Enhanced pathological angiogenesis in mice lacking β_3 integrin or β_3 and β_5 integrins

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Inhibition of $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin function has been reported to suppress neovascularization and tumor growth, suggesting that these integrins are critical modulators of angiogenesis. Here we report that mice lacking β_3 integrins or both β_3 and β_5 integrins not only support tumorigenesis, but have enhanced tumor growth as well. Moreover, the tumors in these integrin-deficient mice display enhanced angiogenesis, strongly suggesting that neither β_3 nor β_5 integrins are essential for neovascularization. We also observed that angiogenic responses to hypoxia and vascular endothelial growth factor (VEGF) are augmented significantly in the absence of β_3 integrins. We found no evidence that the expression or functions of other integrins were altered as a consequence of the β_3 deficiency, but we did observe elevated levels of VEGF receptor-2 (also called Flk-1) in β_3-null endothelial cells. These data indicate that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are not essential for vascular development or pathological angiogenesis and highlight the need for further evaluation of the mechanisms of action of α_v-integrin antagonists in anti-angiogenic therapeutics.

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, involves coordinated endothelial-cell proliferation, migration and tube formation. This process is influenced both by growth factors, such as vascular endothelial growth factor (VEGF), and by cell adhesion molecules such as integrins[1,2]. Angiogenesis is a hallmark of cancer, as well as various ischemic diseases such as retinopathy of prematurity[3], implying that anti-angiogenic drugs are likely to be of importance in the treatment of these diseases. Elucidating the precise molecular mechanisms of angiogenic regulation is therefore important in determining rational strategies for such anti-angiogenic approaches.

VEGF has been identified as a major angiogenic factor acting through endothelial cell-specific receptors, including VEGF receptor-2 (VEGFR-2; also called Flk-1)[4]. The importance of the VEGF/VEGFR-2 system in angiogenesis is supported strongly by the lack of vascular development and early embryonic lethality in mice both heterozygous for and deficient in VEGFR-2 (refs. 4–6). In many tumors and ischemic diseases, VEGF production is elevated, inducing adult pathological angiogenesis and further emphasizing the importance of VEGF in neovascularization. Strategies to block VEGF and VEGF receptor signaling and function have resulted in significant inhibition of tumor angiogenesis and such reagents are presently in clinical trials[1,7,8]. Several members of the integrin family are also implicated in angiogenesis[9–18]. The largest body of data has linked $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (both receptors for vitronectin and other extracellular matrix molecules) with blood-vessel development[11–18]. Particular attention has been paid to the role of $\alpha_v\beta_3$ integrin in angiogenesis as it is prominent on proliferating vascular endothelial cells[13,14]. Furthermore, blockade of $\alpha_v\beta_3$ integrin with monoclonal antibodies or low-molecular-weight antagonists inhibits blood-vessel formation in a variety of *in vivo* models[17], including tumor angiogenesis[11–13] and neovascularization during oxygen-induced retinopathy[19]. In a recent report, a single small-molecule inhibitor of both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins inhibited tumor angiogenesis in animal models[20]. Taken together, these inhibition data suggest critical roles for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in angiogenesis, and highlight their importance as potential targets in anti-angiogenic therapy. In fact, the $\alpha_v\beta_3$-integrin antagonist, Vitaxin, is presently in clinical trials[21].

In contrast with these inhibitor studies, mice lacking α_v, β_3 or β_5 integrins exhibit extensive developmental angiogenesis[22–24]. All α_v-null mice have extensive sprouting angiogenesis and develop normally until embryonic day 9.5 and approximately 20% of them survive to birth[22]. β_3-null mice are both viable and fertile and developmental angiogenesis, including postnatal neovascularization of the retina, appears to be β_3-independent[23]. β_5-null mice are also viable and fertile and have no defects in wound healing, suggesting that adult angiogenesis is unaffected in these animals[24]. These results indicate that the precise role of α_v integrins in angiogenesis is likely to be more complex than initially thought and raise the question of the importance of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in adult pathological angiogenic processes. Using genetically defi-

