The role of heterodimerization between VEGFR-1 and VEGFR-2 in the regulation of endothelial cell homeostasis


Digital Object Identifier (DOI):
10.1038/ncomms1977

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Nature Communications

Publisher Rights Statement:
This work is licensed under a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/3.0/

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
VEGF-A activity is tightly regulated by ligand and receptor availability. Here we investigate the physiological function of heterodimers between VEGF receptor-1 (VEGFR-1; Flt-1) and VEGFR-2 (KDR; Flk-1) (VEGFR-1−2) in endothelial cells with a synthetic ligand that binds specifically to VEGFR-1−2. The dimeric ligand comprises one VEGFR-2-specific monomer (VEGF-E) and a VEGFR-1-specific monomer (PIGF-1). Here we show that VEGFR-1−2 activation mediates VEGFR phosphorylation, endothelial cell migration, sustained in vitro tube formation and vasorelaxation via the nitric oxide pathway. VEGFR-1−2 activation does not mediate proliferation or elicit endothelial tissue factor production, confirming that these functions are controlled by VEGFR-2 homodimers. We further demonstrate that activation of VEGFR-1−2 inhibits VEGF-A-induced prostacyclin release, phosphorylation of ERK1/2 MAP kinase and mobilization of intracellular calcium from primary endothelial cells. These findings indicate that VEGFR-1 subunits modulate VEGF activity predominantly by forming heterodimer receptors with VEGFR-2 subunits and such heterodimers regulate endothelial cell homeostasis.
vascular endothelial growth factor-A (VEGF-A), a multifunctional cytokine induced by hypoxic stress, initiates the assembly of endothelial cells into new blood vessels, a process known as angiogenesis, via the activation of two receptor tyrosine kinases (RTKs), VEGF receptor-1 (VEGFR-1; Flt-1) and VEGFR-2 (KDR; Flk-1).

VEGF-A levels must be maintained within a narrow concentration range to ensure proper cardiovascular development and survival of the embryo\(^5\). The effects of VEGF-A can be deleterious if uncontrolled. Excess VEGF-A increases leakiness of blood vessels, which can cause severe oedema, loss of limb and death in animals\(^6\). Overexpression of VEGF-A in skeletal muscle causes haemangiomas (vascular tumours)\(^7\). By contrast, reduced VEGF-A activity in preeclampsia\(^8\), due to increased production of soluble VEGF-1 (sFlt-1)\(^9\), decreases angiogenesis\(^10\).

The varied functional activities of VEGF-A necessitate several regulatory mechanisms. In addition to sFlt-1 controlling VEGF activity\(^11\), membrane-bound VEGF-1 homeric receptor negatively regulates VEGF-2-mediated endothelial cell proliferation\(^12\) and tumour growth\(^13\). VEGF-1 can transactivate VEGFR-2 (ref. 19) and an absolute requirement for VEGF receptor subtype interaction exists during the development of different blood vessels in the zebrafish embryo\(^20\). Heteromerization of receptor subunits is commonly observed for G-protein-coupled receptors and in RTK systems\(^21\). Heteromerization has been identified in the platelet-derived growth factor (PDGF)\(^22\) and epidermal growth factor (EGF)\(^23\) systems. Moreover, the EGF receptor subunit heterodimerizes with the PDGF-β-receptor subunit in unstimulated cells and is required for PDGF-mediated ERK1/2 phosphorylation\(^24\). The role of heterodimer receptors within the structurally related VEGF RTK system is ill-defined. Pre-assembled heterodimeric receptors of VEGFR-2 and VEGF-3 subunits have been isolated from primary lymphatic endothelial cells\(^25\) and detected in situ on angiogenic sprouts\(^26\). Heterodimerization between VEGF-1 and VEGF-2 subunits (VEGFR\(_1\)-\(_2\)) has been detected in cell-free systems\(^13\) and in endothelial cell lines\(^19\). A computational model of VEGF receptor subunit dimerization concluded that a tenfold excess of one VEGF receptor subunit would result in minimal homodimerization of the less abundant receptor\(^27\). On the endothelial cell surface, subunits of VEGFR-1 are up to tenfold less abundant than those of VEGFR-2 (refs 35,36). This observation implies that the normal physiological state for VEGFR-1 subunits within the endothelium may be as part of heterodimers with VEGFR-2, and that VEGFR-1 in homodimeric form may be relatively rare.

Extensive elucidation of VEGF receptor homodimeric-specific function has been possible owing to the existence of VEGF family ligands that act selectively on the individual receptor homodimers. VEGF-B and PlGF only bind to VEGFR-1, whereas VEGF-E, a non-mammalian viral protein, is a selective agonist for VEGFR-2. The native forms of VEGF-C and VEGF-D are VEGFR-3-specific. In vivo, VEGFR\(_1\)-\(_2\) is activated by VEGF-A and the VEGF-A:PlGF-1 heterodimer, but these ligands also simultaneously activate VEGF homodimeric receptors. Currently, no natural ligands are known that exclusively activate VEGFR\(_1\)-\(_2\) without activating VEGFR-1 homodimers. Therefore, dissection of VEGFR\(_1\)-\(_2\) heterodimeric-specific function has been challenging. Receptor subunit knockdown and use of cell lines expressing specific receptors indicate that prostacyclin release\(^33\) and biphasic calcium induction\(^32\) is likely to be the result of VEGFR\(_1\)-\(_2\) activation.

