LETTERS

A mechanosensory complex that mediates the endothelial cell response to fluid shear stress

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Shear stress is a fundamental determinant of vascular homeostasis, regulating vascular remodelling, cardiac development and atherogenesis[1], but the mechanisms of transduction are poorly understood. Previous work showed that the conversion of integrins to a high-affinity state mediates a subset of shear responses, including cell alignment and gene expression[2–4]. Here we investigate the pathway upstream of integrin activation. PECAM-1 (which directly transmits mechanical force), vascular endothelial cell cadherin (which functions as an adaptor) and VEGFR2 (which activates phosphatidylinositol-3-OH kinase) comprise a mechanosensory complex. Together, these receptors are sufficient to confer responsiveness to flow in heterologous cells. In support of the relevance of this pathway *in vivo*, PECAM-1-knockout mice do not activate NF-κB and downstream inflammatory genes in regions of disturbed flow. Therefore, this mechanosensing pathway is required for the earliest-known events in atherogenesis.

Atherosclerotic lesions occur preferentially in regions of low or disturbed shear stress at vessel branch points, bifurcations and regions of high curvature, whereas high laminar shear stress is atheroprotective[1]. It has been proposed that shear stress is transmitted from the apical surface of the endothelial cell through the cytoskeleton to points of attachment at cell–cell and cell–matrix adhesions; therefore, these adherens junctions experience changes in mechanical tension and could serve as mechanotransducers[1]. The endothelium contains adherens junctions that depend on vascular endothelial cell cadherin (VE-cadherin), and tight junctions involving claudins and occludins. Endothelial cells also contain three widely expressed junctional adhesion molecules, and the immunoglobulin family receptor platelet endothelial cell adhesion molecule (PECAM)-1. PECAM-1 is a homophilic adhesion receptor whose cytoplasmic domain binds to β- and γ-catenins and contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) that, in its phosphorylated form, binds to SH2-domain-containing protein tyrosine phosphatase (SHP)-2 (ref. 5).

Acute onset of shear stress triggers a number of events[1], including the activation or induction of ion channels, Src-family and vascular endothelial growth factor receptor 2 (VEGFR2) tyrosine kinases, extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs), p38 mitogen-activated protein kinase and AKT (v-*akt* murine thymoma viral oncogene homologue) serine/threonine kinases, and transcription factors such as NF-κB (nuclear factor of kappa light chain gene enhancer in B cells) and activator protein (AP)-1. VE-cadherin is implicated in activation of AKT kinases and NF-κB by flow[6]. The onset of flow also triggers phosphorylation of PECAM-1 ITIM tyrosines, and direct application of force to PECAM-1 can induce ERK activation[7]. In laminar shear, vascular endothelial cells adapt to the flow so that after the initial stimulation these events are downregulated. In contrast, in disturbed shear, where flow magnitude and direction are continually changing, these pathways are activated in a sustained manner[8]. As a result, NF-κB is activated and NF-κB-dependent genes, encoding proteins such as intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, endothelial (E)-selectin and platelet-derived growth factor (PDGF), are expressed at atherosclerosis-prone sites *in vivo* before, or in the absence of, other markers for atherosclerosis[9]. Stimulation of these pathways by changes in flow is thought to contribute to the initiation of atherosclerosis, so the acute onset of laminar shear is commonly used to investigate fundamental mechanisms of mechanotransduction.

Although many of the signalling events stimulated by shear have been identified, an understanding of shear sensing has not emerged and the primary transducer(s) that mediate both adaptive responses and atherogenesis have not been identified. The conformational activation of integrins initiates both alignment (an adaptive response to laminar shear) and activation of NF-κB (which promotes atherogenesis in disturbed shear[2,3]), so we investigated how the onset of flow induces integrin activation.

Phosphatidylinositol-3-OH kinase (PI(3)K) has been implicated in integrin activation in many cell types[10]. In endothelial cells, phosphorylation of the p85 subunit of PI(3)K was detected within 15 seconds after the onset of flow, and continued to increase for several minutes (Supplementary Fig. S1a). When phosphatidylinositol-3,4,5-trisphosphate, the product of PI(3)K, was assayed by examining the membrane translocation of the AKT pleckstrin homology domain fused to green fluorescent protein (GFP–AKT PH), rapid activation by the onset of flow was observed (Supplementary Fig. S1b). When the activation of integrin $\alpha_v\beta_3$ by shear stress was assayed by binding of WOW-1, a monoclonal antibody Fab fragment that binds selectively to high-affinity α_v integrins[2,11], a complete block in integrin activation was observed in the presence of the PI(3)K inhibitors LY294002 (Supplementary Fig. S1c) and wortmannin (data not shown). Thus, the stimulation of PI(3)K mediates integrin activation.

