Regulation of lymphatic-blood vessel separation by endothelial Rac1


There was an error published in the supplementary material for Development 136, 4043-4053.

In the legends for Figs S1, S2, S4, S5, S6 and S9, Rac\textsuperscript{fl/fl} was incorrectly stated as Rac\textsuperscript{+}. 

In the title of Fig. S5, Vegfr3 was incorrectly stated as VR3.

The supplementary figure legends have now been corrected.

We apologise to authors and readers for this mistake.
Regulation of lymphatic-blood vessel separation by endothelial Rac1

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Sprouting angiogenesis and lymphatic-blood vessel segregation both involve the migration of endothelial cells, but the precise migratory molecules that govern the decision of blood vascular endothelial cells to segregate into lymphatic vasculature are unknown. Here, we deleted endothelial Rac1 in mice (Tie1-Cre;Rac1+/-) and revealed, unexpectedly, that whereas blood vessel morphology appeared normal, lymphatic-blood vessel separation was impaired, with corresponding edema, haemorrhage and embryonic lethality. Importantly, normal levels of Rac1 were essential for directed endothelial cell migratory responses to lymphatic-inductive signals. Our studies identify Rac1 as a crucial part of the migratory machinery required for endothelial cells to separate and form lymphatic vasculature.

KEY WORDS: Rac1 conditional knockout, Lymphangiogenesis, Vegfr3 (Flt4), Tie1-Cre, Blood-filled lymphatics

INTRODUCTION

Sprouting angiogenesis in the developing mouse embryo involves endothelial cell activation and migration to form vascular tubes and occurs after embryonic day (E) 8.5, whereas early heart development and vasculogenesis occur before this stage (Risau, 1997; Risau and Flamme, 1995). By E11.5, endothelial progenitors, stimulated by vascular endothelial growth factor C (Vegf-C), migrate away from the cardinal vein and begin to form the lymphatic vascular system (Alitalo et al., 2002; Karkkainen et al., 2004; Oliver and Altalto, 2005). Thus, endothelial cell migration is an essential process for both angiogenesis and lymphangiogenesis, but the endothelial migratory machinery that determines the precise coordination of these processes is unknown.

Rho GTPases play key roles in coordinating the cellular responses required for cell migration. In particular, Rac1 is considered essential for endothelial cell migration in vitro (Garrett et al., 2007; Nobes and Hall, 1999; Ridley et al., 2003; Soga et al., 2001; Zeng et al., 2002), and for lumen and tube formation in vitro (Bayless and Davis, 2002; Cascone et al., 2003; Connolly et al., 2002; Davis and Bayless, 2003; Koh et al., 2008). The constitutive ablation of Rac1 results in embryonic lethality owing to defects during gastrulation (Sugihara et al., 1998). Nevertheless, theloxP-Cre-based conditional gene-targeting approach has been extensively applied to delete Rac1 in a tissue- and cell-type-specific manner (Wang and Zheng, 2007).

Both Tie1-Cre and Tie2 (Tek)-Cre transgenic mice show Cre recombinase activity in the developing endothelial cells of embryos (Gustafsson et al., 2001; Ilijin et al., 2002; Kisanuki et al., 2001; Schlaeger et al., 1997). Although early, tissue-specific deletion of Rac1 in Tie2-Cre mice has revealed a role for this molecule in heart development and early vasculogenesis (Tan et al., 2008), because these embryos display an arrested phenotype at E8.5 and begin to die at E9.5 an examination of the role of Rac1 in developmental sprouting angiogenesis and lymphangiogenesis was not possible.

Here, we show by deleting Rac1 in an alternative Cre-expressing model, Tie1-Cre mice, that embryo survival is increased, such that the role of Rac1 in both sprouting angiogenesis and lymphangiogenesis could be investigated. We demonstrate that when endothelial Rac1 is deleted, blood vessels appear normal, but we reveal a previously unknown role for this Rho GTPase in regulating lymphatic-blood vessel separation during embryogenesis.

MATERIALS AND METHODS

Mice

Heterozygous (Rac1+/−) and homozygous (Rac1−/−) floxed mice (Walsmley et al., 2003) were intercrossed with Tie1-Cre transgenic mice (Gustafsson et al., 2001) (provided by Prof. R. Fässler, Max-Planck Institute of Biochemistry, Germany) to generate Tie1-Cre;Rac1−/− mice with conditional deletion of Rac1 in endothelial cells. Tie1-Cre;Rac1−/− and Tie1-Cre;Rac1−/− were used as controls and no differences were observed between them (data not shown). The developmental stage of mouse embryos was determined by considering 10.00 h on the day of the vaginal plug as E0.5. For Rac1 PCR genotyping, the following primers were used: forward primer 1, 5′-ATTTTGTGCCAAGGACAGTGACAAGCT-3′; forward primer 2, 5′-GAAGGAGAAGAAGCTGACTCCCATC-3′ and reverse primer 3, 5′-CACCCACAGGCCAATGAGTGTTC-3′. Products are 300 bp (endogenous Rac1 locus), 328 bp (Rac1 CRISPR locus: flox allele) and 175 bp (Cre-excised Rac1 locus: null allele). PCR analysis for Tie1-Cre transgenesis was performed in parallel. All procedures on mice were in accordance with United Kingdom Home Office regulations.

