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Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors

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Inhibitors of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin have entered clinical trials as antiangiogenic agents for cancer treatment but generally have been unsuccessful. Here we present *in vivo* evidence that low (nanomolar) concentrations of RGD-mimetic $\alpha_v\beta_3$ and $\alpha_v\beta_5$ inhibitors can paradoxically stimulate tumor growth and tumor angiogenesis. We show that low concentrations of these inhibitors promote VEGF-mediated angiogenesis by altering $\alpha_v\beta_3$ integrin and vascular endothelial growth factor receptor-2 trafficking, thereby promoting endothelial cell migration to VEGF. The proangiogenic effects of low concentrations of RGD-mimetic integrin inhibitors could compromise their efficacy as anticancer agents and have major implications for the use of RGD-mimetic compounds in humans.

Inhibitors of angiogenesis can suppress tumor growth in preclinical models and have entered the clinic as prospective anticancer therapeutics[1,2]. Tumor angiogenesis is predominantly driven by vascular endothelial growth factor (VEGF), a proangiogenic growth factor expressed by many solid cancers[3,4]. VEGF stimulates angiogenesis through VEGF receptor-2 (VEGFR2), a tyrosine kinase receptor expressed by endothelial cells[4,5]. Inhibitors of the VEGF signaling pathway can extend progression-free survival in colorectal, lung and breast cancers when used in combination with chemotherapy[6–8] and in renal cancer when used as a monotherapy[9]. Angiogenesis is also regulated by endothelial cell adhesion molecules, such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins[10,11]. Inhibitors of these integrins ($\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors) suppress tumor growth in certain pre-clinical models[12–14], but $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors have failed to produce significant results in the majority of humans tested[15,16]. The reason for this lack of efficacy is currently not clear.

The integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ can be expressed by both tumor cells and tumor endothelial cells[10]. It is speculated that drugs that inhibit the adhesive function of these integrins can inhibit tumor growth in at least two ways: by targeting the tumor cells directly and by inhibiting tumor angiogenesis[10,11,17–20]. Existing $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors include the RGD-mimetic cyclic peptide, cilengitide (also called EMD 121974)[21,22] and RGD-mimetic small molecules such as S 36578 (refs. 23,24). Cilengitide is currently in phase 1 and 2 clinical trials for cancer therapy. Although a small proportion of patients with glioma respond to cilengitide when it is administered at high doses[25], there is currently little evidence that cilengitide is effective in the treatment of other human cancers[26–28]. $\alpha_v\beta_3$ integrin–expressing glioma cells are highly sensitive to cilengitide; thus, the efficacy of cilengitide in gliomas may be largely attributable to direct effects on α_v integrin–expressing glioma cells[29,30] rather than antiangiogenic effects *per se*. In support of these observations, there is to our knowledge currently no published data demonstrating that RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors can suppress angiogenesis in any type of human tumor, including glioma[15,16]. There is, therefore, a need to address why integrin inhibitors have proven to be largely ineffective in the majority of human cancers.

Here we present evidence for a mechanism that may compromise the efficacy of RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors. We show that, to our surprise, nanomolar concentrations of RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors can actually enhance the growth of tumors *in vivo* by promoting VEGF-mediated angiogenesis. This may seriously compromise the activity of these agents as anticancer drugs and may provide an explanation as to why therapy with RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors is usually ineffective in humans.

RESULTS

Integrin inhibitors can enhance tumor growth and angiogenesis

Clinical trials have shown that the majority of human tumors are refractory to integrin inhibitor therapy. To model this scenario, we used two tumor models that are refractory to integrin inhibitor therapy. Daily administration of 200 mg $kg^{-1}d^{-1}$ S 36578 or cilengitide

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did not suppress tumor growth or angiogenesis significantly in mouse B16F0 melanoma or Lewis lung carcinoma (LLC) tumor grafts (**Fig. 1a**,**b**), despite expression of $\alpha_v\beta_3$ integrin in the vasculature of both tumor types (**Fig. 1c**). Thus, although both tumor types express the drug target ($\alpha_v\beta_3$ integrin) in their vasculature, B16F0 and LLC tumor grafts seem refractory to $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor monotherapy, a scenario that mirrors the situation often observed in clinical trials. Notably, within a few hours after administration of an integrin inhibitor bolus (200 mg kg^{-1}), plasma concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor fell rapidly from micromolar to nanomolar levels (**Supplementary Fig. 1a** online). Furthermore, we observed sustained nanomolar plasma concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor for 16–24 h after administration (**Supplementary Fig. 1a**). Therefore, we investigated whether nanomolar doses of integrin inhibitors could have different effects on tumor growth and angiogenesis as compared to micromolar doses of integrin inhibitors. To our surprise, when we maintained low plasma concentrations of 1.7 ± 0.8 nM S 36578 in mice (using osmotic minipumps loaded with 0.1 mg ml^{-1} S 36578, **Supplementary Fig. 1b**), the growth of B16F0 melanomas was enhanced significantly compared to that in vehicle-treated mice ($P < 0.05$; **Fig. 1d**). In contrast, when we maintained plasma concentrations of S 36578 at higher levels of 4 ± 1.5 μM (using osmotic minipumps loaded with 100 mg ml^{-1} S 36578, **Supplementary Fig. 1b**), tumor volumes were similar to those in vehicle–treated mice (**Fig. 1d**). Low plasma concentrations of 1.7 ± 0.8 nM S 36578 also enhanced growth of LLC tumors ($P < 0.001$; **Fig. 1d**).

Enhanced B16F0 and LLC tumor growth also occurred when we maintained low plasma concentrations (<17 nM) of cilengitide in mice (using osmotic minipumps loaded with 0.1 mg ml^{-1} cilengitide, B16F0 $P < 0.05$; LLC $P < 0.01$; **Fig. 1e**). Concentrations of

