Integrin β4 signaling promotes tumor angiogenesis

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Summary

Mice carrying a targeted deletion of the signaling portion of the integrin β4 subunit display drastically reduced angiogenesis in response to bFGF in the Matrigel plug assay and to hypoxia in the retinal neovascularization model. Molecular cytology indicates that α6β4 signaling promotes branching of β4$^+$ medium- and small-size vessels into β4$^-$ microvessels without exerting a direct effect on endothelial cell proliferation or survival. Signaling studies reveal that α6β4 signaling induces endothelial cell migration and invasion by promoting nuclear translocation of P-ERK and NF-κB. Upon subcutaneous implantation of various cancer cells, the mutant mice develop smaller and significantly less vascularized tumors than wild-type controls. These results provide genetic evidence that α6β4 signaling promotes the onset of the invasive phase of pathological angiogenesis and hence identify a novel target for antiangiogenic therapy.

Introduction

The possibility of ameliorating or even suppressing the progression of cancer with antiangiogenic drugs has attracted vivid interest (Hanahan and Folkman, 1996). Studies on transgenic mouse models of multistage carcinogenesis have revealed the existence of a discrete angiogenic step. In RIP-Tag and K14-HPV16 mice, which develop islet cell and epidermal squamous cell carcinoma, respectively (Arbeit et al., 1994; Hanahan, 1985), enhanced angiogenesis precedes the transition from carcinoma in situ to invasive carcinoma (Arbeit et al., 1996; Folkman et al., 1989), and mutations that impair angiogenesis inhibit disease progression (Bergers et al., 2000; Coussens et al., 2000; Inoue et al., 2002). Thus, the angiogenic step precedes and is potentially rate limiting for tumor invasion and growth. Small metastatic lesions co-opt existing host vessels rather than eliciting angiogenesis, but these vessels eventually regress, and subsequent tumor expansion requires robust neoangiogenesis (Holash et al., 1999). These observations suggest that angiogenesis is required both during initial tumor invasion and growth and during metastatic spread.

Tumor cells elicit angiogenesis through both enhanced production of proangiogenic factors, generally VEGF and bFGF, and decreased generation of angiogenesis inhibitors (Hanahan and Folkman, 1996). As a result, host vessels in the vicinity of the tumor are destabilized, and specific endothelial cells acquire an invasive phenotype. Upon detaching from adjacent cells and penetrating the underlying basement membrane, these cells proliferate and migrate as cords in the interstitial matrix. During the last phase of the process, the endothelial cells acquire a quiescent, differentiated phenotype: they deposit a basement membrane and acquire polarity, coincident with the formation of a lumen. Pericytes and smooth muscle cells are finally recruited to ensheathe the newly formed vessels. These steps are repeated in an iterative manner, as mature vessels become locally destabilized and groups of endothelial cells reacquire an invasive phenotype to generate a new vascular branch (reviewed in Risau, 1997).

Multiple integrins are likely to contribute to tumor angiogenesis. The integrins mediate adhesion to the extracellular matrix and regulate cell survival, proliferation, and migration (Giancotti and Ruoslahti, 1999; Miranti and Brugge, 2002). Known angiogenic factors, such as bFGF and VEGF, enhance the expression and activity of endothelial integrins (Byzova et al., 2000; Klein et al., 1993), whereas negative regulators of angiogenesis, such as class 3 semaphorins, promote vascular remodeling by inhibiting integrin function (Serini et al., 2003). Studies with adhesion blocking reagents and knockout mice have implicated α5β1 and αv integrins in angiogenesis (Eliceiri and Cheresh, 1999; Hynes, 2002). However, the mechanisms by which these and possibly other integrins function in angiogenesis are not clear (Sheppard, 2002). In addition to playing an adhesive role, the integrins may play a signaling role during tumor angiogenesis. Integrin-specific signals impart a stringent control to the action of receptor tyrosine kinases (RTKs), determining whether cells proliferate or undergo growth arrest, migrate or remain stationary, and live or undergo apoptosis when adhering to a specific matrix (Giancotti and Tarone, 2003). Hence, integrin signals can