Antibodies and immunohistochemical analysis

Antibodies used were: rabbit anti-mouse Lyve1 (gift from Prof. K. Alitalo, Biomedicum Helsinki, Finland), Syrian Hamster anti-mouse podoplanin (Acris), rabbit anti-mouse Proxl (Abcam), rat anti-mouse endomucin (gift...
from Prof. D. Vestweber, Max Planck Institute of Molecular Biomedicine, Germany), rabbit anti-laminin (Sigma), Cy3-conjugated mouse anti- 
smooth muscle actin (α-SMA; Acta2 – Mouse Genome Informatics; Sigma), rat anti-mouse Ki67 (Dako) and mouse anti-Rac1 (clone 23A8; 
Upstate Biotechnology). For Rac1 immunostaining, embryos were snap-

frozen and 5 µm sections were processed as described (Benitah et al., 2005). 
For all other immunostaining, embryos were paraffin-embedded and 5 µm 
sections were treated with sodium citrate buffer (pH 6.0) or trypsin retrieval 
solutions. Fluorescent or 3,3-diaminobenzidine (DAB, Sigma)- 
chromogenic detections were carried out using fluorochrome-conjugated (Molecular Probes) or biotin-conjugated (Vector Laboratories) secondary antibodies, respectively. For DAB detection, the ABC Vectastain Elite 
Peroxidase-based Kit was also used (Vector Laboratories), and sections were 
counterstained with hematoxylin, cleared and mounted in Permount (Sigma). Fluorescently-stained sections were incubated with DAPI 
(Invitrongen) and mounted with Gelvatol (Calbiochem) containing anti-fade 
DABCO (Sigma). Immunostaining was examined either using a confocal 
laser-scanning microscope (Zeiss) with accompanying LMS 510 software, 
or a bright-field microscope (BX41, Olympus) with an Olympus camera 
(DP70) and DP version 1.2.1.108 software. Images were processed with 
Adobe Photoshop CS2.

Whole-mounts and vessel quantitation

E10.5 whole embryos, and yolk sacs and tissues from E12.5 embryos, were 
fixed in 4% paraformaldehyde (PFA), blocked in 0.3% Triton X-100 in PBS 
containing 10% normal goat serum, and incubated with rat monochonal anti-
mouse Pecam1 (clone MEC 13.3, Pharmingen) or rat anti-mouse endomucin 
antibodies. After incubation with Alexa Fluor 488-conjugated anti-rat 
antibodies (Molecular Probes), samples were analysed using a fluorescence 
stereomicroscope (M2 Bio Quad, Carl Zeiss) equipped with a colour digital 
camera (AxioCam HRc, Carl Zeiss) and multi-channel software 
(Axiovision 4.4, Carl Zeiss). Images were processed with Adobe 
Photoshop CS2. Whole-mount Pecam1 staining of E12.5 hindbrains was 
performed as described (Ruhberg et al., 2002). For each genotype, the 
number of sprouting microvessels on the pial side and the number of vessel 
branch-points on the subventricular side were determined in six randomly 
chosen 0.25 mm² fields.

X-Gal staining in transgenic embryos

Tie1-Cre;Rac1fl/+ mice were intercrossed with ROSA26R reporter mice 
(Soriano, 1999) (The Jackson Laboratory, ME, USA) and E9.5 and E10.5 
embryos were used for whole-mount X-Gal staining to test for Cre activity 
(Gustafsson et al., 2001).

Three-dimensional (3D) embryonic vasculature reconstructions

Approximately 100 serial 5 µm transverse sections through comparable 
levels of the jugular region of E12.5 Tie1-Cre;Rac1fl/fl and Tie1-

Cre;Rac1fl/fl embryos, cranial to caudal orientated, were stained for Lyve1. 
Photographs were taken at 20X magnification using a Nikon E1000 with a 
DXM 1200 camera and associated Eclipse software (Nikon). Images were 
loaded and aligned using Autoaligner and Imaris software (Bitplane AG, 
Zurich, Switzerland). Left lymphatic sac and cardinal vein perieters were 
outlined to create the solid contour surface of these vessels. Snapshots at 
various x, y and z positions of 3D rotational views were taken to illustrate the 
juxtapositional distance between the jugular lymph sac and the cardinal 
vein.

India ink visualisation of blood vessels

India ink (2-4 µl) was injected into the left ventricle, embryos fixed in 4% 
PFA, dehydrated for 30 minutes each in ascending concentrations of ethanol 
and cleared in 100% methyl salicilate (Sigma), and then photographs taken.

Isolation of embryonic endothelial cells

E12.5 embryos were dissected and livers and spleens removed to exclude 
haematopoietic cells. Embryos were digested in 2.4 U/ml Dispase/0.1% 
collagenase 1 (both Gibco) to produce single-cell suspensions. Cells were 
pelleted by low-speed centrifugation, resuspended in MLEC medium 
(Reynolds and Hodivala-Dilke, 2006) and plated onto tissue culture plates 
coated with 0.1% gelatin containing 10 µg/ml fibronectin (Sigma) and 10 
µg/ml Vitrogen (Cohesion). At ~80% confluency, macrophages were 
removed from the culture by immunosorting using rat anti-mouse 
CD16/CD32 (Fcg3r3/Fcg2b) Fcy receptor antibodies with a subsequent sort 
to positively select for endothelial cells using rat anti-mouse Icam2 
antibodies (Reynolds and Hodivala-Dilke, 2006). Endothelial cell purity was 
98% as assessed by Pecam1 expression. For some of our Rac1 siRNA and 
Rac1 activity assays we isolated primary mouse lung endothelial cells 
(MLECs) from Vegfr3+/− and wild-type 4- to 6-week-old mice as reported 
(Reynolds and Hodivala-Dilke, 2006). Vegfr3−/− mice (Dumont et al., 1998) 
were provided by Dr T. Tammela and Prof. K. Alitalo (Biomedical 
Helsinki, Finland).