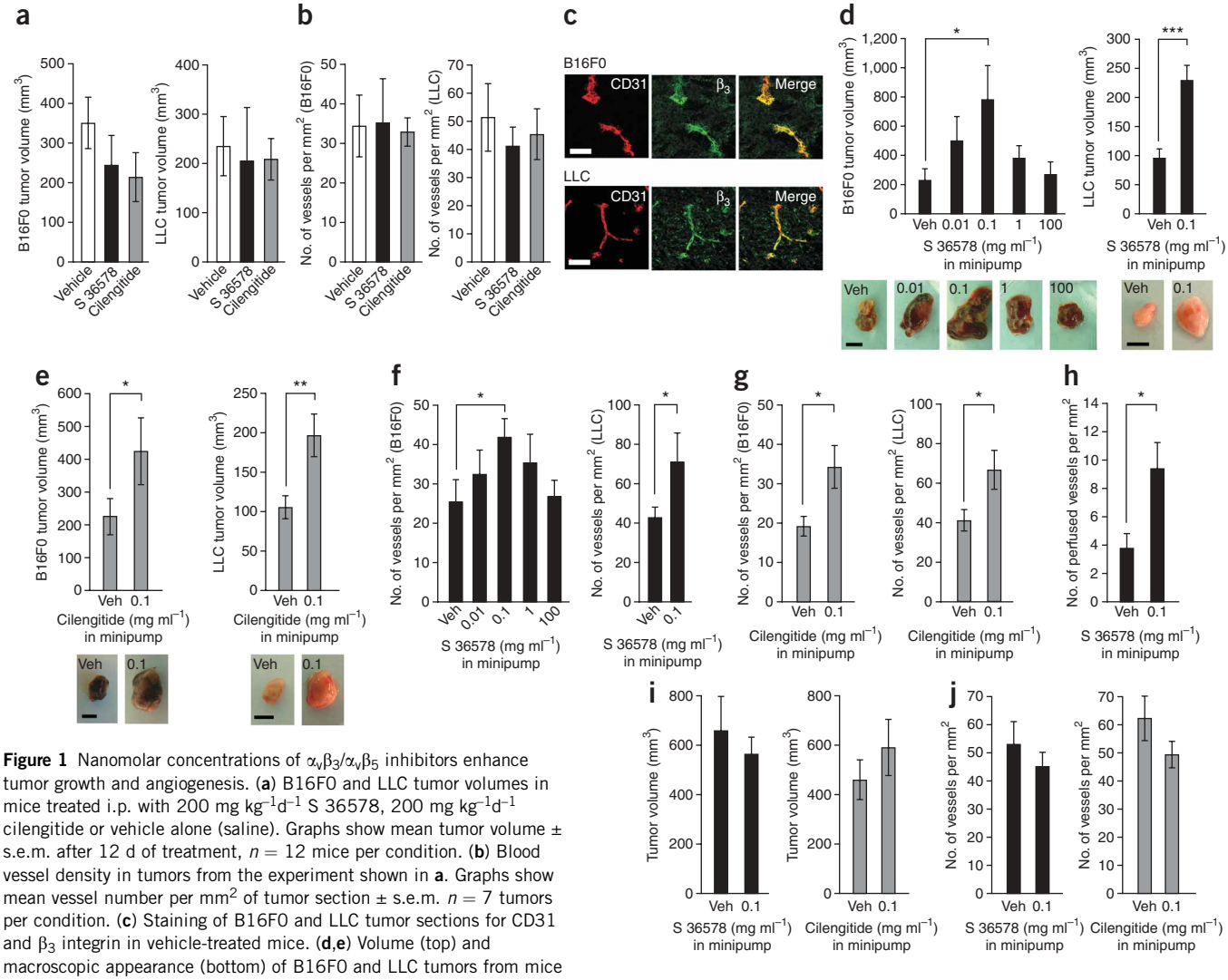

Figure 1 Nanomolar concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors enhance tumor growth and angiogenesis. (**a**) B16F0 and LLC tumor volumes in mice treated i.p. with 200 mg kg^{-1}d^{-1} S 36578, 200 mg kg^{-1}d^{-1} cilengitide or vehicle alone (saline). Graphs show mean tumor volume ± s.e.m. after 12 d of treatment, $n = 12$ mice per condition. (**b**) Blood vessel density in tumors from the experiment shown in **a**. Graphs show mean vessel number per mm^2 of tumor section ± s.e.m. $n = 7$ tumors per condition. (**c**) Staining of B16F0 and LLC tumor sections for CD31 and β_3 integrin in vehicle-treated mice. (**d**,**e**) Volume (top) and macroscopic appearance (bottom) of B16F0 and LLC tumors from mice treated via minipumps loaded with vehicle alone (veh) or the indicated concentrations of S 36578 (**d**) or cilengitide (**e**). Graphs show mean tumor volume ± s.e.m. at 10 d (B16F0) or 13 d (LLC) of treatment, $n = 10$–15 mice per condition. (**f**,**g**) Blood vessel density in tumors from the experiments shown in **d** and **e**. Graphs show mean vessel number per mm^2 of tumor section ± s.e.m. $n = 5$ or 6 tumors per condition. (**h**) Quantification of perfused blood vessels in B16F0 tumors from mice treated via minipumps loaded with vehicle or 0.1 mg ml^{-1} S 36578. Graph shows mean number of perfused vessels per mm^2 of tumor section ± s.e.m. $n = 7$ tumors per condition. (**i**) B16F0 tumor volumes in β_3 and β_5 integrin doubly deficient mice treated via minipumps loaded with vehicle, 0.1 mg ml^{-1} S 36578 or 0.1 mg ml^{-1} cilengitide. Graphs show mean tumor volume ± s.e.m. after 10 d of treatment, $n = 10$–15 mice per condition. (**j**) Blood vessel density in tumors from the experiment shown in **i**. Graphs show mean number of blood vessels per mm^2 of tumor section ± s.e.m. $n = 5$ or 6 tumors per condition. Scale bars, 30 μM in **c** and 5 mm in **d** and **e**. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ (Student's t test).

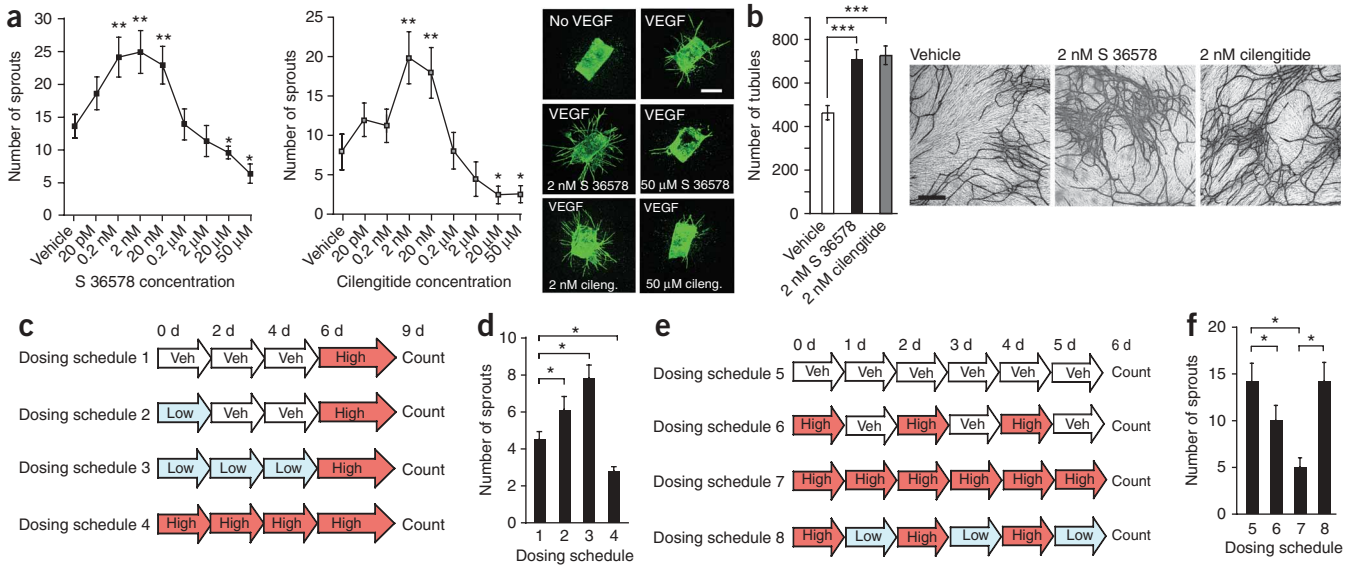

Figure 2 Low concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors promote VEGF-mediated angiogenesis and compromise antiangiogenic effects. (**a**) Microvessel sprouting of mouse aortic rings incubated with VEGF and the indicated concentrations of either S 36578 or cilengitide. The culture medium was changed every 48 h. Graphs show the number of sprouts per aortic ring after 7 d of culture ± s.e.m. $n = 20$–30 aortic ring explants per condition. The macroscopic appearance of FITC–BS1 lectin–stained aortic rings, treated with or without VEGF and the indicated concentration of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor, is shown at right. (**b**) Quantification of tubule formation in a fibroblast-HUVEC co-culture model of human angiogenesis in the presence of vehicle, 2 nM S 36578 or 2 nM cilengitide. Representative fields of tubules from each condition tested are shown at right. $n = 18$ fields of vessels per condition. (**c**) Experimental setup in which aortic rings were treated with one of four different dosing schedules (1–4) by changing of the culture medium every day. (**d**) Microvessel sprouting of aortic rings treated as in **c** after 9 d of treatment. (**e**) Experimental setup in which aortic rings were treated with one of four different dosing schedules (5–8) by changing of the culture medium every day. (**f**) Microvessel sprouting of aortic rings treated as in **e** after 6 d of treatment. 'Veh' arrows, vehicle; 'low' arrows, a low (2 nM) S 36578 dose; 'high' arrows, a high (20 μM) S 36578 dose. $n = 20$–30 aortic ring explants per condition for **d** and **f**. Scale bars, 400 μm in **a** and 200 μm in **b**. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's t test).