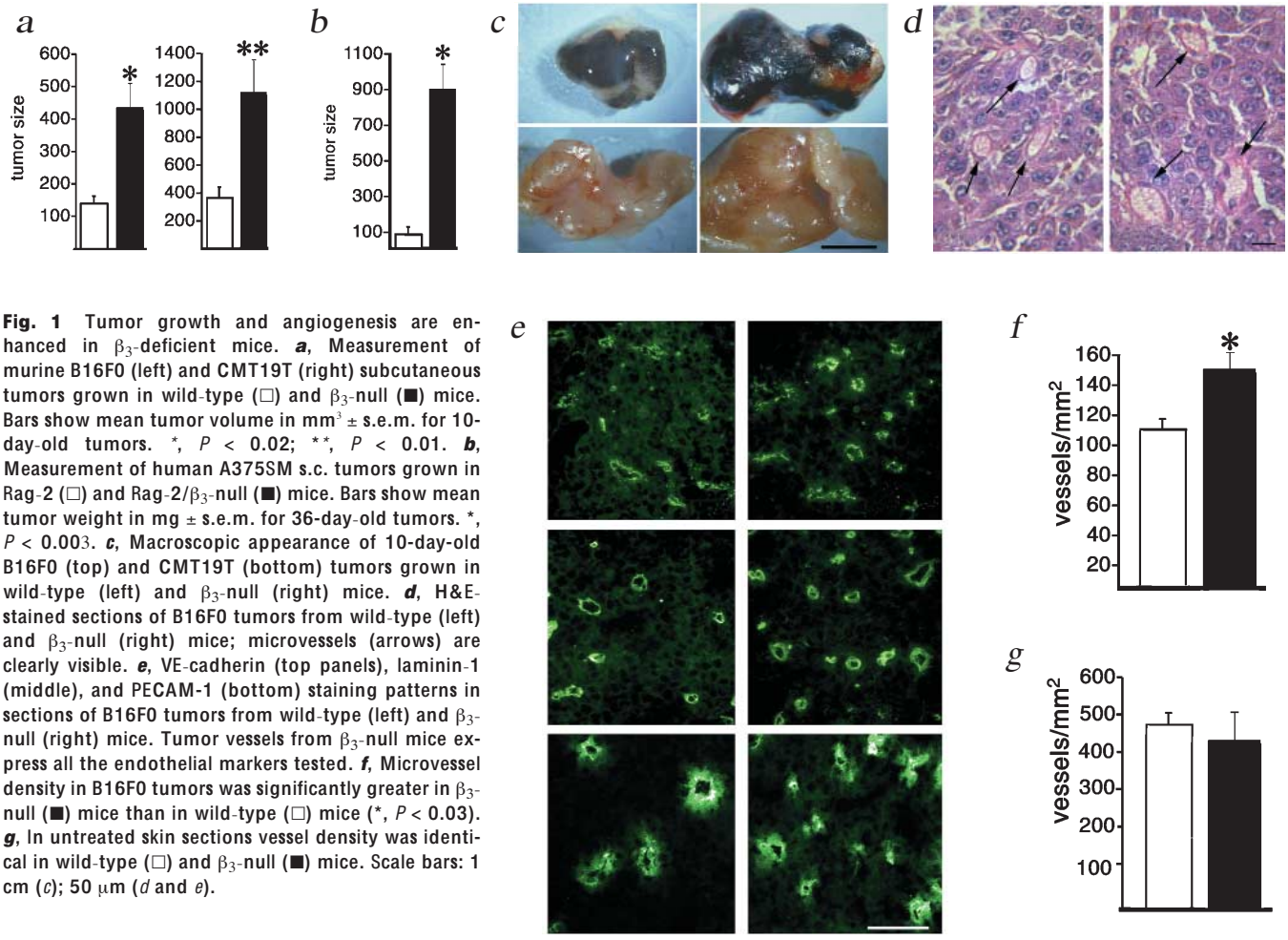

Fig. 1 Tumor growth and angiogenesis are enhanced in β_3-deficient mice. ***a***, Measurement of murine B16F0 (left) and CMT19T (right) subcutaneous tumors grown in wild-type (□) and β_3-null (■) mice. Bars show mean tumor volume in mm^3 ± s.e.m. for 10-day-old tumors. *, $P < 0.02$; **, $P < 0.01$. ***b***, Measurement of human A375SM s.c. tumors grown in Rag-2 (□) and Rag-2/β_3-null (■) mice. Bars show mean tumor weight in mg ± s.e.m. for 36-day-old tumors. *, $P < 0.003$. ***c***, Macroscopic appearance of 10-day-old B16F0 (top) and CMT19T (bottom) tumors grown in wild-type (left) and β_3-null (right) mice. ***d***, H&E-stained sections of B16F0 tumors from wild-type (left) and β_3-null (right) mice; microvessels (arrows) are clearly visible. ***e***, VE-cadherin (top panels), laminin-1 (middle), and PECAM-1 (bottom) staining patterns in sections of B16F0 tumors from wild-type (left) and β_3-null (right) mice. Tumor vessels from β_3-null mice express all the endothelial markers tested. ***f***, Microvessel density in B16F0 tumors was significantly greater in β_3-null (■) mice than in wild-type (□) mice (*, $P < 0.03$). ***g***, In untreated skin sections vessel density was identical in wild-type (□) and β_3-null (■) mice. Scale bars: 1 cm (*c*); 50 μm (*d* and *e*).

cient mice, we have tested the requirements for β_3, or the combination of β_3 and β_5 integrins, in adult neovascularization by monitoring pathological angiogenesis. Here we report the following results: 1) tumor growth and angiogenesis not only are supported, but were even enhanced in β_3-deficient and β_3/β_5–doubly deficient mice; 2) despite the parallel roles of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in angiogenesis, $\alpha_v\beta_5$ and other integrins tested were not upregulated in response to β_3 deficiency in endothelial cells; and 3) enhanced VEGF-induced angiogenic responses in β_3-null mice may be due, at least in part, to the elevated VEGFR-2 levels observed in β_3-deficient endothelial cells.

Tumor angiogenesis is enhanced in β_3-deficient mice

Given that adult tumor angiogenesis can be inhibited by blockade of $\alpha_v\beta_3$ integrin[11–13,17], we investigated whether β_3 deficiency had any effect on adult tumor angiogenesis. β_3-null and wild-type mice were injected subcutaneously with murine tumor cells; either melanoma (B16F0) or lung carcinoma (CMT19T) cells. As shown in Fig. 1*a*, tumors not only grew in both lines, but tumor size was enhanced significantly in the β_3-deficient mice when compared with controls ($P < 0.02$ for B16F0 and $P < 0.01$ for CMT19T). Representative examples of B16F0 and CMT19T tumors are shown in Fig. 1*c*. The growth of a human melanoma (A375SM) cell line was also tested by subcutaneous injection into immunocompromised (Rag-2) mice and Rag-2/β_3-null mice (Fig. 1*b*). In concordance with the other tumor models, A375SM tumors were significantly larger in Rag-2/β_3-null mice ($P < 0.003$) (Fig. 1*b*).

Sections of tumors were stained with hematoxylin and eosin (H&E). Microvessels were evident in tumors in both wild-type and β_3-null mice (Fig. 1*d*). Immunostaining with antibodies to various endothelial markers, including vascular endothelial (VE)-cadherin, laminin-1 and platelet endothelial-cell adhesion molecule-1 (PECAM-1) (Fig. 1*e*), VEGFR-2 and Tek (data not shown), established that the blood vessels in the β_3-null mice expressed all of the tested markers of endothelial cells. Blood-vessel density was quantified by counting the number of vessels per unit area across entire tumor sections. β_3-null mice had significantly elevated numbers of vessels per square millimeter of tumor when compared with wild-type controls ($P < 0.03$) (Fig. 1*f*). Importantly, vessel density in non tumor-burdened adult scruff-skin was comparable between β_3-null and wild-type mice (Fig. 1*g*) suggesting that the enhanced tumor angiogenic response was not due to elevated blood-vessel density in untreated β_3-null skin.