SIGNIFICANCE

To analyze the physiological role of α6β4 signaling in the absence of the potentially confounding effect of loss of adhesion, we have generated mice carrying a targeted deletion of the C-terminal signaling portion of the integrin β4 subunit. Our analysis of these mice provides genetic evidence that α6β4 signaling controls pathological angiogenesis by promoting the acquisition of an invasive phenotype by angiogenic endothelial cells. Since it is known that α6β4 signaling also promotes carcinoma cell invasion, its inhibition may be especially beneficial for cancer therapy.

potentially affect various phases of angiogenesis. In accordance with this hypothesis, studies on signaling molecules that function downstream of integrins and RTKs, such as focal adhesion kinase (FAK), Src, Shc, and ILK, have documented a general role for joint integrin-RTK signaling in angiogenesis (Hood et al., 2003; Lai and Pawson, 2000; Tan et al., 2004).

The α6β4 integrin—a receptor for laminin-5—has been studied predominantly in the context of epithelial and tumor biology studies. α6β4 signaling proceeds through Src family kinase-mediated phosphorylation of the large cytoplasmic tail of β4, recruitment of Shc, and activation of Ras (Dans et al., 2001; Gagnoux-Palacios et al., 2003; Mainiero et al., 1995) and PI-3K (Shaw et al., 1997). In stratified and transitional epithelia, α6β4 mediates, upon cessation of signaling, assembly of hemidesmosomes (Dans et al., 2001; Murgia et al., 1998; Spinardi et al., 1993). Activation of the EGF-R and Ron RTKs enhances phosphorylation of β4, causing disruption of hemidesmosomes and increased epithelial cell migration (Dans et al., 2001; Santoro et al., 2003; Trusolino et al., 2001), suggesting that these RTKs decrease the ability of α6β4 to mediate stable adhesion but increase its signaling function. Deregulation of α6β4-RTK cosignaling contributes to carcinoma invasion and growth (Gambaletta et al., 2000; Trusolino et al., 2001). Although it is conceivable that similar mechanisms underlie the invasive phase of angiogenesis, the observation that β4 null embryos do not display defective vasculogenesis or developmental angiogenesis (Dowling et al., 1996; van der Neut et al., 1996) has discouraged an examination of the role of α6β4 during angiogenesis.

In this study, we have used a genetic approach to examine the role of α6β4 signaling in postnatal angiogenesis. Prior studies had shown that mice carrying a targeted deletion of the entire cytoplasmic domain of β4 lack hemidesmosomes and, like β4 null mice, die at birth due to extensive blistering of the skin and upper gastrointestinal tract (Murgia et al., 1998). To analyze the role of α6β4 signaling in the absence of the effect of loss of adhesion strengthening, we have generated mice carrying a deletion of the C-terminal signaling segment of the β4 tail. These mice are viable and fertile and do not display signs of epidermal fragility. Through an analysis of these mice, we provide evidence that α6β4 signaling promotes pathological and tumor angiogenesis.

Results

Targeted deletion of the integrin β4 substrate domain impairs signaling to ERK and AKT

Two developments made it possible to address the role of β4 signaling in postnatal life in the absence of potentially confounding effects of loss of adhesion. First, it became clear that the N-terminal part of the cytoplasmic domain of β4 to amino acid 1355 is sufficient for interaction with the plakin HD-1/plectin and hence for association with the keratin cytoskeleton (Schaapveld et al., 1998). Second, mapping studies revealed that the five major tyrosine phosphorylation sites of β4, including those involved in the recruitment of Shc and PI-3K, are located in the C-terminal portion of the β4 tail, downstream of amino acid 1355 (Dans et al., 2001). We thus reasoned that a deletion of the C-terminal portion of the β4 cytoplasmic domain (henceforth referred to as "substrate domain") would suppress α6β4 signaling without interfering with adhesion strengthening.

