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Heterodimerisation between VEGFR-1 and VEGFR-2 and not the homodimers of VEGFR-1 inhibit VEGFR-2 activity

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Abstract

Vascular endothelial growth factor (VEGF) signaling is tightly regulated by specific VEGF receptors (VEGF-R). Recently, we identified heterodimerisation between VEGFR-1 and VEGFR-2 ($VEGFR_{1\text{-}2}$) to regulate VEGFR-2 function. However, both the mechanism of action and the relationship with VEGFR-1 homodimers remain unknown. The current study shows that activation of $VEGFR_{1\text{-}2}$, but not VEGFR-1 homodimers, inhibits VEGFR-2 receptor phosphorylation under VEGF stimulation in human endothelial cells. Furthermore, inhibition of phosphatidylinositol 3-kinase (PI3K) increases VEGFR-2 phosphorylation under VEGF stimulation. More importantly, inhibition of PI3K pathway abolishes the $VEGFR_{1\text{-}2}$ mediated inhibition of VEGFR-2 phosphorylation. We further demonstrate that inhibition of PI3K pathway promotes capillary tube formation. Finally, the inhibition of PI3K abrogates the inhibition of *in vitro* angiogenesis mediated by $VEGFR_{1\text{-}2}$ heterodimers. These findings demonstrate that $VEGFR_{1\text{-}2}$ heterodimers and not VEGFR-1 homodimers inhibit VEGF-VEGFR-2 signaling by suppressing VEGFR-2 phosphorylation via PI3K pathway.

(138 words)

Key words: Vascular endothelial growth factor (VEGF), VEGF Receptors, heterodimers, phosphatidylinositol 3-kinase (PI3K), angiogenesis

Introduction

Patients with ischemic limb and cardiac disease suffer from vascular insufficiencies. There is, therefore, a need to increase blood flow and promote functional collateral circulation in these patients. The discovery of vascular endothelial growth factor (VEGF) as a stimulator of blood vessel formation provided a new avenue for the treatment of vascular insufficiencies. The VEGF signaling pathway promotes angiogenesis [1]. The major ligand VEGF-A binds to and activates VEGFR-1, VEGFR-2 and $VEGFR_{1-2}$ receptors. Unlike VEGF-A, placental growth factor (PlGF-1) specifically activates VEGFR-1, but not neuropilin or VEGFR-2, thus serves a unique tool for studying VEGFR-1 signaling (Fig. 1A) [2, 3]. Although PlGF-1 was demonstrated to regulate inter- and intramolecular cross talk between the VEGFR-1 and VEGFR-2, this regulation is mediated through an indirect pathway rather than direct binding to VEGFR-2 [4]. Similarly, VEGF-E, a virus-origin VEGF homologue, exclusively binds to and activates VEGFR-2, and not VEGFR-1 or VEGFR-3 (Fig. 1A) [5, 6, 7]. However, no natural ligands are identified that activate $VEGFR_{1-2}$ without activating VEGFR-1 homodimers.

The VEGF signaling pathway is tightly regulated as the process of angiogenesis regulates blood vessel formation and wound healing, and dysregulation leads to tumor growth and ischemic disease [8, 9, 10, 11]. VEGF-A levels are maintained within a narrow concentration range to ensure successful function [11, 12]. A soluble form of VEGFR-1 (sFlt-1) acts as a natural antagonist to VEGF-A and PlGF-1 to suppress VEGF signaling [13,

14]. Prior to the development of tools to study $VEGFR_{1-2}$ heterodimer function, it was reported that VEGFR-1 receptor acts as a negative regulator of VEGF signaling pathway. The activation of VEGFR-1 receptor limited trophoblast cell DNA synthesis [15], inhibited VEGFR-2 mitogenic activity [16], downregulated endothelial cell proliferation [17] and angiogenesis [18]. However, it is not clear whether VEGFR-1 homomeric or $VEGFR_{1-2}$ heteromeric receptor mediates the inhibition of VEGFR-2 activity. To dissect the function of $VEGFR_{1-2}$ in endothelial cells, we have generated a unique $VEGFR_{1-2}$-specific ligand VEGF-E:PlGF-1 (VE:Pl) that comprises of one monomer of VEGFR-2-specific ligand (VEGF-E) and one monomer of VEGFR-1-specific ligand (PlGF-1) [19]. A recent study revealed that the VEGF-E:PlGF-1 ligand specifically activates heterodimeric $VEGFR_{1-2}$ to inhibit VEGF-A-induced prostacyclin release, phosphorylation of ERK1/2 MAP kinase and mobilization of intracellular calcium in endothelial cells [19]. This inhibition is exclusively conducted through the $VEGFR_{1-2}$ heterodimer as VEGF-E:PlGF-1 does not activate VEGFR-1 or VEGFR-2 homodimers. We know this to be the case because we engineered porcine aortic endothelial (PAE) cells, which do not express VEGF receptors, to express either VEGFR-1 or VEGFR-2 [19]. In these cells, VEGF-E:PlGF-1 had no effect on VEGF receptor phosphorylation [19]. However, both the mechanism of how $VEGFR_{1-2}$ modulates VEGF signaling and the relationship with VEGFR-1 homodimers remain unknown. Our novel VEGF-E:PlGF-1 ligand which binds only to the $VEGFR_{1-2}$ heterodimer receptor (Fig. 1A) provides the unique opportunity to address the role of $VEGFR_{1-2}$.

Although VEGFR-1 signaling activates many pathways including phosphatidylinositol-3 kinase (PI3K), Akt, p38, and ERK1/2, PI3K appears to be a central regulator in specific cells [20]. Stimulation of VEGF in the cells exclusively expressing VEGFR-1 resulted in a strong increase of PI3K/Akt phosphorylation [21] and inhibition of VEGFR-1 activity suppressed PI3K-Akt signaling in mice [22]. Our previous study showed that VEGFR-1 activates the PI3K/Akt pathway to regulate nitric oxide release in endothelial cells [23]. Moreover, VEGFR-2 preferentially utilizes the PLCγ-PKC-MAPK pathway, but not PI3K/Akt, for signaling, further confirming the preference of PI3K in the VEGFR-1 mediated signaling pathway [24]. Therefore, we tested the possibility that $VEGFR_{1\text{-}2}$ heterodimer activates PI3K to modulate VEGFR-2 activity to negatively regulate VEGF signaling in endothelial cells.

In the present study, we demonstrated that the activation of $VEGFR_{1\text{-}2}$ inhibited, while inhibition of PI3K, promoted VEGFR-2 phosphorylation. Furthermore, inhibition of PI3K abrogated the $VEGFR_{1\text{-}2}$ mediated inhibition of VEGFR-2 phosphorylation. Finally, we confirmed functionally that the activation of $VEGFR_{1\text{-}2}$ significantly suppressed VEGFR-2 induced *in vitro* angiogenesis and this suppression could be abolished by inhibition of PI3K pathway.