Lymphatic microvascular endothelial cell culture

Dermal-derived normal neonatal human primary lymphatic microvascular 
endothelial cells (HMVEC-dLy-Neo, Lonza) were maintained in EGM 
2-MV medium supplemented with appropriate BulletKit (Lonza) and used 
prior to passage 5.

Rac1 siRNA

siGENOME ON-TARGETplus SMARTpool reagents (Dharmacon) were used. 
MLECs and HMVEC-dLy-Neo cells were transfected with anti-mouse and 
animal and human Rac1 siRNA, respectively, using Oligofectamine 
(Invitrogen). The final concentration of oligonucleotides in the transfection 
mixture was 100 nM and gene expression was examined 48 hours post-

transfection by RT-PCR and/or western blot. In all transfections, scrambled 
siRNA (Dharmacon)-transfected cell samples were used as controls.

Western blot analyses

Cells were grown to ~80% confluency and lysed with RIPA buffer (50 mM 
Tris-HCl pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 
mM EDTA, 1 mM Na3VO4, 1 mM NaF, 1 mM PMSF) supplemented with 
a protease inhibitor cocktail (Calbiochem). Mouse antibodies against Rac1, 
Cdc42 (Chemicon), RhoA (Santa Cruz) and human VEGFR3 (clone 9DF9, 
a gift from Prof. K. Alitalo, Biomedical Helsinki, Finland) were used. A 
western blot for Vegfr3 identifies three bands (Bando et al., 2004). 
Membranes were stripped (Chemicon) and re-probed with mouse anti-

Hsc70 (Hspa8) antibody (Santa Cruz). Densitometry was performed using 
a gel acquisition and analysis set-up (UV Products). Band densities were 
normalised to Hsc70.

Semi-quantitative RT-PCR

Rac1 isoform-specific primers and RT-PCR conditions were as described 
(Wells et al., 2004). As controls in Rac2 and Rac3 reactions, cDNA was 
synthesised from total RNA from spleen and brain tissues. RT-PCR of actin 
provided a loading control.

GTPase activity assays

A construct encoding glutathione S-transferase linked to amino acid residues 
57 to 141 of the PAK-CRIB domain (gift from Drs J. Collard, King’s College 
and V. Braga, Imperial College, London, UK) was used to pull down active 
Rac1 and Cdc42 (Nyström et al., 2006) in MLECs transfected with 
scrambled or Rac1 siRNA. For Rac1 activity in Vegfr3−/− endothelial cells, 
the cells were stimulated with 500 ng/ml Vegf-C156S for 30 minutes. RhoA 
activity was assessed using the G-LISA RhoA Activation Assay Biochem 
Kit (Cytoskeleton, CO, USA).

Separation of embryonic endothelial cell populations by FACS

E11.5 embryos were digested as above. Erythrocytes were excluded by 
treatment with Lymphoprep (Axis-Shield). Fcyreceptors were blocked with 
rat anti-mouse CD16/CD32 antibody. Cells were stained with the following 
buffers at 1 µg/105 cells on ice for 30 minutes: mouse anti-mouse CD45 
(clone 104), peridinin chlorophyll protein (PerCP) cyanine 5.5 (Cy5.5)- 
conjugated; rat anti-mouse CD31 (Pecam1) (clone MEC 13.3), fluorescein 
isothiocyanate (FITC)-conjugated; rat anti-mouse CD34 (clone RAM 34), 
R-phycocythrin (R–PE)-conjugated (all from Pharmingen); biotinylated 
anti-mouse Lvyel1 (eBiosciences) and goat anti-mouse Vegfr3 (R&D 
Systems). Cells were incubated with allopurinol (APC)-conjugated 
streptavidin (Pharmingen) to visualise Lvyel1-positive cells and with donkey 
anti-goat IgG F(ab ′)2-APC-Cy7 (Santa Cruz) to visualise Vegfr3. Cell
populations were analysed using an LSRII flow cytometer or a FACSAria cell sorter (Becton Dickinson) and data analysed with FACS Diva software (BD Immunocytometry Systems). Several controls, including isotope antibodies, fluorescence-minus-one (Perfetto et al., 2004) and unstained samples were performed to determine appropriate gates, voltages and compensations.

**Cytospin**

CD45+Pecam1+ FACS-sorted cells were cytospun, fixed in acetone, probed with rabbit anti-mouse Prox1, followed by anti-rabbit EnVision (Dako) and DAB staining. Cells were counterstained with Hematoxylin and Eosin.

**Migration assays**

siRNA-treated HMVEC-dLy-Neo cells were plated at 375 cells/cm² on precoated tissue culture plastic and incubated for 18-36 hours in complete growth media. Before time-lapse microscopy, cells were serum-starved and stimulated, or not, with VEGF-C156S (500 ng/ml). Phase-contrast micrographs of live cells were taken using a 768×576 pixel 8-bit CCD camera and 10× Plan/Neofluor objective (Zeiss). Data sets were collected using kinetic imaging motion analysis software. Images were collected every 5 minutes for 8 hours. Tracking of at least 25 cells/sample was performed using MetaMorph software. Analyses of cell speed and persistence were carried out using Mathematica workbooks. Scratch assays were performed as described (Reynolds et al., 2004).