To investigate the function of VEGFR\(_1\)-\(_2\) in endothelial cells, we have generated a novel, VEGFR\(_1\)-\(_2\)-specific ligand (VEGF-E:PlGF-1), comprising one monomer of VEGFR-2-specific ligand (VEGF-E) and one monomer of PlGF-1, a specific ligand for VEGFR-1. We show that the heterodimer mediates responses previously shown to involve VEGFR-1 homodimers and negatively regulates some VEGFR-2-mediated functions. Thus, VEGFR-1 subunits seem to regulate VEGF activity predominantly by forming heterodimer receptors with VEGFR-2 subunits to maintain endothelial cell homeostasis. Receptor nomenclature in this manuscript follows the recommendations from Ferre and co-workers\(^37\). A receptor is defined as the minimal functional, signal-transducing unit and, for RTKs, this normally comprises two subunits. Therefore, a ‘homomer/homodimer receptor’ is the minimal functional unit comprised of two or more of the same subunits that are not functional on their own.

Results

VEGFR-1 and VEGFR-2 form heterodimers in endothelial cells. Heterodimerization between VEGFR-1 and VEGFR-2 subunits in preassembled complexes has been identified in cell-free systems\(^13\) and in porcine\(^29\), bovine\(^30\) and murine\(^9\) endothelial cell lines. We confirmed VEGF receptor subunit heterodimerization in porcine aortic endothelial cells (PAEC) overexpressing human VEGFR-1 and VEGFR-2 (PAER-1:R-2), by immunoprecipitation (Fig. 1; Supplementary Fig. S1 for full-length blot). To date, evidence for VEGFR\(_1\)-\(_2\) heterodimers in primary human endothelial cells has been lacking. Using the immunoprecipitation procedure optimized with the PAEC we successfully demonstrated that VEGFR-1 and VEGFR-2 subunits do heterodimerize in an immortalized human endothelial cell line (HMEC-1) and in primary human endothelial cells (HUVEC) (Fig. 1b). In addition, VEGFR-2 could be detected in VEGF-1 immunoprecipitates (Fig. 1c) and co-localization of the two receptors in HUVEC was shown by confocal microscopy (Fig. 1d). Enzyme-linked immunosorbent assay (ELISA) further confirmed the presence of preassembled VEGFR\(_1\)-\(_2\) heterodimers in human endothelial cells (Fig. 1e) and in the mouse liver, lung and kidney (Fig. 1f).

Production and purification of a VEGFR\(_1\)-\(_2\)-specific ligand. To characterize the function of the VEGFR\(_1\)-\(_2\) heterodimer, we engineered a dimeric ligand that exclusively binds to this receptor. The ligand was produced using the insect cell-baculovirus system and comprises one monomer of VEGFR-2-specific VEGF-E and one monomer of His-Tagged, VEGFR-1-specific, human PlGF-1. VEGF-E is a viral protein, identified in a number of strains of the orb virus, which has the ability to specifically bind to and activate VEGFR-2 (ref. 38). The VEGF-E isoform used, in this study, was isolated from the D1701 strain\(^39\) and was engineered to contain the VEGF-A-heparin-binding domain\(^40\). This protein binds to both heparin and neuruplin-1 like VEGF-A.\(^40\) Purification of the novel recombinant heterodimeric ligand was achieved by sequential application of the medium to Ni-NTA and Heparin-Sepharose columns. The antibodies against VEGF-E and PlGF did not cross-react and the two proteins migrated differently (PlGF as two bands at 21 kDa and VEGF-E at 23 kDa; Fig. 2a). Both proteins were expressed after simultaneous infection of the same cells with both viruses (Fig. 2b) and ELISA demonstrated heterodimerization of VEGF-E subunits with those of PlGF-1 after the infection (Fig. 2c). Samples of the purified proteins of PlGF-1, VEGF-E:PlGF-1 and VEGF-E were run adjacently on SDS–PAGE under reduced and non-reduced conditions to ascertain protein size and reactivity with the PlGF and VEGF-E antibodies. Silver staining showed that the heterodimer preparation was ~95% pure (Fig. 2d). Under non-reducing conditions, the novel ligand migrated to an intermediate distance between PlGF-1 and VEGF-E at 43 kDa (Fig. 2d) and it reacted with antibodies against PlGF-1 and VEGF-E (Fig. 2d), confirming the purification of the heterodimeric ligand. To assess the binding capacity of the novel ligand to endothelial cells, PAER-1:R-2 were incubated with VEGF-E:PlGF-1 and binding detected using an anti-His-Tag antibody by fluorescence-activated cell sorting (FACS). Endothelial cells express PlGF, so the anti-His-Tag antibody was used to distinguish endogenous cell surface PlGF from...
the novel ligand. The presence of VEGF-E:PIGF-1 was demonstrated on the cell surface of PAER-1:R-2 (Fig. 2e, bottom right), but not on PAEC, which lack VEGF receptors (Fig. 2e, top left), PAER-1 (Fig. 2e, top right) or PAER-2 (Fig. 2e, bottom left), demonstrating that VEGF-E:PIGF-1 specifically binds to the VEGFR1-2 heterodimer receptor.

**VEGF-E:PIGF-1 induces VEGFR-2 and ERK1/2 phosphorylation.**

To assess whether the VEGF-E:PIGF-1 ligand increases VEGFR1-2 heterodimers to levels above those already preassembled, lysates of HUVEC stimulated with VEGF-E:PIGF-1 were immunoprecipitated with anti-VEGFR-2 and immunoblotted with anti-VEGF (Fig. 3a), or subjected to the human VEGFR1-2 ELISA (Fig. 3b). No increase in VEGFR1-2 was detected after addition of VEGF-E:PIGF-1 in either assay. It is possible that our assays were not sensitive enough to detect an increase in heterodimerization. However, these findings support the computational model of VEGF receptor dimerization, which states that when an excess of one receptor subunit is present, most of the less abundant receptor subunit will be present as preassembled heterodimers with it.

