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BRIEF COMMUNICATION

Involvement of $\alpha_v\beta_3$ integrin in gremlin-induced angiogenesis

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Abstract $\alpha_v\beta_3$ integrin modulates pro-angiogenic endothelial cell (EC) responses following vascular endothelial growth factor receptor-2 (VEGFR2) engagement. The bone morphogenic protein antagonist gremlin is a novel non-canonical VEGFR2 ligand that promotes the acquisition of a pro-angiogenic phenotype in ECs. Here we investigated the role of $\alpha_v\beta_3$ and extracellular matrix components on EC activation induced by gremlin. Gremlin triggers VEGFR2 phosphorylation and cell motility in ECs adherent to the $\alpha_v\beta_3$ ligand fibrinogen but not in ECs adherent to type-I collagen or fibronectin. Also, gremlin and VEGF-A stimulate the formation of VEGFR2/$\alpha_v\beta_3$ integrin complexes as shown by co-immunoprecipitation experiments and fluorescence resonance energy transfer analysis of β_3-ECFP/VEGFR2-EYFP co-transfected ECs. Accordingly, anti-β_3 antibodies block the angiogenic activity exerted by gremlin or VEGF-A in vitro, *ex vivo* and in vivo. The results demonstrate a non-redundant role for $\alpha_v\beta_3$ in gremlin-induced angiogenesis and emphasize its contribution to the formation of functional multi-molecular VEGFR2 complexes responsible for the neovascularization events triggered by canonical and non-canonical pro-angiogenic VEGFR2 ligands.

Keywords Angiogenesis · Endothelium · Extracellular matrix · Gremlin · Integrin · VEGFR2

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Introduction

Angiogenesis plays a key role in various physiological and pathological conditions, including embryonic development, wound repair, inflammation, and tumor growth [1]. The uncontrolled release of angiogenic growth factors is responsible for the endothelial cell (EC) proliferation that takes place during tumor neovascularization and angiogenesis-dependent diseases [2]. Numerous inducers of angiogenesis, including vascular endothelial growth factor-A_{165} (VEGF-A_{165}) and other members of the VEGF family, are produced by tumor or inflammatory cells, accumulate in extracellular matrix (ECM) and eventually interact with tyrosine kinase receptors (TKRs) expressed on EC surface [3]. TKR activity is modulated by integrin receptors that regulate EC response to angiogenic growth factors by binding various cell adhesive ECM proteins, including collagens, vitronectin, fibronectin (FN), and fibrin (ogen) (FG) [4]. Thus, the EC response to angiogenic growth factors is regulated in a complex fashion by distinct sets of inputs conveyed by TKRs and different co-receptors, including integrins.

VEGF-A_{165} interacts directly with VEGF receptor-2 (VEGFR2), the main transducer of VEGF-mediated angiogenic signals in ECs, heparan sulfate proteoglycans (HSPGs) and neuropilins (NRP-1 and -2) [5]. Also, VEGF-A_{165} may induce different intracellular events by inducing the formation of multi-molecular VEGFR2 complexes as a consequence of the association of this TKR with $\alpha_v\beta_3$ integrin, neuropilins, vascular endothelial-cadherin, and ephrin-B2 ([6] and references therein). In particular, interaction between VEGFR2 and $\alpha_v\beta_3$ integrin is required

for a full phosphorylation of VEGFR2 and to drive the VEGF-dependent activation of motogenic pathways [7]. Conversely, the formation of VEGFR2/$\alpha_v\beta_3$ integrin complexes results in an increase of $\alpha_v\beta_3$ integrin affinity for ECM substrata [8]. Also, experimental evidences suggest that VEGF-A_{165} may support EC adhesion, sprouting and survival in a TK independent manner by binding $\alpha_3\beta_1$ and $\alpha_v\beta_3$ integrins [9]. Finally, β_3 integrin can regulate negatively VEGF-mediated angiogenesis by limiting the interaction of VEGFR2 with the co-receptor NRP-1 [10].

Gremlin belongs to the CAN (Cerberus and Dan) family of cystine-knot secreted proteins [11, 12]. It binds various bone morphogenic proteins (BMPs), thus preventing their interaction with the cognate TGFβ family receptors [13]. The BMP antagonist activity of gremlin is thought to play a role in embryonic development of various organ systems as bone, kidney and lung [14–16]. Also, gremlin has been implicated in the pathogenesis of human diseases, including pulmonary hypertension, idiopathic pulmonary fibrosis [17] and diabetic nephropathy [18]. Moreover, gremlin is produced by human tumors [19, 20] and is expressed by pro-angiogenic ECs in vitro and tumor endothelium in vivo [21]. Gremlin stimulates EC intracellular signalling and migration in vitro, leading to a potent angiogenic response in vivo [21, 22]. This is due to its capacity to bind and activate VEGFR2 in a BMP-independent manner [23]. Thus, gremlin acts as a non-canonical VEGFR2 agonist distinct from VEGF family members that may play paracrine/autocrine functions in tumor neovascularization. Interestingly, similarly to VEGF-A_{165}, gremlin binds HSPGs on the EC surface and ECM. However, at variance with canonical heparin-binding VEGFs, gremlin does not interact with NRP-1 co-receptor [24]. Thus, gremlin differs from canonical heparin-binding VEGFs in the ability to co-opt VEGFR2 co-receptors and appears to exert a VEGFR2-dependent pro-angiogenic activation of ECs with mechanisms that are, at least in part, different from VEGFs.

On this basis, to further characterize the multi-molecular VEGFR2 complexes induced by gremlin on EC surface, we investigated the role of $\alpha_v\beta_3$ integrin in the angiogenic response triggered by gremlin. The data demonstrate that gremlin induces the formation of VEGFR2/$\alpha_v\beta_3$ integrin complexes in ECs and that $\alpha_v\beta_3$ integrin engagement by ECM ligands modulates VEGFR2 activation by gremlin and consequent EC responses in vitro and in vivo.