Shear stress is known to activate c-Src within seconds[12,13]. Antibodies against both activating (Y418) and inhibitory (Y527) phosphorylation sites showed that Src was maximally activated within 15 s of the initiation of flow (Supplementary Fig. S1d); it is thus slightly faster than PI(3)K phosphorylation. Both the phosphorylation of the

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PI(3)K p85 subunit and integrin activation were prevented by the Src kinase inhibitors PP2 (Supplementary Fig. S1e) or SU6656 (data not shown). Therefore, a Src family kinase is upstream of PI(3)K-dependent integrin activation in response to shear stress.

To address the requirement for junctional receptors in flow-induced integrin activation, VE-cadherin$^{-/-}$ and PECAM-1$^{-/-}$ endothelial cells were tested for WOW-1 binding. Responses in reconstituted null cells, in which VE-cadherin or PECAM-1 was ectopically re-expressed, were similar to those in wild-type bovine and human endothelial cells (data not shown). VE-cadherin$^{-/-}$ and PECAM-1$^{-/-}$ endothelial cells failed to show strong activation of integrins after the onset of flow, whereas null cells that had been engineered to re-express the relevant receptors showed normal levels of integrin activation (Fig. 1a). Basal levels of WOW-1-binding in VE-cadherin$^{-/-}$ and PECAM-1$^{-/-}$ endothelial cells were similar to those in reconstituted cells (data not shown). Sparse endothelial cells, cultured at low cell-density and lacking cell–cell junctions, also showed no increase in WOW-1 binding after the onset of flow (data not shown).

We also assessed the events upstream of integrin activation in response to shear stress. VE-cadherin$^{-/-}$ endothelial cells showed no significant increase in PI(3)K phosphorylation or activation of AKT, a PI(3)K-dependent event (Fig. 1b), consistent with a previous analysis of AKT in these cells[6]. Src activation was delayed but not substantially inhibited in VE-cadherin$^{-/-}$ cells (Fig. 1c). However, PECAM-1$^{-/-}$ endothelial cells showed no activation of PI(3)K, AKT or Src family kinases (Fig. 1a–c). Therefore, PECAM-1 is required for Src activation, while VE-cadherin is required for the transmission of that signal to PI(3)K.

Consistent with the idea that the integrin pathway mediates both cell alignment in the direction of flow and the transient induction of NF-κB by flow[3], neither VE-cadherin$^{-/-}$ nor PECAM-1$^{-/-}$ cell lines

