The interaction of uPAR with VEGFR2 promotes VEGF-induced angiogenesis*

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ABSTRACT

In endothelial cells, binding of vascular endothelial growth factor (VEGF) to the receptor VEGFR2 activates multiple signaling pathways that trigger processes such as proliferation, survival, and migration that are necessary for angiogenesis. VEGF-bound VEGFR2 becomes internalized, which is a key step in the proangiogenic signal..Here, we showed that the urokinase plasminogen activator receptor (uPAR) interacted with VEGFR2 and described the mechanism by which this interaction mediated VEGF signaling and promoted angiogenesis. Knockdown of uPAR in human umbilical vein endothelial cells impaired VEGFR2 signaling and uPAR deficiency in mice prevented VEGF-induced angiogenesis. Upon exposure of HUVECs to VEGF, uPAR recruited the low density lipoprotein receptor related protein 1 (LRP-1) to VEGFR2, which induced VEGFR2 internalization. Thus, the uPAR-VEGFR2 interaction is crucial for VEGF signaling in endothelial cells.

INTRODUCTION

Angiogenesis, the process by which new blood vessels are formed from pre-existing ones, plays an essential role in tumor growth, invasion and metastasis(*1*). Angiogenesis is predominantly regulated by vascular endothelial growth factor (VEGF), which binds to the tyrosine kinase receptor VEGFR2, inducing the dimerization and phosphorylation of the receptor (*2-6*). VEGFR2 is phosphorylated at several intracellular phosphorylation sites (including tyrosine residues 951, 1054, 1059, 1175 and 1214) (*7-9*), but the phosphorylation of Tyr^{1175} is required for angiogenic functions (*5, 10-13*). Different VEGFR2 phosphorylation events induce distinct signaling pathways such as mitogen-activated protein kinases (MAPKs) (*12*), phosphatidylinositol-3-kinase (PI3K)/AKT (*14*), and nitric oxide production (*15*), resulting in the regulation of proliferation, survival, migration and vascular permeability of endothelial cells. Upon activation by the binding of specific ligands, growth factor receptors are internalized, which can lead to receptor degradation and the termination of signaling (*16*). However, internalized VEGFR2 continues to signal from the endosomal compartments in endothelial cells (*17*).

The urokinase plasminogen activator receptor (uPAR; also known as CD87) also contributes to angiogenesis (*18, 19*). uPAR is a glycosylphosphatidylinositol (GPI)-anchored protein (*20*) that contains three homologous domains, designated as D1, D2 and D3, which are connected by short linkers (*21*). At the cell surface, uPAR is the receptor for the inactive precursor pro-urokinase (pro-uPA) (*22*). VEGF can induce endothelial cell migration by activating pro-uPA bound to uPAR (*23*), which requires the internalization and redistribution of uPAR and integrins (*24, 25*). Because uPAR lacks transmembrane and cytosolic domains, it must interact with other partners such as vitronectin (*26*), caveolin (*27*), the G protein-coupled receptor FPLR1 (*28*), various receptor tyrosine kinases, including EGFR (*29, 30*) and PDGFR (*31*), integrins (*32-36*) and LDL receptor related protein-1 (LRP-1) (*37-39*) to transduce signaling and to modulate angiogenic effects (*33, 40-42*).

One way in which angiogenesis is tightly regulated is through the interaction of VEGFR2 with its co-receptors (*43-46*). We show here that VEGF induced the formation of a complex between VEGFR2, β1 integrin, uPAR and LRP-1 at the cell surface of endothelial cells.

We also demonstrate that LRP-1-dependent-internalization of this complex upon VEGF stimulation is crucial for VEGFR2 signaling and its biological effects.

RESULTS

VEGF induces the interaction of VEGFR2 with uPAR

Previous studies have reported that uPAR participates in the VEGF-induced migration of endothelial cells (*24, 25*). To determine the mechanism by which uPAR regulates VEGF signaling, we performed proximity ligation assays (PLAs) to determine whether VEGFR2 forms a complex with uPAR on the cell membrane in HUVECs after VEGF stimulation. The red dots in the PLA images indicate that uPAR and VEGFR2 were located less than 40 nm from each other after 10 min of VEGF stimulation (Fig. 1A). To confirm that VEGFR2 formed a complex with uPAR, we performed co-immunoprecipitation experiments. In HUVECs, the association of VEGFR2 with uPAR was enhanced upon VEGF treatment (Figure 1B). We wondered whether VEGFR2 also interacted with other binding partners of uPAR, such as LRP-1 and integrins (*34, 38, 47, 48*). PLAs indicated that within 10 min of stimulation with VEGF, VEGFR2 formed complexes with β1 integrin and LRP-1 complexes (Fig. 1A). Co-immunoprecipitation analysis confirmed that VEGF stimulation induced an increase in the amount of VEGFR2 or uPAR that associated with β1 integrin, which was not due to increases in the overall abundance of VEGFR2, uPAR, or β1 integrin as assessed by immunoblotting of cell lysates (Figure 1B-D). Previous studies have reported that β3 integrin binds to VEGFR2 (*49-52*). However, in our study, VEGF treatment did not induce the formation of a VEGFR2/β3 integrin complex (Figure 1A). Consistent with these results, PLAs revealed that VEGF induced the formation of uPAR/β1integrin and uPAR/LRP-1 complexes but not of an uPAR/β3 integrin complex (Figure 1E). Together these data indicate that uPAR, β1 integrin, and LRP-1 interact with VEGFR2 upon VEGF treatment.