Materials and methods

Cell cultures

Human umbilical vein endothelial cells (HUVECs) were grown in M199 medium (Grand Island, NY) supplemented with 20 % fetal calf serum (FCS, Gibco Life Technologies), endothelial cell growth factor (100 μg/ml) (Sigma Chemical Co., St. Louis, MO) and porcine heparin (Sigma) (100 μg/ml). HUVECs were used at early passages (I–IV) and grown on plastic surface coated with porcine gelatin (Sigma). Fetal bovine aortic endothelial GM7373 cells [25] were grown in Eagle's minimal essential medium (MEM, Gibco Life Technologies) containing 10 % FCS, vitamins, essential and non-essential amino acids. GM7373 cells were co-transfected with a pcDNA3/Enhanced Yellow Fluorescent Protein (EYFP) vector harboring the extracellular domain of murine VEGFR2 (ECD-VEGFR2) cDNA (kindly provided by K. Ballmer-Hofer, PSI, Villigen) and with the pcDNA3/β_3-ECFP vector to generate β_3-ECFP/ECD-VEGFR2-EYFP GM7373 cells for FRET experiments [26]. Parental and $\alpha_v\beta_3$-overexpressing HEK-293 cells were obtained from C.S. Kumar (Schering-Plough Research Institute, USA) and grown in Dulbecco modified Eagle's medium (DMEM) containing 10 % FCS, vitamins, essential and non-essential amino acids. Human adenocarcinoma breast cancer MCF7 cells (HTB-22, ATCC) were grown in DMEM containing 10 % FCS, vitamins, essential and non essential amino acids. MCF7 cells were stably transfected with a pcDNA3 vector harboring human gremlin cDNA by standard procedures to generate gremlin-MCF7 cells.

Chemotaxis

Cells were seeded at 1.0×10^6 cells/ml in the upper compartment of a Boyden chamber containing gelatin-coated PVP-free polycarbonate filters (5 μm pore size, Costar, Cambridge, MA) in presence of anti-β_3 (BV4) and anti-β_1 (BV7) integrin antibodies (Immunological Sciences, Rome, Italy) or irrelevant IgG (20 μg/ml). Murine gremlin (50 ng/ml, R&D System, Minneapolis, MN) or human VEGF-A_{165} (provided by K. Ballmer-Hofer, PSI, Villigen) were dissolved in DMEM with 1 % FCS and placed in the lower compartment. Medium containing 1 % FCS was used as negative control. After 4 h of incubation at 37 °C, cells migrated to the lower side of the filter were stained with Diff-Quik (Dade-Behring, Milan, Italy). Five random fields were counted for each triplicate sample.

Cell adhesion assay

5×10^5 cells were seeded onto wells pre-coated with 2 μg/ml of FG, FN, type-I collagen (CO) (all from Sigma) or gremlin. Cell adhesion was allowed to occur for 2 h at 37 °C. Then, wells were washed with PBS. Adherent cells were fixed and stained with methylene blue/Azur II (1:1, v/v) and solubilized with acetic acid. Plates were read with a microplate reader at 595 nm.

Cell motility assay

EC motility was assessed by time lapse video-microscopy. To this purpose, ECs were seeded on immobilized FG, CO or FN (all at 2 μg/ml) at 150 cells/mm^2 in 24 well-plates for 1 h and then stimulated with 50 ng/ml of gremlin or 30 ng/ml of VEGF-A dissolved in M199 *plus* 5 % FCS. Constant temperature (37 °C) and 5 % CO_2 were maintained throughout the experimental period by means of a heatable stage and climate chamber. Cells were observed under an inverted photomicroscope (Zeiss Axiovert 200 M) and phase-contrast snap photographs (one every 10 min) were digitally recorded for 8 h. Cell paths (20–25 cells per experimental point) were generated from centroid positions and migration parameters were analysed with the "Chemotaxis and Migration Tool" of ImageJ Software (http://rsbweb.nih.gov/ij).

Small interfering RNA and transfection

A pool of 4 small interfering RNAs specific for human β_3 integrin subunit (β_3-siRNA) (Gene solution siRNA ITGβ_3) and control nontargeting siRNA (nt-siRNA) were purchased from Qiagen (Chatsworth, CA). HUVEC transfections were carried out using the HiPerfect system (Qiagen) in accordance to the manufacturer's protocol [26]. After 24 h, cells were tested for integrin down-modulation by FACS analysis and used in chemotaxis assays as described above.

FACS analysis

HUVECs were incubated on ice with 2 μg/ml of anti-β_3 integrin antibody (BV4 clone) and with anti-mouse Alexafluor 488 IgG (Invitrogen, Carlsbad, CA). The secondary Ab alone was used as a control. FACS analysis was performed with a CyFlow Partec flow cytometer (Partec Italia, Milano, Italy) and data were analysed with FlowJo software (Ashland, OR).

Sandwich ELISA assay of VEGFR2/β_3 integrin complex

Ninety-six-well plates were coated for 16 h at 4 °C with 6.5 μg/ml of anti-VEGFR2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS followed by a 1 h blocking step with 1 % bovine serum albumin (Sigma). Next, lysates of HUVECs that had been stimulated with gremlin or VEGF-A in absence or presence of BV4 antibody were added to the wells and incubated for 1 h at room temperature. Then, wells were further incubated for 1 h at room temperature with 2 μg/ml of anti-β_3 integrin BV4 antibody in PBS. Finally, wells were incubated for 45 min at room temperature with a secondary anti-mouse horseradish peroxidase–conjugated antibody (Sigma).

Western blotting and receptor cross-linking assay

Confluent HUVECs were detached from culture plates, resuspended in M199 medium containing 5 % FCS, allowed to adhere on plastic coated with CO, FG or FN or on uncoated wells for 2 h, and stimulated with 50 ng/ml gremlin. After 15 min, cells were lysed in lysis buffer [50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 % Triton X-100, 1.0 mM Na_3VO_4, and protease and phosphatase inhibitors (Sigma)] and 50 μg of total cellular lysate were separated by SDS–PAGE and probed with anti-phospho-VEGFR2 antibody (pTyr1175, Cell Signaling Technology, Boston, MA) and anti-VEGFR2 antibody (Santa Cruz Biotechnology Santa Cruz, CA) in a Western blot.

HUVEC surface biotinylation was performed as previously described [7]. Briefly, confluent cells were incubated for 2 h at 4 °C with biotin-3-sulfo-N-hydroxy-succinimide ester sodium salt (biotin-NHS) (Sigma) dissolved in Hanks' Balanced Salt Solution (HBSS) at 0.5 mg/ml. Cells were then stimulated with 50 ng/ml gremlin or 30 ng/ml VEGF-A for 15 min at room temperature and lysed in lysis buffer. Then, samples (1.0 mg of protein) were immunoprecipitated with anti-VEGFR2 or anti-β_3 integrin (clone BV4) antibodies and separated on SDS-PAGE under non-reducing conditions. Analysis of the biotinylated immunocomplexes was performed using horseradish peroxidase-conjugated streptavidin (HRP-streptavidin).