β_3/β_5-deficient mice have enhanced tumor angiogenesis

Because $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are related receptors and are both thought to be involved in angiogenesis[15,20], it is possible

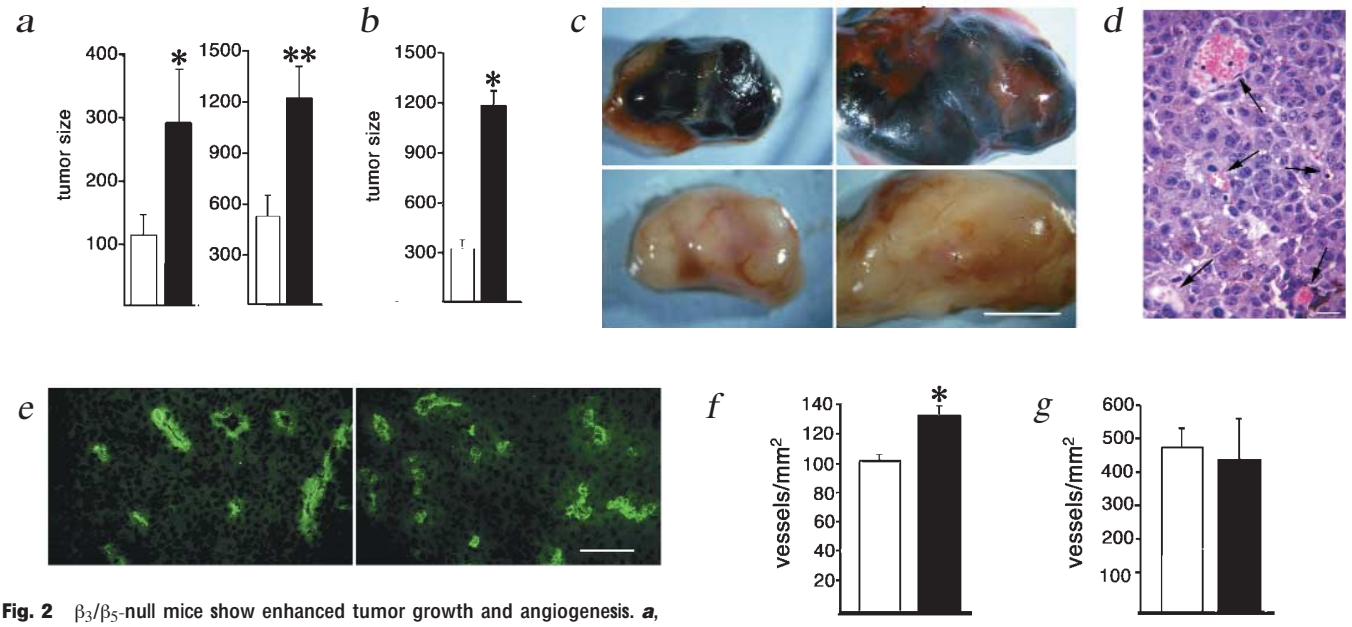

Fig. 2 β_3/β_5-null mice show enhanced tumor growth and angiogenesis. ***a***, Measurement of B16F0 (left) and CMT19T (right) s.c. tumors in wt (□) and β_3/β_5-null (■) mice. The mean tumor volume in mm^3 ± s.e.m. is given for 10-day-old tumors. *, $P < 0.05$; **, $P < 0.007$. ***b***, Measurement of human LS180 s.c. tumors grown in Rag-2 (□) and Rag-2/β_3/β_5-null (■) mice. The mean tumor weight in mg ± s.e.m. is presented for 21-day-old LS180 tumors (*, $P < 0.003$). ***c***, Macroscopic appearance of 10-day-old B16F0 (top) and CMT19T (bottom) tumors grown in control (left) and β_3/β_5-null (right) mice. ***d***, In H&E-stained sections of tumors from β_3/β_5-null mice microvessels are clearly visible (arrows). ***e***, Microvessels were detected by PECAM immunostaining of tumors in control (left) and β_3/β_5-null (right) mice. ***f***, Microvessel density in B16F0 tumors was significantly higher in β_3/β_5-null (■) mice than in wild-type (□) mice. *, $P < 0.008$. ***g***, In untreated skin sections vessel density was the same in wild-type (□) and β_3/β_5-null (■) mice. Scale bars: 1 cm (*c*); 50 μm (*d* and *e*).

that in the absence of both β_3 and β_5 integrins angiogenesis would be completely blocked. To test this, we examined subcutaneous B16F0 and CMT19T tumors in β_3/β_5–doubly deficient mice. Results showed that tumor growth was not only supported in these mice, but also that tumors were significantly larger when compared with controls (Fig. 2*a*). Similarly, subcutaneous growth of colon carcinoma (LS180) in Rag-2/β_3/β_5-null mice was significantly enhanced compared with controls ($P < 0.014$) (Fig. 2*b*). Representative examples of B16F0 and CMT19T tumors are shown in Fig. 2*c*. Histological analysis of an H&E-stained tumor section (Fig. 2*d*) and immunodetection of PECAM-1 (Fig. 2*e*), VE-cadherin, laminin-1 and VEGFR-2 (data not shown) revealed that angiogenic blood vessels were clearly detectable in the β_3/β_5-null mice. Moreover, tumors from these mice were significantly hyper-vascularized when compared with controls ($P < 0.008$) (Fig. 2*f*). In contrast, blood-vessel density in unchallenged β_3/β_5-null skin was comparable to control skin