Methods and Materials

Reagents and antibodies

Recombinant growth factors, VEGF-A, VEGF-E and PlGF-1, were purchased from RELIA*Tech* (Brauschweig, Germany). VEGF-E:PlGF-1 was generated in-house as described previously [19]. LY294002 was purchased from Sigma-Aldrich (USA).

Rabbit antibodies against phosphorylated VEGF receptor-2 at tyrosine Y951 and Y1175, phospho-tyrosine of VEGFR-2, phospho ERK1/2 (Thr-202/Tyr-204), phosphor-Akt (Ser-473), total ERK and Akt were obtained from Cell Signaling Technology (Danvers, MA 01923, United States). Rabbit antibodies anti-VEGF receptor-1 and 2 were purchased from Santa Cruz Biotechnology (Texas, USA). Mouse monoclonal anti-β-Actin antibody was obtained from Sigma-Aldrich (A5441, St.Louis, MO, Uinited States). Growth factor reduced Matrigel was purchased from Becton Dickinson (Oxford, UK). M199 and Ham F-12 medium were purchased from Invitrogen (Paisley, UK). Calcein AM Fluorescent Dye was purchased from BD Bioscience (P.O. Box 999 Sparks, MD, USA 21152). EZ-ECL kit was purchased from Geneflow Ltd (Staffordshire, UK).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Cellworks (Buckingham, UK) and cultured in M199 medium as described previously [18]. Experiments were performed on third or fourth passage HUVECs. The engineered stable porcine aortic endothelial cells (PAECs) containing human VEGF receptor 2 (PAER-2) or both VEGF receptor 1 and 2 (PAER-1:R-2) were maintained in Ham F-12 supplemented with 10 % FCS with the addition of G418 [25].

siRNA against VEGFR-1

The small interfering RNA (siRNA) against human VEGFR-1 and a mismatch universal control siRNA were described previously [19]. The sequences of siRNA duplex to VEGFR-1 are sense, 5′-UGAUGGCCUUACACUGAAAtt-3′; antisense, 5′-UUUCAGUGUAAGGCCAUCAtt-3′. The non-targeting control siRNA (D-001206-13) was purchased from Dharmacon.

In vitro cell transfection

The siRNA was transfected into HUVECs using an AMAXA nucleofector machine with Primary Endothelial Cells transfection kit (Cat. No. VPI-1001, Amaxa).Briefly, HUVECs were trypsinized, and $\approx 1 \times 10^6$ cells were electroporated with ≈ 3 μg siVEGFR-1 or control siRNA according to

manufacturer's instructions. Transfected cells were incubated overnight before treatment at 37°C incubator.

Western Blotting

The procedure for western blot was described previously [26]. Briefly, total protein from PAE cells or HUVECs was lysed in RIPA buffer. Protein concentration was analyzed using Bradford assay. 30 µg RIPA lysate was separated on 8-12 % SDS-PAGE and transferred into Hybond N+ membrane. Primary antibody diluted 1:1000 was used to incubate with the membrane for 4-6 h and then washed with TBS-T buffer three times of 15 mins. Secondary antibody diluted 1: 5000-10,000 was used to incubate with the membrane at room temperature for 1 h and then followed by three 20 mins washes. Finally, the membrane was exposed in a dark room for 1-5 mins using EZ-ECL kit. Densitometric analysis was performed across three independent experiments using ImageJ software. Protein densitometric levels were normalized to respective loading controls.

Cell proliferation assay

Cell proliferation assay was carried out using CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen). HUVECs were seeded with a density of 2000 cells/well in a 96-well plate and rested for overnight. The cells were subsequently pre-treated with LY294002, VEGF-E:PlGF-1 alone or in combination for 30 minutes. Following pre-treatment, cells were stimulated with VEGF-E:PlGF-1 (50 ng/ml), VEGF-E (20 ng/ml) alone or in combination for 48-72 hours. The cell number was determined by measuring cellular DNA

according to manufacturer's instruction. Fluorescence density values were measured with excitation at 485 nm and emission detection at 530 nm.

In vitro tube formation assay

Formation of capillary-like structures on growth factor reduced Matrigel was determined as previously described [18]. Briefly, cells (1 x 10^4 cells/well) were seeded onto a Matrigel-coated 96-well plate in culture medium containing 10% fetal calf serum (FCS). After cells had attached to the Matrigel (1 hours at 37°C), the FCS containing medium was removed and VEGF-A (20 ng/ml) or VEGF-E (20ng/ml) in medium containing 0.2% bovine serum albumin was added. After stimulation at 37°C for 6-8 hours, calcein AM Flurorescent Dye (BD Bioscience) was added with concentration of 4 μg/ml and incubated in 37°C for 30 mins for fluorescence microscope observation. Total tube length was calculated using Image-Pro plus software and data were collected from three independent experiments.

Statistical analysis

All data were collected from at least three independent experiments and expressed as a mean ± S.E.M. Statistical comparisons were performed using Student's t-test or one-way ANOVA. Statistical significance was set at a value of $p<0.05$.

Results

Activation of $VEGFR_{1-2}$ heterodimeric and not VEGFR-1 homomeric receptor inhibits VEGFR-2 receptor phosphorylation

The VEGFR-2 phosphorylation upon VEGF-A stimulation was measured at different time points. The phosphorylation of VEGFR-2 was detected 5 minutes after stimulation and remained for least 15 minutes without a significant decrease (Fig. S1). Thus, the following experiments, if not stated differently, were performed within a 5 to 10 minute range after growth factor stimulation.

To investigate whether deletion of VEGFR-1 affects VEGFR-2 activation, the phosphorylation of VEGFR-2 was measured in HUVECs with transfection of siVEGFR-1 under the stimulation of VEGF-A. HUVECs transfected with siVEGFR-1 showed an increase in phosphorylation at the two major tyrosine phosphorylation sites (Y951 and Y1175) in VEGFR-2 compared to the control siRNA transfected cells (Fig. 1B). This data implies that the presence of VEGFR-1 inhibits VEGFR-2 phosphorylation under VEGF stimulation.

As knock-down of VEGFR-1 affects both the VEGFR-1 homodimer and $VEGFR_{1-2}$ heterodimer, we further investigated whether the homodimer or heterodimer of VEGFR-1 negatively regulates VEGFR-2 phosphorylation. HUVECs were pre-treated with PlGF-1 (which only stimulates VEGFR-1 homodimer, Fig 1A) and subsequently stimulated with a vehicle or VEGF-E.

