-irradiated K14-apelin mice exhibited decreased macrophage infiltration in the dermis (Figure 2H). Morphometric analyses confirmed a decreased number of infiltrating macrophages in the dermis of UVB-irradiated K14-apelin mouse ears, compared with WT mice after UVB-irradiation ($P < 0.01$) (Figure 2J).

Activation of Apelin/APJ Pathway Inhibits UVB-Induced Inflammation by Blocking Abnormal Enlargement of Lymphatic Vessels and Blood Vessels

To investigate how activation of apelin/APJ signaling attenuates edema formation and inflammation induced