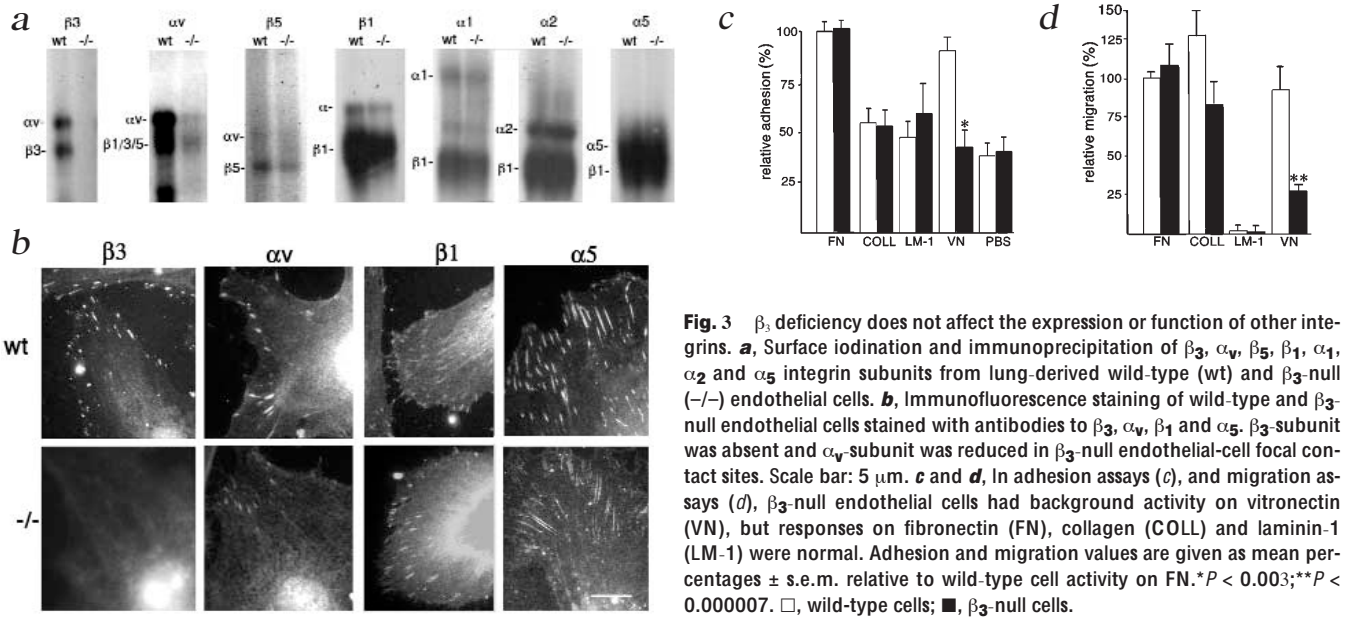

Fig. 3 β_3 deficiency does not affect the expression or function of other integrins. ***a***, Surface iodination and immunoprecipitation of β_3, α_v, β_5, β_1, α_1, α_2 and α_5 integrin subunits from lung-derived wild-type (wt) and β_3-null (–/–) endothelial cells. ***b***, Immunofluorescence staining of wild-type and β_3-null endothelial cells stained with antibodies to β_3, α_v, β_1 and α_5. β_3-subunit was absent and α_v-subunit was reduced in β_3-null endothelial-cell focal contact sites. Scale bar: 5 μm. ***c*** and ***d***, In adhesion assays (*c*), and migration assays (*d*), β_3-null endothelial cells had background activity on vitronectin (VN), but responses on fibronectin (FN), collagen (COLL) and laminin-1 (LM-1) were normal. Adhesion and migration values are given as mean percentages ± s.e.m. relative to wild-type cell activity on FN.*$P < 0.003$;**$P < 0.000007$. □, wild-type cells; ■, β_3-null cells.

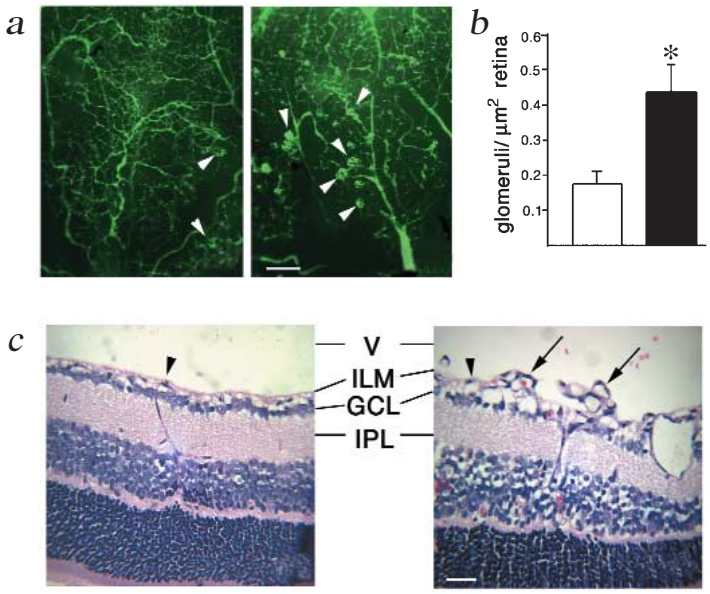

Fig. 4 Hypoxia-induced retinal angiogenesis is enhanced in β_3-null mice. ***a***, Flat mounts of retinas from wild-type (left) and β_3-null (right) mice that have undergone hypoxia-induced retinal angiogenesis show extensive areas of neovascularization with excessive development of vascular glomeruli (arrowheads) in the β_3-null retinas. ***b***, Quantification of vascular glomeruli in wild-type (□) and β_3-null (■) retinas (n = 9–12 mice per genotype; *, $P < 0.02$). ***c***, Cross-sections of eyes from wild-type (left) and β_3-null (right) mice showing retinal neovascularization internal to the inner limiting membrane in both genotypes (arrowheads) with excessive development of vascular glomeruli in the β_3-null retinas (arrows). V, vitreous; ILM, inner limiting membrane; GCL, ganglion cell layer; IPL, inner plexiform layer. Scale bars: 100 µm (*a*); 50 µm (*c*).

(Fig. 2*g*). Thus, our data provide strong evidence that deficiency of either β_3 alone, or both β_3 and β_5 integrins, can enhance tumor growth and angiogenesis. These data demonstrate that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are not essential for tumor angiogenesis.

β_3 deficiency does not affect other integrin subunits

Apart from the two receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, other integrins such as the fibronectin receptor $\alpha_5\beta_1$ and the collagen receptors $\alpha_2\beta_1$ and $\alpha_1\beta_1$ have also been suggested to play roles in angiogenesis[9–14,25]. This raises the possibility that other integrins may compensate for β_3 deficiency. We compared the expression profiles and functions of integrins in wild-type and β_3-null endothelial cells. Surface iodination followed by immunoprecipitation of β_3 and α_v integrins showed a complete loss of β_3 integrin and reduced levels of α_v integrin in the β_3-null endothelial cells. However, no changes in surface expression of β_5, β_1, α_1, α_2 or α_5 integrins were detected by surface iodination and immunoprecipitation (Fig. 3*a*). Western-blot analysis also showed no difference in total β_5-subunit levels in endothelial cells (data not shown). Focal contact distribution of integrins in the β_3-null endothelial cells was examined by immunofluorescence using antibodies to various integrins, including β_3, α_v, β_1 and α_5 integrins. Apart from the absence of detectable β_3 integrin in the β_3-null endothelial cells, α_v expression appeared to be somewhat reduced and there were no changes in β_1 or α_5 integrin patterns (Fig. 3*b*).