$\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors that enhanced tumor growth also promoted tumor vascularization, as determined by quantification of vessel numbers in tumor sections ($P < 0.05$; **Fig. 1f,g** and **Supplementary Fig. 2** online) and by assessment of functional tumor vascularization (**Fig. 1h**). However, low doses of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors did not enhance vascular endothelial growth factor–A (VEGF-A) messenger RNA or protein expression in either B16F0 or LLC tumors (**Supplementary Fig. 3a,b** online). Low doses of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors did not enhance the vascularity of unchallenged mouse skin (**Supplementary Fig. 3c**), indicating that the effects of the inhibitors on vascularization were tumor specific. We also tested the effects of low concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors on large established tumors. We allowed LLC tumors to reach a size of approximately 200 mm^3, at which point we implanted mice with minipumps loaded with vehicle or 0.1 mg ml^{-1} $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor. We found that low plasma concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor still enhanced tumor growth and tumor vascularization in these large established tumors (**Supplementary Fig. 3d,e**).

In contrast to B16F0 and LLC tumors, the growth of A375 tumors *in vivo* was significantly suppressed by 200 mg $kg^{-1}d^{-1}$ S 36578, indicating that A375 tumors are an integrin inhibitor–responsive tumor type (**Supplementary Fig. 3f**). These results are in agreement with previously published data showing that the growth of A375 tumors *in vivo* is suppressed by integrin inhibitors[12]. However, we found that a low dose of RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor promoted both A375 tumor growth and angiogenesis *in vivo* (**Supplementary Fig. 3g,h**). These data show that low concentrations of RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors can stimulate tumor growth and tumor angiogenesis *in vivo* in both integrin inhibitor–refractory tumors, such as B16F0 and LLC tumors, and in integrin inhibitor–responsive tumors, such as A375 tumors.

Integrin inhibitors can promote VEGF-mediated angiogenesis

We next tested the direct effects of integrin inhibitors on B16F0 and LLC tumor cell growth *in vitro*. Although high doses (≥2 μM) of either S 36578 or cilengitide inhibited the growth of B16F0 and LLC tumor cells *in vitro*, low concentrations (20 pM–0.2 μM) did not promote tumor cell growth under the same conditions (**Supplementary Fig. 4** online). We next examined the effect of low concentrations of S 36578 and cilengitide on tumor growth and angiogenesis in β_3 and β_5 integrin doubly deficient mice. We did not observe any enhancement in tumor growth or tumor angiogenesis when we treated β_3 and β_5 integrin doubly deficient mice with low concentrations of S 36578 or cilengitide (**Fig. 1i,j**). These data suggest that low doses of integrin inhibitors do not promote tumor cell growth directly but instead promote tumor growth by acting on a host cell type that expresses α_v integrins, such as tumor endothelial cells.

We therefore examined the effects of integrin inhibitors within two model systems of angiogenesis that closely recapitulate the processes of sprouting angiogenesis and tube formation observed *in vivo*. Using the *ex vivo* aortic ring assay, we observed a biphasic dose response to the $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors. Low concentrations (0.2–20 nM) of S 36578 or cilengitide enhanced VEGF-stimulated angiogenesis ($P < 0.01$; **Fig. 2a**), whereas inhibition of angiogenesis was observed only in aortic rings incubated with ≥20 μM of these inhibitors ($P < 0.05$; **Fig. 2a**). Low concentrations of the $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors also promoted tubule formation in an *in vitro* angiogenesis assay using human endothelial cells (**Fig. 2b**). Notably, angiogenesis was not stimulated by

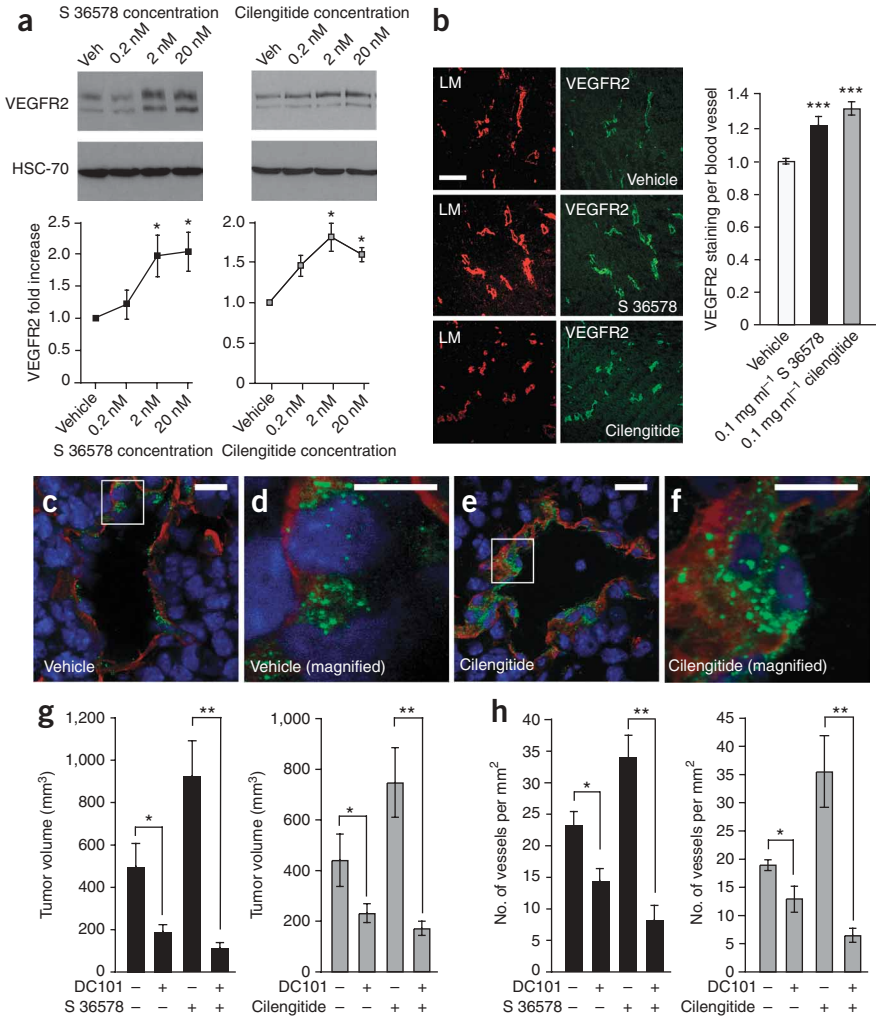

Figure 3 VEGFR2 is required for the effects of low dose $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors. (**a**) VEGFR2 protein abundance in primary mouse endothelial cells incubated for 24 h with vehicle or the indicated concentration of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor. Graphs show mean fold increase in VEGFR2 protein ± s.e.m. HSC-70 was used as a loading control. $n = 3$ experiments. (**b**) Left, staining for laminin (LM) and VEGFR2 in B16F0 tumor sections from mice treated via minipumps loaded with vehicle alone (top), 0.1 mg ml^{-1} S 36578 (middle) or 0.1 mg ml^{-1} cilengitide (bottom). Right, quantification of VEGFR2 staining in blood vessels. $n = 3$ or 4 tumors per condition. (**c**–**f**) High magnification views of laminin and VEGFR2 staining in B16F0 tumors from mice treated with vehicle alone (**c**,**d**) or low dose cilengitide (**e**,**f**). Panels **d** and **f** are magnified images of the boxed areas in **c** and **e**, respectively. (**g**) B16F0 tumor volumes from mice treated via minipumps loaded with vehicle alone, 0.1 mg ml^{-1} S 36578 or 0.1 mg ml^{-1} cilengitide in combination with i.p. injections of DC101 or vehicle (saline), as indicated. Graphs show mean B16F0 tumor volume after 13 d of treatment ± s.e.m. $n = 10$–15 mice per condition. (**h**) Blood vessel densities in tumors from the experiment shown in **g**. Graphs show mean vessel density per mm^2 in tumor section ± s.e.m. $n = 5$ or 6 tumors per condition. Scale bars: 50 μM in **b**, 20 μM in **c** and **e** and 10 μM in **d** and **f**. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's t test).

low concentrations of integrin inhibitors in the absence of VEGF, by low doses of negative control compounds (**Supplementary Fig. 5a** online) or in β_3 and β_5 integrin doubly deficient aortic rings (**Supplementary Fig. 5b**). These data suggest that, although high ($\geq$20 μM) concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors can inhibit VEGF-stimulated angiogenesis, exposure to low concentrations (0.2–20 nM) of these agents can promote VEGF-stimulated angiogenesis.