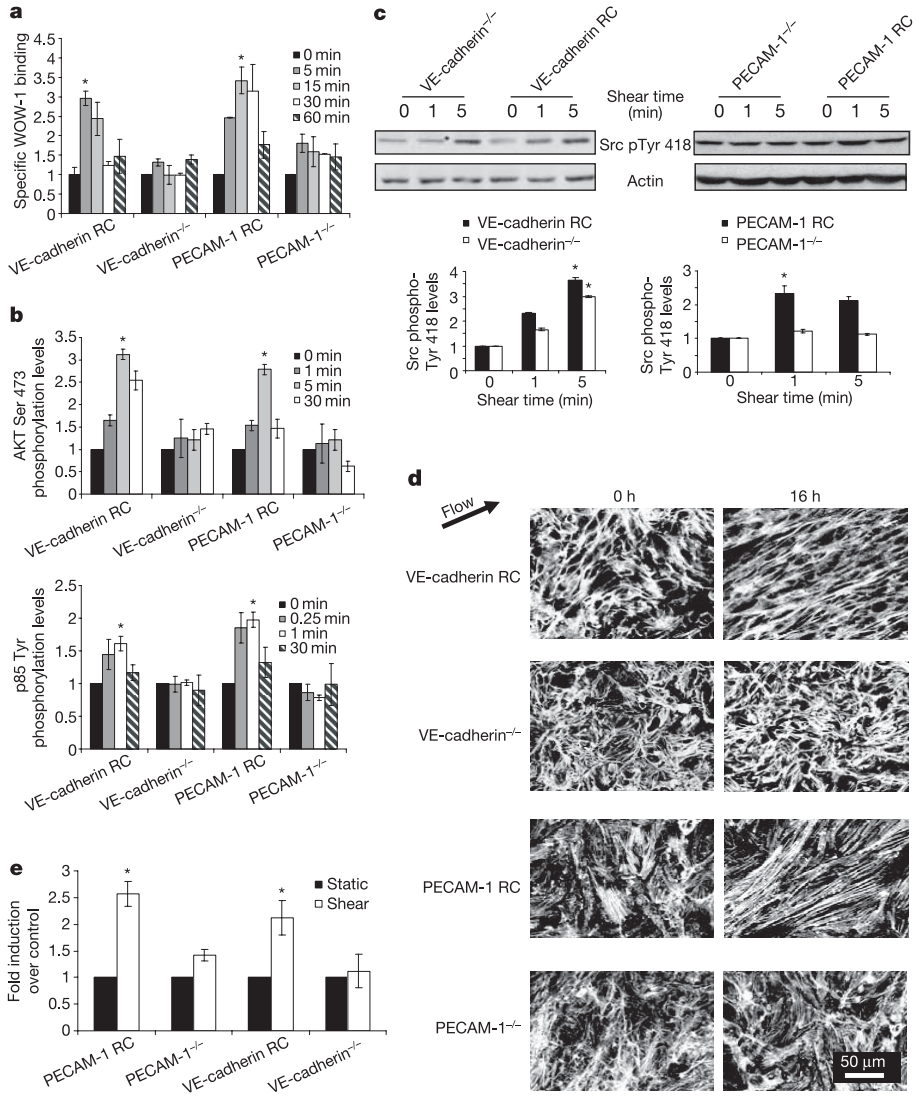

Figure 1 | Responses of PECAM-1$^{-/-}$ and VE-cadherin$^{-/-}$ cell lines to shear stress. a, PECAM-1$^{-/-}$ and PECAM-1-reconstituted (PECAM-1 RC) cells, and VE-cadherin$^{-/-}$ and VE-cadherin-reconstituted (VE-cadherin RC) cells, were subjected to shear stress for the times indicated and assayed for integrin activation by measuring WOW-1 binding. **b**, AKT activation was assayed by western blotting for phosphorylated AKT and total AKT. Values are means ± s.e.m.; $n = 3$. Phosphorylation of the PI(3)K p85 subunit was assayed by immunoprecipitation and western blotting for phosphorylated p85 as described in the Methods. **c**, Activity of Src family kinases was determined by western blotting for phospho-Tyr 418. Values are means ± s.e.m.; $n = 3$. **d**, Cells were subjected to shear for 16 h then fixed and stained with TRITC-phalloidin to visualize actin filaments. The arrow indicates the direction of flow. **e**, The indicated endothelial cells were co-transfected with a vector containing the PDGF-A/SSRE regulating the expression of firefly luciferase, together with *Renilla* luciferase, under the control of a minimal promoter and subjected to shear for 60 min. Values represent firefly luciferase activity normalized to *Renilla* luciferase (mean ± s.e.m.; $n = 4$). Statistical significance as determined by Student's *t*-test is indicated by an asterisk ($P < 0.01$).

showed an alignment of actin filaments in the direction of flow (Fig. 1d), or an induction of luciferase under the control of an NF-κB-dependent promoter[14] (Fig. 1e). These data confirm the requirement for these adhesion receptors in the activation of the integrin pathway and downstream events by shear stress.

To test whether these receptors can directly transduce force, sparse endothelial cells were incubated with 4.5-μm-diameter magnetic beads coated with antibodies to cell adhesion receptors, followed by the application of a magnetic field. Antibody-coated beads induced receptor binding, clustering and downstream signalling as assessed by their ability to recruit actin (Supplementary Fig. S2). The magnet exerts a maximum force on a 4.5-μm-diameter bead of ~130 pN, which is ~10% of the drag force exerted by shear stress at 12 dyn cm^{-2} on a 30 μm × 30 μm endothelial cell (~1.1 nN). Anti-PECAM-1-coated beads were bound to isolated bovine aortic endothelial cells expressing the GFP–AKT PH fusion protein as a sensor for PI(3)-lipids. Local activation of integrins was assessed by staining with the WOW-1 Fab fragment. The application of magnetic force for as little as 15 s triggered the activation of PI(3)K and integrins around the anti-PECAM-1-coated beads (Fig. 2a). The pretreatment of cells with PP2 or LY294002 abolished this response. Application of the same magnetic force to cells with bound anti-CD44- or anti-VE-cadherin-coated beads had little or no effect on activation levels (Fig. 2b). We also noted that when confluent endothelial cells were exposed to flow for 1 min, WOW-1 staining was higher near cell–cell junctions (Supplementary Fig. S3). However, at later timepoints WOW-1 stained the basal surface uniformly[2], indicating that either the active integrins or the activating signal could diffuse. We conclude that PECAM-1 is the direct transducer of mechanical force in this system.