For cross-linking experiments, HUVECs were incubated for 2 h at 4 °C in the absence or in the presence of 50 ng/ml gremlin and BS3 cross-linker (Bis (sulfosuccinimidyl) suberate, Thermo Scientific, Rockford IL). After Tris-HCl saturation, cells were lysed and cell lysates (1.0 mg of protein) were immunoprecipitated with anti-β_3 integrin (BV4 clone) antibody, separated on a SDS-PAGE gel under reducing conditions, and probed with anti-gremlin or anti-β_3 integrin antibodies (R&D System, Minneapolis, MN) in a Western blot.

Fluorescence resonance energy transfer (FRET) analysis

FRET experiments were performed as previously described [26]. Briefly, β_3-ECFP/ECD-VEGFR2-EYFP GM7373 cells were stimulated with 50 ng/ml gremlin or 30 ng/ml VEGF-A for 10 min, fixed with 4 % paraformaldehyde and subjected to FRET analysis. In FRET analysis, a region of interest (ROI 1) was selected and photobleached by applying 100 % intensity of 514 nm laser. FRET efficiency was calculated by using the formula: FRET $= (D_{post} - D_{pre})/D_{post}$,

where D_{post} and D_{pre} represent the donor (ECFP) emission intensities before and after photobleaching, respectively. FRET efficiency was also measured in a non-photobleached region (ROI 2) of the same cell as an in situ control. In all experiments, cells transfected with a CFP-YFP fusion protein were used as FRET positive controls [27].

Human artery ring assay

One-mm human umbilical artery rings were embedded in fibrin gel [22] and cultured in human serum-free EC medium (Gibco Life Technologies) with 50 ng/ml gremlin or 30 ng/ml VEGF-A in the absence or in the in presence of anti-β_3 (BV4) or anti-β_1 (BV7) integrin antibodies (both at 10 μg/ml). After 3 days, EC sprouts, morphologically distinguishable from scattering fibroblasts/smooth muscle cells, were counted under an inverted microscope at 200× magnification and photographed at day 6 of incubation.

Chick embryo chorionallantoic membrane (CAM) assay

Alginate beads (5 μl) containing vehicle, gremlin or VEGF-A (both at 100 ng per embryo) in the absence or in the presence of 800 ng of the anti-$\alpha_v\beta_3$ integrin antibody LM609 (Millipore, Billerica MA) were placed on the CAM of fertilized White Leghorn chicken eggs at day 11 of incubation [22]. In a second set of experiments, conditioned media (CM) from MCF7 and gremlin-MCF7 cells were collected and concentrated fivefold using Centricon YM-10 filters (Millipore). Alginate beads containing concentrated CM (3 μl) were placed on the CAM in the absence or in the presence of 200 ng of anti-hVEGF-A antibody (R&D System) or 1.5 μg of LM609 antibody (Millipore) [22]. After 72 h, newly formed blood vessels converging towards the implant were counted at 5× magnification using a STEMI SR stereomicroscope equipped with an objective *f* equal to 100 mm with adapter ring 475070 (Zeiss).

Data representation

Data are expressed as mean ± S.D. Statistical analyses were done using Student's *t* test. The significance level was set at $p < 0.05$.

Results and discussion

$\alpha_v\beta_3$ integrin plays an important role in modulating pro-angiogenic EC responses following VEGFR2 engagement by VEGF [28]. Recent observations have shown that gremlin is a pro-angiogenic, neuropilin-independent non-canonical VEGFR2 ligand [23, 24]. On this basis, we performed a preliminary set of experiments to evaluate whether $\alpha_v\beta_3$ integrin function may mediate also the pro-angiogenic activity of gremlin. To this purpose, the capacity of gremlin to induce a chemotactic response in ECs was assessed in the absence or in the presence of antibodies directed against the β_3 or the β_1 integrin subunit (clones BV4 and BV7, respectively). As shown in Fig. 1a, the anti-β_3 antibody BV4, but not the anti-β_1 antibody BV7, significantly reduced the chemotactic activity exerted by gremlin and by the VEGFR2 canonical ligand VEGF-A on HUVECs. It must be pointed out that at the concentration tested the BV4 antibody inhibits $\alpha_v\beta_3$ integrin signalling without affecting EC adhesion to the substratum [7]. A similar reduction in the chemotactic response to the two VEGFR2 ligands was obtained when β_3 integrin expression in HUVECs was downregulated by β_3-siRNA transfection (Online Resource 1, Fig. S1a,b). Thus, our observations indicate that $\alpha_v\beta_3$ integrin may play an active role in gremlin-induced EC stimulation.

These results prompted us to investigate the ability of gremlin to induce a productive crosstalk between VEGFR2 and $\alpha_v\beta_3$ integrin receptors in activated ECs, leading to the formation of VEGFR2/$\alpha_v\beta_3$ integrin complexes [7]. To this purpose, the cell membrane of HUVEC cultures was biotinylated and cells were challenged with gremlin or VEGF-A. Next, cells were lysed and immunoprecipitated with an anti-VEGFR2 antibody. Biotinylated proteins were analyzed by SDS-PAGE followed by blotting with HRP-streptavidin. Two biotinylated bands representing α_v and β_3 integrin subunits were present in VEGFR2-immune complexes from stimulated but not from unstimulated HUVECs. The identity of the two bands was confirmed by immunoprecipitation of biotinylated membrane proteins with an anti-β_3 integrin antibody (Fig. 1b). On this basis, we compared the kinetics of VEGFR2/$\alpha_v\beta_3$ integrin complex formation in gremlin- and VEGF-A-stimulated HUVECs. The two VEGFR2 agonists induce the formation of VEGFR2/$\alpha_v\beta_3$ integrin complexes with similar kinetics, as assessed by a sandwich ELISA assay of HUVEC lysates, with a peak at 15 min after stimulation (Online Resource 1, Fig. S2a). Of note, VEGFR2/$\alpha_v\beta_3$ integrin complex formation was fully prevented when HUVECs were pre-incubated with the anti-β_3 BV4 antibody before ligand administration (Online Resource 1, Fig. S2b).