We used homologous recombination in ES cells to introduce such a mutation in mice. To construct the vector, we cloned the sequences encoding the cytoplasmic domain of β4 up to amino acid 1355, a stop codon, a SV40 polyadenylation signal, and a neomycin resistance gene, immediately downstream of the exon encoding the transmembrane segment of the protein (Figure 1A). Southern blotting and PCR analysis indicated successful introduction of the mutation in mice (Figures 1B and 1C). Analysis of the intercrosses between heterozygous mice carrying the targeted deletion revealed that the mutation was transmitted with the expected Mendelian frequency. Both homozygous and heterozygous β4 mutant mice were found to be viable and fertile and to not manifest skin fragility. Histological analysis of the skin did not reveal any defect in epidermal adhesion to the basement membrane (data not shown). Thus, deletion of the signaling domain of β4 has no obvious effect on embryonic and postnatal development.

Immunoprecipitation and FACS analysis on primary keratinocytes from wild-type and mutant mice indicated that the β4-1355T subunit associates with α6 and is expressed at the cell surface as well as wild-type β4 (Figures 1D and 1E). To test the adhesive ability of the mutant integrin, wild-type and mutant keratinocytes were plated on laminin-5 at 4°C. At this temperature, the function of α3β1, which also binds to laminin-5, is inactivated, and adhesion proceeds only through α6β4 (Gagnoux-Palacios et al., 2003; Xia et al., 1996). The mutant keratinocytes attached to laminin-5 at 4°C as efficiently as wild-type keratinocytes, suggesting that the mutant integrin retains intact ligand binding capacity (Figure 1F). In accordance with the absence of a skin fragility phenotype, transmission electron microscopy (EM) revealed that the skin of mutant mice contained well-structured hemidesmosomes (C. Puri, C. Tacchetti, and F.G.G., unpublished data). Thus, deletion of the C-terminal signaling domain of β4 does not affect the ability of α6β4 to establish a transmembrane connection between laminin-5 and the hemidesmosomal cytoskeleton and to mediate stable epidermal adhesion in vivo.

To examine the effect of deletion of the β4 substrate domain on signaling, primary keratinocytes isolated from wild-type and mutant mice were plated on laminin-5 or, as a control, on collagen I in the presence of serum and subjected to immunoblotting with anti-phospho-ERK and anti-phospho-AKT antibodies. As shown in Figure 1G, adhesion to laminin-5 induced significant phosphorylation of ERK in wild-type but not in mutant keratinocytes, whereas adhesion to collagen I caused similarly high activation of ERK in both types of cells. This result is consistent with the role of the β4 substrate domain recruitment of Shc and activation of Ras to ERK signaling (Dans et al., 2001; Mainiero et al., 1997). In addition, adhesion to laminin-5 led to significant phosphorylation of AKT in wild-type keratinocytes, but it induced a much more limited effect in mutant keratinocytes (Figure 1G), in agreement with the hypothesis that the β4 substrate domain activates PI-3K to AKT signaling (Shaw et al., 1997). We concluded that targeted deletion of the C-terminal segment of the β4 tail impairs α6β4-dependent signaling through ERK and AKT, but it does not affect adhesion to laminin-5 and assembly of hemidesmosomes.

α6β4 and its ligand, laminin-5, are expressed in tumor vasculature

The mutant mice did not display any macroscopic defect suggestive of defective cardiovascular development, indicating that