We next examined whether stimulation of angiogenesis by low doses of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors could compromise the efficacy of a subsequent high dose of the same inhibitor. We found that exposure of aortic rings to a nanomolar dose of S 36578 suppressed the ability of a subsequently applied micromolar dose of S 36578 to inhibit angiogenesis in this assay (**Fig. 2c,d**). We used the aortic ring assay to model how exposure to fluctuating concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor, such as those that might occur during repeated bolus administration, affects the ability of the inhibitor to suppress angiogenesis. Intermittent exposure of aortic rings to a nanomolar dose of S 36578 dose in between high (micromolar) doses suppressed the ability of the high dose to inhibit angiogenesis (**Fig. 2e,f**). Thus, in this *ex vivo* model, exposure to low concentrations of an $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor counteracted the antiangiogenic effects of high concentrations of the same inhibitor.

Nanomolar concentrations of cyclic RGD peptides have been shown to promote the binding of soluble recombinant $\alpha_v\beta_3$ integrin to immobilized vitronectin[31]. We therefore deemed it possible that nanomolar concentrations of RGD-mimetic integrin inhibitors could promote angiogenesis by altering the adhesive function of $\alpha_v\beta_3$ integrin. However, we found that low doses (20 pM–200 nM) of these inhibitors neither promoted cell adhesion (**Supplementary Fig. 6** online) nor promoted $\alpha_v\beta_3$ integrin activation (**Supplementary Fig. 7** online). These data suggest that low doses of these inhibitors do not enhance angiogenesis by promoting integrin–mediated cell adhesion or by causing activation of surface integrin on endothelial cells. In contrast, we found that micromolar concentrations (2–20 μM) of S 36578 or cilengitide can suppress the adhesion of endothelial cells to vitronectin (**Supplementary Fig. 6**) and led to $\alpha_v\beta_3$ integrin activation (**Supplementary Fig. 7**). Because high doses of inhibitors are apparently required to induce integrin activation, these data support the concept that low doses of integrin inhibitors do not enhance angiogenesis by promoting activation of surface $\alpha_v\beta_3$ integrin on endothelial cells.

Integrin inhibitors stimulate VEGFR2 and β_3 integrin recycling

Because VEGF-mediated angiogenesis was promoted by low concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors, we examined the effects of these inhibitors on the expression of VEGFR2. Exposure of mouse endothelial cells to nanomolar concentrations (0.2–20 nM) of S 36578 or cilengitide induced an increase in VEGFR2 protein expression ($P < 0.05$, **Fig. 3a**). At concentrations of $\geq$200 nM, these inhibitors caused endothelial cells to detach from their substrate precluding analysis of VEGFR2 protein amounts at these higher doses. Low doses of the inhibitors did not affect levels of VEGFR2 mRNA (**Supplementary Fig. 8a** online), suggesting that their effects on VEGFR2 expression occur at a post-transcriptional level. We also found that,

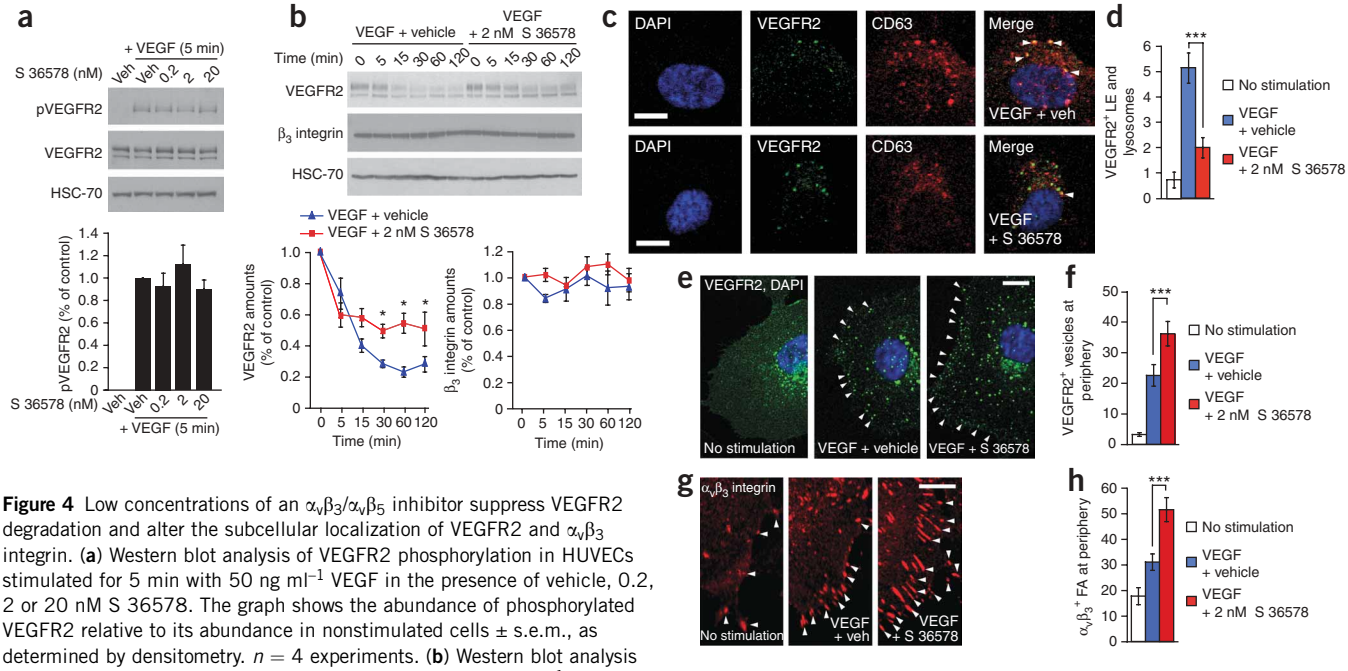

Figure 4 Low concentrations of an $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor suppress VEGFR2 degradation and alter the subcellular localization of VEGFR2 and $\alpha_v\beta_3$ integrin. (**a**) Western blot analysis of VEGFR2 phosphorylation in HUVECs stimulated for 5 min with 50 ng ml^{-1} VEGF in the presence of vehicle, 0.2, 2 or 20 nM S 36578. The graph shows the abundance of phosphorylated VEGFR2 relative to its abundance in nonstimulated cells ± s.e.m., as determined by densitometry. $n = 4$ experiments. (**b**) Western blot analysis of total VEGFR2 amounts in HUVECs stimulated with 50 ng ml^{-1} VEGF plus vehicle alone or 2 nM S 36578 for the indicated times. Graphs show total VEGFR2 and β_3 integrin protein amounts relative to nonstimulated cells ± s.e.m. $n = 3$ experiments. (**c**–**h**) HUVECs were stimulated with 50 ng ml^{-1} VEGF plus vehicle or 2 nM S 36578 for 30 min and then stained for VEGFR2 and CD63 (**c**), VEGFR2 (**e**) or $\alpha_v\beta_3$ integrin (**g**). Arrowheads indicate examples of VEGFR2–positive late endosomes–lysosomes (**c**), areas at the cell periphery containing recycled vesicles of VEGFR2 (**e**) or $\alpha_v\beta_3$ integrin–positive focal adhesions (**g**). Graphs show the number of CD63–positive late endosomes (LE) and lysosomes staining for VEGFR2 (**d**), the number of VEGFR2–positive recycling vesicles at the cell periphery (**f**) or the number of $\alpha_v\beta_3$ integrin–positive focal adhesions (FA) at the cell periphery (**h**) in cells treated as indicated. $n = 20$ cells per condition. Scale bars, 10 μM in **c** and **e** and 5 μM in **g**. $^*P < 0.05$ and $^{***}P < 0.001$ (Student's t test).