uPAR promotes VEGFR2 signal transduction and its biological effects on endothelial cells *in vitro* and neovascularization *in vivo*

We next investigated how uPAR impacts VEGF-induced angiogenesis. Transfection of HUVECs with an uPAR siRNA duplex reduced uPAR protein abundance by 50% (Figure

2A).To analyze whether VEGF-induced endothelial cell migration requires uPAR, wound closure assays in HUVECs were performed, which showed that uPAR knockdown abolished VEGF-induced migration (Fig. 2B). In addition, we evaluated whether uPAR is essential for VEGF-induced proliferation by measuring BrdU incorporation. The proliferation induced by VEGF was significantly reduced in uPAR-knockdown HUVECs compared to HUVECs transfected with control siRNA (Figure 2C). Because VEGF increases vascular permeability (*15, 53*), we examined the influence of uPAR on VEGF-induced permeability and found that transfection of uPAR siRNA decreased the ability of VEGF to induce permeability in HUVECs (Figure 2D). Effects on migration and proliferation were confirmed using a second siRNA for uPAR (Figure S1A,B). As a second strategy to inhibit uPAR, we used blocking antibodies designed against D1 or D2 domain of uPAR. Although preincubation of HUVEC with either U1 or U2 antibody alone did not alter HUVEC migration, the combination of both resulted in reduced VEGF-induced migration (Figure S1C-E). These results suggest that blocking both domains is required to prevent VEGFR2 function.

To further determine whether uPAR silencing also impacts neovascularization *in vivo*, we investigated the role of uPAR in the angiogenic effects of VEGF in the retinal vasculature of *uPAR*$^{-/-}$ and wild-type mice. The retina is avascular at birth, and a layer of blood vessels grows from the optic nerve toward the periphery from postnatal day 1 until postnatal day 7 (*54*). During the extension of the developing superficial vascular plexus (Fig. 2E), the retinal surface was colonized by vessels, and the complexity, which is characterized by the number of branch points in the vascular network, was significantly increased in wild-type newborn pups that were intraocularly injected with recombinant VEGF compared to pups injected with vehicle. This increase was abrogated in *uPAR*$^{-/-}$ pups that were injected with VEGF (Fig. 2F-H). To further analyze the impact of silencing uPAR in another model of neovascularization, we perfomed Matrigel plug assays. Inclusion of VEGF in matrigels induced vessel ingrowth into Matrigel plugs (as assessed by measuring the hemoglobin content (*55*)) in wild-type mice but to a lesser extent in *uPAR*$^{-/-}$ mice (Figure 2I). These data suggest that uPAR enables VEGFR2 to mediate its angiogenic effects *in vitro* and *in vivo*.

We next examined the effects of uPAR silencing on VEGF signaling. In HUVECs with uPAR knockdown, the phosphorylation of VEGFR2 at Tyr^{1175} following VEGF stimulation was decreased by 40% compared to control cells (si-Ctrl). uPAR knockdown also decreased the downstream induction of ERK phosphorylation by VEGF, (45%), but not that of AKT phosphorylation (Figure 3A). These results suggest that uPAR is a binding partner of VEGFR2.

VEGF induces the internalization of VEGFR2, uPAR, β1 integrin, and LRP-1 in endothelial cells

Because internalization of VEGFR2 is crucial for its signaling, we analyzed the impact of uPAR on the VEGFR2 internalization process. VEGFR2 interacted with LRP-1 (Fig. 1), a protein that is involved in uPAR internalization (*39, 56*); therefore, we determined whether VEGFR2, uPAR, β1 integrin, and LRP-1 was internalized following short-term VEGF treatment. Flow cytometry analysis on non-permeabilized HUVECs indicated that 15 min of stimulation with VEGF decreased the surface abundance of VEGFR2 (33%), uPAR (47%), β1 integrin (38%) and LRP-1 (13%) (Figs. 3B-E). As expected, β3 integrin, which was not detected in this complex (Fig. 1A), did not show differences in cell surface abundance after VEGF stimulation (Fig. 3F). To determine whether these decreases reflected the internalization of these proteins, we performed biotinylation experiments. HUVECs were surface-biotinylated and then subjected to VEGF treatment for 10 min. Biotin attached to the proteins remaining at the cell surface was selectively cleaved using a membrane-impermeable reducing agent, and internalized biotinylated pool was detected using labeled streptavidin. 15 min of treatment with VEGF induced the internalization of VEGFR2, uPAR and β1 integrin (Fig. 3G). Together, these data suggest that VEGF induces the internalization of the components of the VEGFR2/uPAR/β1 integrin/LRP-1 complex.