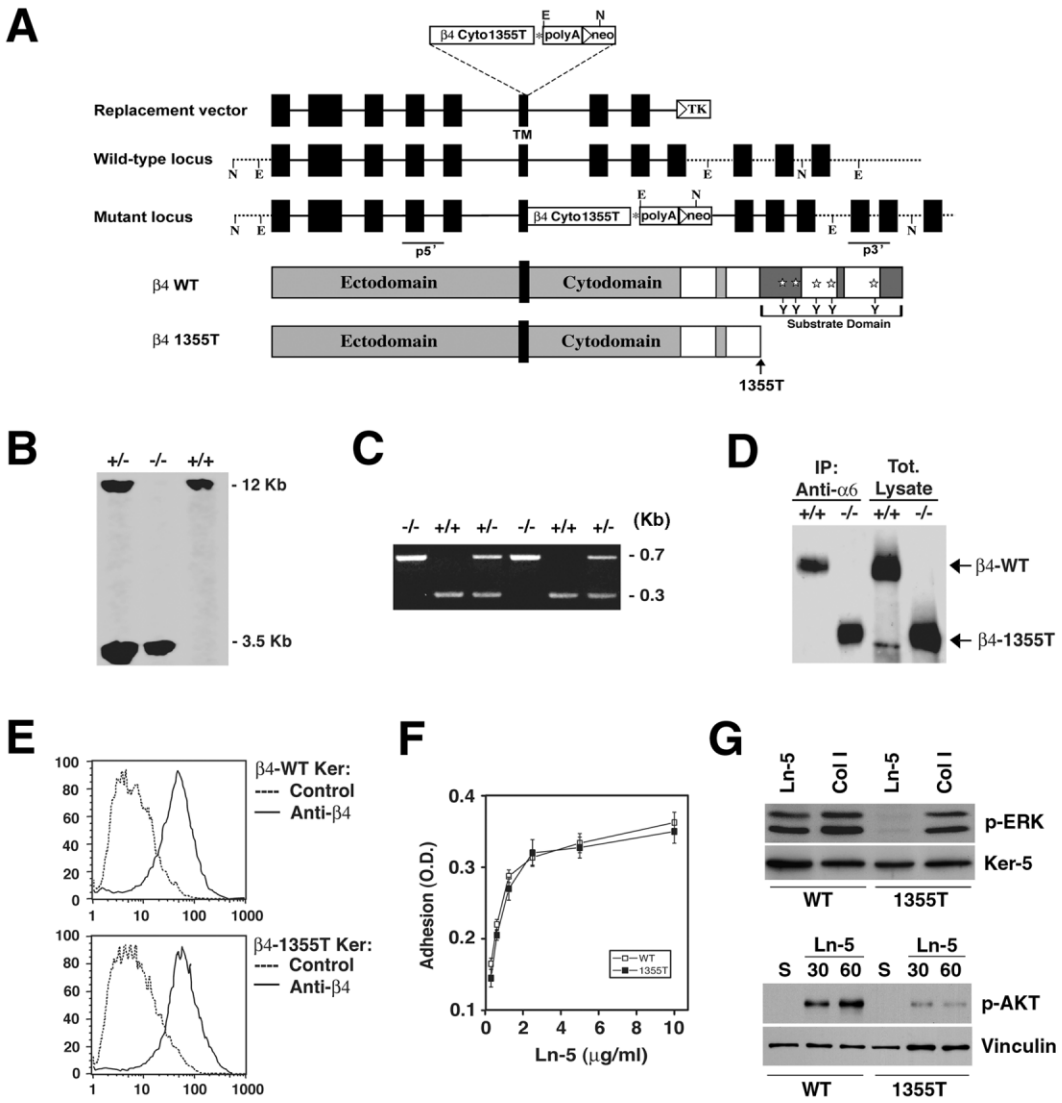

Figure 1. Targeted deletion of the β4 substrate domain

A: Replacement vector, wild-type locus, and mutant locus are shown above. Solid boxes, exons; TM, exon encoding the transmembrane segment; open boxes, cDNA sequences; solid asterisk, stop codon; polyA, SV40 polyadenylation signal; neo, neomycin resistance cassette; TK, thymidine kinase; E, EcoRI; N, NcoI; p5′ and p3′, probes for Southern blotting. Wild-type protein (β4 WT) and truncated mutant (β4 1355T) are shown below. White boxes, fibronectin type-III repeats; open asterisks, tyrosine phosphorylation sites.

B: Southern blotting on genomic DNA from wild-type (+/+), homozygous (−/−), and heterozygous mutant (+/−) mice. Samples were digested with NcoI and probed with a 500 bp radioactive cDNA probe complementary to sequences in the extracellular domain of β4.