The phosphorylation levels of VEGFR-2 at tyrosine Y951 and Y1175 were measured by Western blot. As expected, PlGF-1 treatment did not cause phosphorylation of VEGFR-2 (Fig. 1C). Interestingly, cells pre-treated with PlGF-1 did not show any difference in VEGFR-2 phosphorylation at both tyrosine sites under VEGF-E stimulation compared to VEGF-E only treated cells (Fig. 1C). Therefore it demonstrated that activation of the VEGFR-1 homodimer does not affect VEGFR-2 phosphorylation. We went on to test whether activation of $VEGFR_{1-2}$ heterodimer negatively regulates VEGFR-2 phosphorylation using VEGF-E:PlGF-1 ligand (which only activates $VEGFR_{1-2}$ heterodimer, Fig 1A). HUVECs were either (i) pre-treated with VEGF-E:PlGF-1 ligand and then stimulated with VEGF-E; or (ii) simultaneously treated with VEGF-E:PlGF-1 and VEGF-E. These conditions were compared to VEGF-E only treated cells (Fig. 1D). VEGF-E:PlGF-1 pre-treatment significantly reduced VEGFR-2 phosphorylation at both Y951 and Y1175 sites compared to cells treated with VEGF-E alone. Interestingly, simultaneous treatment of VEGFE and VEGF-E:PlGF-1 had no inhibitory effect on VEGFR-2 phosphorylation (Fig. 1D). Notably, the phosphorylation level of VEGFR-2 was higher in the VEGF-E:PlGF-1 and VEGF-E co-treated cells compared to VEGF-E only treated cells, probably due to the additive effect caused by VEGF-E:PlGF-1 (Fig. 1D). Taken together, the data suggest that $VEGFR_{1-2}$ rather than VEGFR-1 homomeric receptor negatively regulates VEGFR-2 phosphorylation (Fig. 1E).

Inhibition of PI3K pathway increases VEGFR-2 phosphorylation

Evidence from earlier studies suggest that the PI3K signaling pathway is involved in the downstream signaling of VEGFR-1 to regulate VEGFR-2 activity [22, 23]. Thus we tested whether inhibition of PI3K could alter the phosphorylation level of VEGFR-2 under stimulation of VEGF-A, VEGF-E and VEGF-E:PlGF-1 separately or in combination. HUVECs were pre-treated with the PI3K inhibitor LY294002 and subsequently treated with VEGF-A. VEGFR-2 phosphorylation levels at both Y951 and Y1175 sites were significantly increased in the cells pre-treated with LY294002 (Fig. 2A).

Since VEGF-A can activate both VEGFR-1 homodimeric and $VEGFR_{1\text{-}2}$ heterodimeric receptors, we further investigated which receptor was signaling through PI3K to prevent VEGFR-2 phosphorylation. As before, HUVECs were pre-treated with LY294002 and PlGF-1 separately or in combination. The pre-treated cells were subsequently stimulated with VEGF-E. Not surprisingly, the phosphorylation level of VEGFR-2 in the cells pre-treated with LY294002 and PlGF-1 did not show a significant difference compared to cells pre-treated with PlGF-1 alone (Fig. 2B). However, VEGF-E stimulation induced a dramatic increase in phosphorylation of VEGFR-2 at both Y951 and Y1175 sites in HUVECs co-pre-treated with LY294002 and VEGF-E:PlGF-1 compared to those only pre-treated with VEGF-E:PlGF-1 (Fig. 2C). This data suggests that $VEGFR_{1\text{-}2}$ heterodimeric and not VEGFR-1 homodimer receptor regulate VEGFR-2 phosphorylation via PI3K (Fig. 2D).

Inhibition of PI3K abrogates the VEGF-E:PlGF-1

mediated inhibition of VEGFR-2 signaling

Since our data suggest that $VEGFR_{1-2}$ heterodimeric receptor regulates VEGFR-2 phosphorylation via PI3K, we tested whether inhibition of PI3K could abrogate the VEGF-E:PlGF-1 mediated inhibition of VEGFR-2 signaling. Firstly, we confirmed that pre-treatment of VEGF-E:PlGF-1 is capable of activating PI3K/Akt signaling pathway as measured by Akt (Ser473) phosphorylation in endothelial cells (Fig. 3A, 1st panel, red squares). Densitometric analysis of phospho-Akt indicated that VEGF-E:PlGF-1 significantly induced Akt phosphorylation and the addition of LY294002 dramatically decreased Akt phosphorylation level ($P<0.01$, Fig. 3B). As expected, phosphorylation of VEGFR-2 at Y951 and Y1175 under VEGF-A and VEGF-E stimulation was reduced in the HUVECs pre-treated with VEGF-E:PlGF-1 compared to those without pre-treatment (Fig. 3A, 3rd and 4th panels). However, the reduction of VEGFR-2 phosphorylation was abolished, at least partially, in the cells additionally pre-treated with LY294002 (Fig. 3A, 3rd and 4th panels, red square). The densitometric analysis revealed that phosphorylation of VEGFR-2 at Y951 and Y1175 in HUVECs co-pre-treated with VEGF-E:PlGF-1 and LY294002 was significantly increased compared to cells pre-treated with VEGF-E:PlGF-1 under VEGF-A stimulation ($P<0.001$, Fig. 3C and D). Moreover, HUVECs receiving LY294002 pre-treatment also exhibited a significant increase in phosphorylation of VEGFR-2 at Y1175 under VEGF-E stimulation ($P=0.0003$, Fig. 3D). A similar trend was observed on Y951 although not significant ($P=0.18$, Fig. 3C). Furthermore, we examined the level of total phospho-tyrosine of VEGFR-2 under similar

treatment conditions. HUVECs pre-treated with VEGF-E:PlGF-1 exhibited a notable reduction in total phospho-tyrosine of VEGFR-2 under VEGF-E stimulation compared to those without pre-treatment. The inhibition was lost when cells were simultaneously stimulated with VEGF-E and VEGF-E:PlGF-1 (Fig. 3F, red square). As expected, HUVECs which had been co-pre-treated with VEGF-E:PlGF-1 and LY294002 showed increased total phospho-tyrosine of VEGFR-2 compared to cells pre-treated with VEGF-E:PlGF-1 only (Fig. 3F, the 5^{th} and 7^{th} lanes). Taken together, the data implies that tyrosine residues on VEGFR-2, including Y951 and Y1175, may be involved in the $VEGFR_{1\text{-}2}$ mediated inhibition of VEGFR-2 activation.

ERK is the downstream effector in VEGFR-2 mediated signaling [27]. We further investigated the ERK phosphorylation under pre-treatment of VEGF-E:PlGF-1 and LY294002. Phosphorylation of ERK was reduced in the HUVECs pre-treated with VEGF-E:PlGF-1 under VEGF-E stimulation compared to non-pre-treated cells. The reduction in ERK phosphorylation was abrogated by additional pre-treatment with LY294002 (Fig 3A, 6^{th} and 7^{th} panel, red square). The densitometric analysis further confirmed that HUVECs co-pre-treated with VEGF-E:PlGF-1 and LY294002 showed a significant increase in ERK1/2 phosphorylation compared to cells pre-treated with VEGF-E:PlGF-1 only both under VEGF-A and VEGF-E stimulation ($P<0.05$, Fig. 3E).