**Analysis of statistical significance**

Data sets were analysed using Student’s t-test. P<0.05 was considered statistically significant. Results shown are the mean ± s.e.m. from at least three independent experiments.

**RESULTS**

**Rac1 deletion in endothelial cells**

To test whether Rac1 deficiency in endothelial cells affects developmental angiogenesis and lymphangiogenesis, Rac1-floxed mice (Rac1<sup>fl/fl</sup>) were bred with mice expressing Cre recombinase under control of the Tie1 promoter (Tie1-Cre) (Gustafsson et al., 2001). To confirm the onset of active Cre expression, Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> mice were intercrossed with the reporter strain Rosa26R (R26R) (Soriano, 1999) and tissues examined for Cre activity by X-Gal staining of whole embryos. It has been reported that Tie1-driven Cre activity is detectable in the developing heart tube, dorsal aorta and head mesenchyme as early as E8.5 (Gustafsson et al., 2001). Here we showed that, by E9.5, all Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> transgenic mice revealed that Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> mice did not survive to birth (see Fig. S1E in the supplementary material). To determine the onset of lethality, embryos were analysed at various gestational stages from E9.5 onwards. Up to, and including, E12.5, all Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> embryos were viable and morphologically indistinguishable from Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> controls at the gross level. By contrast, after E13.5, Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> embryo survival was compromised, with only 64% of mutant mice surviving at E13.5 and 50% at E15.5. By E17.5, no mutant embryos were found alive.

**Blood vessel morphology appears normal in Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> embryos**

To determine the cause of death in Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> embryos, we examined their vasculature at E10.5, when Rac1 was clearly deleted from blood vessels (see Fig. S1B-D in the supplementary material). Whole-mount staining for endomucin revealed that the vascular pattern in E10.5 mutants was normal (Fig. 1). Since Rac1 has been postulated to be required for endothelial cell migration, at least in vitro, we hypothesised that Rac1 is likely to play a role in blood vessel formation. Surprisingly, the endocardium, perineural plexus and intersomitic vessels were all present and well developed in E10.5 Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> embryos (Fig. 1A). Furthermore, high-magnification examination revealed that blood vessel branching and sprouting patterns in the perineural plexus (Fig. 1B) and intersomitic vasculature (Fig. 1C) were normal in the mutant embryos.

Further analysis revealed that at E12.5, the normal appearance of Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> embryos (Fig. 2A) correlated with normal vasculature both in the forelimbs and intestine (Fig. 2B). Yolk sac vasculature was also analysed by whole-mount staining for endomucin, which revealed that this vascular network was also normal in E10.5 Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> embryos (Fig. 1A). Furthermore, high-magnification examination revealed that blood vessel branching and sprouting patterns in the perineural plexus (Fig. 1B) and intersomitic vasculature (Fig. 1C) were normal in the mutant embryos.

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Furthermore, we quantitated the degree of angiogenesis in Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> embryos. During development, the cerebral cortex is vascularised by sprouting angiogenesis and produces a vascular plexus that contains a highly reproducible number of vessels and branch-points in mouse embryos (Ruhberg et al., 2002). Examining the vasculature in the hindbrains of E12.5 embryos by immunostaining for Pecam1 (a pan-endothelial cell marker (Vecchi et al., 1994)) revealed that the number and patterning of blood vessels is normal in Tie1-Cre<sup>−/−</sup>Rac1<sup>fl/fl</sup> embryos.
Cre;Rac1fl/fl and controls (Fig. 3A). In addition, the number of branch-points per unit area on the subventricular side of the hindbrains was also comparable between the genotypes (Fig. 3B). These data corroborated our previous results and showed that the degree of angiogenesis in E12.5 Tie1-Cre;Rac1fl/fl embryos is normal.

Other Rho GTPases do not compensate for the loss of Rac1 in endothelial cells

It is conceivable that the absence of an angiogenic phenotype in the Tie1-Cre;Rac1fl/fl mice is due to compensation by other Rho-related GTPases. To test this, levels of Rac2, Rac3, Cdc42 and RhoA were analysed in wild-type and Rac1-depleted microvascular endothelial cells. RT-PCR analysis of Rac1-depleted endothelial cells showed significantly reduced Rac1 mRNA levels (P<0.01; see Fig. S3A in the supplementary material). Similar analysis showed that Rac2 and Rac3 mRNAs were not detectable in isolated lung endothelial cells, although positive expression was found by RT-PCR analysis of mRNA extracted from control tissues such as spleen and brain, respectively (see Fig. S3A in the supplementary material). Using western blot analysis and GST-PAK pull-downs, both Rac1 total protein (P<0.001; see Fig. S3B in the supplementary material) and active Rac1 levels (P<0.01, see Fig. S3C in the supplementary material) were reduced significantly in Rac1-depleted endothelial cells. By contrast, transfection of endothelial cells using a scrambled siRNA (Con siRNA) had no effect on Rac1 expression or activity (see Fig. S3A-C in the supplementary material). In addition, Cdc42 and RhoA total protein and active levels were not affected by Rac1 depletion (see Fig. S3C in the supplementary material). These results indicate that other members of the Rho family of small GTPases, at least those tested, do not compensate for the loss of Rac1 in endothelial cells in vitro.