C: PCR analysis on intercrosses between heterozygous mutant (+/−) mice. The 0.7 kb band originates from the homozygous mutant allele, and the 0.3 kb band originates from the wild-type allele.

D: Wild-type (+/+) and homozygous mutant (−/−) keratinocytes were immunoprecipitated with the anti-α6 mAb GoH3 and probed with rabbit anti-β4-exo. Equal amounts of total lysates were directly probed with anti-β4-exo. Arrows point to the expected electrophoretic mobilities of wild-type and mutant β4.

E: Wild-type and mutant keratinocytes were subjected to FACS analysis with mAb 346-11A, which binds to the extracellular domain of mouse β4.

F: Wild-type (WT) and mutant (1355T) keratinocytes were plated for 1 hr on microtiter plates coated with the indicated amounts of laminin-5 at 4°C. Cell adhesion to fibronectin at 4°C was negligible (data not shown).

G: Wild-type (WT) and mutant (1355T) keratinocytes were deprived of growth factors, detached, and plated for 2 hr on laminin-5 (Ln-5) or collagen I (Col I) (top), or they were kept in suspension (S) or plated on laminin-5 for the indicated minutes (bottom). Equal amounts of total proteins were probed with antibodies to activated ERK (p-ERK) and keratin-5 (top) or to activated AKT (p-AKT) and vinculin (bottom).

α6β4 signaling does not play an essential role during embryonic vasculogenesis and angiogenesis. This conclusion is consistent with the observation that α6β4 is expressed in blood vessels only after completion of developmental angiogenesis (Hiran et al., 2003). To examine the potential role of α6β4 in tumor angiogenesis, we first studied the expression of α6β4 in paraffin-embedded sections of human papillary thyroid carcinoma, breast adenocarcinoma, prostate carcinoma, and glioblastoma