To assess whether the novel ligand could induce receptor phosphorylation, PAER-1:R-2 and HUVEC were stimulated with VEGF-A, VEGF-E and VEGF-E:PIGF-1. Lysates were immunoprecipitated with anti-VEGFR-2, or anti-VEGFR-1 and immunoblotted for phosphorylated tyrosine residues. Tyrosine phosphorylation of VEGF-E was detected after stimulation with VEGF-A, VEGF-E and VEGF-E:PIGF-1 in PAER-1:R-2 and in HUVEC (Fig. 3c,d). Stimulation of HUVEC with equimolar concentrations of VEGF-A and VEGF-E induced strong phosphorylation of ERK1/2 MAPK, whereas VEGF-E:PIGF-1 only induced a weak ERK1/2 phosphorylation (Fig. 3d). VEGF-E:PIGF-1 could not induce VEGF-E phosphorylation in PAER-2 cells (Fig. 3e) confirming the absence of contaminating VEGF-E homodimers in the ligand preparation and indicating that the presence of both receptors is required for the signalling activity of VEGF-E:PIGF-1. The PAER-1:R-2 cell line was produced by stably transfecting PAER-2 with human VEGF-1, thus PAER-2 are the parent cells of PAER-1:R-2 cells. However, when equal protein concentrations of lysates were probed for total VEGF-2, VEGF-2 levels were found to be lower in PAER-2 cells compared with PAER-1:R-2 cells (Fig. 3e).

**Figure 1 | VEGFR1-2 heterodimerization in human endothelial cells.** Lysates of (a) PAER-1 cells, and (b) HMEC-1 and HUVEC cells were immunoprecipitated with anti-VEGFR-2 or rabbit IgG and Western-blotted for VEGFR-1 or VEGFR-2. (c) PAER-1:R-2 cells and HUVEC were immunoprecipitated with anti-VEGFR-1 or mouse IgG and western-blotted for VEGFR-1 or VEGFR-2. (d) Confocal microscopy showing a representative image of VEGF-1 co-localized with VEGFR-2, in HUVEC. Scale bar, 20 µm. (e) VEGFR1-2 heterodimerization was assayed in cell lysates by ELISA. (f) VEGFR1-2 heterodimerization was assayed in lysates from mouse organs. Results are expressed as mean (± s.e.m.) of at least three independent experiments. **P < 0.01 versus PAER-2, ***P < 0.001 versus PAER-2.
Neuropilins have no role in VEGF-E:PlGF-1-induced signalling. The heparin-binding domain of VEGF confers the ability to interact with two co-receptors; neuropilin-1 and -2 (NRP-1 and NRP-2). Although, VEGF cannot induce neuropilin phosphorylation or signalling, neuropilins have been shown to associate with the VEGF receptors. This is reported to augment the functional response to VEGF in endothelial cells. To ascertain whether neuropilins have any role in heterodimer receptor function, we used siRNA to knockdown neuropilin-1 and neuropilin-2 in HUVEC and then stimulated with the ligand. Neuropilin-1 knockdown had no effect on VEGFR-2 phosphorylation after VEGF-A or VEGF-E:PlGF-1 administration (Fig. 3g) and knockdown of neuropilin-1 or neuropilin-2 had no effect on ERK1/2 phosphorylation (Fig. 3h). This suggests neuropilins are not having a major role for these signalling functions. (Verification of neuropilin-1 and neuropilin-2 knockdown can be seen in Supplementary Fig. 3c,d). Whether neuropilins are involved at a functional level warrants further enquiry.

Ligand and receptor dynamics. To assess cellular binding and trafficking of VEGF-E:PlGF-1, HUVEC treated with the ligand for 2.5–120 min, were stained with the anti-VEGF-E antibody without permeabilization. Confocal microscopy analysis demonstrated that VEGF-E:PlGF-1 bound to the cell but was undetectable by 30–60 min (Fig. 4a). Following 15 min of stimulation, VEGF-E:PlGF-1 was detected within the cell indicating internalization of the ligand (Fig. 4b). Interestingly, the staining pattern was strikingly different between cells stimulated with VEGF-E and those stimulated with VEGF-E:PlGF-1. VEGF-E seemed to be predominantly located in the nucleus, whereas VEGF-E:PlGF-1 was diffusely distributed throughout the intracellular compartment (Fig. 4b).

HUVEC were treated with VEGF-A, VEGF-E and VEGF-E:PlGF-1, and the distribution of the receptors evaluated using antibodies raised against the amino-termini of VEGFR-1 and VEGFR-2. VEGFR-1 expression remained stable at the cell surface throughout the stimulation period (Fig. 4c). By contrast, VEGFR-2 rapidly disappeared from the cell surface after stimulation with VEGF-E (Fig. 4c), whereas VEGFR-2 levels following stimulation with VEGF-A and VEGF-E:PlGF-1, which bind to VEGFR1-2, remained at the cell surface (Fig. 4c), further supporting to our earlier findings that the activation of VEGFR-1 can stabilize VEGFR-2 levels.

Functional consequences of VEGFR1-2 activation. Existing research using ligands specific for VEGFR-1 or VEGFR-2 homomeric receptors and cell lines expressing only a single receptor subunit type demonstrates that VEGF-A-mediated endothelial cell migration, and induction of VEGFR-1 promoter activity and tissue factor release, are VEGFR-2-mediated functions. However, the limitation of these tools is that they cannot fully delineate the contribution of VEGFR1-2 to these functions. Use of VEGF-E:PlGF-1 in a modified Boyden chamber assay demonstrated that VEGFR1-2 activation is involved in the migration of human primary endothelial cells (Fig. 4a). VEGF-E:PlGF-1 could not induce migration of PAER-2 (Fig. 4b) confirming the absence of VEGF-E phosphorylation was detected by either immunoprecipitation or VEGFR-1 phospho-tyrosine ELISA.
contamination in the purified fraction. VEGF-E:PIGF-1 induced activity of a VEGFR-1 promoter luciferase reporter construct in cells containing both VEGFR-1 and VEGFR-2 subunits, but not in cells only expressing VEGFR-2 (Fig. 5c). VEGF-E:PIGF-1 also caused release of sVEGFR-1 from HUVEC (Fig. 5d), demonstrating the VEGFR₁−₂ signal results in a regulatory function.