To test whether the loss of β_3 integrins had any effect on the functions of other integrins, we performed adhesion and migration assays on fibronectin, collagen, laminin-1 and vitronectin. In adhesion assays, wild-type and β_3-null endothelial cells adhered equally well to fibronectin, collagen and laminin-1. In contrast to wild–type cells, β_3-null cells showed background levels of adhesion to vitronectin (Fig. 3*c*). In migration assays, β_3-null endothelial cells had normal migratory abilities on most substrates, but migration was ablated on vitronectin (Fig. 3*d*). These data show no evidence for compensation by other integrins in response to β_3 deficiency.

β_3 deficiency enhances VEGF-induced blood-vessel growth

Since β_3-null mice exhibited enhanced tumor angiogenesis, we sought to investigate the effect of β_3 deficiency in another adult neovascularization assay. Hypoxia-induced retinal neovascularization in neonatal mice is a model for the ischemic disease retinopathy of prematurity, and it provides a useful

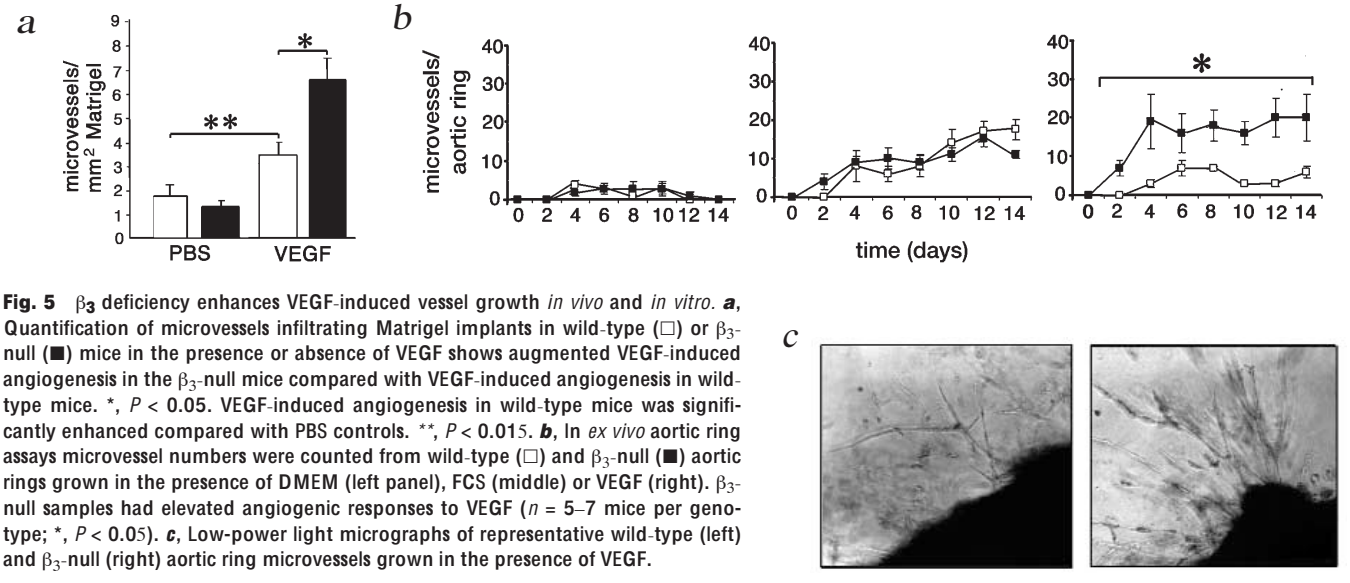

Fig. 5 β_3 deficiency enhances VEGF-induced vessel growth *in vivo* and *in vitro*. ***a***, Quantification of microvessels infiltrating Matrigel implants in wild-type (□) or β_3-null (■) mice in the presence or absence of VEGF shows augmented VEGF-induced angiogenesis in the β_3-null mice compared with VEGF-induced angiogenesis in wild-type mice. *, $P < 0.05$. VEGF-induced angiogenesis in wild-type mice was significantly enhanced compared with PBS controls. **, $P < 0.015$. ***b***, In *ex vivo* aortic ring assays microvessel numbers were counted from wild-type (□) and β_3-null (■) aortic rings grown in the presence of DMEM (left panel), FCS (middle) or VEGF (right). β_3-null samples had elevated angiogenic responses to VEGF (n = 5–7 mice per genotype; *, $P < 0.05$). ***c***, Low-power light micrographs of representative wild-type (left) and β_3-null (right) aortic ring microvessels grown in the presence of VEGF.