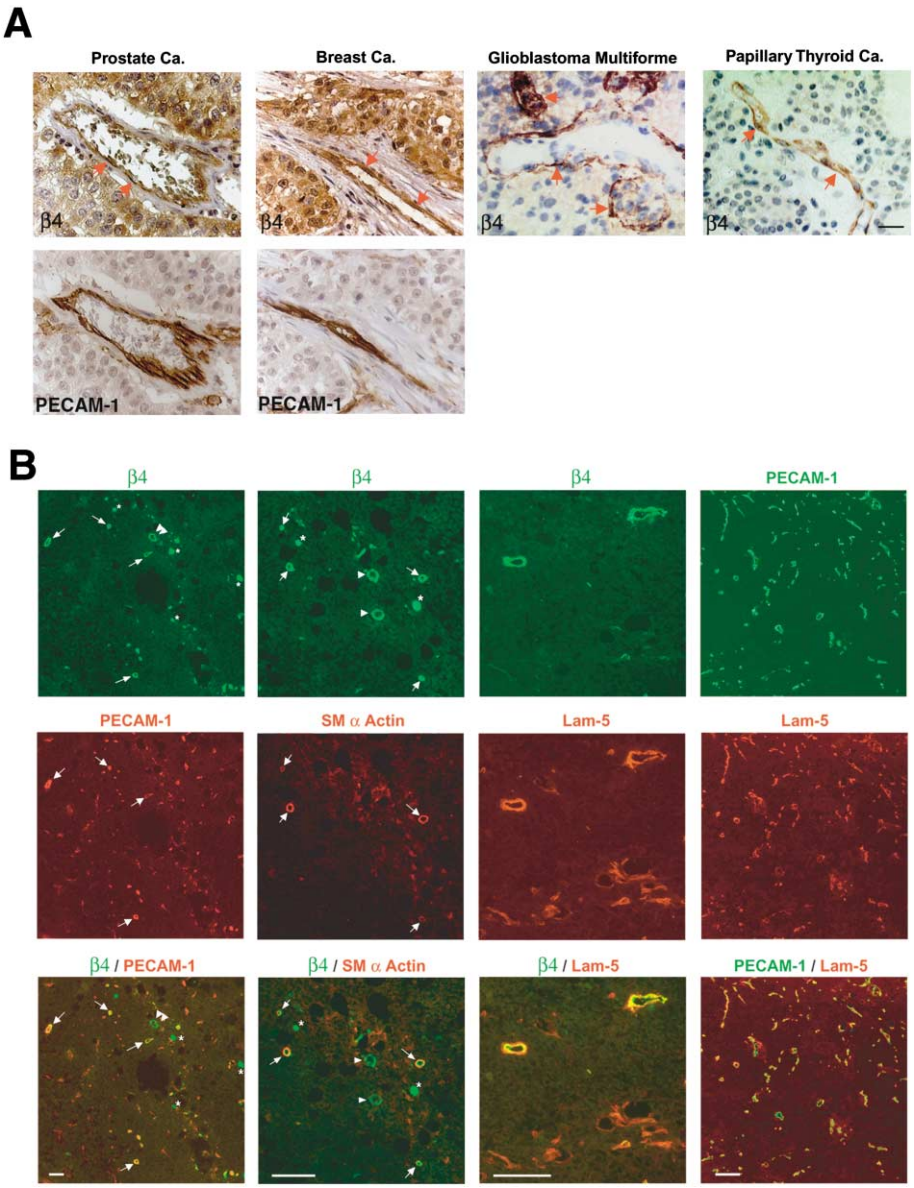

Figure 2. Expression of α6β4 in tumor vasculature

A: Consecutive paraffin-embedded sections of the indicated human tumors were subjected to immunohistochemistry with goat anti-β4 and rat anti-PECAM-1 or stained with anti-β4 alone. Scale bar, 10 μm.

B: Frozen sections of B16F0 melanoma tumors from wild-type mice were doubly stained with rat anti-β4 (green) and goat anti-PECAM-1, mouse anti-smooth muscle α-actin, or rabbit anti-Laminin-5 (red) (far left and two center columns) or doubly stained with goat anti-PECAM-1 (green) and rabbit anti-Laminin-5 (red) (far right column). In the far left column, the arrows point to β4-positive vessels, and the double arrowhead points to a presumptive lymphatic vessel. In the center left column, the arrows point to β4-positive vessels ensheathed by mural cells, and the arrowheads point to β4-positive vessels lacking mural cells. Asterisks indicate $\beta4^+$ peripheral nerves. Scale bar, 40 μm.

multiforme. Significant levels of α6β4 were detected in medium- and small-size vessels in all these tumors (Figure 2A). Since tumor cells in breast and prostate cancer samples expressed high levels of α6β4, these samples were subjected to anti-PECAM-1 staining to unequivocally identify tumor vessels (Figure 2A).

To further characterize the expression of α6β4 during tumor angiogenesis, we examined frozen sections of B16F0 melanoma xenografts. Double staining with antibodies to β4 and PECAM-1 showed that α6β4 is expressed in these tumors in medium- (arrows) and small-size vessels, but not in microvessels (Figure 2B). The anti-β4 antibodies also reacted with structures resembling peripheral nerves (Figure 2B, asterisks). Double staining with antibodies to β4 and to the neurofilament protein S-100 confirmed the identification of these structures as peripheral nerves (data not shown). This observation is consistent with the known expression of α6β4 in Schwann cells (Einheber et al., 1993) and the increasing evidence that tumors, including melanoma, are innervated (Seifert and Spitznas, 2002). Notably, the anti-β4 antibodies also stained vessel-like structures that reacted with anti-PECAM-1 very weakly (Figure 2B, double arrowhead). These structures reacted with antibodies to the lymphatic endothelial hyaluronan receptor (LYVE-1) (data not shown), suggesting that α6β4 is also expressed in tumor lymphatics.