When VE-cadherin$^{-/-}$ endothelial cells were examined in this assay, the application of a magnetic force to bound anti-PECAM-1-coated beads failed to induce signalling (Fig. 2b), whereas reconstituted cells showed responses similar to bovine aortic endothelial cells (data not shown). This result is surprising, because no ligand for VE-cadherin is present on the anti-PECAM-1-coated beads. To determine whether the role of VE-cadherin function in confluent monolayers under flow is also ligation-independent, VE-cadherin-reconstituted cells were mixed with a much larger number of VE-cadherin$^{-/-}$ cells. Under these conditions, VE-cadherin cannot participate in homophilic adhesion. After 16 h of flow, isolated VE-cadherin-positive cells aligned normally, whereas surrounding VE-cadherin$^{-/-}$ endothelial cells did not (Fig. 2c, d). Additionally, the treatment of cells in a confluent monolayer with a VE-cadherin-blocking antibody (BV9), which blocks homophilic binding and causes the dispersion of VE-cadherin from junctions to the rest of the cell surface[15], induced the redistribution of VE-cadherin from intercellular junctions, but had no effect on flow-stimulated WOW-1 binding (data not shown). Thus, the involvement of VE-cadherin in shear-stress-dependent signalling is independent of cell–cell adhesion.

VEGFR2 shows rapid, Src-dependent, ligand-independent transactivation in response to flow[16,17]. Staining of cells with an antibody against VEGFR2 phosphotyrosine 1054, which is phosphorylated upon receptor activation, revealed the activation of VEGFR2 within 15 s upon the initiation of flow and localization of a fraction of the activated VEGFR2 to cell–cell junctions (Supplementary Fig. S4a). This signal persisted for at least 5 min and, consistent with previous results[17], was Src-dependent (data not shown). Furthermore, flow-induced activation of VEGFR2 was absent in both PECAM-1$^{-/-}$ and VE-cadherin$^{-/-}$ endothelial cells at all times between 15 s and 5 min (Supplementary Fig S4a; data not shown); reconstituted cells responded similarly to bovine aortic endothelial cells (data not shown). Therefore, VEGFR2 activation by shear stress is also downstream of the junctional receptors. We also noticed that β-catenin may translocate to the nucleus after the onset of flow in PECAM-1$^{-/-}$ and VE-cadherin$^{-/-}$ cells, but this observation was not investigated further. A selective inhibitor of VEGFR2 kinase activity (VTI)