Capillary-like tube formation on growth factor-reduced Matrigel requires endothelial cell differentiation and survival. HUVEC were seeded on matrigel and stimulated with VEGF ligands for 18 h. VEGF-E:PIGF-1 significantly induced in vitro tube formation to similar levels as that observed with VEGF-A and VEGF-E (Fig. 5e,f) suggesting that VEGFR₁−₂ heterodimer signalling is important for angiogenesis. VEGF-A induces endothelial cell proliferation as a direct result of VEGF-2-mediated ERK1/2 MAPK phosphorylation. Stimulation of HUVEC with VEGF-A and VEGF-E resulted in a significant increase in proliferation (Fig. 5g) consistent with the observed strong ERK1/2 phosphorylation (Fig. 3d,g). By contrast, VEGF-E:PIGF-1 induced only weak ERK1/2 phosphorylation (Fig. 3d,g) and did not induce cell proliferation (Fig. 5g), indicating that VEGF-2 homodimer activation is required for the VEGF-mediated proliferative response.

Tissue factor expression and subsequent coagulation has been attributed to VEGF-2 (ref. 44). VEGF-E:PIGF-1 did not induce tissue factor expression, confirming the VEGF-2 specificity of this response (Fig. 5h).

VEGF-E:PIGF-1 modulates vessel reactivity. An important function of VEGF-A is the regulation of vascular tone. After preconstriction, VEGF-E:PIGF-1, PIgf-1 and VEGF-A caused vasorelaxation of arterial tissue (Fig. 6a). Relaxation could be abolished by co-incubation with NAME (Fig. 6b–d), confirming the role of endothelial-derived nitric oxide (NO) in vascular relaxation. Interestingly, activation of the VEGF-2 homodimer by VEGF-E did not significantly alter vascular tone within the same concentration range as the other VEGF ligands (≤1 nM; Fig. 6a); providing evidence, for the first time, that VEGF-A-induced vasorelaxation
may depend on the VEGFR-1 homodimer and/or the VEGFR-1-2 heterodimer and that the VEGFR-1 homodimer is not involved.

It is well established that VEGF-A is important for the release of the vasodilator NO, and that NO is crucial for VEGF-A-mediated angiogenesis. To ascertain whether VEGFR-1-2 has a direct role in NO release, supernatants from VEGF-E:PlGF-1 stimulated HUVEC were assayed for NO content, using a Sievers NOA 280 chemiluminescence analyser. VEGF-E:PlGF-1 induced NO release from HUVEC in a concentration-dependent manner (Fig. 7a) and stimulated eNOS activation as indicated by an increase in phosphorylation of eNOS at serine-1177 (Fig. 7b).

The VEGF-mediated release of prostacyclin from endothelial cells was previously attributed to VEGFR-1-2 activation. Consistent with this report, stimulation of HUVEC with VEGF-E:PlGF-1 induced a time-dependent and concentration-dependent increase in prostacyclin release as measured by the accumulation of its stable break-down product 6-keto PGF\(_{1\alpha}\) (Fig. 7c,d). However, our results also show that VEGF-A and VEGF-E promote much greater (>sixfold) 6-keto PGF\(_{1\alpha}\) release from HUVEC than VEGF-E:PlGF-1 (Fig. 7e). Moreover, VEGF-A induced a tenfold increase in 6-keto PGF\(_{1\alpha}\) release from PAER-2 cells while only eliciting a twofold release from PAER-1-R-2 cells (Fig. 7f). This finding suggests that VEGFR-2 homodimer activation is essential for strong VEGF-A-induced prostacyclin release and the presence of VEGFR-1 in PAER-2 cells negatively regulates prostacyclin release. To determine whether VEGFR-1-2 antagonizes the action of the VEGFR-2 homodimer receptor, we pre-treated HUVEC with VEGF-E:PlGF-1, stimulated them with either VEGF-A or VEGF-E and then assessed prostacyclin production (Fig. 8a). Interestingly, pre-incubation with VEGFR-1-2 significantly reduced the ability of VEGF-A and VEGF-E to stimulate prostacyclin release, whereas PlGF-1 had no inhibitory effect. These data suggest that the inhibitory signal for this response is exclusively via VEGFR-1-2.

Activation of VEGFR-2 mobilizes calcium from intracellular stores, and evidence derived from cell lines expressing specific VEGF receptor subtypes indicated that the heterodimer mediated induction of calcium release in a biphasic manner. In our study, VEGF-A caused a rapid, but transient calcium release in HUVEC (Fig. 8b). Pre-incubation of HUVEC with VEGF-E:PlGF-1 before VEGF-A addition delayed calcium mobilization in response to VEGF-A, and was followed by a slower and more-sustained release of calcium (Fig. 8b). Finally, pre-incubation of HUVEC with VEGF-E:PlGF-1 inhibited phosphorylation of ERK1/2 by VEGF-A and VEGF-E (Fig. 8c).