VEGF requires internalization mediated by LRP-1 for its effects on endothelial cells

We next investigated whether VEGF requires internalization of the VEGFR2/uPAR/β1 integrin/LRP-1 complex to mediate its angiogenic effects. To that aim, we incubated HUVECs with dynasore to block dynamin-dependent endocytosis (*57*) before VEGF

treatment. VEGF-mediated phosphorylation of VEGFR2 and ERK, but not that of AKT, was reduced after dynasore pre-incubation compared to control cells (Fig. 4A). The presence of LRP-1 in the complex led us to postulate that the internalization mediated by VEGF was LRP-1 dependent. To verify this hypothesis, we incubated HUVECs with receptor-associated protein (RAP), a LRP-1 antagonist that inhibits LRP-1-dependent internalization, prior to VEGF stimulation (*56*). The ability of VEGF to induce migration and proliferation was reduced in the presence of RAP compared to control HUVECs (Figure 4B-C). The permeability induced by VEGF was also significantly reduced in HUVECs pretreated with RAP (Figure 4D). We next analyzed whether LRP-1 deficiency impacts VEGF signaling. In control HUVECs, VEGF-mediated phosphorylation of VEGFR2 and ERK, but not that of AKT, were decreased in the presence of RAP compared to untreated control cells (Fig. 4E). Together, these data suggest that VEGF requires LRP-1-dependent internalization to mediate its angiogenic effects.

uPAR is required for VEGFR2 internalization

Our previous observations showed that uPAR enables VEGF to induce its effects. Moreover, we showed that VEGFR2 requires internalization that is mediated by LRP-1 to exert its angiogenic effects. Because LRP-1 interacts with uPAR, we performed PLAs to test our hypothesis that uPAR was the principal mediator of VEGFR2 internalization. HUVECs with uPAR knockdown contained lower amounts of the VEGFR2/LRP-1 complex after VEGF treatment, suggesting a role for uPAR in the induction of the interaction of VEGFR2 with LRP-1. In addition, cell surface biotinylation assays showed that VEGFR2 and β1 integrin internalization in response to VEGF was reduced in HUVECs transfected uPAR siRNA compared to those transfected with control siRNA (Fig. 4G). These differences in internalization were not due to changes in the abundance of VEGFR2 and β1 integrin (Fig. 4B).

DISCUSSION

Our results demonstrate that VEGFR2, a major mediator of angiogenic signaling, interacts with uPAR at the cell surface. Silencing of uPAR disrupted VEGFR2 signaling downstream of VEGFR2, leading to impaired angiogenesis in vivo. After uPAR binding, VEGFR2 was

internalized in the cell in a LRP-1-dependent manner. VEGFR2 signaling is the consequence of several steps that are initiated at the cell surface upon ligand binding. VEGF binding to VEGFR2 leads to the recruitment of co-receptors that might modulate downstream signaling and angiogenic functions. For example, after VEGF stimulation, NRP-1 recruits the synectin-myosin VI complex to VEGFR2 to induce its internalization, which perpetuates the signals involved in survival, migration and permeability. Another VEGFR2 binding partner, ephrin B2, regulates tip cell filopodia extension by inducing VEGFR2 internalization (*44, 46*). Moreover, we found that uPAR enhanced the ability of VEGF to induce migration, proliferation and permeability of endothelial cells and angiogenesis *in vivo.*

We here show that uPAR is required for VEGFR2 signaling and function *in vitro*. *In vivo*, although significant, the effect in *uPAR*$^{-/-}$ mice is less impressive. Such discrepancy has been described in the past (*40*). The strongest effect observed *in vitro* is unlikely due to our siRNA silencing methodology since endothelial cells isolated from *uPAR*$^{-/-}$ mice showed similar level of reduced proliferation and migration capacities (*18*). For a long time it was thought that the *uPAR*$^{-/-}$ mice do not have physiological abnormalities as they appeared normal and were fertile (*58*). Indeed our results did not shown any difference in neovascularization in the retina in *uPAR*$^{-/-}$ *vs* WT mice without VEGF administration. Similar lack of effect was observed in untreated matrigels. Consistent with our findings, matrigel data obtained by Larusch and collaborators showed that VEGF-induced angiogenesis is affected by uPAR depletion while no alteration was observed in the absence of VEGF treatment (*29*). Thus in general, *uPAR*$^{-/-}$ mice do not show a strong angiogenic phenotype. However, several recent investigations have documented that *uPAR*$^{-/-}$ mice show severe reduced microvessel density in the myocardium (*59*) and in the dermis (*60*) highlighting a potential role for uPAR in systemic sclerosis. Those observations suggest that, depending on the microenvironment, the impact of the absence of uPAR on vascularization could be more impressive. The observation that *uPAR*$^{-/-}$ endothelial cells show strong phenotype *in vitro* is also in favor for compensatory mechanisms that are microenvironment-dependent. One explanation could be that uPA compensates the lack of uPAR by activating other signalings. In agreement with this, is the observation that uPA by its kringle domain interacts specifically with αvβ3 integrin in