Lymphatic vessels of E13.5 Tie1-Cre;Rac1fl/fl embryos are filled with blood

Until E12.5, Tie1-Cre;Rac1fl/fl embryos appeared morphologically normal. By E13.5, however, embryo survival was reduced to 64%. From E13.5 onwards, 39% of Tie1-Cre;Rac1fl/fl embryos could be identified by a combination of phenotypes including the appearance of edema, blood-filled lymphatics, blood vessel tortuosity,
The perineurial vascular network was visualised at E12.5 by whole-mount Pecam1 staining on mouse hindbrains. Quantification of (A) the number of sprouting vessels on the pial side and (B) the microvessel branch-points on the subventricular side in random areas of 0.25 mm² Tie1-Cre;Rac1<sup>fl/fl</sup> and Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> hindbrains shows no significant differences. n=8 embryos per genotype. Scale bars: 100 μm.

Fig. 3. Sprouting angiogenesis does not rely on endothelial Rac1 expression in vivo. (A, B) The perineurial vascular network was visualised at E12.5 by whole-mount Pecam1 staining on mouse hindbrains. Quantification of (A) the number of sprouting vessels on the pial side and (B) the microvessel branch-points on the subventricular side in random areas of 0.25 mm² Tie1-Cre;Rac1<sup>fl/fl</sup> and Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> hindbrains shows no significant differences. n=8 embryos per genotype. Scale bars: 100 μm.

Haemorrhage and involuting limbs (Fig. 4A). Analysis of H&E-stained sections of these embryos confirmed edema in the dorsal skin and haemorrhage in the mesenchyme of the developing forelimbs (Fig. 4B). In addition, the blood vessels in these involuting forelimbs, detected by immunostaining for laminin, appeared collapsed (Fig. 4B). Additional examination of aorta by double immunostaining for laminin and α-smooth muscle actin (a mural cell marker (Gerhardt and Betsholtz, 2003)) revealed that these blood vessels also appeared collapsed in the Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos and were of significantly smaller diameter than those of controls (P<0.001; Fig. 4C). These defects in blood vessel morphology might reflect either a secondary response to the haemorrhage in these mice, as they were not observed in non-haemorrhagic mutant mice, or the onset of increased fragility. Together, these results raised the question as to why Rac1 deletion in endothelial cells might cause this phenotype at this stage of development, especially considering that angiogenesis is normal in Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos.

In vertebrates, the lymphatic vasculature develops after the blood vasculature has formed. Edema is a read-out of the malfunction of the blood vessels or lymphatic vasculature, but because we did not detect any changes in the blood vasculature until E12.5, we focused our attention on the lymphatics. As originally proposed by Sabin, a subpopulation of endothelial cells migrates centrifugally from the cardinal veins to form lymph sacs and finally develops into the lymphatic network (Sabin, 1902; Sabin, 1904). In adult mice, lymphatic and blood vessels do not communicate, except at the point where the thoracic duct enters the subclavian vein, and aberrant, persistent connections between the two systems lead to blood-congested lymphatic vessels (Abtahan et al., 2003). Immunohistochemical examination of sections at comparable levels of the thoracic region from E13.5 embryos for the expression of Lyve1 and podoplanin [lymphatic endothelial cell markers (Kaiapin et al., 1995; Prevo et al., 2001)] and endomucin (a blood vascular, but not lymphatic, endothelial cell marker), showed the presence of red blood cells not only in blood vessels, but also in the lymphatic vasculature of Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos. Important, this was not evident in any of the control embryos examined (Fig. 4D). In contrast to control Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos, blood-congested lymphatic vessels were apparent in E13.5 Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos (see Fig. S4A-C in the supplementary material). Furthermore, we found blood-filled lymphatic vessels not only in Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos that displayed haemorrhage, but also in Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos that did not (Fig. 4D). These data indicated that the appearance of blood-filled lymphatic vasculature in Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos was unlikely to be due to the bleeding observed in these animals at this developmental stage. However, it is interesting to speculate that this phenotype might reflect the presence of abnormal, persistent, direct veno-lymphatic communications between blood vessels and lymphatic vasculature. It is conceivable that the haemorrhage observed might be caused by a combination of increased blood vessel fragility and the rupture of blood-congested lymphatic vessels. Although no blood vessel defects were evident at E12.5 (see Fig. 3 and Fig. S2 in the supplementary material), some loss of blood vessel integrity was observed at E13.5 (see Fig. 4B,C). Moreover, the rupture of blood-congested lymphatic vessels and subsequent haemorrhage into the surrounding mesenchyme was detected in Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos (see Fig. S4D in the supplementary material).

Given that we observed blood in lymphatic vessels in E13.5 Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos, even in the absence of any overt bleeding, this suggested that somehow blood was entering the lymphatics directly from the blood vasculature and that this might be due to a defect in the separation of blood vessels and lymphatic vessels. Indeed, the separation of the jugular lymph sac from the parental cardinal vein in mice is normally considered to be complete by E12.5 (Oliver, 2004; Oliver and Alitalo, 2005), so blood should not be detectable in the lymphatics at E12.5. To investigate the possibility that endothelial Rac1 deletion affects lymphatic-blood vessel separation, we analysed Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos at E12.5. Similar to observations made at E13.5, in E12.5 embryos red blood cells were evident in both endomucin-positive cardinal veins and Lyve1- and podoplanin-positive jugular lymphatic sacs of Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos, but never in controls (Fig. 5A). Furthermore, the distance between the cardinal veins and the laterally located jugular lymph sacs was reduced significantly in Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos compared with controls at similar thoracic levels (P<0.0001; Fig. 5A). It is important to note that although a common reason for the appearance of blood in the lymphatics is overt haemorrhage into tissue struma, our results are distinct from this: in Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos, the presence of blood in the lymphatics is not due to prior bleeding, and blood-filled lymphatic vessels appear before haemorrhage in these mice.