Furthermore, we tested our concept in the PAE cells which were engineered to only express VEGFR-2 receptors [19]. PAE-R2 cells co-pre-treated with VEGF-E:PlGF-1 and LY294002 did not show a significant difference in the

phosphorylation of VEGFR-2 under VEGF-E stimulation compared to VEGF-E:PlGF-1 pre-treated cells (Fig. 3G). The densitometric analysis also showed no significant difference between cells pre-treated with VEGF-E:PlGF-1 and LY294002 and those pre-treated with VEGF-E:PlGF-1 only (Fig. 3H). Notably, a VEGF-E:PlGF-1 stimulation did not change the phosphorylation level of VEGFR-2 compared to vehicle control (Fig. 3G, 1st and 3rd lanes). This data confirm that VEGF-E:PlGF-1 does not activate VEGFR-2 homodimers as reported earlier [19].

Inhibition of PI3K enhances growth factor-mediated angiogenesis

Previous data demonstrated that PI3K regulates VEGFR-2 and ERK phosphorylation [28, 29]. We therefore tested whether inhibition of PI3K could enhance endothelial cell biological function. As a main feature of endothelial cells, the *in vitro* angiogenic ability was investigated in the cells with inhibited PI3K. PAER-1:R-2 cells were pre-treated with LY294002 and plated on Matrigel under stimulation of VEGF-A (Fig. S2A). The total tube length of cells pre-treated with LY294002 was significantly increased compared to non-pre-treated cells (Fig. S2B), further confirming that inhibition of PI3K could enhance endothelial cell biological function.

Inhibition of PI3K partially abrogates VEGF-E:PlGF-1

mediated inhibition of cellular activities

To prove VEGF-E:PlGF-1 acts through PI3K to inhibit VEGFR-2 activity, we investigated whether inhibition of PI3K could abrogate the VEGF-E:PlGF-1 mediated inhibition of VEGFR-2 biological activity in endothelial cells. HUVECs were seeded with a density of 2000 cells/well in a 96-well plate and pre-treated with LY294002 and VEGF-E:PlGF-1, alone or in combination. Following pre-treatment, cells were stimulated with VEGF-E or in combination with VEGF-E:PlGF-1 for 48-72 hours. The cell numbers were measured by cellular DNA density after stimulation. The cell number in the cells pre-treated with VEGF-E:PlGF-1 was significantly reduced compared to cells without pre-treatment under VEGF-E stimulation (Figure 4A, 3rd and 7th bars). Interestingly, the cells simultaneously stimulated with VEGF-E:PlGF-1 and VEGF-E also exhibited significant reduction in cell number compared to cells stimulated with VEGF-E only (Figure 4A). However, the additional pre-treatment of LY294002 abrogated the reduction in cell number and increased cell number significantly to a level similar to VEGF-E alone stimulated cells (Figure 4A, 3rd and 9th /10th bars).

To further test our theory in angiogenesis, HUVECs were pre-treated with VEGF-E:PlGF-1 alone or with LY294002 and subsequently plated on Matrigel under stimulation of VEGF-E (Fig. 4B). The total tube length formed in the cells pre-treated with VEGF-E:PlGF-1 was decreased under VEGF-E stimulation compared to non-pre-treated or VEGF-E:PlGF-1 and VEGF-E simultaneously treated cells (Fig. 4C). In line with our hypothesis, HUVECs

co-pre-treated with LY294002 and VEGF-E:PlGF-1 showed a significant increase in total tube length compared to cells only pre-treated with VEGF-E:PlGF-1 only under VEGF-E stimulation (Fig. 4C, 5th and 6th bars). The data confirmed that inhibition of PI3K by LY294002 restores the tube formation to a comparable level to cells treated with VEGF-E and VEGF-E:PlGF-1 simultaneously (Fig. 4C, the 4th and 6th bars). Interestingly, total tube length in the VEGF-E:PlGF-1 and VEGF-E simultaneously treated cells was significantly decreased compared to VEGF-E treated cells (P=0.01, Fig. 4C, 2nd and 4th bars). Notably, the simultaneous treatment of VEGF-E:PlGF-1 and VEGF-E did not show an inhibitory effect on VEGFR-2 phosphorylation (Fig. 1D). The inhibition of tube formation in simultaneously treated cells was probably due to the prolonged incubation with VEGF-E:PlGF-1 (6-8 hours to 5-10 minutes).

Discussion

VEGF-A has a lower affinity for VEGFR-2 but this receptor is responsible for endothelial cell proliferation and migration [30], whereas VEGFR-1 homomeric receptor is reported to negatively regulate VEGF signaling [15, 16, 17, 18]. The density of VEGFR-1 on the cell surface is less than tenfold compares to those of VEGFR-2 [31] and therefore the majority of VEGFR-1 subunits form $VEGFR_{1\text{-}2}$ heteromeric receptors [32]. The role of VEGF signaling via the $VEGFR_{1\text{-}2}$ has eluded us due to the lack of a ligand that specifically binds to $VEGFR_{1\text{-}2}$ receptor. The generation of VEGF-E:PlGF-1 ligand by our group provided the opportunity to dissertate the $VEGFR_{1\text{-}2}$ receptor function [19]. To the best of our knowledge, this study demonstrated for the first time that activation of $VEGFR_{1\text{-}2}$ decreased VEGFR-2 phosphorylation under VEGF stimulation. This is the first direct evidence to demonstrate the interplay between VEGFR-1 and VEGFR-2 receptor-mediated signaling pathways in endothelial cells. It has been demonstrated that VEGF, through VEGFR-1 stimulates PI3K [21, 33, 34, 35]. However it is not clear whether PI3K is activated by VEGFR-1 homomeric receptor or $VEGFR_{1\text{-}2}$ heteromeric receptor. In this study, we identified that $VEGFR_{1\text{-}2}$ receptor and not VEGFR-1 homomeric receptor activates PI3K to suppress VEGFR-2 receptor activity and the inhibition of PI3K could restore the $VEGFR_{1\text{-}2}$ receptor mediated inhibition of VEGFR-2 activity.

The mechanism by which $VEGFR_{1\text{-}2}$ inhibits VEGFR-2 activity is still not clear. Our study confirms the ability of $VEGFR_{1\text{-}2}$ itself to induce biological signaling