uPAR-depleted CHO cells (*61*) and that the isolated uPA kringle domain binds other integrins such as α4β1 and α9β1 (*62*). Nerveless, the precise role of uPAR in modulating vascularization in vivo warrant further investigation.

uPAR silencing affected VEGFR2 signaling by reducing the phosphorylation of VEGFR2 at Tyr^{1175}, one of the first steps in VEGFR2 signaling. ERK phosphorylation induced by VEGF was also affected in the uPAR-deficient cells. These data agree with a previous study showing that the phosphorylation of VEGFR2 at Tyr^{1175} is crucial for the binding and activation of PLC-γ, which is a major effector of ERK activation (*12*). These data show that uPAR is a binding partner of VEGFR2.

Our results also showed that β1 integrin is recruited to the VEGFR2/uPAR complex at the cell surface. β1 integrin interacts with the D2 and D3 domains of uPAR (*34, 47, 48*) and also binds VEGFR2 (*43*). We therefore suggest that β1 integrin acts as an adaptor protein between uPAR and VEGFR2. A similar model has been proposed for the interaction between β1 integrin, uPAR and another tyrosine kinase receptor, EGFR, in endothelial and cancer cells (*29, 30, 63*).

The recruitment of β1 integrin and uPAR to VEGFR2 is likely to occur in lipid rafts microdomain at the cell membrane. Various studies have shown that VEGFR2 colocalizes with caveolin-1 in lipid rafts and have suggested that VEGFR2 localization to lipid rafts is necessary for VEGFR2 dimerization and endocytosis and endothelial cell migration and proliferation (*64-69*). In addition, activation of both β1 integrin (*70*) and uPAR (*71*) requires recruitment to lipid rafts.

Currently, the internalization of VEGFR2 is considered to be an important mechanism by which cells control the intensity and duration of downstream signaling. An important question that is raised by our results is how uPAR mediates VEGFR2 internalization. Our data showed that VEGF stimulation led to the recruitment of LRP-1 in the VEGFR2/β1 integrin/uPAR complex at the endothelial cell surface. By interacting with the D3 domain of uPAR, LRP-1 can also induce the internalization of uPAR-associated proteins, such as integrins, its ligand urokinase-type plasminogen activator (uPA) and its inhibitor plasminogen activatory inhibitory type 1 (PAI-1) (*38, 56, 72*). Using flow cytometry and cell surface biotinylation assays, we demonstrated that all members of this complex were internalized following short-term VEGF treatment.

To date, no binding sites for LRP-1 have been described on VEGFR2. Because LRP-1 is required for uPAR internalization (*56, 72*), we speculate that uPAR brings LRP-1 into the complex, subsequently leading to VEGFR2 internalization. Supporting this hypothesis is the observation that under some conditions (for example, when bound to PAI-1 and uPA), uPAR can interact with LRP-1 on the plasma membrane through its D3 domain (*38*). Based on these observations, we suggest that PAI-1 and uPA may also be members of the VEGFR2 complex at the cell surface.

Then, we wondered whether VEGFR2 internalization mediated by LRP1 was required for VEGFR2 signal transduction and its biological effects on endothelial cells. We found that RAP, an inhibitor of LRP-1-dependant internalization, impaired VEGF-mediated phosphorylation of VEGFR2 at Tyr^{1175}, activation of the VEGFR2 downstream effector ERK (but not that of AKT), and angiogenesis in HUVECs. Together, these data reinforce that internalization is crucial for the angiogenic role of VEGF. Although some initial studies support the view that internalization is a crucial step for signal termination, recent studies favor the role of VEGFR2 internalization in controlling the intensity and duration of the signal. For example, internalized VEGFR2 is not degraded but rather signals from early endosomes (*17*). Moreover, VEGFR2 internalization induces signaling and filopodia extension through an ephrin B2-mediated mechanism in endothelial cells from the mouse retina (*44*).

In summary, we propose a model in which VEGF induces VEGFR2/β1 integrin/uPAR/LRP-1 complex formation at the cell surface. We believe that β1 integrins enables the association between VEGFR2 and uPAR. In this model, uPAR brings LRP-1 to VEGFR2, and LRP-1 mediates VEGFR2 internalization, thereby promoting signaling by this receptor and angiogenesis (Figure 5).