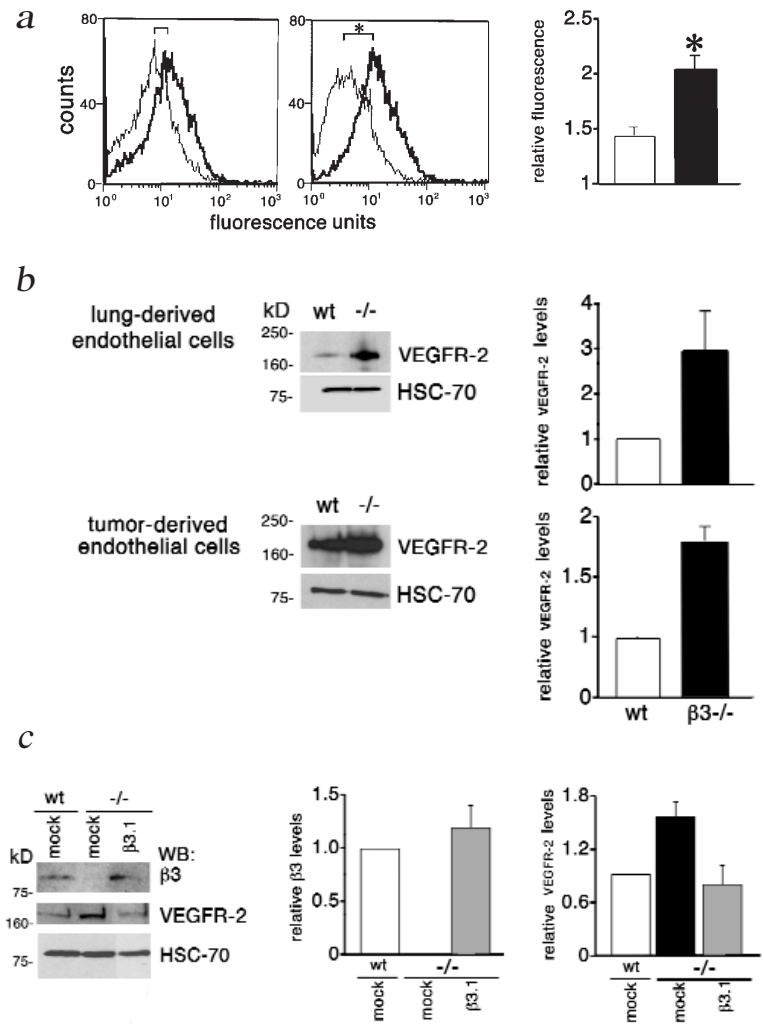

Fig. 6 Enhanced VEGFR-2 expression on β_3-null endothelial cells. ***a***, Flow-cytometric analysis of VEGFR-2 surface-expression levels showed that β_3-null (right) endothelial cells expressed significantly higher levels of VEGFR-2 relative to wild-type controls (left). Bold line, VEGFR-2-labeled cells; thin line, negative control. Bar chart shows means ± s.e.m. of relative surface VEGFR-2 expression in wild-type (□) and β_3-null (■) endothelial cells compared with negative control (n = 3; *, $P < 0.014$). ***b***, Western-blot analysis of VEGFR-2 levels in wild-type and β_3-null endothelial cells that were either grown in culture or isolated from tumors directly. Bar charts represent densitometry results (means ± s.e.m.) from 2–4 independent experiments that show that β_3-null (■) endothelial cells express 2–3-fold more VEGFR-2 than wild-type controls (□). Western blotting for HSC-70 provided loading controls. ***c***, β_3-null endothelial cells were infected with control retrovirus (mock), or with virus containing human β_3 integrin ($\beta_{3.1}$) and mock-infected wild-type cells were used as controls. Western-blot analyses confirmed that β_3 levels were restored in β_3-null cells infected with $\beta_{3.1}$. In β_3-null cells infected with $\beta_{3.1}$, VEGFR-2 expression was reduced to wild-type levels. Western blotting for HSC-70 provided loading controls. Bar charts represent densitometry results (means ± s.e.m., n = 3) of relative β_3 integrin and VEGFR-2 levels.

method to quantify neovascularization in β_3-null mice. Postnatal day 7 (P7) mice were placed in 75% oxygen for five days causing central avascularization of both wild-type and β_3-null retinas (data not shown). This incubation was followed by housing the mice for five further days (until P17) under normoxic conditions after which neovascularization was detected by perfusion of the entire vasculature with a non-diffusible fluorescein-dextran solution. In flat-mounted wild-type and β_3-null retinas, we detected areas of neovascularization and vascular glomeruli (Fig. 4*a*). Vascular glomeruli are highly proliferative clusters of tortuous vessels that are produced in response to angiogenic stimuli and protrude through the inner limiting membrane. The numbers of glomeruli were counted to compare retinal neovascularization in wild-type and β_3-null mice. We observed significantly elevated numbers of vascular glomeruli in β_3-null mice at P17 (Fig. 4*a* and *b*) and in retinal cross-sections (Fig. 4*c*). These data provide evidence that β_3-null mice show enhanced neovascularization not only in tumors but also in hypoxia-induced retinopathy.

VEGF expression is elevated in hypoxia-induced retinal angiogenesis and is thought to be the major angiogenesis-stimulating factor in this system[26,27]. Because hypoxia-induced retinal neovascularization was enhanced in the β_3-null mice, we hypothesized that VEGF-stimulated angiogenesis per se might be elevated in β_3-deficient mice. Matrigel implants impregnated with VEGF or PBS (in the presence of heparin[44]) were administered subcutaneously to wild-type and β_3-null mice and blood-vessel infiltration of the implants was quantified. In contrast to PBS controls, VEGF induced angiogenic responses in both wild-type and β_3-null mice and this response was significantly elevated in β_3-null mice when compared with controls (Fig. 5*a*).

Further analysis of VEGF-induced neovascularization responses was carried out using *ex vivo* aortic ring assays. Wild-type and β_3-null aortic rings were embedded in Matrigel in the presence or absence of serum or VEGF and the numbers of vascular sprouts per aortic ring over a period of 14 days in culture were assessed. In the absence of serum or VEGF, very little vessel outgrowth was detected in either wild-type or β_3-null samples, whereas serum induced neovascularization to a similar extent in both. However, in the presence of VEGF, the total number of vessel sprouts was significantly higher for β_3-null samples when compared with wild-type controls ($P < 0.05$) (Fig. 5*b*). Representative micrographs of vessels sprouting from aortic rings grown in the presence of VEGF are shown in Fig. 5*c*.

Elevated VEGFR-2 levels in β_3-null endothelial cells

Since β_3-null mice had elevated responses to VEGF, we tested whether the β_3-null endothelial cells had elevated levels of VEGFR-2. Flow cytometric analysis showed that β_3-null endothelial cells expressed significantly higher levels of surface VEGFR-2 than wild-type control endothelial cells ($P < 0.014$) (Fig. 6*a*) and densitometric analyses of western blots approximately three-fold more total VEGFR-2 (Fig. 6*b*). In addition, tumor-derived endothelial cells from β_3-null mice also had elevated levels of total VEGFR-2 (Fig. 6*b*). Tumor-derived endothelial cells from wild-type and β_3-null mice both had consistently elevated total VEGFR-2 levels when compared with lung-derived endothelial cells. Moreover, we showed that transduction of β_3-null endothelial cells with human β_3 integrin not only in-

duced β_3 expression in these cells, but also reduced the aberrant levels of VEGFR-2 to wild-type levels (Fig. 6*c*), suggesting that β_3 integrin can regulate VEGFR-2 expression.

Discussion

Our observations, that β_3-null or β_3/β_5-null mice not only support tumor angiogenesis and tumor growth, but even exhibit augmented responses, were unexpected and call for a reconsideration of the functions of α_v integrins in angiogenesis.