In addition, by performing unilateral three-dimensional reconstructions of the jugular lymphatic sac and cardinal vein using over one hundred 5 μm serial sections from E12.5 Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> and Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos, we demonstrated that endothelial Rac1 deletion substantially reduced the distance between these two vessels (Fig. 5B). These results suggested that expression of Rac1 in endothelial cells is crucial for proper lymphatic-blood vessel separation during embryonic development.

**Endothelial Rac1 deficiency impairs the transition from blood endothelial cell to lymphatic endothelial cell**

We then sought to determine the mechanism underlying the poor separation between the lymphatic and blood vasculature in the Tie1-Cre<sup>+</sup>;Rac1<sup>fl/fl</sup> embryos. A pre-requisite for lymphatic vessel
formation and separation is the transition of a subset of committed venous endothelial cells to differentiate into lymphatic endothelial cells (Alitalo et al., 2005; Oliver, 2004). This occurs at ~E11.5 and involves a stage in which some cells transiently express both endothelial and lymphatic vessel markers. Previous work has shown that Spred1/2 doubly deficient mice have lymphatic defects, which correlates with increased numbers of Lyve1/CD34 double-positive cells (Taniguchi et al., 2007). To examine whether Rac1 expression in endothelial cell populations [CD45– (Ptprc)/Pecam1+] from E11.5 embryos affected the proportion of cells expressing either blood endothelial cell (BEC) markers (Lyve1–/CD34+), lymphatic endothelial cell (LEC) markers (Lyve1+/CD34–) or both [Lyve1/CD34 double-positive], we carried out FACS analysis of CD45–/Pecam1+ endothelial cells for Lyve1 and CD34. The proportion of cells that were CD45–/Pecam1+ was similar in both genotypes (see Fig. S5A in the supplementary material). In Tie1-Cre+;Rac1fl/fl embryos, although the percentage of BECs and LECs appeared normal, a significantly increased Lyve1/CD34 double-positive cell population, which was likely to represent a transition state of LECs from BECs, was found in mutant embryos when compared with controls (P<0.03; see Fig. S5B in the supplementary material).

Vegf receptor 3 (Vegfr3; Flt4) identifies blood vasculature during early embryonic development, but at ~E11.5-12.5 its expression becomes restricted to the lymphatic vasculature (Kaipainen et al., 1995). We performed similar assays replacing analysis of Lyve1 with that of Vegfr3 and showed that loss of Rac1 in endothelial cells leads to an increase in Vegfr3/CD34 double-positive cells (P<0.05; see Fig. S5C in the supplementary material). This could reflect either a delay in maturation of BECs or a delay of committed differentiating LECs in downregulating BEC markers. Since we did not observe any change in blood vascular morphology or function, our data suggest that Rac1 might be responsible for regulating, at least temporarily, the degree of transition of BECs to LECs.
Rac1 regulates budding of Prox1-expressing endothelial cells from the cardinal vein

Lymphatic vessel development involves the migration of differentiating LECs from the cardinal vein into the mesenchyme in a process known as budding. This process can be morphologically identified at E11.5 when Prox1-expressing LEC progenitors migrate away from the cardinal vein and begin to organise into primitive lymph sacs (Wigle and Oliver, 1999; Oliver and Alitalo, 2005). We hypothesised that the abnormal lymphatic-blood vessel separation found in Tie1-Cre^+/Rac1^fl/fl embryos might reflect abnormal cell migration during budding, and examined the expression patterns of Prox1-positive cells in E11.5 Tie1-Cre^+/Rac1^fl/fl and control embryos. In both genotypes, Prox1-positive staining was restricted to a subpopulation of endothelial cells located on one side of, and budding off, the cardinal vein. However, analysis of Prox1-stained sections revealed that the percentage of Prox1-positive endothelial cells within the wall of the cardinal vein was reduced significantly in mutant embryos (P<0.0001; Fig. 6A-C). Furthermore, the distribution patterns of budding Prox1-positive cells were altered in Tie1-Cre^+/Rac1^fl/fl embryos. Whereas in control embryos Prox1-positive cells appeared to follow a narrow path of migration, the distribution pattern of the majority of Prox1-positive cells in Tie1-Cre^+/Rac1^fl/fl embryos appeared to be wider and more random (Fig. 6A,B). Analysis of these areas showed that the average maximum width to maximum length ratio was elevated significantly in Tie1-Cre^+/Rac1^fl/fl embryos when compared with controls (P<0.005; Fig. 6B). In addition, the number of Prox1-positive cells in these areas was reduced significantly in Tie1-Cre^+/Rac1^fl/fl embryos (P<0.01; Fig. 6A-C). Ki67/Prox1 double immunostaining of this region showed that this reduction in Prox1-positive cells did not correlate with a decrease in the percentage of Ki67-positive proliferating cells (see Fig. S6A,B in the supplementary material). Moreover, quantitation of the numbers of Prox1-positive cells in cytospins of CD45^-/Pecam1^+ FACS-sorted cells from E11.5 embryos showed no significant difference between genotypes (see Fig. S6C in the supplementary material). This indicated that the total number of Prox1-positive cells in the CD45^-/Pecam1^+ population was not affected by Rac1 deletion. Taken together, these data
suggest that endothelial Rac1 deficiency not only appears to alter the degree of transition of BECs to LECs, but also that it is sufficient to impair the pattern of cell migration during lymphatic budding.