when mice were treated with low doses of the inhibitors, VEGFR2 expression was increased in tumor blood vessels *in vivo* (**Fig. 3b**). Moreover, analysis of blood vessels at higher power revealed that VEGFR2 staining was largely concentrated within vesicular structures that bore a close resemblance to the endosomal compartment (**Fig. 3c–f**). Blockade of VEGFR2 function with DC101, a VEGFR2 inhibitory antibody[32,33], suppressed the ability of nanomolar inhibitor concentrations to promote VEGF-mediated angiogenesis *ex vivo* (**Supplementary Fig. 8b**) and tumor growth and angiogenesis *in vivo* (**Fig. 3g,h** and **Supplementary Fig. 8c,d**). Thus, VEGFR2 function is essential for $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors to enhance VEGF-mediated angiogenesis, tumor growth and tumor angiogenesis.

VEGF stimulation of endothelial cells promotes VEGFR2 phosphorylation, VEGFR2 degradation and the accumulation of internalized VEGFR2 in endosomes and lysosomes[34–37]. A low dose of S 36578 did not alter phosphorylation of VEGFR2 in human umbilical vein endothelial cells (HUVECs; **Fig. 4a**). However, a low dose of this inhibitor did attenuate the VEGF-induced degradation of VEGFR2 without affecting β_3 integrin protein amounts ($P < 0.05$; **Fig. 4b**) and suppressed the VEGF-induced accumulation of VEGFR2 in late endosomes and lysosomes ($P < 0.01$, **Fig. 4c,d**). VEGF also stimulates the recycling of internalized VEGFR2, and this recycled population is not targeted for degradation but instead accumulates in small vesicles at the periphery of endothelial cells[36,37]. A low dose of S 36578 strongly promoted accumulation of VEGFR2 in this population of recycling vesicles at the periphery of endothelial cells ($P < 0.01$, **Fig. 4e,f**). Furthermore, we found that low doses enhanced the VEGF-induced delivery of $\alpha_v\beta_3$ to focal adhesions at the cell periphery (**Fig. 4g,h**).

We then measured the effect of an $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor on the internalization and recycling rates of VEGFR2 and $\alpha_v\beta_3$ integrin in endothelial cells. Although internalization was unaffected by S 36578 treatment (**Fig. 5a**), recycling of both VEGFR2 and $\alpha_v\beta_3$ was strongly promoted by low concentrations of both S 36578 and cilengitide ($P < 0.01$–0.05; **Fig. 5b** and **Supplementary Fig. 9a,b** online). In agreement with previous findings[37], we found that a significant proportion of internalized VEGFR2 colocalized with Rab4 (**Fig. 5c,d**), a GTPase known to control recycling of $\alpha_v\beta_3$ integrin and growth factor receptors[38,39]. We found that small interfering RNA (siRNA)-mediated inhibition of Rab4A in HUVECs (**Supplementary Fig. 9c**) ablated the effects of low dose S 36578 on both VEGFR2 and $\alpha_v\beta_3$ recycling (**Fig. 5e,f**), as well as on VEGFR2 degradation (**Fig. 5g,h**). In contrast, recycling of the transferrin receptor was not altered by low dose S 36578 (**Supplementary Fig. 9e**), and Rab11A-specific siRNA did not prevent VEGFR2 or $\alpha_v\beta_3$ recycling (**Supplementary Fig. 9f**). At concentrations of ≥200 nM, S 36578 and cilengitide caused HUVECs to detach from their substrate, precluding an analysis of trafficking at these higher doses. These data indicate that Rab4A is required for low concentrations of an $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor to promote VEGFR2 and $\alpha_v\beta_3$ recycling, and the consequences of this increased recycling are reduced VEGFR2 degradation and recruitment of $\alpha_v\beta_3$ to focal adhesions.

We next examined whether low doses of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors can alter the functional response of endothelial cells to VEGF. Although low concentrations of S 36578 or cilengitide did not promote the proliferation of endothelial cells significantly (**Fig. 6a**), they did promote VEGF-mediated endothelial cell migration in a dose-dependent manner (**Fig. 6b**). These low inhibitor concentrations did not enhance HUVEC migration to other growth factors (**Fig. 6c**), nor did