MATERIAL AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated as previously described (*73*). Briefly, umbilical veins were washed with PBS, filled with trypsin solution (0.05% trypsin (Difco), 0.2% EDTA, PBS, pH 7.6), clamped and incubated in a 37°C water bath for 20 min. Each umbilical vein was washed with PBS, and the eluate was centrifuged to

harvest the cells. The HUVECs were seeded into gelatin-coated culture dishes and cultured in EGM-2 medium (EGM-2 BulletKit medium (CC-3162, Lonza) supplemented with 5% fetal bovine serum (FBS)).

siRNA transfection and internalization inhibitors treatment

For the siRNA experiments, HUVECs were transfected with uPAR or scramble siRNA (50 nM). Assays were performed 72 h post-transfection. For the experiments using internalization inhibitors, the cells were preincubated for 1 h with RAP (200 nM) (Calbiochem) or dynasore (20 µM) (Sigma) before VEGF treatment. Small interfering RNA (siRNA) duplexes were obtained from Integrated DNA Technologies, including two siRNAs targeting *uPAR* and one negative control. The sequences of the uPAR siRNA (IDT-DNA) were uPAR si-RNA 1:5'- GGUGAAGAAGGGCGUCCAA-3'; and the corresponding control uPAR si-RNA: 5'- AACCUGCGGGAAGAAGUGG-3'. Similar results were obtained with another uPAR si-RNA 2:5'-CAUUUCCUGUGGCUCAUCA-3'. Cells were transfected using the $Ca_3(PO_4)_2$ method. Briefly, $2.5x10^5$ HUVECs were seeded into each well of a 6-well plate in serum free medium (SFM) supplemented with 5 ng/ml basic fibroblast growth factor (bFGF) and allowed to adhere overnight. siRNA-$CaCl_2$ complexes were prepared by first combining the siRNA with 10 µl of 2.5 M $CaCl_2$. One hundred microliters of HBSP (280 mM NaCl, 1.9 mM Na_2HPO_4, 12 mM glucose, 10 mM KCl, 50 mM HEPES, pH 7.05) was added, and the mixture was incubated for 1 min at room temperature. The mixture was added dropwise to the cells, and the cells were incubated for 16 h. The cells were then collected and seeded for further tests. Antibodies blocking different domains of uPAR were used: domain D1 (U1 antibody; R&D System) or D2 of uPAR (U2 antibody:American Diagnostica).

Scratch wound migration assay

In total, $8×10^4$ cells were seeded into each well of a 48-well plate in 350 µl SFM (Lonza) with 5 ng/ml bFGF (Promega) and incubated to reach confluence. Using a pipette tip, a “wound” was made in the monolayer (at time 0). The cells were then washed with PBS and incubated with SFM containing 50 ng/ml of recombinant VEGF (RELIATech GmbH) for 6 h. In the experiments using internalization inhibitors, the cells were preincubated for

1 h with RAP (200 nM) (Calbiochem) or dynasore (20 μM) (Sigma) before VEGF treatment. For the siRNA experiments, HUVECs were transfected with uPAR or scramble siRNA (50 nM) using the $Ca_3(PO_4)_2$ method for 72 h. After 24 h of transfection, the HUVECs were seeded in a 48-well plate in SFM containing bFGF (5 ng/ml).

Cell proliferation assay

HUVECs were transfected with siRNA as described above and then trypsinized 24 h post-transfection. A total of 1×10^4 cells were seeded into each well of a 96-well plate in 100 μl SFM (Lonza) containing 5 ng/ml bFGF (Promega) and incubated for 48 h. SFM supplemented with recombinant VEGF (50 ng/ml) (RELIATech GmbH) was added to the cells, followed by BrdU 8 h later, and the culture was incubated for 16 h. BrdU incorporation was measured with the Cell Proliferation ELISA BrdU (chemiluminescence) kit (Roche Applied Sciences) according to the manufacturer's protocol.

Permeability assay

Permeability was analyzed *in vitro* using the diffusion of 75-kDa fluorescein isothiocyanate (FITC)-dextran (Sigma) through the endothelial monolayer. A total of $3x10^4$ cells were seeded in SFM supplemented with bFGF (5 ng/ml) on transwell inserts (0.4 μM) (Greiner) coated with fibronectin (7 μg/ml) and incubated for 72 h. The medium in the upper compartment was replaced with SFM containing FITC-dextran and recombinant VEGF (50 ng/ml) (RELIATech GmbH). The amount of FITC-dextran that diffused through the endothelial monolayer into the lower compartment was measured. For the experiments using internalization inhibitors, the cells were preincubated for 1 h with RAP (200 nM) (Calbiochem) or dynasore (20 μM) (Sigma) before VEGF treatment. For the siRNA experiments, HUVECs were transfected with uPAR or scramble siRNA (50 nM) using the $Ca_3(PO_4)_2$ method. After 16 h of transfection, the HUVECs were seeded in SFM containing bFGF (5 ng/ml) on transwell inserts coated with fibronectin.