**Discussion**

This study demonstrates the existence of preassembled VEGFR-1-2 heterodimer receptors in human primary endothelial cells and animal tissues, and identifies their physiological function. Recent computer modelling of VEGF receptor subunit dimerization predicted that heterodimerization would be inevitable when cells express both VEGFR-1 and VEGFR-2 subunits. Furthermore,
the model demonstrated that an excess of one VEGF receptor subunit would result in minimal homodimerization of the less abundant receptor subunit. VEGFR-1 is known to be up to tenfold less abundant than VEGFR-2 on the endothelial cell surface, implying that the majority of VEGFR-1 in endothelial cells exists as preformed heterodimers and preferentially bind VEGF-A heterodimers and thereby stoichiometrically block the binding of other VEGF ligands.

To investigate the function of VEGFR1-2 heterodimers in endothelial cells, we generated a selective ligand, comprising one monomer of VEGF-E (VEGFR-2-specific ligand) and one monomer of PlGF-1 (VEGFR-1-specific ligand). Use of PAEC expressing VEGFR-1, VEGFR-2, or both receptor subunits, confirmed the specificity of the ligand for the heterodimer receptor in binding and function. In addition, the FACS binding assay discounted the possibility that the respective monomers of VEGF-E:PlGF-1 would bind to homodimer receptors and thereby stoichiometrically block the binding of other VEGF ligands.

The stimulation of murine lung capillary endothelial cells with VEGF-A was reported to increase the amount of VEGFR-1 associating with VEGFR-2 (ref. 19). In this study, we were unable to demonstrate an increase in receptor heterodimerization following ligand stimulation. This suggests that maximal heterodimerization occurs under basal conditions providing further evidence that the majority of VEGFR-1 subunits exist as preformed heterodimers with VEGFR-2 in HUVEC, as predicted by MacGabhann and Pope’s computational model. Although VEGF-A has a lower affinity for VEGFR-2 than VEGFR-1 (ref. 49), it is present in up to a tenfold excess of VEGFR-1. Therefore, VEGFR-1 subunits should exist as VEGFR1-2 heterodimers and preferentially bind VEGF-A
ahead of VEGFR-2 homodimers. We suggest that the presence of VEGFR-1-specific ligands such as PlGF and VEGF-B may be a mechanism for regulating VEGF receptor heterodimerization. Tyrosine phosphorylation of VEGFR-2, but not of VEGFR-1, could readily be detected after stimulation of VEGF-E:PlGF-1. Detecting VEGFR-1 phosphorylation consistently under native conditions is particularly difficult. The fact that VEGF-E:PlGF-1 induces a significant increase in VEGFR-2 phosphorylation without

**Figure 6 | Vasorelaxation induced by VEGF ligands in isolated rat aorta.** (a) VEGF-E:PlGF-1, PlGF-1 and VEGF-A, but not VEGF-E, cause vasorelaxation. The relaxation to (b) VEGF-A, (c) PlGF-1 and (d) VEGF-E:PIGF-1 is inhibited by Nω-Nitro-arginine methyl ester (l-NAME; 200 μM) supporting a role for NO in this response. Control (open circles), l-NAME (black circles). Vessels are precontracted with ECA α-phenylephrine and relaxation expressed as a percentage of the precontraction. Data points are means s.e.m. Results are expressed as mean (+ s.e.m.) of at least three independent experiments.

**Figure 7 | VEGF-E:PlGF-1 causes NO and prostacyclin release.** (a) Human umbilical vein endothelial cells (HUVEC) were rested overnight before stimulation with vehicle, VEGF-A or VEGF-E:PlGF-1 for 1 h and supernatant analysed for NO content using a Sievers noA 280 chemiluminescence analyzer. (b) HUVEC lysates were then subjected to SDS–PAGE and western-blotted with anti phospho-eNOS serine 1177 (p-eNOS (serine 1177)) and eNOS. (c) HUVEC were incubated with increasing concentrations of VEGF-E:PlGF-1 or VEGF-A, with VEGF-E:PlGF-1 (50 ng ml−1) for increasing time periods and 6-keto PGF1α was assessed in the supernatants by ELISA. **P < 0.01 versus vehicle. ***P < 0.001 versus vehicle. (d) Treatment with vehicle, VEGF-A, PlGF-1, VEGF-E and VEGF-E:PIGF-1 (VE:Pl). After 30 min, supernatant was collected and assayed for 6-keto-PGF1α by ELISA. **P < 0.01 or ***P < 0.0001 versus vehicle. (f) PAER-2 and PAER-1R-2 were rested for 24 h before incubation with VEGF-A for 30 min. Supernatant was collected and 6-keto PGF1α was assessed by ELISA. Results are expressed as mean (+ s.e.m.) of at least three independent experiments.
binding to VEGFR-2 homodimers demonstrates that the VEGFR-2 subunit undergoes tyrosine phosphorylation when dimerized with a VEGFR-1 subunit.

To assess the physiological effect of the VEGF-E:PlGF-1 heterodimer, we performed various in vitro assays with endothelial cells. The novel ligand mediated endothelial cell migration and VEGFR-1 promoter activity, and caused sustained in vitro tube formation to an extent comparable with VEGF-A and VEGF-E. These results suggest a role for VEGFR1-2 in endothelial cell differentiation and maintenance. VEGFR1-2 activation resulted in induction of ERK1/2 phosphorylation, but had no significant effect on cell proliferation, confirming previous findings that VEGF-A-induced proliferation is a VEGFR-2-mediated event41.

VEGF-A regulates vessel tone via NO production, which is dependent on eNOS activation. In addition, eNOS phosphorylation and NO release are crucial for VEGF-mediated angiogenesis16,50,51. VEGF-A, PlGF and VEGF-E:PlGF-1 caused significant relaxation of vessels precontracted with phenylephrine, whereas activation of the VEGFR-2 homodimer, using VEGF-E, did not increase vessel relaxation. This provides the first evidence that VEGF-A-induced vasorelaxation depends on the VEGFR-1 homodimer and/or the VEGFR1-2 heterodimer, and does not require the VEGFR-2 homodimer. Endothelial VEGFR1-2 activation also resulted in eNOS phosphorylation and NO release.