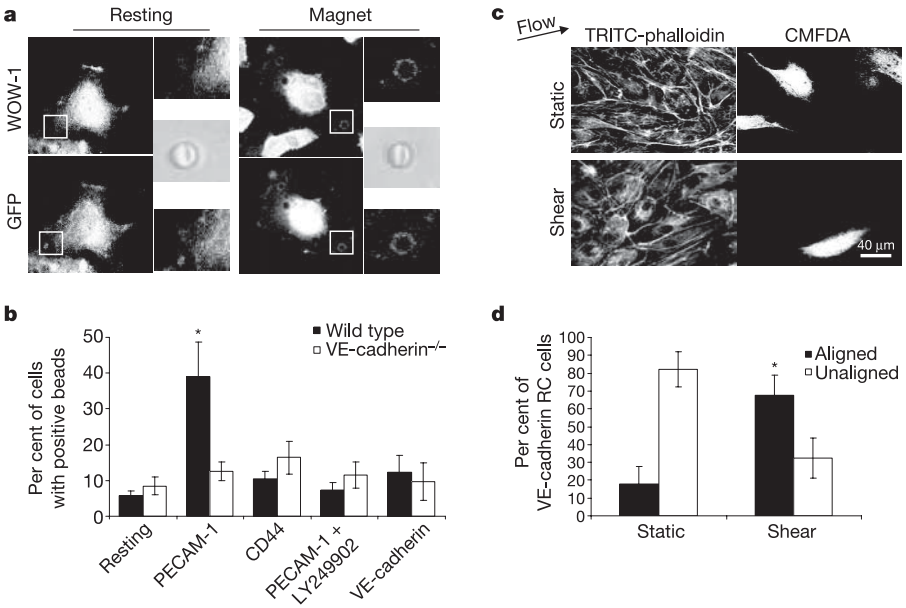

Figure 2 | Direct mechanotransduction and adhesion-independent role for VE-cadherin. **a**, **b**, Bovine aortic endothelial cells at low density and expressing the GFP–AKT PH fusion protein were incubated with antibody-coated magnetic beads for 30 min, in some cases after pretreatment with LY249902 to inhibit PI(3)K activity. Magnetic force was applied parallel to the coverslip for 15 s. **a**, Cells were fixed, stained with WOW-1 and examined for fluorescence. Insets show areas within the same field of view at a higher magnification. **b**, Antibody-coated beads were scored for a ring of WOW-1 or GFP–AKT PH fluorescence above background. Beads coated with anti-VE-cadherin antibody and VE-cadherin$^{-/-}$ cells were also analysed (>300 cells per condition). **c**, VE-cadherin-reconstituted cells loaded with CellTracker Green CMFDA dye and unlabelled VE-cadherin$^{-/-}$ cells were mixed at a ratio of 1:100. Cells were sheared for 16 h, fixed and then stained with TRITC-phalloidin. Green cells were scored for alignment in the direction of flow (indicated by the arrow). **d**, Quantification of the alignment of VE-cadherin-reconstituted cells (200 cells counted). For experiments **a**–**c**, $n = 4$; for **d**, $n = 3$; values are mean ± s.e.m.; asterisk, $P < 0.01$.