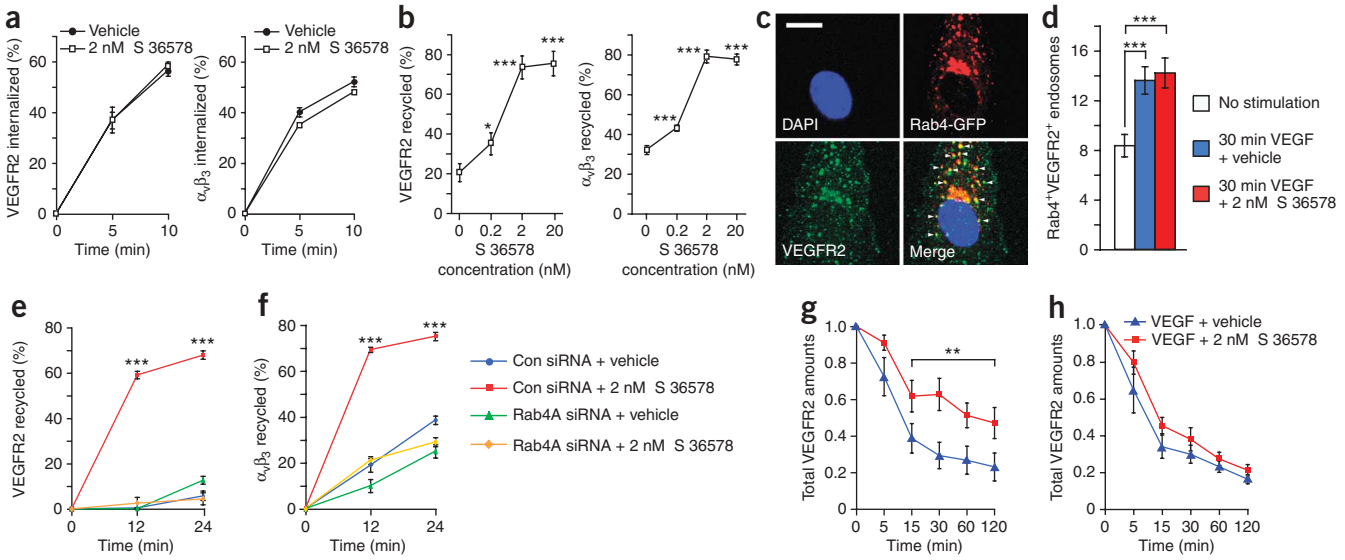

Figure 5 Low concentrations of an $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor promote Rab4A-mediated recycling of VEGFR2 and $\alpha_v\beta_3$ integrin. (**a**) VEGFR2 and β_3 integrin internalization in HUVECs exposed to vehicle or 2 nM S 36578. Graphs show the mean percentage of surface VEGFR2 (left) or β_3 integrin protein (right) internalized ± s.e.m. $n = 3$ experiments. (**b**) VEGFR2 and β_3 integrin recycling in HUVECs exposed to vehicle or the indicated concentration of S 36578. Graphs show percentage of VEGFR2 (left) or β_3 integrin (right) recycled from the internal pool ± s.e.m. $n = 3$ experiments. (**c**) Colocalization of VEGFR2 with Rab4-positive endosomes in HUVECs stimulated with VEGF for 30 min. Rab4 was expressed as a fusion protein with GFP. (**d**) Quantification of structures doubly positive for VEGFR2 and Rab4 in HUVECs treated as indicated ± s.e.m. $n = 20$ cells per condition. (**e**,**f**) Quantification of VEGFR2 (**e**) and β_3 integrin (**f**) recycling in HUVECs transfected with control (Con) or Rab4A-specific siRNA oligonucleotides. Graphs show the percentage of VEGFR2 (**e**) and β_3 integrin (**f**) protein recycled from the internal pool ± s.e.m. $n = 3$ independent experiments. (**g**,**h**) Quantification of VEGFR2 total protein amounts in HUVECs transfected with control (**g**) or Rab4A-specific siRNA (**h**) oligonucleotides after VEGF stimulation in the presence of vehicle or 2 nM S 36578. Graphs show VEGFR2 protein amounts relative to nonstimulated cells ± s.e.m., as determined by western blot densitometry. $n = 3$ experiments. Scale bar, 10 μM in **c**. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ (Student's *t* test).

they induce basal migration in the absence of added growth factor (**Fig. 6c**). Transfection with Rab4A-specific siRNA (**Supplementary Fig. 9c,d**) ablated the ability of low concentrations of S 36578 or cilengitide to promote VEGF-dependent migration (**Fig. 6d**) and VEGF-mediated angiogenesis (**Fig. 6e**). These data suggest that low doses of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors promote angiogenesis by enhancing VEGF-stimulated migration of endothelial cells and that this is dependent on their capacity to promote the Rab4A-mediated recycling of VEGFR2 and $\alpha_v\beta_3$ integrin.

DISCUSSION

Inhibitors of $\alpha_v\beta_3$ integrin–mediated cell adhesion[17–19] or function-blocking mutations in the β_3–integrin cytoplasmic domain[40] have been reported to suppress pathological angiogenesis in mice, supporting a functional role for $\alpha_v\beta_3$ integrin in neovascularization. Conversely, the enhanced tumor growth and angiogenesis shown by β_3 and β_5 integrin doubly deficient mice strongly suggests that these integrins are not actually required for pathological angiogenesis[41]. Moreover, although complete and partial responses to $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors have been observed in some individuals with glioma[25], evidence that these inhibitors can suppress angiogenesis in human cancers has yet to be obtained[26–28]. Clearly, the role of $\alpha_v\beta_3$ integrin in tumor angiogenesis is more complex than originally thought, and there is a need to reevaluate the precise effects that $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors have on this process[42,43].

In most clinical trials, $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors are administered as infusions of short duration twice weekly. Under these circumstances, plasma drug concentrations fall to nanomolar levels between administration sessions as a consequence of drug metabolism[26,28]. Some compounds that inhibit tumor growth at high concentrations can stimulate tumor growth at lower concentrations—that is, a hormetic or 'bell-shaped' dose-response curve is observed[44]. However, to our knowledge, the effects of nanomolar concentrations of integrin inhibitors on tumor growth and angiogenesis *in vivo* have not been previously explored.

Here we demonstrate that nanomolar concentrations of RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors can unexpectedly enhance tumor growth and tumor angiogenesis *in vivo*. Because low concentrations of these inhibitors did not promote the growth of tumor cells *in vitro* and did not promote tumor growth in β_3 and β_5 integrin doubly deficient mice, we inferred that these inhibitors promote tumor growth by acting on a host-derived cell type that expresses α_v integrins, such as tumor endothelial cells. We present several lines of evidence strongly suggesting that low concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors promote tumor growth by directly stimulating VEGF-mediated tumor angiogenesis. Low concentrations of these inhibitors promoted increases in functional tumor vascularization, *ex vivo* VEGF-mediated angiogenesis and VEGFR2 expression both *in vitro* and *in vivo*. In addition, blockade of VEGFR2 function prevented low doses of these inhibitors from promoting VEGF-mediated angiogenesis *ex vivo* and tumor growth and angiogenesis *in vivo*.

We found that RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors promote VEGF-stimulated endothelial cell migration, providing a mechanistic explanation for how these inhibitors promote VEGF-mediated angiogenesis. Growth factor–stimulated cell migration is known to be influenced by recycling of internalized integrins and growth factor receptors[39,45–48]. Notably, we found that low concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors promote recycling of both $\alpha_v\beta_3$ integrin and VEGFR2 in endothelial cells. This increased recycling was associated with attenuated degradation of VEGFR2, accumulation of VEGFR2 in