The integrin receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, have been implicated in angiogenesis by their expression in vascular sprouts and by experiments blocking angiogenesis stimulated by growth factors, by tumors[11–13] or in hypoxia-induced retinal angiogenesis[16,19]. These data have been interpreted to suggest that antagonists of $\alpha_v\beta_3$ integrin inhibit angiogenesis by blocking its ligand-binding functions with consequences such as reduced proliferation and migration and increased apoptosis of endothelial cells[11–18]. Thus $\alpha_v\beta_3$ integrin has been thought to be essential for both developmental and pathological angiogenesis, and this has led to the generation of $\alpha_v\beta_3$ antagonists as potential anti-angiogenic therapeutics[21].

However, it is also known that mice lacking α_v integrins[22] and others deficient in β_3 or β_5 integrins[24,25], can support developmental angiogenesis. What was not known thus far is whether the absence of these integrins has any impact on pathological angiogenesis. In contrast with the inhibitor data, our results show not only that neither $\alpha_v\beta_3$ nor $\alpha_v\beta_5$ integrin is required for such angiogenesis, but also that, in their absence, there is an increased angiogenic response. Furthermore, the enhanced angiogenic responses in the β_3-null and β_3/β_5-null mice lead to enlarged tumors, again in marked contrast with the blocking data where $\alpha_v\beta_3$ antagonists inhibit tumor growth. Our *in vivo* findings appear to be supported by recent *in vitro* observations that $\alpha_v\beta_3$ antagonists enhance the differentiation of endothelial cells into tubes[28]. In order to generate vessels, endothelial cells need to proliferate, survive and migrate into avascular tissue. As discussed above, these processes were previously thought to be dependent on $\alpha_v\beta_3$ integrin, but our data indicate that this is not the case. How can these discrepancies between the blocking and genetic ablation data be explained?

A first possibility is that genetic ablation underestimates the importance of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins because of some sort of compensatory response by other adhesion molecules. We failed to detect upregulation in levels or activities of other integrins including $\alpha_v\beta_5$ in β_3-null endothelial cells. Additionally, β_3/β_5-null mice show the same enhanced tumor growth and angiogenesis as do β_3-null mice. This indicates that $\alpha_v\beta_5$ integrin is unlikely to compensate for the absence of $\alpha_v\beta_3$ integrin and that neither $\alpha_v\beta_3$ nor $\alpha_v\beta_5$ integrin are essential in angiogenesis. However, other mechanisms of compensation cannot be ruled out.

A second possible explanation for the discrepancy is that the blocking experiments may overestimate the contributions of $\alpha_v\beta_3$ in angiogenesis. Assessment of the impact of known $\alpha_v\beta_3$ antagonists such as LM609 (ref. 17) in β_3-null and wild-type mice would be of great interest. However, LM609 does not react with mouse $\alpha_v\beta_3$ and thus use of peptide antagonists may better address specificity of these reagents in future studies. Cross-talk between $\alpha_v\beta_3$ and other integrins such as $\alpha_5\beta_1$ has been suggested to affect angiogenesis[10,25]. Trans-dominant inhibition of other integrins by interference with β_3 integrins has also been demonstrated[29]. In fact, cross-talk among integrins is a well established phenomenon[30–33]. Based on these prior results, it is possible that the antibodies and low-molecular-weight antagonists of integrins, however specific they are, could be inhibiting other integrins, or even other functions, indirectly. Blocking and other experiments have highlighted the importance of α_5, α_1 and α_2 in angiogenesis[10,12–14,25,34] and it is clear that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are not the only integrins involved in angiogenesis.

A third possible explanation could be an altered mechanism of angiogenesis in the β_3-null mice. Recent data have indicated that in pathological angiogenesis, some endothelial cells in newly formed blood vessels can originate from bone marrow–derived stem cells[35–38]. The differences between $\alpha_v\beta_3$ blocking and genetic ablation results may relate to differences in stem-cell mobilization. Notably, in a recent study of placental growth factor (PlGF)-deficient mice, developmental angiogenesis was normal, but loss of PlGF impaired pathological angiogenesis. Here the differences were attributed to inhibition of mobilization of bone marrow–derived endothelial cells in the PlGF-null mice[39]. While mobilization of bone marrow could be enhanced in β_3-null mice, such a possibility would not explain our *ex vivo* results and the precise mechanism would require further elucidation especially concerning the role of VEGFR-2. An alternative mechanism is that absence of α_v integrins could compromise recruitment of leukocytes and that this in turn could allow faster tumor growth.

Our observation that tumors are actually larger in mice lacking $\alpha_v\beta_3$ or both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and that angiogenesis is actually enhanced in these mice suggest that, rather than being required for angiogenesis, these integrins might normally have a role in limiting angiogenesis *in vivo*. To cite one potential example, during the growth of new vessels, ligated and unligated integrins might provide positive and negative signals for endothelial cell growth and/or survival, only allowing the formation of vessels in the presence of appropriate extracellular matrix ligands. Integrin antagonists could then mimic the unligated state and inhibit vessel growth. In this scenario, the absence of these integrins would result in a loss of the negative signals and could lead to enhanced angiogenesis, as we observed.

Our observation of elevated levels of VEGFR-2 expression on β_3-null endothelial cells suggests one mechanism by which $\alpha_v\beta_3$ integrin could negatively regulate angiogenesis. On the one hand, this could simply be some kind of angiogenic compensatory response to the absence of β_3 integrins; on the other hand, this result could reflect a mechanism normally in play. Indeed, these two possibilities could represent two aspects of the same mechanism. Interactions between β_3 integrins and VEGFR-2 have been reported[40,41]. If a normal function of $\alpha_v\beta_3$ (and perhaps also $\alpha_v\beta_5$) integrin were regulation of the VEGF/VEGFR-2 signaling pathway, then absence of $\alpha_v\beta_3$ could lead to its dysregulation. One possibility, among many that could be imagined, is that $\alpha_v\beta_3$ normally acts as a negative regulator of VEGFR-2 expression and function and ablation of $\alpha_v\beta_3$ causes upregulation of VEGFR-2 (as we observe). Inhibitors of $\alpha_v\beta_3$ could then act by causing dysregulation of VEGFR-2. Although beyond the scope of this study, these results raise some important questions. For example, considering the primacy of VEGF/VEGFR-2 signaling in angiogenic processes, the true function of $\alpha_v\beta_3$ could be to

act as a regulatory balance between inducing and inhibiting angiogenesis.