VEGF-A-induced permeability requires the release of prostacyclin, an event initially attributed to VEGFR-2 homodimer action, because PlGF does not increase prostacyclin release52. However, use of VEGF-C and antisense knockdown of VEGF receptors led Neagoe and colleagues33 to propose that VEGF-A-induced prostacyclin release was a VEGFR1-2-specific phenomenon. This study shows that VEGF-E, which exclusively activates the VEGFR-2 homodimer, induces ~15-fold increase in prostacyclin release from HUVEC. This indicates that VEGFR-2 homodimerization per se, can cause prostacyclin release from endothelial cells, whereas the magnitude of the VEGF-E:PlGF-1-induced increase in prostacyclin release was only threefold. This clearly demonstrates that although prostacyclin release can be mediated exclusively by the heterodimer receptor activation, it is predominantly a VEGFR-2 homodimer receptor function. Additionally, VEGF-A stimulation of PAER-2 induced a tenfold rise in prostacyclin release, whereas only a two-fold release was apparent after VEGF-A stimulation of PAER-1:R-2. This finding suggests that VEGFR-1 negatively regulates prostacyclin release from endothelial cells. The convoluted approach taken by Neagoe and colleagues33 to study VEGFR1-2 heterodimerization further illustrates the benefit of this specific ligand.

VEGF-A regulates its own bioactivity by a number of negative feedback systems; two examples are NO production14 and sVEGFR-1 release53. In this study, we have shown that both of these functions are positively mediated by VEGFR1-2 activation. Several groups have established that VEGFR-1 is a negative regulator of VEGF-2-mediated endothelial and tumour cell proliferation14–18. Thus, we hypothesized that the overall function of VEGF-1 forming heterodimeric receptors with VEGFR-2 may be to negatively regulate VEGF-2 homodimer function in endothelial cells. Preincubation of HUVEC with VEGF-E:PlGF-1 abrogated VEGF-A and VEGF-E-mediated prostacyclin release, whereas preincubation with PlGF-1 had no effect. In addition, preincubation of cells with VEGF-E:PlGF-1 delayed the normally rapid and transient VEGF-A-mediated mobilization of intracellular cellular calcium and the eventual rise in calcium entry was much slower, but more sustained. Rapid increases in intracellular calcium generally equate to release from intracellular stores, whereas slower rises in intracellular calcium are due to influx from an extracellular source. The data suggest that VEGF-A alone, via VEGFR-2 can activate these intracellular stores. In contrast, VEGFR1-2 activation elicits an inhibitory signal that delays this release.

A subsequent dimension to receptor heterodimer function is the regulation of the activating ligand, be that VEGF-A or VEGF-A:PlGF. For the endogenous heterodimeric ligand (VEGF-A:PlGF) to be produced, there must be co-expression of both proteins in the same cell. A highly relevant publication has demonstrated that when this does occur, in a mouse tumour model, branching angiogenesis persists and monocyte migration into the tumours is enhanced54, thus showing a biological relevance of the heterodimer ligand and receptor. However, the contribution of VEGF-A and/or VEGF-A:PlGF to heterodimer receptor activation and cell function is likely to
be regulated in a complicated spatial and temporal manner, which warrants future in-depth study in *in vivo* systems.

In conclusion, data gathered using VEGF-E:PlGF-1 demonstrate that VEGFR1-2 activation is functional in endothelial cells and that the heterodimer receptor serves as a negative regulator of the VEGFR-2 homodimer to regulate angiogenesis. This adds another layer of complexity to the regulation of VEGF-A function. It is possible that the low threshold homeostatic function of VEGF-A is achieved by the formation of the VEGFR1-2 heterodimer, whereas pro-angiogenic and proliferative function is mediated by VEGFR-2 homodimers. We conclude that heterodimerization between VEGFR-1 and VEGFR-2 subunits, negatively regulates both the signaling and cell response of the VEGFR-2 homodimer. This may be significant for angiogenesis and endothelial cell homeostasis. If validated in *in vivo*, this concept would be a dramatic paradigm shift in the current understanding of VEGF receptor biology. Future studies should establish whether this mechanism is apparent *in vivo* and if heterodimerization is dysregulated in pathologies.

**Methods**

**Reagents and antibodies.** Growth factors and receptors were purchased from RELIAtech (Brauschweig, Germany). Mouse anti-VEGFR-1 (Flk-11) was purchased from Sigma (Poole, UK). Mouse IgG, mouse anti-His-Tag, mouse anti-PY99 and rabbit anti-VEGFR-2 (C-1158) were obtained from Autogen Bioclear (Calne, UK). Rabbit polyclonal antibodies against ERK1/2, phospho-ERK1/2 (tyr 202/thr 204), Akt, phospho-Akt (ser 473), endothelial nitric oxide synthase (eNOS), and phospho-eNOS (ser 1177) were obtained from NEB (Hitchin, UK). Mouse goat anti-VEGFR-1 and VEGFR-2; and biotinylated goat anti-VEGFR-1, VEGFR-2 and phospho-tyrosine antibodies were purchased from R&D Systems (Abingdon, UK). Anti-PlGF (5 clone 172) and anti-VEGF-E (E2) were gifts from Dr Weich. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) thiazolyl blue was purchased from Sigma-Aldrich (Poole, UK) and growth factor-reduced Matrigel obtained from Becton Dickinson (Oxford, UK). SE121 cells, TC-100 and SF 90 II medium were purchased from Invitrogen (Paisley, UK).