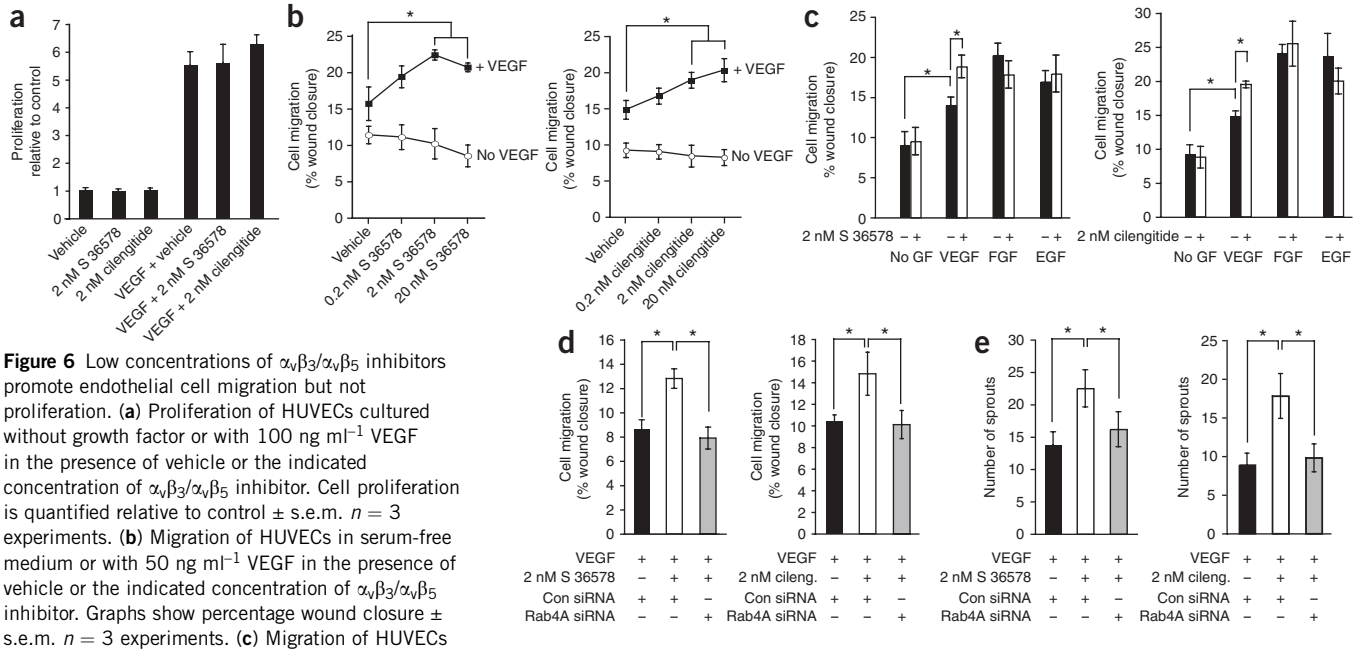

Figure 6 Low concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors promote endothelial cell migration but not proliferation. (**a**) Proliferation of HUVECs cultured without growth factor or with 100 ng ml^{-1} VEGF in the presence of vehicle or the indicated concentration of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor. Cell proliferation is quantified relative to control ± s.e.m. $n = 3$ experiments. (**b**) Migration of HUVECs in serum-free medium or with 50 ng ml^{-1} VEGF in the presence of vehicle or the indicated concentration of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor. Graphs show percentage wound closure ± s.e.m. $n = 3$ experiments. (**c**) Migration of HUVECs in growth factor–free conditions or with 50 ng ml^{-1} VEGF, 50 ng ml^{-1} fibroblast growth factor (FGF) or 50 ng ml^{-1} epidermal growth factor (EGF) in the presence of vehicle, 2 nM S 36578 or 2 nM cilengitide, as indicated. Graphs show percentage wound closure ± s.e.m. $n = 3$ experiments. (**d**) VEGF-induced migration of HUVECs transfected with control or Rab4A-specific siRNA oligonucleotides in the presence of vehicle, 2 nM S 36578 or 2 nM cilengitide, as indicated. Graphs show percentage wound closure ± s.e.m. $n = 3$ experiments. (**e**) VEGF-induced sprouting angiogenesis in aortic rings transfected with control or Rab4A-specific siRNA oligonucleotides in the presence of vehicle, 2 nM S 36578 or 2 nM cilengitide, as indicated. Graphs show the number of sprouts per aortic ring after 7 d ± s.e.m. $n = 20$–30 aortic ring explants per condition. * $P < 0.05$ (Student's t test).

carrier vesicles that probably represent recycling intermediates, and enhanced recruitment of $\alpha_v\beta_3$ integrin into focal adhesions at the cell periphery. The Rab4A recycling pathway has been implicated in the recycling of growth factor receptors and integrins[38,39], and we found that suppression of Rab4A clearly opposes the effects that low doses of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors have on VEGFR2 and $\alpha_v\beta_3$ integrin recycling, VEGFR2 degradation, endothelial cell migration and angiogenesis. Taken together, these data suggest that low doses of RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors promote endothelial cell migration to VEGF by promoting the Rab4A-mediated recycling of both $\alpha_v\beta_3$ integrin and VEGFR2. However, we cannot rule out the possibility that enhanced Rab4A-mediated recycling of other membrane proteins may also be occurring and that this could also promote angiogenesis in response to low concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors. Notably, a recently published study showed that cilengitide can promote tumor cell invasion by altering the recycling kinetics of integrins and the epidermal growth factor receptor[49]. Thus, the recycling kinetics of membrane receptors has a key role in the processes of cell migration and invasion in both cancer cells and endothelial cells.

Stimulation of angiogenesis by low concentrations of RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors in both integrin inhibitor–refractory and integrin inhibitor–responsive tumor types has major implications for the therapeutic use of the inhibitors in humans. On the basis of the reported half-life and C_{max} (the maximal plasma drug concentration obtained after drug administration) of cilengitide in humans[26,27], pharmacokinetic analysis predicts that, 30 h after administration, plasma concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor fall to the angiogenesis-promoting levels (0.2–20 nM) used in our studies and that these concentrations can persist in plasma for several hours. In an *ex vivo* model of angiogenesis, we show that exposure to a low inhibitor concentration, in between high-dose exposures, compromises the antiangiogenic activity of the inhibitor. Therefore, in humans, a low concentration of an $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor might counteract the antiangiogenic properties of a high dose of the inhibitor, leading to poor overall antiangiogenic activity. As our studies address the effects of only RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors, it remains to be seen whether other types of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors, for example, integrin function–blocking antibodies, elicit similar effects on tumor growth and angiogenesis.

We believe that this phenomenon may represent a major mechanism compromising the efficacy of RGD-mimetic $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors and that the dosing and administration of these integrin inhibitors in the clinic should be reevaluated. Our results suggest that it would be advantageous to maintain high plasma concentrations of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors and to avoid low circulating concentrations. Theoretically, this could be achieved with a prolonged infusion of $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors through a pump-based system, a method of administration that is presently being tested in a phase 1 clinical trial of cilengitide (http://clinicaltrials.gov/show/NCT00077155/). Alternatively, the problem could be overcome by designing new $\alpha_v\beta_3/\alpha_v\beta_5$–targeted agents that do not show proangiogenic properties or by combining $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitors with antagonists of VEGF signaling. Our findings might also have consequences for tumor imaging approaches currently in development. Infusion of labeled RGD peptides at low concentrations has been used to image tumors in mice[50,51]. Given the proangiogenic effects that we have observed with RGD ligand mimetics, the use of RGD–based contrast agents at low doses in humans should be approached with caution. In conclusion, this work highlights the need for a more complete understanding of how antiangiogenic agents function *in vivo*, which will be essential in order to use these drugs to their maximum therapeutic potential.

METHODS

Inhibitors. The integrin inhibitors S 36578 and cilengitide were supplied by Institut de Recherches Servier and Merck KGaA. DC101 was supplied by Imclone Systems Incorporated.

***In vivo* tumor growth and treatment.** We gave C57/BL6 mice (Charles River) a subcutaneous injection of 1×10^6 B16F0 cells, 0.5×10^6 LLC cells or 2×10^6 A375 cells (American Type Culture Collection). We administered integrin inhibitors by intraperitoneal (i.p.) injection (4 mg of S 36578 or cilengitide in 200 µl) or by osmotic minipump (model 2002, Alzet). We loaded minipumps with various concentrations of S 36578, cilengitide or vehicle alone (saline) and equilibrated them with saline overnight at 4 °C. The next day, or when tumors had reached a size of ~200 mm^3 (as indicated in the Results section), we anesthetized the mice with halothane and implanted the minipumps i.p. In experiments where mice were also treated with DC101, we gave the mice 800 µg DC101 in 100 µl of PBS (or 100 µl of PBS alone) via i.p. injection. We performed DC101 injections on the day before tumor cell and minipump implantation and on days 3, 6, 9 and 12 thereafter. We killed the mice by terminal anesthesia and dissected out tumors 10–14 d after injection of tumor cells or when tumors began to meet the legal size limit (as indicated in the Results section). We measured tumor size and snap-froze them immediately after resection.

Measurement of S 36578 and cilengitide concentrations in plasma. We measured concentrations of S 36578 and cilengitide in plasma by liquid chromatography–coupled tandem mass spectrometry as described in the **Supplementary Methods** online.

Quantification of vessel density and VEGFR2 expression in tumor sections. We snap froze freshly excised tumors, cryosectioned them and then stained them with the appropriate antibodies. We obtained vessel densities by manual counting, whereas we measured VEGFR2 expression by image analysis. Detailed methods are presented in the **Supplementary Methods.**

Measurement of VEGF protein in tumors. We lysed frozen tumor sections and determined VEGF levels by ELISA assay (R&D Systems). Detailed methods are presented in the **Supplementary Methods.**

Quantitative PCR. We extracted RNA from primary mouse endothelial cells and tumor sections by either the Trizol method (Invitrogen) or with an RNA isolation kit (Qiagen). Details of the methods used for quantitative PCR are presented in **Supplementary Methods.**

***In vitro* tumor cell growth assays.** We measured the growth of B16F0 and LLC cells *in vitro* in the presence of different concentrations of integrin inhibitors. Detailed methods are presented in the **Supplementary Methods.**