Surface biotinylation and immunoblotting

To analyze surface protein turnover, HUVECs in PBS were treated with 2 mg/ml of thiol-cleavable Sulfo-NHS-SS-Biotin (Pierce) for 30 min on ice. Next, 4 ml SFM containing 100

ng/ml VEGF were added, and the cells were incubated at 37°C for 15 min. The cells were then incubated twice with 45 mM of the membrane-impermeable reducing agent GSH (Sigma) in 75 mM NaCl, 75 mM NaOH and 1% BSA for 20 min. HUVECs were lysed on ice with lysis buffer (25 mM HEPES, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol and a cocktail of protease inhibitors (Roche)). The biotinylated proteins were adsorbed onto streptavidin-coated sepharose beads for 2 h at 4°C. The adsorbed proteins were eluted by boiling in reducing sample buffer. The proteins were analyzed by SDS-PAGE, followed by Western blotting.

Immunoprecipitation

A total of 1.5×10^6 cells were seeded in T75 flasks in 10 ml of SFM (Lonza) supplemented with 5 ng/ml bFGF (Promega). After 48 h, the HUVECs were incubated with VEGF (50 ng/ml) for 10 min at 37°C. Following this treatment, the cells were incubated with a cross-linking agent (DTSSP, Thermo Scientific) for 2 h at 4°C according to the manufacturer's protocol. The cells were lysed with 500 µl lysis buffer. The HUVEC extracts were incubated overnight with mouse anti-uPAR (R&D Systems), goat anti-human VEGFR2 (R&D Systems) or a rabbit anti-β1 integrin antibody (Millipore) (5 µg/ml) under rotation. Fifty microliters of protein A agarose were added and incubated for 1 h at 4°C under rotation. After centrifugation for 2 min at 3000 rpm, the supernatants were discarded and the pellets were washed three times with the lysis buffer. After centrifugation, the immunoprecipitated proteins were eluted by boiling in reducing sample buffer. The proteins were analyzed by SDS-PAGE, followed by Western blotting.

Western blot analysis

Cells were lysed in lysis buffer and heated at 95°C for 10 min. Equal amounts of protein were resolved by 6-10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes according to the manufacturer's protocol. The blots were blocked for 1 h at room temperature with 8% milk in Tris-buffered saline with 0.1% Tween 20 and probed overnight at 4°C with 1/1000 mouse anti-uPAR (R&D systems), rabbit anti-ERK 1,2 (Millipore), rabbit anti-VEGFR2, rabbit anti-phospho-tyr 1175 VEGFR2, rabbit anti-β1 integrin, rabbit anti-phospho-ERK 1,2 (Thr 202/Tyr 204), rabbit anti-phospho-AKT or rabbit

anti-AKT (Cell Signaling). After three washes with Tris-buffered saline containing 0.1% Tween 20, the appropriate secondary antibody at a 1/4000 dilution was added for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence with an ECL kit (Pierce). Quantification was performed with ImageJ software

Flow cytometry

Following VEGF stimulation (50 ng/ml, 15 min), HUVECs were scraped off of the plates in PBS containing 5% BSA. After blocking in this solution, the appropriate primary antibody was added for 1h at 4°C to VEGFR2 (Cell Signaling), β1 integrin (Millipore), β3 integrin (Millipore), uPAR (R&D System) and LRP-1 (Calbiochem).Then, the appropriate FITC-conjugated secondary antibody was added for 1 h at 4°C. The cells were analyzed with a BD FACSCalibur flow cytometer.

***In situ* proximity ligation assay (PLA)**

HUVECs were cultured in 35-mm Petri dishes coated with gelatin. After incubation for 10 min with VEGF (50 ng/ml), the cells were washed with cold PBS, fixed with 4% paraformaldehyde for 15 min at 4°C, blocked and incubated overnight with 1/100 mouse anti-uPAR (R&D systems), rabbit anti-uPAR (American Diagnostica), rabbit anti-VEGFR2 (Cell Signaling), mouse anti-LRP-1 (Calbiochem), mouse anti-β1 integrin or anti-β3 integrin (Millipore). The PLA was performed according to the manufacturer's instructions using the Duolink Detection Kit 563 (Olink Bioscience) with anti-mouse MINUS and anti-rabbit PLUS PLA probes. DAPI was used to stain the nuclei. The slides were analyzed by fluorescence microscopy (Nikon Eclipse 90I).