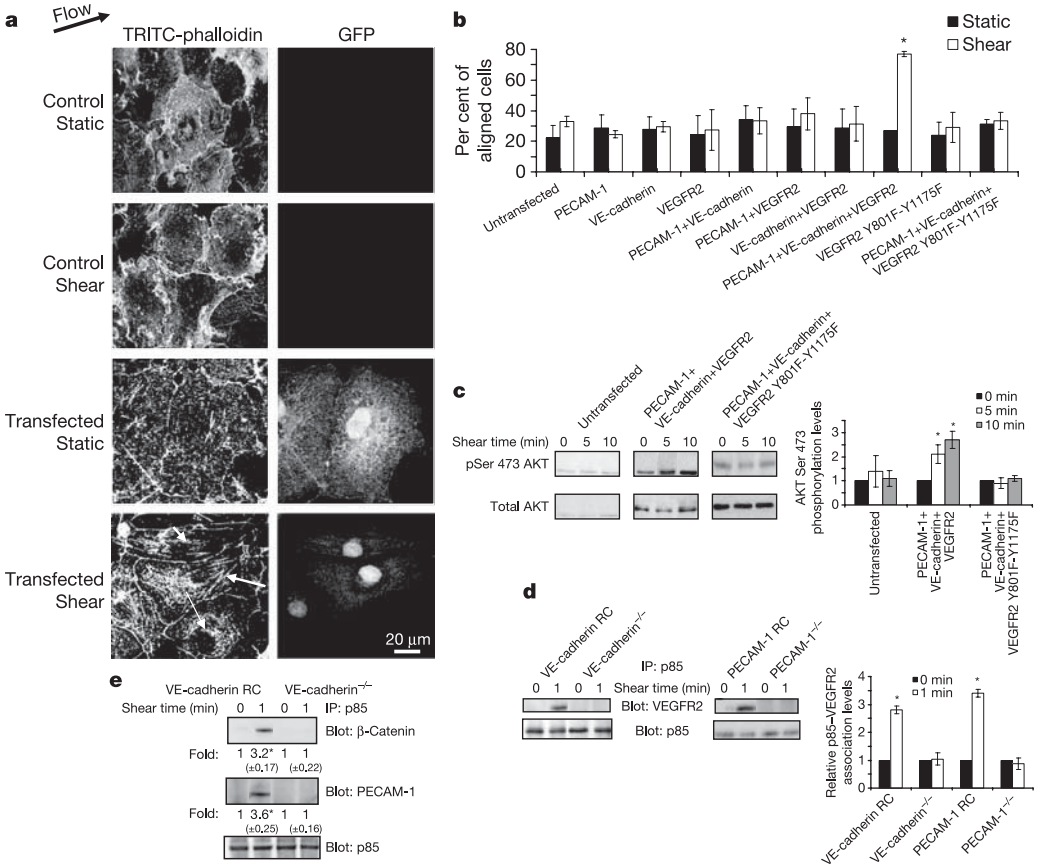

Figure 3 | PECAM-1, VE-cadherin and VEGFR2 form a mechanosensory complex. a, COS-7 cells transfected with constructs encoding PECAM-1, VE-cadherin and VEGFR2, together with GFP, were sheared for 16 h, fixed and stained with TRITC-phalloidin. Direction of flow is indicated by the arrow. White arrows show aligned stress fibres. **b**, Cells transfected with various combinations of receptor constructs, including the VEGFR2 Y801F-Y1175F double mutant, were analysed as in panel **a** and quantified. Values are means ± range; $n = 2$; >100 cells were scored per condition. **c**, COS-7 cells were transiently transfected with PECAM-1, VE-cadherin and wild-type or mutant VEGFR2, and subjected to shear stress. Activation of AKT was assayed by western blotting for phosphorylated AKT and total AKT. Values are means ± s.e.m.; $n = 3$. **d**, Lysates from VE-cadherin$^{-/-}$ and VE-cadherin-reconstituted cells with or without shear were immunoprecipitated (IP) with an anti-PI(3)K p85 subunit antibody. VEGFR2 and p85 levels were assessed by western blotting. Values are means ± s.e.m.; $n = 3$. **e**, VE-cadherin$^{-/-}$ and VE-cadherin-reconstituted cells were sheared, lysed and PI(3)K-immunoprecipitated. The fold induction of the indicated proteins was analysed by western blotting. Values are means ± s.e.m.; $n = 3$. For all experiments, an asterisk indicates $P < 0.01$.

blocked activation of PI(3)K in response to shear stress (Supplementary Fig. S4b). Flow-induced VEGFR2 activation is therefore required for PI(3)K activation.

The alignment of cells in the direction of flow is specific to endothelial cells[18]. To test whether PECAM-1, VE-cadherin and VEGFR2 are sufficient to transduce shear stress, COS-7 African green monkey cells transfected with various combinations of plasmids encoding PECAM-1, VE-cadherin and VEGFR2 were subjected to flow. Transfected cells expressed all three receptors (Supplementary Fig. S5a, b). Untransfected COS-7 cells did not align after 16 h of shear (Fig. 3a, b), nor did they activate AKT after shorter periods of shear exposure (Fig. 3c). In contrast, cells co-expressing all three proteins activated AKT and aligned in the direction of flow (Fig. 3a–c). None of the single- or double-transfectants showed any response in these assays. Thus, PECAM-1, VE-cadherin and VEGFR2 represent the essential endothelial-specific components needed for the alignment of cells by fluid flow.

VEGFR2 binds PI(3)K directly through phosphorylation sites at Tyr 801 and Tyr 1175 on the receptor, leading to the phosphorylation and activation of PI(3)K[19]. To test whether VEGFR2 interacts directly with PI(3)K in shear stress signalling, a Y801F-Y1175F double mutant was examined in COS-7 cells. This mutant receptor failed to induce AKT activation or cell alignment (Fig. 3b, c), suggesting that PI(3)K interacts directly with activated VEGFR2. To further explore intermolecular associations, PI(3)K immunoprecipitates were analysed. VEGFR2 associated with PI(3)K only in cells that expressed VE-cadherin (Fig. 3d). Shear stress also induced association of PECAM-1 and β-catenin with the PI(3)K p85 subunit in a VE-cadherin-dependent manner (Fig. 3e). The association between VE-cadherin and VEGFR2 is thought to be indirect, mediated by β-catenin[20,21]. β-catenin$^{-/-}$ endothelial cells failed to show increased WOW-1 binding under flow (Supplementary Fig. S6). These results indicate that VE-cadherin and its binding partner β-catenin are required for the formation of signalling complexes that correlate with PI(3)K activation. Thus, VE-cadherin appears to function as an adaptor protein within this complex.