To assess whether the formation of co-immunoprecipitable VEGFR2/$\alpha_v\beta_3$ integrin complexes is the result of a direct VEGFR2-β_3 integrin interaction, endothelial GM7373 cells were co-transfected with the β_3 integrin subunit and the ECD-VEGFR2 fused to ECFP (donor) and EYFP (acceptor) protein tags, respectively. Then, transfected cells were subjected to FRET analysis following gremlin stimulation. The analysis, performed by acceptor

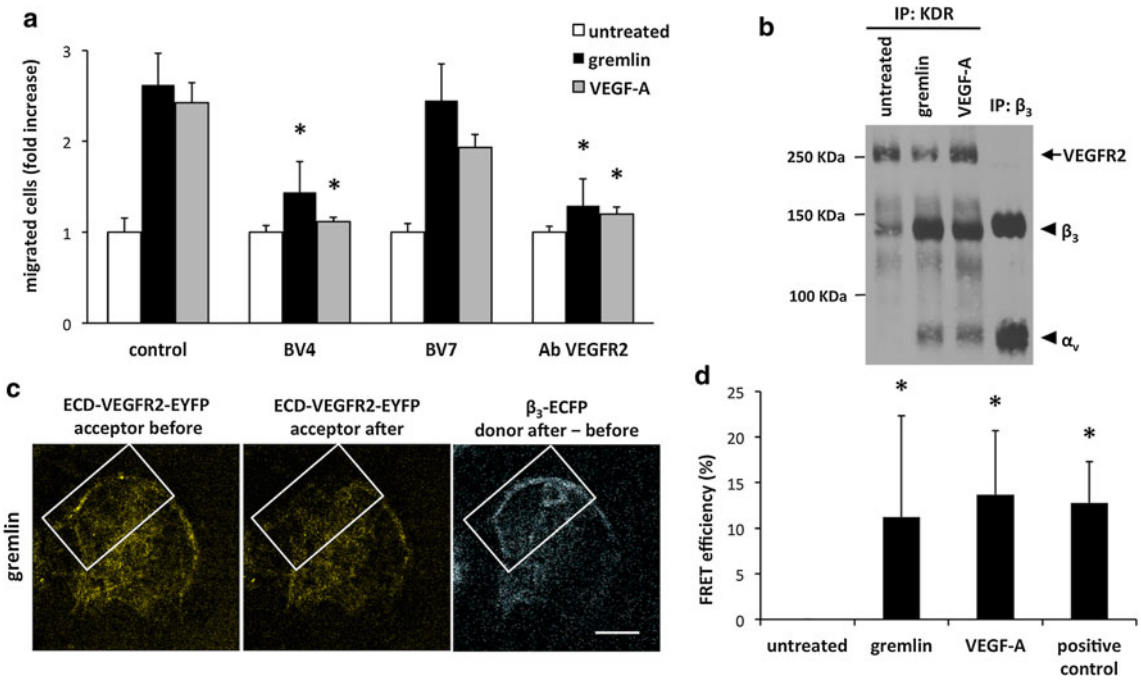

Fig. 1 Gremlin induces VEGFR2/$\alpha_v\beta_3$ integrin complex formation in ECs. **a** HUVECs were assessed for their capacity to migrate in response to 50 ng/ml of gremlin or 30 ng/ml VEGF-A in a Boyden chamber assay in the presence of irrelevant Ig (control), anti-β_3 antibody BV4, anti-β_1 antibody BV7 or neutralizing anti-VEGFR2 antibody (all at 20 μg/ml). After 4 h, cells migrated to the lower side of the filter were counted and data were expressed as fold increase *versus* cells migrated in the absence of a chemotactic stimulus. Both anti-β_3 antibody BV4 and anti-VEGFR2 antibody suppress the chemotactic activity of gremlin and VEGF-A (*, $p < 0.05$, Student's *t* test). **b** Surface HUVEC membrane proteins were labeled with NHS-biotin. Then, cells were incubated for 15 min at room temperature with 50 ng/ml gremlin or 30 ng/ml VEGF-A and VEGFR2 immunocomplexes were immunoprecipited (IP) and analyzed by SDS-PAGE followed by blotting with HRP-streptavidin. The two biotinylated bands of approximately 75 kDa and 125 kDa (arrowheads) significantly increased in gremlin-treated cells represent the α_V and β_3 integrin subunits, respectively, as confirmed by anti-β_3 immunoprecipitation of biotinylated HUVEC membranes. Note the similar content of the biotinylated ~250 kDa VEGFR2 band (arrow) in both VEGFR2 immunoprecipitates from untreated and gremlin- VEGF-A-treated cells. **c**, **d** β_3-ECFP/ECD-VEGFR2-EYFP GM7373 cells were seeded on glass coverslips and starved overnight in serum-free medium. Then cells were stimulated with 50 ng/ml gremlin, 30 ng/ml VEGF-A or left untreated. After 10 min, cells were fixed and subjected to FRET analysis, performed by acceptor photobleaching technology, using a Zeiss LSM 510 confocal microscope. **c** Sequentially representative acceptor photobleaching images from a gremlin-stimulated EC (from left to right: acceptor channel before bleaching, acceptor channel after bleaching, and donor channel before bleaching subtracted to donor channel after bleaching). *Scale bar* = 20 μm. **d** FRET efficiency was calculated in 20 cells per experimental point using the formula: E_F = (Donor intensity after − Donor intensity before)/Donor intensity after. FRET efficiency was measured in a non-photobleached region of the same cell as an in situ negative control. No significant positive FRET signal was observed in untreated cells. As a FRET positive control, cells were transfected with a CFP-YFP fusion protein [27]. (*, $p < 0.0001$, Student's *t* test)

photobleaching technology, showed a significant increase of the direct interaction between VEGFR2 and β_3 integrin in gremlin-stimulated β_3-ECFP/ECD-VEGFR2-EYFP GM7373 cells when compared to control cells (n = 20; *, $p < 0.0001$, Student's *t* test). A similar interaction was observed when cells were stimulated with VEGF-A (n = 20; *, $p < 0.0001$, Student's *t* test) (Fig. 1c, d).