**Cell culture.** HUVEC were isolated and cultured as described previously. Experiments were performed on third or fourth passage HUVEC. Human dermal microvascular endothelial cells (HMEC-1), immortalized by transformation with microvascular endothelial cells (HMEC-1), immortalized by transformation with HRAS neu (PAER-2) were maintained in medium supplemented with zeocin. SF 21 insect cells were expanded (PAER-2) were maintained in medium supplemented with G418. PAER-1:R-2 were Atlanta, USA. The PAEC expressing human VEGFR-1 (PAER-1) or VEGFR-2 (PAER-2) were maintained in medium supplemented with 5% FCS, then stimulated with growth factors for 96 h. MTT (5 mg ml⁻¹) was added and the cells incubated in the dark at 37 °C for 4 h. MTT was aspirated and reconstituted in dimethylsulphoxide. Optical density values were measured at 540 and 690 nm.

**Immunoprecipitation and western Blotting.** PAER-1:R-2, HMEC-1 and HUVEC lysed in radio immunoprecipitation assay buffer were immunoprecipitated using rabbit anti-VEGFR-2 (C-1158) or mouse anti-VEGFR-1. Membranes were incubated with mouse anti-VEGFR-1 (Flk-11), goat anti-VEGFR-1 and rabbit anti-VEGFR-2 (C-1158). After stimulations, HUVEC lysate was separated by 10% SDS-PAGE and membranes probed with rabbit polyclonal antibodies, against signalling molecules, at 4 °C over night. Supernatants from baculoviral infections were run on 15% gels and membranes probed with rabbit anti-PIGF-1 (1 in 100) or rabbit anti-VEGF-E (1 in 2,000). The same antibodies were used to detect PIGF and VEGF-E post purification.

**ELISA assay.** The ELISA for VEGFR1-2 used anti-VEGFR-1 capture antibodies against either, the human or mouse protein and biotinylated anti-VEGF-2 detection antibodies (R&D Systems). The ELISA for VEGF-E:PIGF-1 heterodimers used an anti-His-Tag capture antibody, an anti-VEGF-E detection and a goat anti-rabbit HRP conjugate. Phosphorylated VEGFR-2 was detected using a mouse anti-VEGFR-2 capture antibody and a biotinylated phospho-tyrosine detection antibody. The SVEGF-1 concentration in cell supernatants was determined as described previously. VEGFR-2 was measured in cell lysates using the VEGF-2 ECD DuoSet ELISA (R&D Systems). 6-keto PGF₁α, was measured in cell supernatants using the 6-keto PGF1α ELA kit (Biogenesis, Poole, UK).

**Expression and purification of recombinant VEGF-E:PIGF-1.** The VEGF-E isoform used was isolated from the D1701 strain and engineered to contain the VEGF-A/heparin-binding domain. The cloning, expression and purification of 6His-tagged PIGF-1 and heparin-binding VEGF-E using a baculoviral system has already been described. To produce VEGF-E:PIGF-1, 1 SF 21 insect cells were infected with baculoviruses encoding VEGF-E and PIGF-1. Clarified supernatant was then applied to a nickel chelating column (Ni-NTA; Qiagen, Crawley, UK) and bound proteins eluted with imidazole. The fractions containing PIGF-1 homodimers or VEGFR-1 heterodimers were identified by immunoblotting. VEGF-E-positive fractions were pooled, dialysed, loaded on a Heparin-Sepharose column (HiTrap Heparin; GE Healthcare, Amersham, UK), and eluted with a sodium chloride gradient. After immunoblotting fractions positive for both proteins were pooled, dialysed against PBS and used for *in vitro* experiments.

**FACS analysis.** PAEC were incubated with VEGF-E:PIGF-1 (0.5 µg per 10⁶ cells ml⁻¹). Binding was detected using an anti-His-Tag and then a secondary FITC-conjugated anti-mouse. Flow cytometry was performed using a FACSscan flow cytometer with CELLQuest software (Becton Dickinson, Oxford, UK) and the 488 nm argon laser.

**siRNA transfection.** HUVEC were electroporated with siRNA duplexes to VEGFR-1 (sense, 5′-UAGAUAGCCUUCACUGAAAtt-3′; antisense, 5′-UUCAUG UGAAGGCUCAUAtt-3′), VEGFR-2 (sense, 5′-GGAUUUCCGUAAGACGU Att-3′; antisense, 5′-UAUCGGCUUCAGAAUUCUAtt-3′), and PlGF-1 (sense, 5′-GUAAAGCUCCGAAGAAAtt-3′; antisense, 5′-UUUACUUGACCGAACCGATT-3′), PlGF-2 (sense, 5′-CCAGAAGAUUGCUCAUAtt-3′; antisense, 5′-GGUAGGACAACUUCUGGAtt-3′) or universal control siRNA (Dharmacon) using the HUVEC kit II and AMAXA nucleofector (Lonza, Germany).

**Real-time PCR.** Total RNA of cultured cells was isolated using the total RNA Purification Kit (Norgen Biotek Corporation, Thorold, Canada) and 1 µg RNA was reverse-transcribed. Real-time PCR was performed for neuropilin-1 (sense 5′-GCCTCCGCCTGAACTACCC-3′; antisense, 5′-TGAGTGCCCGCTGAGG-3′), neuropilin-2 (sense 5′-GCTGCAGGAAAGCCGAA; anti-sense GCCCCCAAGGAGGCGCCGAGT) and β-actin (5′-CTGCTTGCTGATGTTTG-3′) using SYBR green and SensiMix (JT DNA Kit (Quantace, London, UK) on a RotorGene 3000 (Corbett Research, Sydney, Australia). For each run, a standard curve was prepared alongside the sample complementary DNAs. Relative neuropilin-1/2 mRNA expression was calculated from the ΔΔCt values following normalization to β-actin levels. PCR products were run on 2% agarose gel to verify product size (neuropilin-1: 521 bp; neuropilin-2: 447 bp) and purity.