Retinal murine neovascularization model

Mice with an inactivation of the gene encoding uPAR (*uPAR*$^{-/-}$) and corresponding wild type mice littermates were used in this study (*74*). Mice are on a mixed C57BL/6 (75%)×129 (25%) background. To analyze postnatal neovascularization in the mouse retina, *uPAR*$^{-/-}$ and wild-type pups were intraocularly injected at P1 with 50 ng/eye VEGF or vehicle and sacrificed at P5. The eyes were fixed in 4% paraformaldehyde in PBS for 45 min, and the retinas were dissected, incubated with biotinylated Isolectin B4 (Vector

laboratories) and stained with streptavidin-Alexa 488 Invitrogen) before being flat-mounted as previously described (*54*). The retinal radius (from the optic nerve to the edge of the retina) and the vascular radius (from the optic nerve to the vascular front) of each petal of the retina were measured. The value for each retina was calculated as the mean of the radii for all petals. The vascular coverage was calculated as the ratio between the vascular radius and the retinal radius. Four or five pups were used in each group. The animal experiment protocol was approved by the Institutional Ethics Committee of the University of Liège.

Matrigel plug assay.

Matrigel plug implantation was performed as previously described(*75*). Matrigel (BD Biosciences), supplemented with 250 ng/ml of fibroblast growth factor-2 (FGF-2), VEGF (300 ng/ml) and 0.0025 U ml^{-1} of heparin. 400 µl of Matrigel was implanted subcutaneously into the right and left flank of WT and *uPAR*$^{-/-}$ from either sex under anesthesia. After 10 days, the plugs were dissected from the mice and their weight was determined. Matrigels were ground in the presence of dispase solution. The concentration of hemoglobin in the supernatant was then determined directly by absorbance at 405 nm and compared with a standard curve of purified hemoglobin (Sigma). The animal experiment protocol used was approved by the Institutional Ethics Committee of the University of Liège.

Statistical analysis

Results were displayed graphically (bar diagrams) as mean and standard error (SE). For immunoprecipitation, Western blotting and flow cytometry, normalized VEGF treated values were tested for significance by the one-sample t-test. For PLAs, control and VEGF-treated cells were compared by the Student t-test or the Mann-Whitney U-test. When comparing several conditions, one way analysis of variance (or the Kruskal-Wallis test) was applied followed by Dunn's post hoc multiple testing. When experiments were repeated under the same conditions (e.g. for *in vitro* migration, proliferation and permeability assays and in vivo experiments), data were treated by analysis of variance for factorial designs (general linear model) with fixed and random effects. All statistical analyses were carried out with Prism 5.0 (GraphPad Software, San Diego, CA, USA) and

Statistica 10 (StatSoft, France) software packages. Results were considered significant at the 5% critical level ($P<0.05$). *: $P<0.05$, **: $P<0.01$,***: $P<0.001$.

SUPPLEMENTARY MATERIALS

Figure S1: uPAR antibody or siRNA-mediated knockdown of uPAR prevents VEGF-induced migration

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FIGURE LEGENDS

Figure 1: VEGF induces VEGFR2/uPAR/ β1 integrin/LRP-1 complex formation at the cell surface of endothelial cells.

A. Complex formation detected by proximity ligation assay (PLA) on HUVECs stimulated with VEGF. Red spots indicate the proximity of the two proteins (DAPI: blue). The graphs show quantification of number of dots/cell from 2 independent experiments.

B-D. Immunoprecipitation assays showing that endogenous VEGFR2 interacts with uPAR HUVECs after VEGF stimulation. HUVEC lysates were immunoprecipitated with a mouse anti-uPAR antibody (**B**, IP:uPAR), a goat anti-VEGFR2 antibody (**C**, IP:VEGFR2) or a rabbit anti-β1 integrin antibody (**D, IP:** β1 integrin) and analyzed by Western blotting using rabbit anti-uPAR antibody, a rabbit anti-β1 integrin antibody and a rabbit anti-VEGFR2 antibody. Right panels show cell lysates prior to immunoprecipitation **E**. PLAs for uPAR and LRP-1, β1 integrin, or β3 integrin were performed as described in (A). PLA: images show representative experiment; graphs are mean and SE (*N*=4-10 images, 2 independent experiments). IP: images show representative experiment; graphs are mean and SE (*N*=3, 3 immunoprecipitation experiments except for IP: uPAR, WB: VEGFR2, *N*=5 immunoprecipitation experiments).