Previous observations had shown that VEGF-A may support EC adhesion, sprouting and survival in a TK independent manner by binding $\alpha_3\beta_1$ and $\alpha_v\beta_3$ integrins [9]. On this basis, we evaluated the possibility that also gremlin may bind directly to $\alpha_v\beta_3$ integrin, thus mediating cell adhesion to the immobilized protein. To this purpose, parental and $\alpha_v\beta_3$ integrin-overexpressing HEK 293 cells were seeded on non-tissue culture plastic coated with the ECM proteins FG, CO or FN or with gremlin. As shown in Online Resource 1, Fig. S3a, $\alpha_v\beta_3$ integrin overexpression caused a significant increase of HEK 293 cell adhesion to immobilized FG, but not to CO or FN. Also, $\alpha_v\beta_3$ integrin overexpression did not increase HEK 293 cell adhesion to immobilized gremlin that was devoid of a specific $\alpha_v\beta_3$ integrin-dependent cell adhesive activity. Accordingly, cross-linking experiments performed on gremlin-treated HUVECs failed to detect the presence of a specific gremlin-immunoreactive band in the β_3 integrin immunoprecipitate of the cell lysates (Online Resource 1, Fig. S3b). Taken together, our results rule out the possibility that gremlin can bind directly to $\alpha_v\beta_3$ and indicate that the interaction of gremlin with EC surface causes the formation of VEGFR2/$\alpha_v\beta_3$ integrin complexes by binding to the

extracellular domain of VEGFR2 only, leading to $\alpha_v\beta_3$ integrin recruitment.

To evaluate the role of $\alpha_v\beta_3$ integrin activation following the engagement by its ECM ligands in mediating the VEGFR2-dependent EC response to gremlin, we performed time-lapse video microscopy to measure the chemokinetic response elicited by gremlin and VEGF-A on individual HUVECs adherent to immobilized FG, CO, or FN, ECM proteins with different integrin selectivity. In the absence of stimulation, HUVECs exhibited a similar baseline cell migration rate on the three ECM substrata (ranging between 8.0 ± 3.7 μm/h to 8.5 ± 6.6 μm/h for cells seeded on CO or FN, respectively). Following stimulation with gremlin or VEGF-A, the EC migration rate increased significantly to 14.9 ± 6.7 μm/h and 14.3 ± 4.5 μm/h, respectively, for HUVECs adherent to the $\alpha_v\beta_3$ integrin ligand FG whereas it remained unchanged for cells seeded on the other ECM proteins. This results in a significant increase of the cumulative distance covered by gremlin- and VEGF-A-stimulated HUVECs adherent to FG in respect to the other experimental conditions ($p < 0.005$, Student's t test) (Fig. 2a,b). On this basis, to assess the role of FG-mediated adhesion on VEGFR2 activation by gremlin, we evaluated VEGFR2 phosphorylation in ECs adherent to FG, CO or FN. To this purpose, quiescent HUVECs were allowed to adhere for 2 h on the different substrata and then challenged with gremlin. Even though HUVECs showed a similar adhesive capacity to the different immobilized proteins (data not shown), gremlin triggered VEGFR2 phosphorylation in cells adherent to the $\alpha_v\beta_3$ ligand FG with minor effects on cells seeded on CO or FN, pointing to a major involvement of $\alpha_v\beta_3$ integrin in VEGFR2 activation by gremlin (Fig. 2c). These data extend previous observations about the role of $\alpha_v\beta_3$ integrin in VEGFR2 activation by the canonical ligand VEGF-A [29]. Time course experiments confirmed that VEGFR2 phosphorylation in FG-adherent cells peaked 5 min after stimulation with gremlin to return to basal levels at 30 min whereas no receptor activation occurred in the absence of $\alpha_v\beta_3$ integrin engagement at all the time points investigated (Online Resource 1, Fig. S4).

In keeping with these observations, the anti-β_3 antibody BV4 fully abolished the ability of gremlin and VEGF-A to induce EC sprouting when added *ex vivo* to human umbilical artery rings embedded in a three-dimensional fibrin gel [23] whereas the anti-β_1 antibody BV7 was ineffective (Fig. 3a, b). Also, the anti-$\alpha_v\beta_3$ integrin antibody LM609 [30] prevented the neovascular response triggered in vivo by gremlin and VEGF-A in a chick embryo CAM assay [23] with no effect on the basal vascularization of the membrane (Fig. 3c, d). It must be pointed out that previous observations from our laboratory had shown that both the *ex vivo* and in vivo responses