and activities (Fig. 4A) [19]. However, it is not clear whether $VEGFR_{1-2}$ inhibits VEGFR-2 signaling through its downstream effector or direct interaction. Our present study implies that $VEGFR_{1-2}$ regulates VEGFR-2 signaling through an indirect rather than direct interaction since only pretreatment of VEGF-E:PlGF-1 is able to inhibit VEGFR-2 phosphorylation (Fig. 1D). Notably, the simultaneous stimulation of VEGF-E:PlGF-1 and VEGF-E leads to higher VEGFR-2 phosphorylation compared to VEGF-E only treatment (Fig. 1D). The loss of inhibition on VEGFR-2 phosphorylation by VEGF-E:PlGF-1 is probably due to the short duration (5-10 minutes) stimulation which is not enough to trigger the downstream effectors. Indeed, the simultaneously addition of VEGF-E:PlGF-1 and VEGF-E in a relative long experiment setting, such as tube formation and cell proliferation (6-8 and 48-72 hours), did inhibit VEGF-E mediated biological activity (Fig. 4A and B). Moreover, our data highlights the possibility of PI3K in the inhibition of VEGFR-2 signaling. However, PI3K is also demonstrated to play an important role in regulating endothelial proliferation, migration and survival through VEGFR-2 signaling [36, 37]. Thus, the PI3K pathway may play a complex role in the regulation of VEGF signaling. As mentioned earlier, only cells that received a pre-treatment of VEGF-E:PlGF-1 exhibited an inhibitory effect and not in the cells simultaneously treated with VEGF and VEGF-E:PlGF-1. This observation further implies that temporal activation of PI3K may affect its subsequent biological activity. In line with this, the spatial and temporal regulation of PI3K is demonstrated to be required for proper chemotaxis and hematopoietic stem cell maintenance [38, 39]. In addtion, the pretreatment of VEGF-E:PlGF-1 may also lead to the rapid internalization and endocytosis of cell surface VEGFR-2 receptor thus

inhibiting VEGF mediated biological activity. Our previous study demonstrated the rapid internalization and trafficking of VEGFR-2 receptor upon VEGF-E:PlGF-1 stimulation [19]. Other studies also confirmed that VEGF receptor internalization and endocytosis is required for endothelial cell biological functions, such as ERK1/2 activation [40], angiogenesis [41] and cell recovery after wounding [42]. To gain a better understanding of $VEGFR_{1\text{-}2}$ biological function, further investigation is warranted.

Anti-VEGF therapies via neutralizing VEGF ligand or VEGFR-2 inhibition are in use but effectiveness in cancer treatment is limited [43, 44]. Likewise, therapeutic angiogenesis used to treat limb ischemia [45], peripheral artery [46] and coronary artery disease [47] by promoting VEGF signaling have not been a great success and clinical trials are inconclusive [48, 49]. This is thought to be due to possible “off-target” effects of the currently available VEGF pathway activators and inhibitors [50]. Our study highlights that the role of $VEGFR_{1\text{-}2}$ in the regulation of VEGF signaling should be considered when designing agents which regulate VEGF receptor activity. The agents that regulate VEGFR-2 receptor might also target $VEGFR_{1\text{-}2}$ heterodimeric receptor and subsequently disrupt the natural inhibitory mechanisms designed to regulate VEGFR-2 activity.

In conclusion, our data demonstrates that $VEGFR_{1\text{-}2}$ heteromeric receptor and not VEGFR-1 homomeric receptor is responsible for limiting VEGF signaling through downregulation of VEGFR-2 phosphorylation and thus attenuates VEGFR-2 activity. Furthermore, we confirmed that PI3K pathway acts

downstream of $VEGFR_{1-2}$ receptor and inhibition of PI3K abrogates $VEGFR_{1-2}$ mediated inhibition of VEGF signaling. $VEGFR_{1-2}$ receptor and the downstream PI3K pathway may serve as new targets to modulate VEGF pathway activity in vascular disorders.

Conflict of interest

None to declare.

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Figure Legends

Figure 1. **$VEGFR_{1-2}$ heteroreceptor, but not VEGFR-1 homoreceptor, negatively regulates VEGFR-2 phosphorylation**. (**A**) Schematic diagram showing VEGF signalling pathway. Red bar represents VEGFR-2 receptor and black bar represents VEGFR-1 receptor. (**B**) HUVECs were electroporated with siRNA against VEGFR-1 (siR-1) and subsequently stimulated with VEGF-A (20 ng/ml) after 24 hour recovery. The protein lysates were collected 5-10 min after vehicle or VEGF-A stimulation and assayed with western blot using antibodies against phosphorylated VEGFR-2 at Y951 and Y1175 sites, total VEGFR-2, total VEGFR-1 and beta-actin. (**C**) HUVECs were stimulated for 5-10 min with VEGF-A (20 ng/ml), PlGF-1 (50 ng/ml), VEGF-E (20 ng/ml) or pre-treated with PlGF-1 for 30 min (Pre) and subsequently stimulated with VEGF-E. Cell lysates were western blotted for phosphorylated VEGFR-2 at Y951 and Y1175 sites, total VEGFR-2 and beta-actin. (**D**) HUVECs were stimulated for 5-10 min with VEGF-E:PlGF-1 (VE:Pl, 50 ng/ml), VEGF-E (20 ng/ml), VEGF-E plus VEGF-E:PlGF-1 simultaneously (Sim) or pre-treated for 30 min (Pre). Cell lysates were western blotted for phosphorylated VEGFR-2 at Y951 and Y1175 sites, total VEGFR-2 and beta-actin. (E) Schematic diagram showing $VEGFR_{1-2}$ heteroreceptor negatively regulates VEGFR-2 phosphorylation. Sim, simultaneously stimulation (added together); Pre, pre-treatment as described. Data were collected from three independent experiments.

Figure 2. Inhibition of PI3K increases VEGFR-2 phosphorylation. (**A**) HUVECs were pre-treated with LY294002 (20uM) for 30 min and subsequently treated with vehicle or VEGF-A (20 ng/ml) for 5-10 min. Cell lysates were collected and assayed for western blot using antibodies against phosphorylated VEGFR-2 at Y951 and Y1175 sites, total VEGFR-2 and beta-actin. (**B**) HUEVCs were pre-treated with LY294002 (20uM) or in combination with PlGF-1 (Pre, 50ng/ml) for 30 min and subsequently stimulated with VEGF-E (20ng/ml) for 5-10 min. Cell lysates were western blotted with antibodies against phosphorylated VEGFR-2 at Y951 and Y1175 sites, total VEGFR-2 and beta-actin. (C) HUVECs were pre-treated with LY294002 (20uM) alone or in combination with VEGF-E:PlGF-1 (VE:Pl, Pre, 50ng/ml) for 30 min and subsequently stimulated with VEGF-E (20ng/ml) for 5-10 min. Cell lysates were western blotted with antibodies against phosphorylated VEGFR-2 at Y951 and Y1175 sites, total VEGFR-2 and beta-actin. (**D**) Schematic diagram showing VEGF-R1:R2 heteroreceptor through PI3K pathway to negatively regulates VEGFR-2 phosphorylation. Data were collected from three independent experiments.