Figure 2: uPAR is crucial for the effects of VEGF *in vitro* and *in vivo.*

A. Western blot analysis of uPAR abundance in extracts of HUVECs transfected with non-silencing siRNA (Ctrl siRNA) or uPAR siRNA. (**B-D**) Assays of endothelial cell function on uPAR or Ctrl siRNA-transfected HUVECs stimulated or not with VEGF. Scratch wound migration assay (*N*=12 wells, 3 independent experiments) (B). Proliferation assay (*N*=9-10

wells, 3 independent experiments) (C). Permeability assay: Time response curves are shown from one representative experiment. Graph shows mean and SE at 180 min (*N*=9 wells, 3 independent experiments). B+C values are expressed relative to the control experiment (Ctrl siRNA, No treatment). **E** Isolectin-B4 staining on postnatal day 7 retinas from WT and *uPAR*$^{-/-}$ pups that were injected at postnatal day 1 with vehicle or VEGF. Scale bar: 25 µm. **F-H**. The histograms represent the quantification of the radial length from the optic nerve to the vascular front (**F**), retinal surface colonized by vessels (**G**), number of branch points in the retinal vasculature (**H**). N=8-9 eyes, 2 independent experiments, **P*<0.02 (**F,G),** **P*=0.028. (**H**). F-H values are expressed relative to the respective controls (white bars). (**I**) Photographs and hemoglobin content of matrigel plugs 10 days after implantation of matrigel mixed with vehicle or with r16K PRL into WT and *uPAR*$^{-/-}$ mice (*N*=9-10 mice, 2 independent experiments, ***P*=0.01).

Figure 3: uPAR siRNA inhibits VEGF-induced VEGFR2 phosphorylation and downstream signaling and the internalization of VEGFR2, uPAR, β1 integrin, and LRP-1.

A. HUVECs transfected with non-silencing siRNA (Ctrl siRNA) or uPAR siRNA were stimulated with VEGF. The phosphorylation of VEGFR2, ERK1/2 and AKT were measured by Western blotting and total VEGFR2, ERK1/2, AKT and tubulin were used as internal controls. uPAR abundance was used as a transfection control. Images show one representative experiment, graphs are mean and SE from 3 independent experiments). **B-F**. Cell surface abundance of VEGFR2 (**B**), uPAR (**C**), β1 integrin (**D**), LRP-1 (**E**) and β3 integrin (**F**) in non-permeabilized HUVECs incubated in the absence (black) or presence of VEGF (red) was determined by flow cytometry. Secondary antibody alone (sec Ab, grey) was used as an internal control. The graph summarizes the quantitative analysis of the cell surface protein abundance, which was calculated from the geometric mean of the fluorescence intensities from 3 separate experiments. Images show one representative experiment; graphs are mean and SE from all experiments. **G.** Internalization of VEGFR2, β1 integrin, and uPAR measured after cell surface biotinylation. Biotinylated proteins were recovered by affinity precipitation on streptavidin beads, subjected to Western blot

analysis and quantified by densitometry relative to the unstimulated control. Tubulin in cell lysates was used as an internal control. Images show one representative experiment of two independent experiments.

Figure 4: Blockage of the internalization process mediated by LRP-1 of HUVECs diminishes VEGF-induced signaling and function.

A. Western blot analysis of HUVECs that were pretreated with dynasore before VEGF stimulation. The phosphorylation of VEGFR2, ERK1/2 and AKT was analyzed by Western blotting and total VEGFR2, ERK1/2, AKT and tubulin were used as internal controls. Quantification was performed to show the fold induction of phosphorylation by VEGF. Images show a representative experiment; graphs are mean and SE from 3 independent experiments. **B-C**. Assays of endothelial cell function were performed on HUVECs pretreated with RAP before VEGF stimulation. B. Scratch wound migration assay. (*N*=12 wells, 3 independent experiments) **C**. Proliferation assay. (*N*=12 wells, 3 independent experiments). **D**. Time response curves are shown from one representative experiment. Graph shows mean and SE at 150 min (*N*=12 wells, 4 independent experiments). **P*<0.05. B+C values are expressed relative to the control experiment (wo RAP, control). **E**. Western blot analysis of HUVECs pretreated with RAP before VEGF stimulation. Western blotting and quantification were performed as described in A (*N*= 3 western blots). **F**. PLAs on HUVECs transfected with non-silencing (Ctrl) or uPAR siRNA and stimulated with VEGF. The PLA signals (red dots) show VEGFR2/LRP-1 complexes (DAPI: blue). The graphs show quantification of number of dots/cell (*N*=4-10 images, 2 independent experiments). **G**. Internalization of VEGFR2 and β1 integrin measured after cell surface biotinylation of HUVECs transfected with non-silencing siRNA (Ctrl siRNA) or uPAR siRNA and stimulated with VEGF. Total protein extracts were analyzed by Western blotting to detect VEGFR2, β1 integrin and uPAR. Tubulin was used as an internal control. Images show one representative experiment of two independent experiments.

Figure 5: VEGFR2 internalization requires uPAR.

The binding of VEGF to VEGFR2 induces its dimerization and phosphorylation by tyrosine kinase. By binding to both uPAR and VEGFR2, β1 integrin acts as an adaptor protein to bring uPAR and VEGFR2 in the complex. uPAR further recruits LRP-1 which can trigger internalization. The whole VEGFR2/β1 integrin/uPAR/LRP-1 complex is internalized, which subsequently enhances VEGFR2 signaling inside the cell.