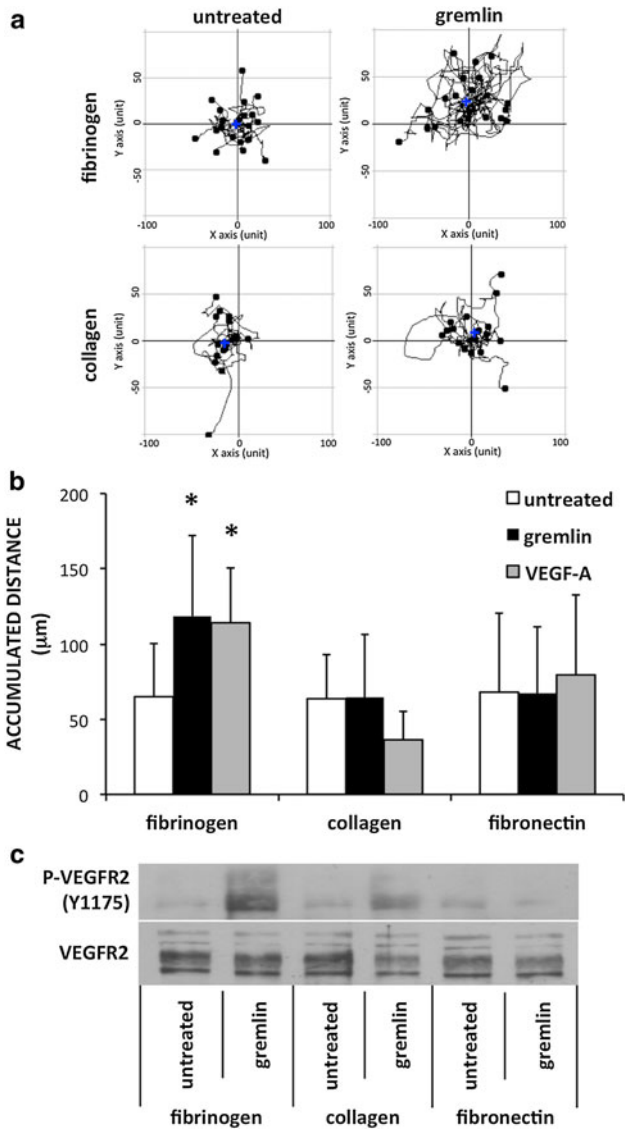

Fig. 2 Effect of $\alpha_v\beta_3$ integrin engagement on gremlin-induced EC motogenesis and VEGFR2 activation. HUVECs were seeded on FG, CO or FN in M199 *plus* 5 % FCS. Cells were stimulated 1 h after plating with 50 ng/ml gremlin or 30 ng/ml VEGF-A and cell motility was assessed by time lapse videomicroscopy using an inverted photomicroscope (Zeiss Axiovert 200 M). Phase-contrast snap photographs were digitally recorded every 10 min for 8 h. Cell paths (20–25 cells per experimental point) were generated from centroid positions and migration parameters were analyzed with the "Chemotaxis and Migration Tool" of ImageJ Software (http://rsbweb.nih.gov/ij). **a** Representative tracked paths of HUVECs adherent to FG or CO and unstimulated or stimulated by 50 ng/ml gremlin. **b** Accumulated distances (in μm) of HUVECs adherent to different ECM proteins and unstimulated or stimulated by gremlin or VEGF-A. Adhesion to FG induced a significant increase of EC motility induced by gremlin and VEGF-A (*, $p < 0.05$, Student's t test). **c** HUVECs were seeded on FG, CO or FN in M199 *plus* 5 % FCS. 2 h after plating, cells were stimulated with 50 ng/ml gremlin for 10 min and total cell lysates (50 μg of proteins) were separated on SDS-PAGE and assessed for VEGFR2 phosphorylation in a Western blot using an anti-phospho-VEGFR2 (pTyr1175) antibody. Gremlin-induced VEGFR2 phosphorylation significantly increased mainly in cells adherent to the $\alpha_v\beta_3$ ligand FG

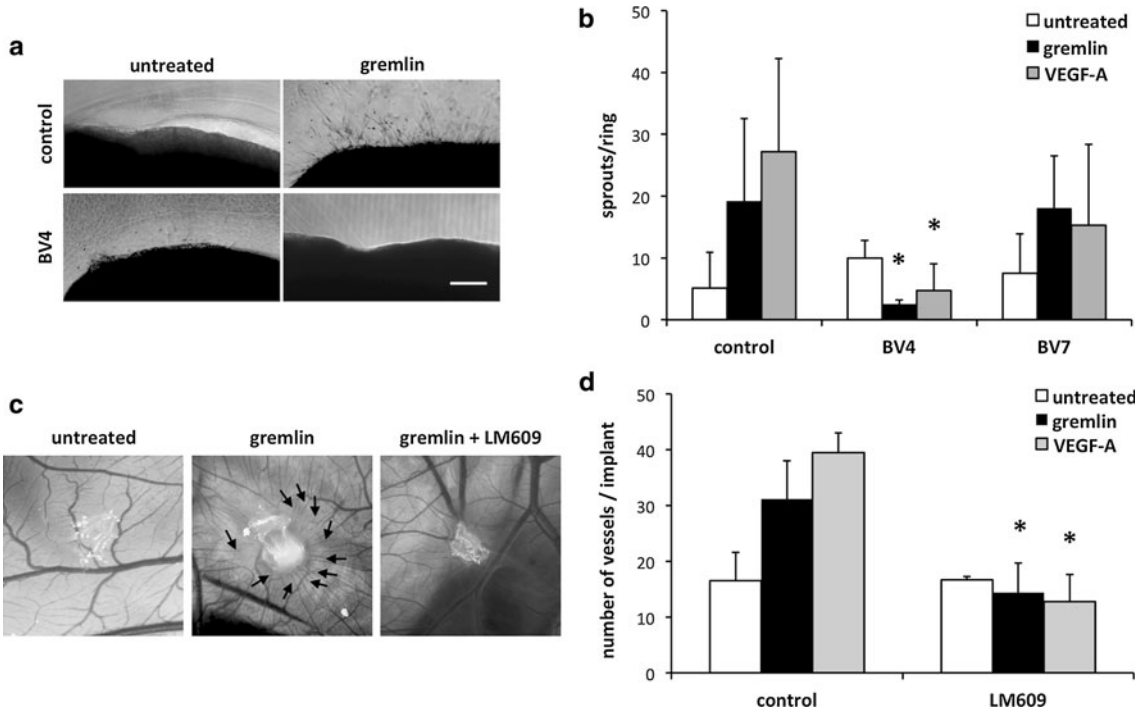

Fig. 3 $\alpha_v\beta_3$ integrin mediates the angiogenic activity of gremlin. **a**, **b** Human umbilical artery rings were embedded in fibrin gel and stimulated with 50 ng/ml gremlin or 30 ng/ml VEGF-A in the absence or in the presence of irrelevant IgG (control), anti-β_3 antibody (BV4) or anti-β_1 antibody (BV7) (all at 10 μg/ml). After 3 days, rings were photographed at 200× magnification using an Axiovert 200 M microscope equipped with a 20× objective (LD A PLAN 20X/0,30PH1, Zeiss) (**a**) and EC sprouts were counted (**b**). Note the presence of numerous EC sprouts in gremlin- and VEGF-A-treated artery rings whose formation is inhibited by the specific anti-β_3 antibody (*, $p < 0.05$, Student's t test). *Scale bar* = 200 μm. **c**, **d** Alginate pellets containing 100 ng of gremlin or VEGF-A alone or added with 800 ng of neutralizing anti-$\alpha_v\beta_3$ integrin LM609 antibody were implanted on the top of chick embryo CAMs at day 11 of development. After 3 days, CAMs were photographed *in ovo* at 5× magnification under a stereomicroscope (**c**) and newly formed microvessels converging *versus* the implant were counted (**d**). The angiogenic response triggered by gremlin or VEGF-A (*arrows* in **c**) was fully abolished by the anti-$\alpha_v\beta_3$ integrin LM609 antibody (*, $p < 0.05$, Student's t test)

triggered by gremlin in these angiogenesis assays were dependent on VEGFR2 engagement and activation [23].