**Aberrant motility of Rac1-depleted LECs is associated with reduced Vegfr3 protein levels**

The migration of Prox1-expressing endothelial cells from the cardinal vein to newly forming lymph sacs is regulated by a concentration gradient of Vegf-C within the surrounding mesenchyme (Karkkainen et al., 2004). Given that the distribution patterns of Prox1-positive cells were altered in Tie1-Cre;Rac1<sup>fl/fl</sup> embryos, we tested whether Rac1 depletion in LECs modestly increased Vegf-C156S-stimulated cell speed and dramatically decreased persistence (*P<0.001). Bar charts represent mean ± s.e.m. n=175-200 cells per condition. Representative migration traces for Con and Rac1 siRNA-transfected LECs are given. (E) Con and Rac1 siRNA-transfected LEC extracts immunoblotted for Vegfr3 and Rac1 show that Rac1 depletion reduces Vegfr3 expression. Hsc70, loading control. Densitometry results (means ± s.e.m.) of relative protein levels. *P<0.01, n=3.
actual distance travelled per cell (persistence), Rac1-depleted LECs were found to exhibit a significantly lower persistence than controls (P<0.001; Fig. 6D). These data indicate that Rac1-depleted LECs have a more random path of VEGF-C156S-stimulated migration than control cells (P=0.001; Fig. 6D). As predicted, knockdown of Rac1, using Rac1-specific siRNA, in endothelial cells inhibited VEGF-A164-mediated two-dimensional migration (see Fig. S7 in the supplementary material). Given this change in VEGF-C156S-mediated migration, we next tested the effect of Rac1 depletion on VEGFR3 expression. Western blot analysis showed that VEGFR3 levels were reduced significantly in Rac1-depleted cells compared with controls (P<0.01; Fig. 6E). By contrast, VEGF-C156S-stimulated VEGFR3(+) endothelial cells showed no change in Rac1 activity, suggesting that although Rac1 can regulate VEGFR3 expression, a substantial loss of VEGFR3 is not sufficient to affect active levels of Rac1 (see Fig. S8 in the supplementary material). It is likely that as LECs were not removed from these preparations, they persist in the endothelial cell cultures. These data suggest that regulation of VEGFR3 expression is a possible mechanism by which the Rac1 Rho GTPase regulates LEC migration and lymphatic-blood vessel separation in vivo.

**DISCUSSION**

Our data provide the first evidence that in vivo deletion of Rac1 in Tie1-Cre mice is not sufficient to impair sprouting angiogenesis, but is required for the correct migration of committed LECs during lymphatic vessel segregation from blood vessels (see Fig. S9 in the supplementary material). Indeed, deletion of Rac1 in Tie1-Cre mice gives rise to poor veno-lymphatic separation, resulting in leakage of blood into the lymphatic system. Although we cannot rule out the possibility that the haemorrhage observed at this stage could be due to a sudden increase in blood vessel fragility, our data on the normal appearance of functional blood vessels would not appear to support this. Despite the generally accepted involvement of Rac1 in the migration of cultured cells (Nobes and Hall, 1999; Ridley et al., 2003), our data indicate that its role during cell migration in the whole organism is not as clear. We show that Rac1 deletion in LECs does not affect the VEGF-C-mediated migration rate, but does affect the persistence of migration. Others have reported that the role of Rho GTPases in the migration of cells in 3D, mimicking the in vivo scenario, versus 2D may not be the same (Sahai et al., 2007; Sahai and Marshall, 2003). Indeed, our data showing an apparent lack of effect of Rac1 deletion on endothelial cell migration during angiogenesis in vivo is reminiscent of other studies that reported that Rac1 deletion in macrophages does not inhibit migration (Wells et al., 2004).