To examine if the expression of β4 in endothelial cells correlated with the presence of vascular smooth muscle cells, we subjected the tumor sections to double staining with antibodies to β4 and to smooth muscle α-actin. As shown in Figure 2B, approximately half of the $\beta4^+$ vessels were found to be ensheathed by smooth muscle cells (arrows), whereas the remainder were not (arrowheads), suggesting that endothelial cells do not express β4 in response to a signal generated by mural cells. Significant amounts of laminin-5 were detected in the basement membrane of both $\beta4^+$ medium- and small-size vessels and

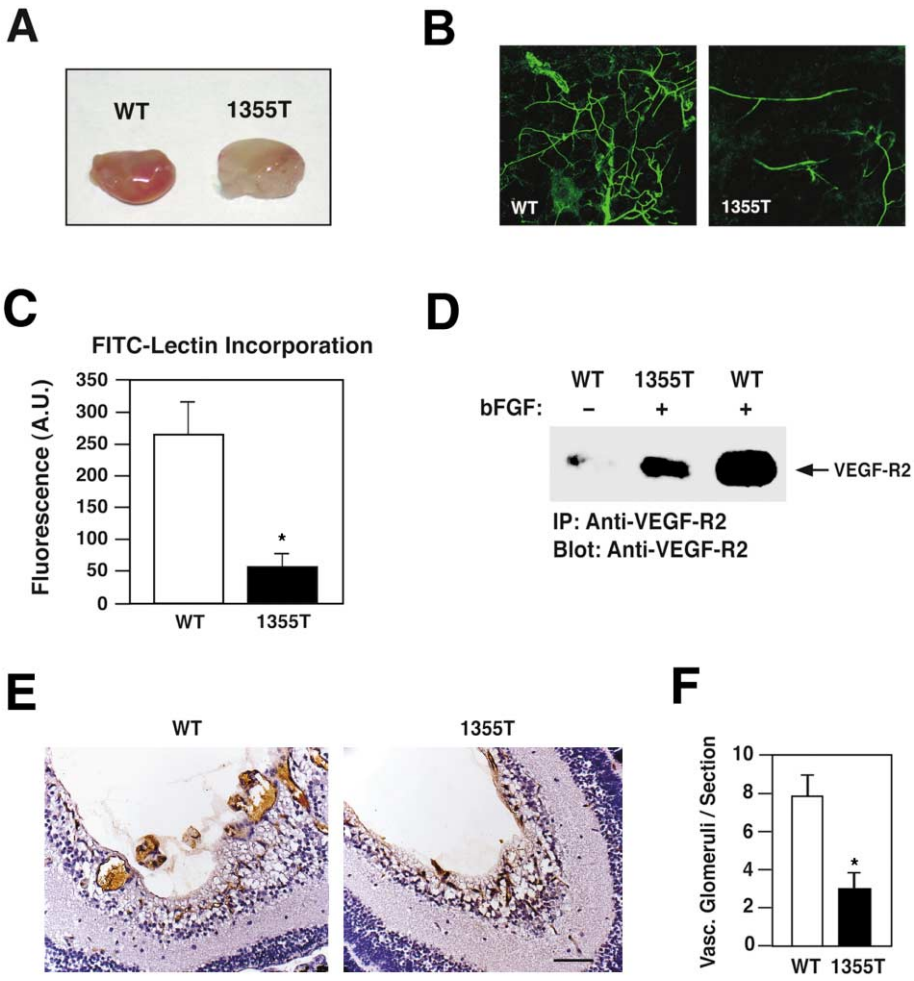

Figure 3. The β4 substrate domain promotes angiogenesis in response to bFGF in the Matrigel plug assay and to hypoxia in the retinal neovascularization model

A: Wild-type (WT) and mutant (1355T) mice were injected s.c. with Matrigel containing PBS or bFGF. After 7 days, the plugs were removed and photographed. The picture shows representative bFGF-containing plugs excised from wild-type and mutant mice.

B: Confocal images of representative bFGF-containing plugs excised from FITC-Lectin-injected wild-type and mutant mice.

C: bFGF-containing plugs from FITC-Lectin-injected wild-type and mutant mice were lysed and subjected to fluorimetry. The graph shows the mean (±SD) from three experiments (*$p < 0.003$).