Aortic ring assays and human angiogenesis assay. We killed mice at 8–12 weeks of age by cervical dislocation. We dissected out the thoracic aortas, cut them into rings approximately 0.5 mm in width, and serum-starved them in OptiMEM medium (Invitrogen) overnight at 37 °C and 8% CO_2. When we performed siRNA transfection, we transfected the aortic rings during this overnight step with 50 nM Rab4A-specific siRNA oligonucleotides or 50 nM control nontargeting oligonucleotides (for details, see **Supplementary Methods**) using Oligofectamine (Invitrogen). The next day, we mounted each ring in 60 µl of 1.1 mg ml^{-1} acid-solubilized rat tail collagen I (BD Bioscience) in DMEM, with one ring per well of a 96-well plate. After collagen polymerization (30 min at 37 °C and 8% CO_2), we added 180 µl of DMEM supplemented with 2.5% FCS and 30 ng ml^{-1} VEGF (R&D Systems) with or without the appropriate inhibitors (S 36578, cilengitide or DC101) or control agents (S 37723, EMD 135981 or rat IgG) to each well. We cultured the rings at 37 °C and 8% CO_2 with medium renewal every 24 h or 48 h as indicated in the legend of **Figure 2**. After 7 d in culture, we fixed the aortic rings with formal saline, permeabilized them with 0.2% Triton X-100 and stained them with 10 µg ml^{-1} FITC-conjugated BS-1 lectin (Sigma). We then counted the number of lectin-stained sprouts grown from each ring. We captured images of the rings with a LSM510 inverted confocal laser-scanning microscope (Zeiss). We performed the human angiogenesis assay with Angiokit (TCS Cell Works) according to the manufacturer's instructions in the presence of vehicle or integrin inhibitor. After 7 d, we fixed the co-cultures, stained them and quantified the number of tubules formed with AngioSys software (TCS Cell Works).

Isolation and culture of primary mouse endothelial cells and human umbilical vein endothelial cells. We isolated and cultured primary mouse endothelial cells essentially as previously described[33,41]. We obtained HUVECs from a commercial source (TCS Cell Works). Detailed methods are presented in the **Supplementary Methods**.

Immunofluorescence and western blotting. The methods used for immunofluorescence and western blotting were based on those previously described[33,41]. Detailed methods are presented in the **Supplementary Methods**.

Adhesion assays. The method used for analysis of endothelial cell adhesion was based on that previously described[52]. A detailed method is presented in the **Supplementary Methods**.

Integrin activation assays. The method used for analysis of integrin activation was based on that previously described[53]. A detailed method is presented in the **Supplementary Methods**.

Internalization and recycling assays. We adapted assays to measure VEGFR2 internalization and recycling from those described previously for integrins[38]. Detailed methods are presented in the **Supplementary Methods**.

Migration and proliferation assays. We measured endothelial cell proliferation in 96-well plates with the Cell Titer Glo assay (Promega). We measured endothelial cell migration by a scratch wounding assay. Detailed methods are presented in the **Supplementary Methods**.

Mice. Ethical approval for animal experimentation was granted by the Cancer Research UK Ethics Committee, and we performed all procedures in accordance with UK Home Office regulations.

Statistical analyses. All numerical data are expressed as the means ± s.e.m. We analyzed data sets for significance with Student's *t* test. We considered *P* values of less than 0.05 as statistically significant.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

A.R.R. conceived of and designed the study, performed the majority of the experiments and co-wrote the manuscript; I.R.H. assisted with *in vivo* experiments and provided comments on the manuscript; A.R.W., R.G.S., V.K. and G.S. assisted with *in vivo* experiments; J.C.W., S.D.R. and D.T.J. assisted with quantitative PCR and angiogenesis assays; G.D.V. made measurements on plasma samples; M.G. and M.C.J. assisted with *in vitro* biochemical assays; M.S. performed the analysis of VEGFR2 staining, F.P.-S. synthesized S 36578; J.C.N. designed and performed some of the receptor trafficking experiments and helped with data interpretation; G.C.T. assisted with the study design and

provided vital research reagents; and K.M.H.-D. supervised the research and co-wrote the manuscript.

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TLR effects on T helper or other cells, ERD did not occur.

In summary, FI-RSV failed to protect primarily as a result of poor avidity, as germline antibodies continued to recognize protective epitopes. Moreover, specifically maturing FI-RSV–specific antibody would not have solved the problem. Last, no nonreplicating vaccine against RSV will be safe for infants if it fails to elicit affinity maturation.

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Will integrin inhibitors have proangiogenic effects in the clinic?

To the Editor:

In a comprehensive analysis, Reynolds *et al.*[1] recently reported that RGD-mimetic agents such as cilengitide may, under certain experimental conditions, promote rather than inhibit angiogenesis. They accordingly express their reservations regarding the clinical exploration of such agents in human patients with cancer.

On the basis of promising phase 2 data[2,3], cilengitide in combination with temozolomide-based radiochemotherapy is currently being explored in a phase 3 registration trial for newly diagnosed glioblastoma with O^6-methylguanine methyltransferase (*MGMT*) promoter methylation (CENTRIC trial, European Organisation for Research and Treatment of Cancer 26071–22072). This new paradigm of seeking approval for a first-in-class agent in a molecularly defined subpopulation of individuals with glioblastoma was based on the observation that the apparent clinical benefit derived from cilengitide in the phase 2 trial was prominent only in this patient population[3]. Do the proangiogenic preclinical data of Reynolds *et al.*[1] raise serious concerns regarding the potential for paradoxical effects of cilengitide in individuals with glioma *in vivo*? We believe that this may not be the case.

First, the clinical importance of the tumor models used by Reynolds *et al.*[1] may be questioned. Although the major target disease of the current clinical development of cilengitide is glioblastoma, no glioma model was studied.

Second, *in vitro* analyses suggest that there are multiple actions of cilengitide that mediate a clinical benefit in glioblastoma, including direct cytolytic effects on tumor cells, cytolytic effects on endothelial cells and inhibition of cell adhesion, migration and invasion[4]. Although the functional consequences of the interactions of cilengitide with its target integrins are probably complex in the context of glioma biology, the overall net effect in the clinic seems to be growth inhibitory rather than growth promoting[2].

Third, in the current clinical setting, cilengitide is used in combination with chemotherapy and radiotherapy, again on the basis of preclinical data showing strong sensitization to radiotherapy in rodent glioma models[5].

Fourth, pulse treatment as used in the clinical trials did not result in adverse effects in any of the models studied by Reynolds *et al.*[1]. In fact, the scheduling claimed to be tumor growth–promoting in their study[1] is not used in humans.

Fifth, cilengitide used at flat doses of 2,000 mg twice weekly results in peak plasma cilengitide concentrations of >200 μM, which, by orders of magnitude, exceed the concentrations shown by Reynolds *et al.*[1] to promote angiogenesis. In fact, simulations based on population pharmacokinetic models show that concentrations in the angiogenesis-promoting range (0.2–20 nM)[1] are not reached in 75% of patients treated with biweekly intravenous infusions of 2,000 mg cilengitide (J. Grevel (Merck Serono), personal communication). Micromolar concentrations of cilengitide have also been measured in the tumor tissue of patients with glioma exposed to the drug before surgery for recurrent disease[6]. Admittedly, the extent of blood-brain and blood-tumor barrier penetration of cilengitide in humans with glioma remains uncertain, and it remains uncertain whether potentially proangiogenic concentrations of cilengitide may be operational at least transiently in the tumor tissue.

Finally, although Reynolds *et al.*[1] suggest that cilengitide mediates angiogenesis by enhancing the effect of vascular endothelial-derived growth factor (VEGF), the striking neuroradiological responses to cilengitide seen in some individuals with glioblastoma[7,8] morphologically closely resemble the effects of VEGF-antagonizing agents such as bevacizumab[9]. On the basis of these considerations, we acknowledge that Reynolds *et al.*[1] have assembled an interesting and unexpected set of data in preclinical models. In fact, a paradoxical proangiogenic effect of cilengitide may be operative in certain settings and contribute to an antitumor effect of cilengitide in combination with radiotherapy or chemotherapy. This consideration relates to the vascular normalization effect of antiangiogenic agents, which we have proposed to underlie the preferential clinical benefit apparently seen in glioblastoma patients with *MGMT* promoter methylation[2]. The clinical importance, however, of the complex effects of cilengitide reported by Reynolds *et al.*[1] as well as by Alghisi *et al.*[10] can be assessed only in appropriately designed clinical trials.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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