Elevated NF-κB activity and expression of NF-κB-dependent genes, including adhesion molecules such as ICAM-1, VCAM-1 and E-selectin, occurs in regions of disturbed flow before other indications of atherosclerosis and is believed to contribute to recruitment of monocytes to the nascent plaque[22,23]. As NF-κB activation and ICAM-1 expression depend on the integrin activation pathway *in vitro* (ref. 3), we examined NF-κB and ICAM-1 at sites of disturbed flow *in vivo* in mice. Wild-type aortas had strong staining for ICAM-1 and nuclear translocation of NF-κB at branch points (Fig. 4), consistent with published data[22]; staining was undetectable in nearby areas where flow is laminar. In contrast, areas near branch points in the PECAM-1$^{-/-}$ aortas showed no detectable activation of

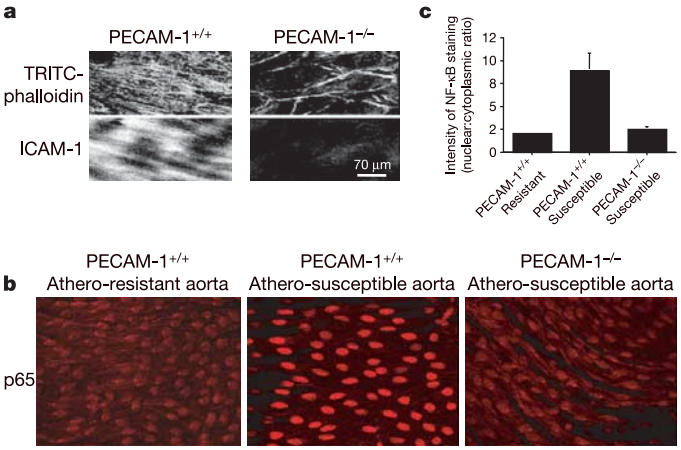

Figure 4 | Responses in PECAM-1$^{-/-}$ mice. a, **b**, Fixed aortas from wild-type and PECAM-1$^{-/-}$ mice were stained for F-actin (using TRITC-phalloidin) and ICAM-1 (**a**), and the p65 subunit of NF-κB (**b**). Images in panel **b** show an athero-resistant region or an athero-susceptible region of the aorta. **c**, NF-κB staining was quantified by determining nuclear and cytoplasmic intensity. Values are mean ± s.e.m.; $n = 3$; 30–60 cells analysed for each area per aorta.

NF-κB or expression of ICAM-1. Additionally, wild-type aortas showed extensive actin stress fibres across the cell, whereas PECAM-1$^{-/-}$ aortas had most of the actin in a circumferential ring (Fig. 4). Thus, flow pathways that are dependent on integrin activation are impaired in PECAM-1$^{-/-}$ mice.

Fluid shear stress triggers the conformational activation of integrins, which mediates both the alignment of endothelial cells in laminar shear and the activation of NF-κB in response to changes in shear[2–4]. We have identified a mechanosensory complex comprised of PECAM-1 (which activates Src), VE-cadherin (which functions as an adaptor) and VEGFR2 (which activates PI(3)K). PI(3)K most probably leads to integrin activation through a conserved pathway shared with other cell types[10]. These events occurred within 15 s, consistent with a direct event. The PECAM-1 cytoplasmic domain can bind Src directly[24], suggesting that changes in this interaction in response to force may mediate mechanotransduction. These proteins represent the essential endothelial-cell-specific components for this pathway. Finally, PECAM-1$^{-/-}$ mice show defects in both F-actin organization and activation of the NF-κB pathway, demonstrating its relevance *in vivo*.

Subconfluent endothelial cells still migrate selectively in the direction of flow[25] and non-endothelial cell types activate a subset of known flow-responsive pathways[26,27], so other mechanisms for shear stress sensing must exist. However, our data define a mechanosensing pathway that is critical for an important subset of known responses to flow. We note that polymorphisms in the PECAM-1 gene are associated with both decreased and increased incidences of artery disease[28]. Therefore, investigating the effects of these mutations on PECAM-1 function in shear stress signalling may be worthwhile.

METHODS

Inhibitors, antibodies and dyes. VEGFR tyrosine kinase inhibitor (VTI, 4-[(4′-chloro-2′-fluoro)phenylamino]-6,7-dimethoxyquinazoline), PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine), SU6656, wortmannin and LY294002 were from Calbiochem. Anti-phosphotyrosine (4G10) and anti-PI(3)K p85 antibodies were from Upstate Biotechnology. The BV9 monoclonal antibody to VE-cadherin[15] and a polyclonal anti-VE-cadherin antibody were from BDBiosciences, the anti-β-catenin antibody was from Sigma, the monoclonal antibody to PECAM-1 was a gift from P. J. Newman. The anti-phospho-Src[pY 418], anti-phospho-Src[pY 527], anti-phospho-AKT[pS 473] and anti-phospho-VEGFR2[pY 1054] antibodies were from Biosource International. The anti-ICAM-1 antibody was from Zymed Laboratories, the 5D2-27 rat anti-mouse-CD44 antibody was from the Developmental Studies Hybridoma Bank, the antibody against the p65 subunit of NF-κB was from Transduction Laboratories, tetramethylrhodamine isothiocyanate (TRITC)- and fluorescein isothiocyanate-labelled goat anti-rabbit antibodies were from Jackson Immunoresearch. CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) dye was from Molecular Probes.