Even though VEGF-A plays a central role in switching on a pro-angiogenic phenotype in most tumors, neoplastic, stromal, and infiltrating cells may produce a plethora of different pro-angiogenic factors, including gremlin [19–21]. This may limit the efficacy of VEGF-targeting antineoplastic strategies and represents a possible mechanism of tumor resistance to anti-VEGF therapies [31]. To assess the impact of gremlin on the neovascular response elicited by VEGF-A-expressing tumor cells, human breast carcinoma MCF7 cells were stably transfected with human gremlin cDNA, thus generating gremlin-MCF7 cells. Parental and gremlin-overexpressing cells release similar amounts of VEGF-A (1.7 ng/10^6 cells/24 h and 1.8 ng/10^6 cells/24 h, respectively) whereas significant levels of gremlin (8.2 ng/10^6 cells/24 h) were detected only in the CM of the transfected cell population. Accordingly, the CM of gremlin-MCF7 cells exerted a more potent neovascular response in the CAM assay when compared to the CM from parental cells both in the absence and in the presence of the anti-VEGF-A antibody that caused only a limited, although significant inhibition of the angiogenic activity of the CM from parental and gremlin-overexpressing cells. Thus, gremlin is able to enhance the angiogenic potential of VEGF-A-expressing tumor cells (Fig. 4). Finally, in agreement with the role of $\alpha_v\beta_3$ integrin in mediating the angiogenic activity of gremlin and VEGF-A, the anti-$\alpha_v\beta_3$ integrin antibody LM609 significantly affected the pro-angiogenic activity of the CM from both parental and gremlin-MCF7 cells (Fig. 4). Further studies will be required to investigate the effect of anti-$\alpha_v\beta_3$ integrin strategies [32] on the tumorigenic potential of gremlin/VEGF-A co-expressing tumors.

Taken together, our data underline the importance of $\alpha_v\beta_3$ integrin in VEGFR2 activation and angiogenesis triggered by gremlin, showing that this non-canonical VEGFR2 ligand is able to induce the formation of VEGFR2/$\alpha_v\beta_3$ integrin complexes. Previous observations had shown that VEGFR2-$\alpha_v\beta_3$ integrin interaction triggered by VEGF-A takes place outside the cell and that the cytoplasmic domain of β_3 integrin is not required for its association with VEGFR2 [33]. However, in apparent contrast with these observations, recent data implicate the

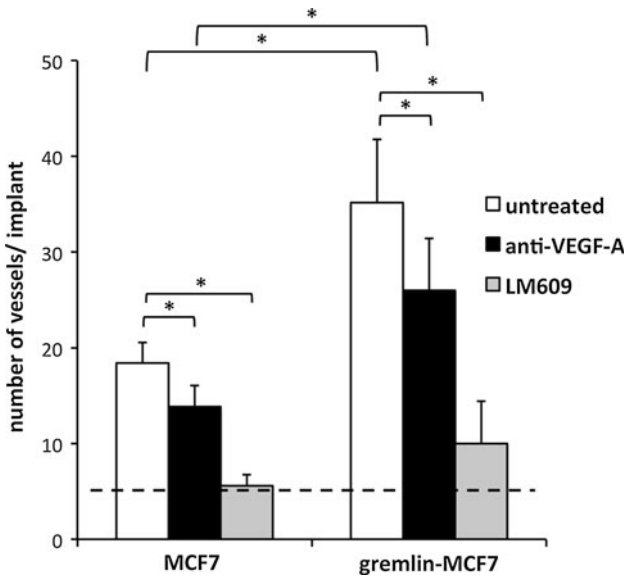

Fig. 4 Gremlin enhances the angiogenic potential of VEGF-A-expressing MCF7 tumor cells. Alginate pellets containing 3 µl of fivefold concentrated CM from parental and gremlin-MCF7 cells were implanted on the top of chick embryo CAMs at day 11 of development in the absence or presence of 200 ng of neutralizing anti-hVEGF-A antibody or of 1.5 µg of neutralizing anti-$\alpha_v\beta_3$ integrin LM609 antibody. After 3 days, newly formed microvessels converging *versus* the implant were counted. Dashed line represents the number of vessels induced by DMEM alone (*, $p < 0.05$, Student's *t* test)

cytoplasmic tail of β_3 integrin in the interaction with a membrane-proximal region in VEGFR2 [34]. Our FRET experiments on β_3-ECFP/ECD-VEGFR2-EYFP EC transfectants extend these findings and demonstrate that the extracellular and transmembrane portion of VEGFR2, devoid of its intracellular domain, is sufficient for $\alpha_v\beta_3$ integrin interaction triggered by gremlin or VEGF-A. Of note, our findings also demonstrate that, at variance with what suggested for VEGF-A [9], gremlin does not interact directly with $\alpha_v\beta_3$, being unable to mediate cell adhesion to the immobilized protein. This suggests that the binding of gremlin to VEGFR2, with consequent VEGFR2 dimerization [23], may induce conformational changes of the extracellular domain of the receptor that allow $\alpha_v\beta_3$ integrin engagement. Indeed, the binding of VEGF-A induces profound conformational changes in ECD-VEGFR2 [35]. Experiments are in progress to assess whether also gremlin interaction may elicit similar conformational modifications in ECD-VEGFR2.

In conclusion, the bulk of experimental evidences indicate that $\alpha_v\beta_3$ integrin plays an important role in the VEGFR2-dependent angiogenic responses induced by gremlin in ECs. These observations emphasize the role of $\alpha_v\beta_3$ integrin in angiogenesis and its contribution to the formation of functional multi-molecular VEGFR2 complexes responsible for the neovascularization events triggered by both canonical and non-canonical pro-angiogenic VEGFR2 ligands.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257
2. Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1:27–31
3. Ferrara N (2010) Binding to the extracellular matrix and proteolytic processing: two key mechanisms regulating vascular endothelial growth factor action. Mol Biol Cell 21:687–690
4. Ingber DE, Folkman J (1987) Regulation of endothelial growth factor action: solid state control by extracellular matrix. Prog Clin Biol Res 249:273–282
5. Bussolino F, Serini G, Mitola S, Bazzoni G, Dejana E (2001) Dynamic modules and heterogeneity of function: a lesson from tyrosine kinase receptors in endothelial cells. EMBO Rep 2:763–767
6. Berger P, Ballmer-Hofer K (2011) The reception and the party after: how vascular endothelial growth factor receptor 2 explores cytoplasmic space. Swiss medical weekly 141:w13318
7. Soldi R, Mitola S, Strasly M, Defilippi P, Tarone G, Bussolino F (1999) Role of alphavbeta3 integrin in the activation of vascular endothelial growth factor receptor-2. The EMBO journal 18:882–892
8. Byzova TV, Goldman CK, Pampori N, Thomas KA, Bett A, Shattil SJ, Plow EF (2000) A mechanism for modulation of cellular responses to VEGF: activation of the integrins. Mol Cell 6:851–860
9. Hutchings H, Ortega N, Plouet J (2003) Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration, and survival through integrin ligation. FASEB journal: official publication of the Federation of American Societies for Experimental Biology 17:1520–1522
10. Robinson SD, Reynolds LE, Kostourou V, Reynolds AR, da Silva RG, Tavora B, Baker M, Marshall JF, Hodivala-Dilke KM (2009) Alphav beta3 integrin limits the contribution of neuropilin-1 to vascular endothelial growth factor-induced angiogenesis. The Journal of biological chemistry 284:33966–33981
11. Pearce JJ, Penny G, Rossant J (1999) A mouse cerberus/Dan-related gene family. Dev Biol 209:98–110
12. Vitt UA, Hsu SY, Hsueh AJ (2001) Evolution and classification of cystine knot-containing hormones and related extracellular signaling molecules. Mol Endocrinol 15:681–694
13. Balemans W, Van Hul W (2002) Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. Dev Biol 250:231–250
14. Khokha MK, Hsu D, Brunet LJ, Dionne MS, Harland RM (2003) Gremlin is the BMP antagonist required for maintenance of Shh and Fgf signals during limb patterning. Nat Genet 34:303–307
15. Lu MM, Yang H, Zhang L, Shu W, Blair DG, Morrisey EE (2001) The bone morphogenic protein antagonist gremlin