**Immunofluorescence imaging.** HUVEC were stimulated with growth factors and fixed with 4% (w/v) paraformaldehyde. To determine cell surface VEGFR-1 and VEGFR-2 expression after stimulations, fixed cells were incubated with primary antibodies goat-anti-VEGFR-1 (R&D Systems; 1 in 10), rabbit-anti-VEGFR-2 (Cell Signaling; 1 in 20), respectively, then FITC-labelled anti-goat (Sigma; 1 in 200) and TRITC-labelled anti-rabbit secondary antibodies (Cell Signaling; 1 in 200). To determine VEGF-E trafficking in HUVECs after stimulation, cells permeabilized with ice-cold methanol were incubated with rabbit-anti-VEGF-E antibody (ReliaTCH) then TRITC-labelled anti-rabbit secondary antibody (Sigma). To determine VEGFR-1 and VEGFR-2 colocalization, permeabilized HUVEC were incubated with both goat anti-VEGFR-1 and rabbit anti-VEGFR-2 primary antibodies and detected with FITC-labelled anti-goat and TRITC-labelled anti-rabbit secondary antibodies. Cells were counterstained with DAPI, and viewed using a Zeiss LSM 510 confocal scanning microscope (Carl Zeiss, Jena, Germany). Images were analysed using LSM 510 software (version 2.3; Carl Zeiss).

**In vitro migration.** Chemotaxis experiments with HUVEC, HMEC, PAEC and PBMC were performed in a modified Boyden chamber. Cells migrated towards growth factor gradients for 6 h at 8 mm peltier plate. Membranes were then fixed in methanol, stained with Diff-Quik (Harleco, USA) and cell migration quantified by counting 8–10 fields of view at X10 magnification.

**Coagulation assay.** Confluent HUVEC monolayers were stimulated with growth factors for 6 h, and a coagulation assay was performed and clotting times converted to tissue factor equivalents.

**In vitro tube formation assay.** Formation of capillary-like structures on growth factor reduced Matrigel was determined, as previously described. MTT assay. Confluent HUVEC were rested overnight in medium containing 5% FCS, then stimulated with growth factors for 96 h. MTT (5 mg ml⁻¹) was added and the cells incubated in the dark at 37 °C for 4 h. MTT was aspirated and reconstituted in dimethylsulphoxide. Optical density values were measured at 540 and 690 nm.
Myography. All experiments were performed according to the Animals Scientific Procedures Act 1986 (U.K. Home Office)35,36. Briefly, adult male Wistar rats (250–400g; Charles River, Margate, UK) were killed by cervical dislocation. The thoracic aorta was dissected free, cleaned and ~5 mm length sections mounted in a multi-myograph system (610M; Danish Myo Technology, Aarhus, Denmark) in Krebs buffer bubbled with 5% CO2/95% O2 at 37°C. A baseline tension of 14.7 mN was gradually applied over 10 min and vessels were allowed to equilibrate for a further 30 min before challenge with 800 mM KCl to assess viability. Phenylenephthrine was used to precontract vessels to ~80% maximum contraction (EC95 ~ 0.2 µM) for each individual aortic ring. Following preconcentration, cumulative concentration response curves to VEGF ligands (0.01–1 nM) were performed in separate vessel segments. Drugs were washed out and concentration response curves were repeated in the presence of 20 min preincubation and presence throughout concentration response curve of the nitric oxide synthase inhibitor Nω-Nitro-l-arginine methyl ester (l-NAME; 200 µM) and the cyclooxygenase inhibitor indomethacin (10 µM). The data from force transducers were processed by a MacLab/4e analog–digital converter and displayed through Chart software, version 3.4.3 (AD Instruments, Sussex, UK).

Analysis of NO release. Confluent HUVEC were incubated with growth factors in medium containing 0.2% BSA, for 1 h at 37°C. Total NO in the culture medium was assayed as nitrite, the stable breakdown product of NO, using a Sievers NO chemiluminescence analyzer (Analytix, Sunderland, UK).

Calcium imaging. HUVEC were grown to 90% confluency on gelatin-coated coverslips, and loaded with the calcium sensitive dye, Fura-2 AM (Molecular Probes, UK), for 30 min. After rinsing, growth factors were added and the change in calcium influx was dynamically assayed over a 2-min period.

Statistical analysis. All data are expressed as mean±s.e.m. Statistical comparisons were performed using one-way ANOVA followed by the Student—Newman–Keuls test as appropriate. Statistical significance was set at a value of P<0.05.

References


Acknowledgements
We thank Professor Christoph Dehio (Biozentrum of University Basel, Basel, Switzerland) for contributing the anti-VEGF-E antibody and Dr Padma-Sheela Jayaraman (Division of Immunity and Infection, University of Birmingham, UK) for her kind gift of the VEGFR-1 reporter construct. We would like to especially acknowledge Mr Steven McLean, Ms Maren Brockelmann and Mr Holger Bernhardt for their excellent technical assistance. This work was supported by the BHF (RG/09/001/25940) and the Medical Research Council (G0601295 and G0700288).

Author contributions

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Cudmore, M.J. *et al.* The role of heterodimerization between VEGFR-1 and VEGFR-2 in the regulation of endothelial cell homeostasis. *Nat. Commun.* **3**:972 doi: 10.1038/ncomms1977 (2012).

License: This work is licensed under a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/3.0/