Figure 3. Inhibition of PI3K abrogates VEGF-E:PlGF-1 mediated inhibition of VEGFR-2 phosphorylation. (**A**) HUVECs were pre-treated with LY294002, VEGF-E:PlGF-1 (VE:Pl) alone or in combination for 30 min (Pre) and subsequently stimulated with vehicle, VEGF-A or VEGF-E for 5-10 min. Cell lysates were blotted with antibodies against phospho-AKT(Ser473), total

AKT, phosphorylated VEGFR-2 at Y951 and Y1175 sites, total VEGFR-2, phospho-ERK (at 2 min and 10 min exposure), total ERK and beat-actin. (**B, C, D, E**) Densitometric analysis of phospho-Akt to total Akt, VEGFR-2 phosphorylation at Y951 and Y1175 to total VEGFR-2 and ERK1/2 phosphorylation to total ERK shown in **A**. (**F**) HUVECs were pre-treated with VEGF-E:PlGF-1 alone or in combination with LY294002 for 30 min (Pre) and subsequently stimulated with VEGF-E. Alternatively, cells were stimulated simultaneously without pre-treatment (Sim) with VEGF-E in combination with VEGF-E:PlGF-1 and/or LY294002 for 5-10 min. Cell lysates were western blotted with antibodies against total phospho-tyrosine of VEGFR-2, total VEGFR-2 and beta-actin. (**G**) PAE-R2 cells were pre-treated with VEGF-E:PlGF-1 or in combination with LY294002 for 30 min (Pre) and subsequently stimulated with VEGF-E or stimulated simultaneously with VEGF-E in combination with VEGF-E:PlGF-1 and/or with LY294002 (Sim) for 5-10 min. Cell lysates were blotted with antibodies against phosphorylated VEGFR-2 at Y951 and Y1175 sites, total VEGFR-2 and beta-actin. (**H**) Densitometric analysis of VEGFR-2 phosphorylation to total VEGFR-2 shown in **G**. Data were collected from three independent experiments.

Figure 4. Inhibition of PI3K pathway rescues VEGF-E:PlGF-1 mediated inhibition of cellular activities. (**A**) HUVECs were seeded with a density of 2000 cells/well in a 96-well plate and pre-treated with LY294002 and VEGF-E:PlGF-1 (VE:Pl), alone or in combination (Pre). The pre-treated cells were then stimulated with VEGF-E or in combination with VEGF-E:PlGF-1 (Sim) for

48-72 hours. The cell numbers were measured using CyQUANT® NF cell proliferation assay kit (Invitrogen) following the manufacturer's instructions. (**B**) HUVECs were pre-treated with LY294002, VEGF-E:PlGF-1 alone or in combination for 30 min (Pre) and subsequently used for capillary-like tube formation assay under VEGF-E stimulation. Alternatively, HUVECs were directly used for tube formation assay under stimulation of VEGF-E or in combination with VEGF-E:PlGF-1 simultaneously without pre-treatment (Sim). Representative fluorescence images of tubular structure were taken after 6-8h incubation. (**C**) The quantification of mean total tube length per X4 field was performed using Image-Pro Plus software. Data are expressed as representative of mean ± SEM of three or more independent experiments performed in triplicate. Scale bar, 250 µm.

S1 Fig. The phosphorylation of VEGFR-2 upon VEGF-A stimulation in endothelial cells. (**A**) HUVECs were stimulated with VEGF-A (20ng/ml) and the phosphorylation of VEGFR-2 of Y951 was measured at 0, 5 and 15 minutes after the stimulation by western blotting.

S2 Fig. Inhibition of PI3K promotes *in vitro* tube formation in endothelial cells. (**A**) PAE-R1:R2 cells were pre-treated with LY294002 for 30 minutes and subsequently used for capillary-like tube formation assay under stimulation of VEGF-A in a 96-well plate. Representative images of tubular structure were taken after 6-8h incubation. (**B**) The quantification of mean total tube length per X10 field was performed using Image-Pro Plus software. Data

are expressed as mean ± SEM of three or more independent experiments performed in triplicate. Scale bar, 25 µm.