regulates proximal-distal patterning of the lung. Dev Dyn 222: 667–680
16. Michos O, Panman L, Vintersten K, Beier K, Zeller R, Zuniga A (2004) Gremlin-mediated BMP antagonism induces the epithelial-mesenchymal feedback signaling controlling metanephric kidney and limb organogenesis. Development 131:3401–3410
17. Costello CM, Cahill E, Martin F, Gaine S, McLoughlin P (2009) Role of gremlin in the lung: development and disease. Am J Respir Cell Mol Biol 42:517–523
18. Lappin DW, McMahon R, Murphy M, Brady HR (2002) Gremlin: an example of the re-emergence of developmental programmes in diabetic nephropathy. Nephrol Dial Transplant 17(Suppl 9):65–67
19. Namkoong H, Shin SM, Kim HK, Ha SA, Cho GW, Hur SY, Kim TE, Kim JW (2006) The bone morphogenetic protein antagonist gremlin 1 is overexpressed in human cancers and interacts with YWHAH protein. BMC Cancer 6:74
20. Sneddon JB, Zhen HH, Montgomery K, van de Rijn M, Tward AD, West R, Gladstone H, Chang HY, Morganroth GS, Oro AE, Brown PO (2006) Bone morphogenetic protein antagonist gremlin 1 is widely expressed by cancer-associated stromal cells and can promote tumor cell proliferation. Proc Natl Acad Sci USA 103:14842–14847
21. Stabile H, Mitola S, Moroni E, Belleri M, Nicoli S, Coltrini D, Peri F, Pessi A, Orsatti L, Talamo F, Castronovo V, Waltregny D, Cotelli F, Ribatti D, Presta M (2007) Bone morphogenic protein antagonist Drm/gremlin is a novel proangiogenic factor. Blood 109:1834–1840
22. Mitola S, Moroni E, Ravelli C, Andres G, Belleri M, Presta M (2008) Angiopoietin-1 mediates the pro-angiogenic activity of the bone morphogenic protein antagonist Drm. Blood 112:1154–1157
23. Mitola S, Ravelli C, Moroni E, Salvi V, Leali D, Ballmer-Hofer K, Zammataro L, Presta M (2010) Gremlin is a novel agonist of the major proangiogenic receptor VEGFR2. Blood 116:3677–3680
24. Chiodelli P, Mitola S, Ravelli C, Oreste P, Rusnati M, Presta M (2011) Heparan sulfate proteoglycans mediate the angiogenic activity of the vascular endothelial growth factor receptor-2 agonist gremlin. Arterioscler Thromb Vasc Biol 31:e116–e127
25. Grinspan JB, Mueller SN, Levine EM (1983) Bovine endothelial cells transformed in vitro by benzo(a)pyrene. J Cell Physiol 114:328–338
26. Urbinati C, Ravelli C, Tanghetti E, Belleri M, Giacopuzzi E, Monti E, Presta M, Rusnati M (2012) Substrate-Immobilized HIV-1 Tat drives VEGFR2/alphavbeta3-integrin complex formation and polarization in endothelial cells. Arteriosclerosis, thrombosis, and vascular biology
27. Karpova TS, Baumann CT, He L, Wu X, Grammer A, Lipsky P, Hager GL, McNally JG (2003) Fluorescence resonance energy transfer from cyan to yellow fluorescent protein detected by acceptor photo bleaching using confocal microscopy and a single laser. J Microsc 209:56–70
28. Somanath PR, Malinin NL, Byzova TV (2009) Cooperation between integrin alphavbeta3 and VEGFR2 in angiogenesis. Angiogenesis 12:177–185
29. Mitola S, Brenchio B, Piccinini M, Tertoolen L, Zammataro L, Breier G, Rinaudo MT, den Hertog J, Arese M, Bussolino F (2006) Type I collagen limits VEGFR-2 signaling by a SHP2 protein-tyrosine phosphatase-dependent mechanism 1. Circ Res 98:45–54
30. Drake CJ, Cheresh DA, Little CD (1995) An antagonist of integrin alpha v beta 3 prevents maturation of blood vessels during embryonic neovascularization. J Cell Sci 108(Pt 7):2655–2661
31. Abdullah SE, Perez-Soler R (2011) Mechanisms of resistance to vascular endothelial growth factor blockade. Cancer
32. Weis SM, Cheresh DA (2011) Tumor angiogenesis: molecular pathways and therapeutic targets. Nat Med 17:1359–1370
33. Borges E, Jan Y, Ruoslahti E (2000) Platelet-derived growth factor receptor beta and vascular endothelial growth factor receptor 2 bind to the beta 3 integrin through its extracellular domain. The Journal of biological chemistry 275:39867–39873
34. West XZ, Meller N, Malinin NL, Deshmukh L, Meller J, Mahabeleshwar GH, Weber ME, Kerr BA, Vinogradova O, Byzova TV (2012) Integrin beta(3) Crosstalk with VEGFR Accommodating Tyrosine Phosphorylation as a Regulatory Switch. PLoS ONE 7:e31071
35. Brozzo MS, Bjelic S, Kisko K, Schleier T, Leppanen VM, Alitalo K, Winkler FK, Ballmer-Hofer K (2012) Thermodynamic and structural description of allosterically regulated VEGFR-2 dimerization. Blood 119:1781–1788