Here we present strong evidence that β_3 and β_3/β_5-null mice support and even show enhanced pathological angiogenic responses and tumor growth. Although blocking studies have implicated $\alpha_v\beta_3$ and $\alpha_v\beta_5$ as having major roles in neovascularization, our findings demonstrate that these integrins are not essential for this process. The efficacy of $\alpha_v\beta_3$ antagonists in angiogenesis inhibition is not in dispute here—such inhibitors may yet prove to be effective therapeutics. However, our data indicate that a more thorough understanding of the precise mechanisms of action of these inhibitors and of the roles of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in angiogenesis is required.

Methods

Antibodies. Antibodies against VEGFR-2, PECAM, VE-cadherin, α_5 integrin and biotinylated antibodies against β_3 integrin were purchased from Pharmingen (Bedford, UK). Rabbit polyclonal antibodies against human β_3 and β_5 integrins were gifts from B. Coller and L. Reichardt, respectively. Rabbit polyclonal antibody against β_1 integrin was as described[42]. Antibody against α_v integrin was either purchased from Chemicon (Harrow, UK) or for immunofluorescence was a gift from E. Ruoslahti. LM609 was purchased from Chemicon. Antibody against laminin-1 was purchased from Sigma (Poole, UK). Antibody against the heat-shock protein HSC-70 was purchased from Autogen Bioclear (Wiltshire, UK). Conjugated secondary antibodies were purchased from Biosource (Nivelles, Belgium).

Extracellular matrix reagents. Fibronectin for cell culture was purchased from Sigma. Rat fibronectin for functional assays was purchased from Gibco BRL (Paisley, UK) and collagen, vitronectin, laminin-1 were obtained from Collaborative Biomedical Research (Bedford, Massachusetts).

Mouse transplants. Age- and sex-matched control and integrin-deficient mice on a mixed genetic background (C57BL6/129Sv) were given single s.c. injections of either 1×10^6 B16F0 or CMT19T cells and collected ten days after injection (n = 9–12 mice per group). Rag2- and Rag2/integrin-deficient mice were given single s.c. injections of either 2×10^6 LS180 or A375SM cells and collected 3–5 wk after injections (n = 3–11 mice per group).

Quantification of blood-vessel density. Blood-vessel density was quantified by counting the total numbers of PECAM$^+$/laminin-1$^+$ blood vessels across whole sections of tumors (n = 3–6 per group).

Primary lung endothelial-cell isolation. Wild-type and β_3-null mouse lungs were minced, collagenase-digested (Gibco), strained and the resulting cell suspension plated on flasks coated with a mixture of 0.1% gelatin (Sigma), 10 mg/ml fibronectin (Sigma) and 30 μg/ml Vitrogen (Collaborative Biomedical Research). Endothelial cells were purified by a single negative (FCγ-RII/III antibody; Pharmingen) and two positive (ICAM-2; Pharmingen) cell sorts using anti-rat IgG-conjugated magnetic beads (Dynal, Wiltshire, UK) producing a >97% pure population (J.C.L., unpublished data).

Immunofluorescence staining. Subconfluent cultures of endothelial cells and frozen tissue sections were immunostained as described[23].

Integrin-surface iodination and immunoprecipitation. These experiments were carried out as described[42,23].

Western blotting. Wild-type and β_3-null endothelial cells were lysed using RIPA buffer, and 100 μg of protein from each lysate were immunoprecipitated using the antibody VEGFR-2 followed by western-blot analysis also using an antibody against VEGFR-2 (ref. 40).

Cell migration and adhesion assays. Cell migration[43,23] and adhesion[23] assays were performed as described.

Hypoxia-induced retinal angiogenesis. This assay was carried out as described[16] using wild-type and β_3-null mice.

***In vivo* Matrigel plug assay.** This assay was carried out as described[44] in wild-type and β_3-null mice. Using 200 μl of growth factor-reduced Matrigel (Becton Dickinson, Beds, UK) containing 60 units/ml of heparin (Sigma), mixed with PBS or VEGF (60 ng/ml, R&D Systems, Oxon, UK). Blood-vessel infiltration in 7-day pellets was quantified by analysis of H&E-stained sections using a Zeiss Axioplan microscope.

Aortic ring assay. Mouse aortic ring assays were performed essentially as described[45]. 1-mm thoracic aortic rings were placed between 2 layers of 50 μl growth factor-reduced Matrigel supplemented with 20 U/ml heparin (Sigma), and overlaid with 100 μl of DMEM with or without VEGF (30 ng/ml). Microvessel outgrowth was visualized by phase microscopy and the number of vessels growing from each aortic ring was counted every 2 d using a Zeiss Axioplan microscope.

FACS analysis. Wild-type and β_3-null endothelial cells were incubated with VEGFR-2 antibody followed by incubation with a FITC-conjugated secondary antibody. Cells were then analyzed using a Becton Dickinson FACSCalibur flow cytometer.

Isolation of endothelial cells from transplants. CMT19T tumors grown subcutaneously in wild-type and β_3-null mice were collected 10 days after injection. Single-cell suspensions were generated by mincing the tumors and digesting them twice for 30 min at 37 °C with a mixture of collagenases (Roche, Lewis, UK; and Gibco). Cell suspensions were stained with an antibody against VE-cadherin (1 μg per 1×10^6 cells) followed by incubation with antibody against rat IgG-FITC. VE-cadherin$^+$ cells were sorted using a MoFlo (Cytomation, Frieburg, Germany) FACS sorter resulting in a ~90% pure population. 2×10^6 VE-cadherin positive endothelial cells were lysed in 200 μl RIPA buffer and used for VEGFR-2 immunoprecipitation as described above.

β_3 transduction. The medium from a cell line (E86β3.1) producing ecotropic retrovirus (gift from J. Marshall) was used to infect endothelial cells with functional human β_3 integrin. After 48 h endothelial cells infected with the β_3-integrin virus were selected from the non-infected cells by magnetic bead (Dynal) sorting using the antibody against human $\alpha_v\beta_3$, LM609. Cells were lysed and VEGFR-2 was immunoprecipitated followed by western-blot analysis as described. Experiments were repeated 3 times.

Animal regulations. All animals were used in accord with United Kingdom Coordination Committee on Cancer Research guidelines and Home Office regulations.

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