Cell culture, shear stress assays and transfections. Bovine aortic endothelial cells were cultured as previously described[2]. VE-cadherin$^{-/-}$ cells and cells reconstituted with human VE-cadherin were prepared as described[20]. PECAM-1$^{-/-}$ cells and cells reconstituted with full-length PECAM-1 were prepared as described[29,30]. Levels of PECAM-1 and VE-cadherin in reconstituted cells are similar to wild-type levels[20,29,30]. Bovine aortic endothelial cells were subjected to shear stress at 12 dyn cm^{-2} in a parallel-plate flow chamber[2–4]. Transfections were performed as described[2]. The PECAM-1 complementary DNA in the pcDNA3 vector was a gift from P.J. Newman.

Alignment experiments. Confluent cells were subjected to flow for 16 h. In mixing experiments, reconstituted VE-cadherin endothelial cells were labelled with CellTracker Green CMFDA according to the manufacturer's instructions, mixed with unlabelled VE-cadherin$^{-/-}$ cells at a ratio of 100:1 and subjected to shear stress for 16 h, then fixed and stained with TRITC-phalloidin.

Fluorescence microscopy. Staining of cells was carried out as described[2]. Images of fixed cells were acquired using a 1024 confocal microscope (BioRad).

Luciferase activity assays. Luciferase activity assays were performed as described[3] using a vector (1.0 μg) containing the PDGF-A-chain shear stress response element (PDGF-A/SSRE) regulating the expression of firefly luciferase, together with *Renilla* luciferase, under the control of a minimal promoter[14]. Cell cultures were electroporated by using the Electro cell manipulator (EC100, Fisher Scientific).

Immunoprecipitations. Cells were washed in ice-cold PBS containing Ca^{2+} and Mg^{2+} and collected in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_3VO_4, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 1 mM aprotinin, 1 mM phenylmethylsulphonyl fluoride, 1 μg ml^{-1} leupeptin, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate). Samples were precleared with protein A or G sepharose (Pharmacia) for 1 h at 4 °C. Supernatants were then incubated with protein A or G sepharose previously conjugated with the appropriate rabbit or mouse antibody for 1 h at 4 °C under continuous mixing. Samples were washed three times in lysis buffer, and immune complexes were eluted in SDS sample buffer.

Direct force application. Magnetic beads (4.5 μm in diameter) conjugated with goat-anti-mouse IgG (Dynabeads; Dynal) were treated with 30 μg ml^{-1} anti-PECAM-1, anti-CD44 or anti-VE-cadherin antibodies for 1 h and washed three times with PBS. Beads (2×10^7) were added for 30 min to sparsely plated cells that had been transiently transfected with 0.5 μg of the construct encoding the GFP–AKT PH fusion protein. A magnet was passed over the cells for 15 s. Cells were fixed in formaldehyde and stained with the WOW-1 antibody Fab fragment and TRITC-anti-mouse IgG. In control experiments, cells were stained with rhodamine-phalloidin. The apparatus used to apply a high magnetic field gradient to the beads was comprised of eight cylindrically shaped NdFeB permanent magnets of 0.5 inches in diameter and 0.5 inches in length (part number 0013; Forcefield; http://www.wondermagnet.com/). Pairs of magnets were combined to make four units 0.5 inches wide and 1 inch long. These units were mounted in a lucite holder to form two columns so that a glass slide covered with cells could be passed through a gap. The magnet poles were configured so that the two magnets above the slide had their poles attracting each other, and the magnets located directly below the slide had their pair of poles oriented so as to produce repulsion between the top and the bottom set of magnets.

Staining aortas. Wild-type and PECAM-1$^{-/-}$ C57BL/6 female mice were deeply anaesthetized with a mixture of ketamine and xylazine, and perfused with 20 ml of 0.9% NaCl into the ascending aorta for 1 min, followed by 100 ml of 4% paraformaldehyde for 5 min. The whole aorta was then removed and continually fixed within 4% paraformaldehyde for 4 h. The fixed aortas were kept in PBS at 4 °C until staining.

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