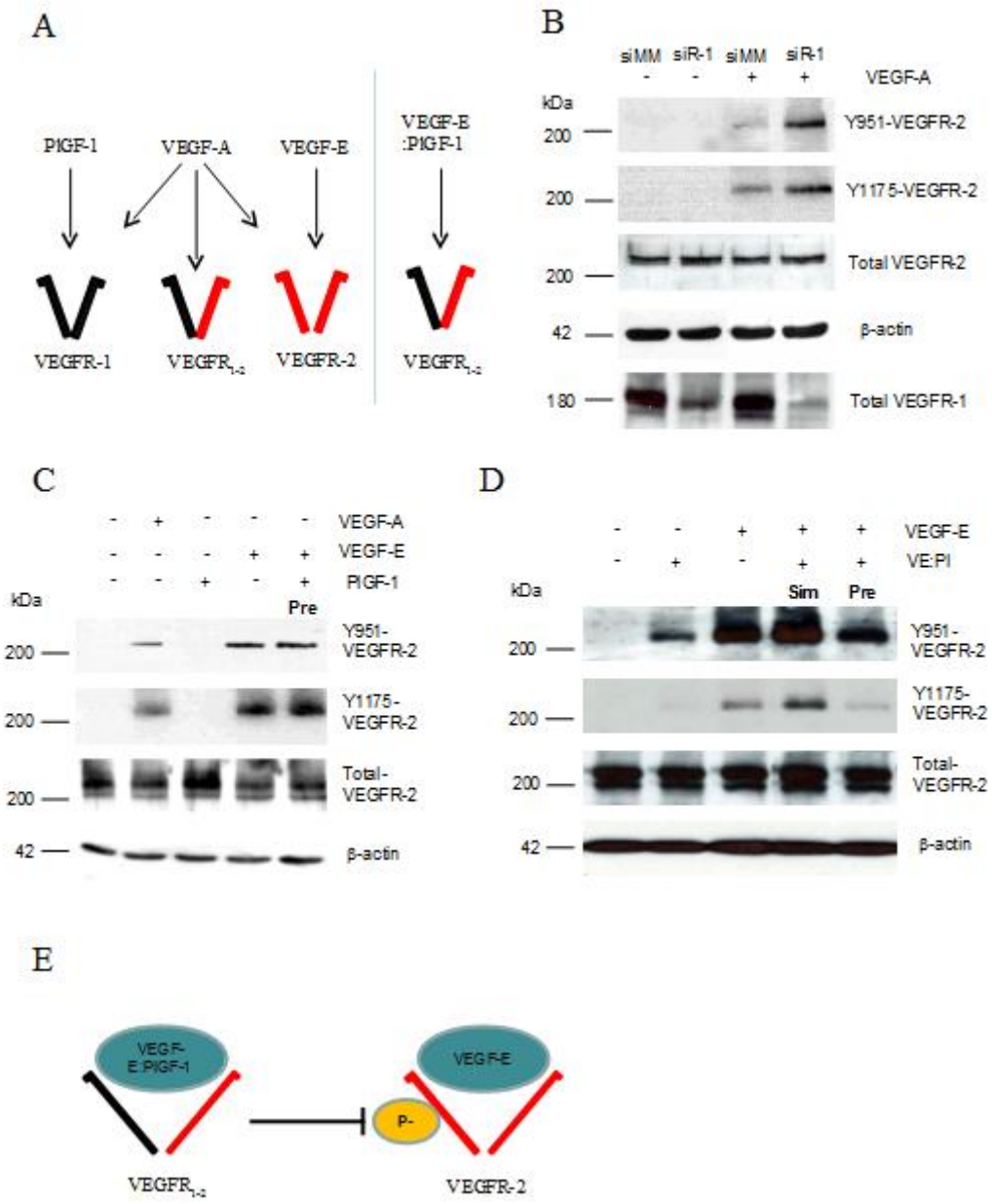


Figure 1

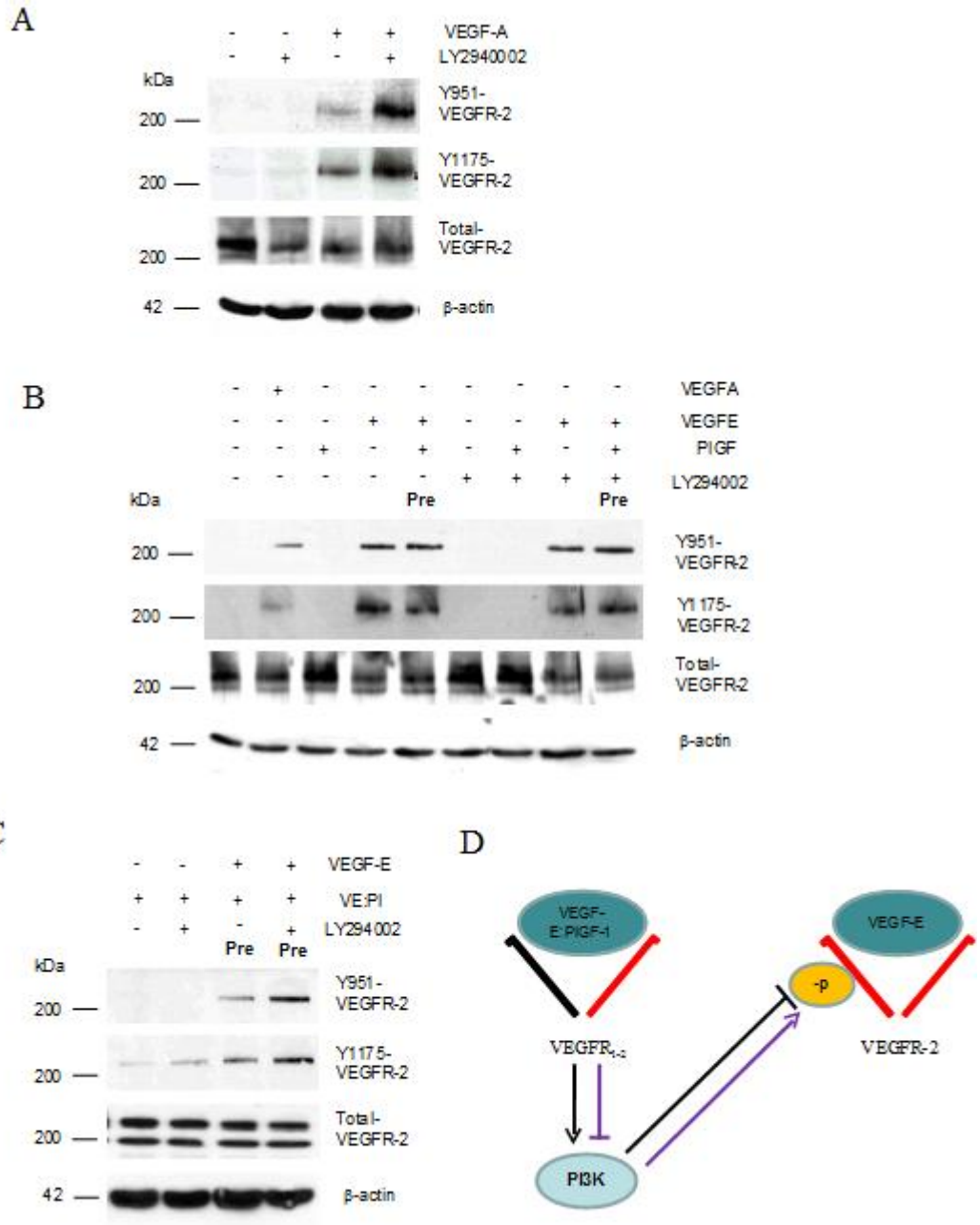
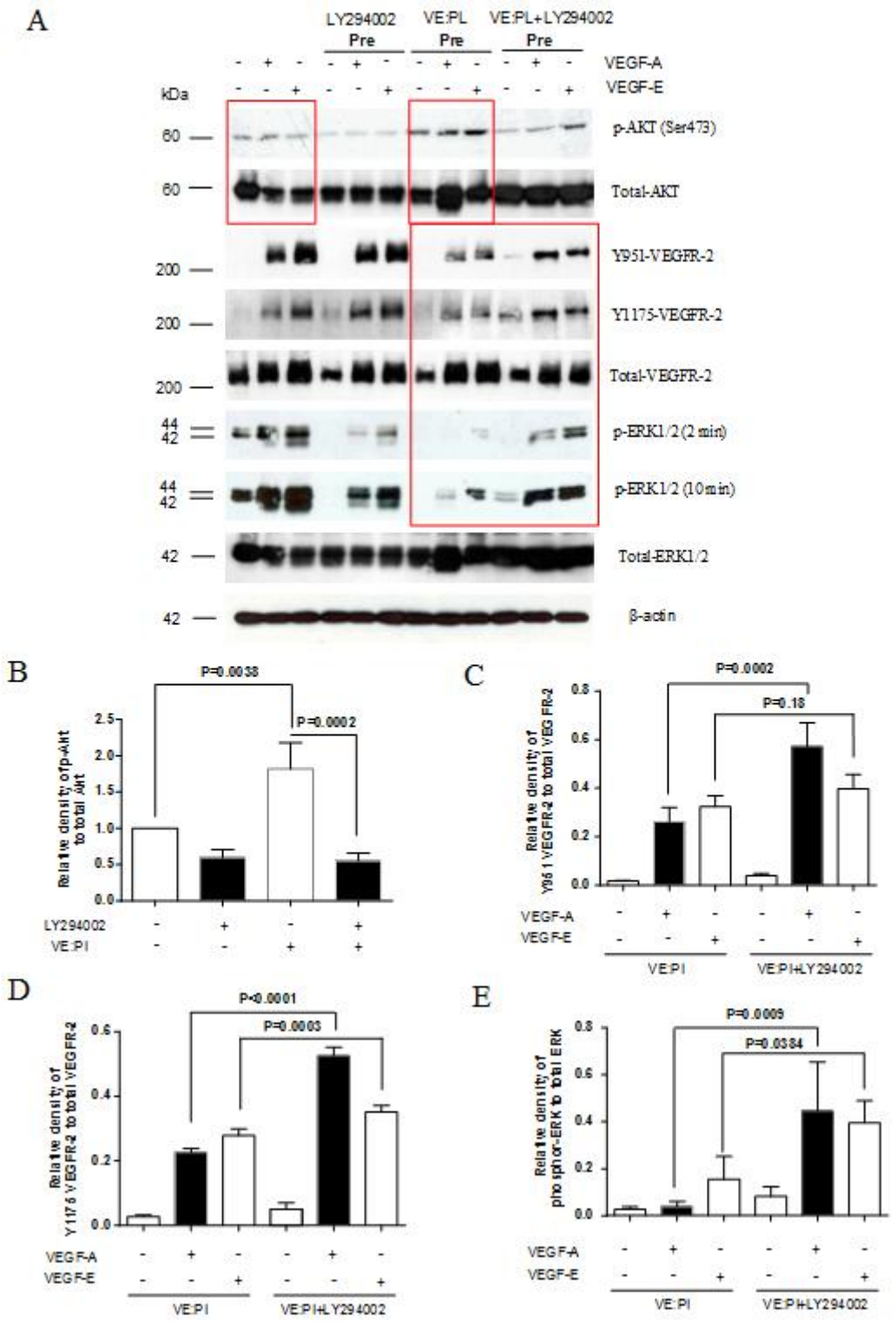