Recently, it has been shown that Rac1 deletion in Tie2-Cre mice results in gross defects in the development of major vessels by E8.5 and embryonic lethality soon after (Tan et al., 2008). By contrast, our studies show that Rac1 deletion in Tie1-Cre mice is not sufficient to impair sprouting angiogenesis. How can we explain the discrepancies between the Tie1-Cre- and Tie2-Cre-driven Rac1 deletion studies? One explanation is that Cre expression in Tie1-Cre mice is sometimes found to be rather patchy, giving rise to a chimeric loss of targeted genes (Engle et al., 2002). However, this appears not to be the case in the Tie1-Cre+;Rac1fl/fl embryos that we have analysed, as we see excellent Cre activity throughout the vasculature and concomitant deletion of Rac1 expression in the endothelial cells of Tie1-Cre+;Rac1fl/fl embryos both in vivo and in vitro. Thus, we believe that insufficient deletion of Rac1 is not the reason for the apparently normal sprouting angiogenesis in Tie1-Cre+;Rac1fl/fl embryos. Another possible explanation includes the involvement of hematopoietic cells in embryonic angiogenesis (Suda and Takakura, 2001). Indeed, the functional role of hematopoietic cells, including platelets, in lymphatic vascular development is a matter of some debate (Sébzda et al., 2006; Srinivasan et al., 2007; Taniguchi et al., 2007). For example, platelets have been implicated as important in the separation of lymphatics from blood vessels (Suzuki-Inoue et al., 2007; Uhrin, 2006), and as Tie1 is known to be expressed in platelets (Batard et al., 1996; Tsiamis et al., 2000), it is possible that Rac1 deficiency in this population might contribute to the poor lymphatic-blood vessel separation that we observed. However, as there is no evidence for Tie1 promoter-driven Cre expression in platelets (Gustafsson et al., 2001) and thus no evidence for Rac1 deficiency in them, we cannot comment upon whether the loss of Rac1 in platelets is responsible for the defect we observed. Since in Tie2-Cre mice over 80% of hematopoietic cells display Cre activity (Constien et al., 2001; Griffin et al., 2008), as opposed to only 13% in Tie1-Cre mice (Gustafsson et al., 2001), we hypothesise that the resultant loss of Rac1 in the vast majority of Tie2-Cre+ positive-developing hematopoietic cells might well be the cause of the gross vascular defects in the Tie2-Cre+;Rac1fl/fl mice, something that the Tie1-Cre+;Rac1fl/fl embryos would be relatively protected from. Unfortunately, there is presently no way to test the functional consequence of the potential deficiency in Rac1 in the 13% of hematopoietic cells on lymphatic separation in E12.5 embryos in vivo. In addition, the lethality of Tie2-Cre+;Rac1fl/fl mice prior to lymphatic vessel development prevents an examination of the role of Rac1 in lymphatic development and thus makes the Tie1-Cre mice a more appropriate model for such studies.

Since Tie1-Cre+;Rac1fl/fl endothelial cells display clear Cre activity and show significant Rac1 deletion both in vivo and in vitro, we have concentrated our investigations on the role of Rac1 in endothelial cells. Importantly, our data show abnormal migration specifically of LECs during the first stages of lymphatic budding, and demonstrate that different subpopulations of endothelial cells can have different dependencies on a single migratory molecule, such as Rac1, in vivo. This differential dependency is manifested in apparently normal vascular endothelial cell migration during sprouting angiogenesis, but abnormal migration during lymphatic vessel segregation. How this is regulated and what stimulates different subpopulations of cells to react differently to their neighbours in vivo will be subjects for future studies. Others have shown that mice carrying one hypomorphic Vegfr3<sup>+</sup> allele have normal blood vessel development but impaired lymphatic vessel development and Vegfr3 levels (Haiko et al., 2008). We show that loss of Vegfr3 does not affect Rac1 expression or activity and this might explain why Vegfr3<sup>+</sup> mice do not display a lymphatic separation defect (Dumont et al., 1998). By contrast, our study shows that the combination of Rac1 deficiency with a reduction in Vegfr3 expression, which is likely to be via signalling control of other molecules, may be sufficient to affect the persistence of Vegfr3-stimulated LEC migration, and therefore might be one mechanism by which this Rho GTPase regulates lymphatic-blood vessel separation.

Our data suggest that Rac1 deletion in Tie1-Cre mice leads to malfunctioning lymphatics, which is likely to give rise to the edema in these mutant embryos. In addition, they suggest that abnormal veno-lymphatic separation might lead to blood-congested lymphatics. Clinically, it is common to see blood-filled lymphatics in lymphatic malformations, but how the blood gets there has never been demonstrated. Furthermore, the presence of excessive blood in the lymphatics could be one reason for the blood vessel collapse...
observed in Tie1-Cre;Rac1^{f/f} embryos at E13.5, as it leads to lymphatic vessel rupture that would result in interstitial haemorrhage, loss of tissue integrity and, finally, to the death of Tie1-Cre;Rac1^{f/f} embryos. Alternatively, we cannot exclude the hypothesis that the gross haemorrhage and loss of blood vessel function might be independent of the lymphatic defect and instead reflect an increase in blood vessel fragility in these mutant mice.

Acknowledgements
We thank Dr Christiana Ruhrberg for assistance with the hindbrain model; Michel Germain for assistance with experiments; Gary Saunders, Colin Wren, Colin Pedram, Claire Darnborough, Claire Watkins and Emma Murray for technical assistance with animals; Drs John Collard and Vania Braga for providing the GST-PAK-CRIB construct; Prof. Dietmar Vestweber for providing the endomucin antibody; Prof. Reinhard Fässler for providing Tie1-Cre mice; Prof. Karin Tammela for providing VegfGFP^{t/m} mice; Takako Makita for technical advice on the India ink injections; and Prof. Peter Mortimer, Prof. Ian Hart and Prof. Tanya Petrova for their critical comments on the manuscript. G.D., E.N., A.R.R., S.D.R., L.E.R., V.K., K.S., D.M., D.A., B.S.-D., D.D., D.D. and K.M.H.-D. were funded by Cancer Research UK. R.G. was supported by the Wellcome Trust; T.M. was funded by The Medical Research Council. D.J.T. was funded by Breast Cancer Campaign. G.T. was funded by the Health Foundation, Royal College of Pathologists. Deposited in PMC after 6 months.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/23/4043/DC1

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