Figure 2


A
LY294002 Pre
VE:PL Pre
VE:PL+LY294002 Pre
VEGF-A
VEGF-E
kDa
p-AKT (Ser473)
Total-AKT
Y951-VEGFR-2
Y1175-VEGFR-2
Total-VEGFR-2
p-ERK1/2 (2 min)
p-ERK1/2 (10 min)
Total-ERK1/2
β-actin
B
P=0.0038
P=0.0002
LY294002
VE:PI
C
P=0.0002
P=0.18
VEGF-A
VEGF-E
VE:PI
VE:PI+LY294002
D
P<0.0001
P=0.0003
VEGF-A
VEGF-E
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VEGF-E
VE:PI
VE:PI+LY294002

Figure 3

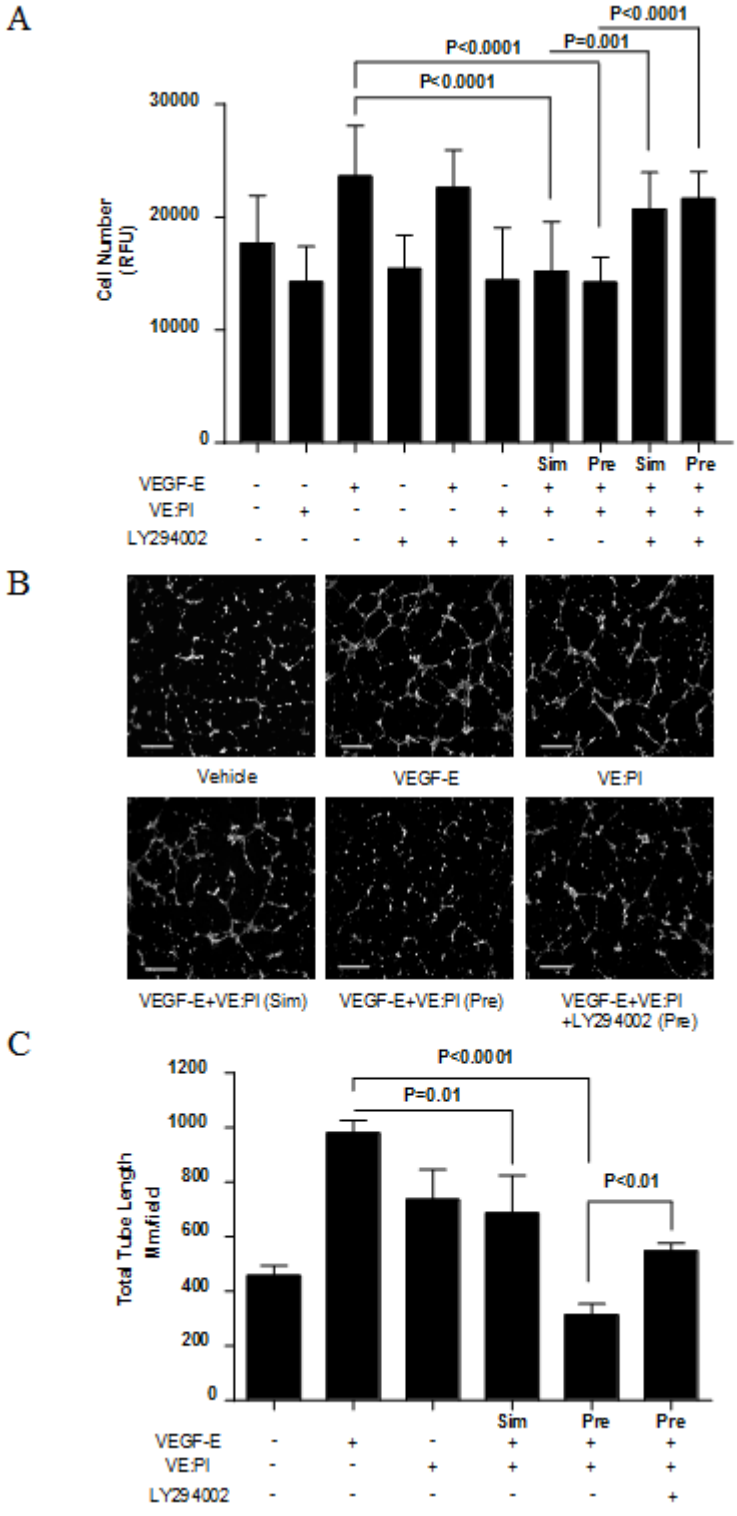
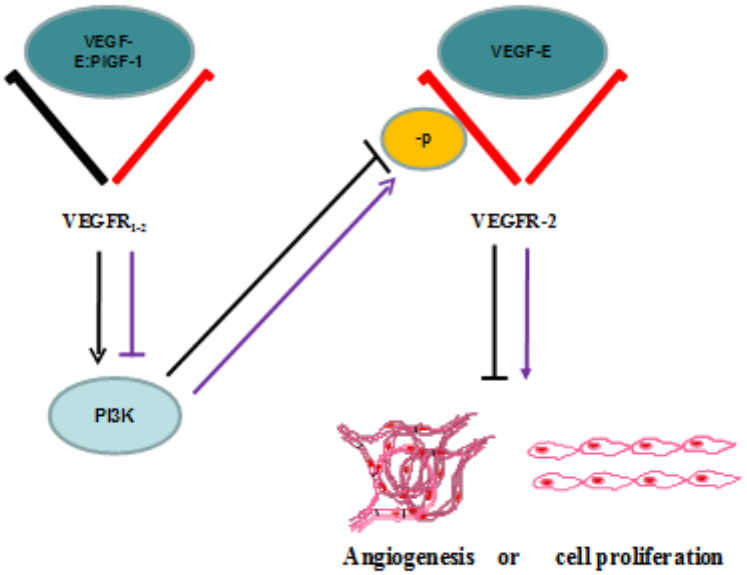


Figure 4



Graphical abstract