D: PBS- and bFGF-containing plugs from wild-type and mutant mice were lysed and subjected to immunoprecipitation and immunoblotting with anti-VEGF-R. Scale bar, 100 μm.

E: P7 wild-type (WT) and mutant (1355T) mice were exposed to hyperoxia and returned to normoxic conditions. Eye cross-sections were stained with anti-PECAM-1 and counterstained with hematoxylin. Scale bar, 50 μm.

F: Quantification of vascular glomeruli abutting the limiting membrane in wild-type (WT) and mutant (1355T) retinas (n = 5 mice per genotype) (*$p < 0.004$).

β4$^-$ microvessels, suggesting the existence of another laminin-5 binding integrin in these smaller vessels. In fact, the staining patterns generated by anti-laminin-5 and anti-PECAM-1 antibodies were virtually identical (Figure 2B). Antibodies to α6 decorated all PECAM-1$^+$ vessels, irrespective of β4 expression, indicating that the β4$^-$ microvessels express α6β1 (data not shown). It is possible that α6β1 or another laminin binding integrin, such as α1β1, mediates endothelial cell adhesion to laminin-5 in microvessels. These results indicate that the endothelial cells of tumor vessels deposit and organize a laminin-5-rich basement membrane and, as they mature, attach to it through α6β4.

The β4 substrate domain promotes bFGF- and VEGF-mediated angiogenesis

To examine if α6β4 signaling plays a role in bFGF-induced angiogenesis, Matrigel plugs containing bFGF were implanted in wild-type and mutant mice and recovered 7 days later. Macroscopic analysis revealed that the plugs from mutant mice were much paler than those from control mice (Figure 3A). To visualize the development of vascular ramifications in the plugs, the mice were injected with an endothelial-specific FITC-labeled Lectin prior to euthanasia. Confocal analysis indicated that the vascular tree was in mutant plugs much less developed and complex than that in wild-type plugs. The medium-size vessels penetrating into these plugs generated significantly fewer branches than expected, and these secondary branches only occasionally formed tertiary ramifications (Figure 3B). Fluorimetry indicated that the mutant plugs had incorporated approximately 5-fold less FITC-Lectin than wild-type controls (Figure 3C). In addition, immunoblotting showed that the mutant plugs contained a much smaller amount of VEGF-R and, by inference, of angiogenic endothelial cells than wild-type plugs (Figure 3D). These observations indicate that loss of β4 signaling impairs bFGF-induced angiogenesis to a significant extent.

We examined if α6β4 signaling is required for angiogenesis in the retinal neovascularization model. In this model, angiogenesis is driven by hypoxia-induced production of VEGF (Shweiki et al., 1992). P7 mice were maintained in 75% oxygen for 5 days to induce central avascularization in the retina and then returned to normoxic conditions for 5 additional days. Histological analysis indicated that numerous vascular glomeruli penetrated the inner limiting membrane and abutted in the vitreous in wild-type mice, whereas the development of these abnormal vessels was significantly blunted in mutant mice (Figure 3F). Quantification of the results confirmed that mutant mice have a significantly reduced angiogenic response to retinal hypoxia (Figure 3G). Taken together, these results indicate that α6β4

signaling promotes both bFGF- and VEGF-induced angiogenesis.

α6β4 signaling is not required for endothelial proliferation or survival

To examine the cellular mechanism by which α6β4 signaling regulates angiogenesis, we conducted immunohistochemical studies on Matrigel plugs from wild-type and mutant mice. Anti-PECAM-1 staining of frozen sections showed that the angiogenic vessels of mutant mice penetrated significantly less into the bFGF-containing Matrigel plugs than those of wild-type mice (Figure 4A). The wild-type plugs contained two types of vessels: small-size vessels, which were detected predominantly at the periphery of the plug, and microvessels, which penetrated inside the plug. By contrast, the mutant plugs contained almost exclusively peripheral small-size vessels, and these were somewhat reduced in number as compared to those of wild-type plugs. While the endothelial cells of small-size vessels expressed α6β4, those of microvessels did not express the integrin (Figure 4A). These observations suggest that deletion of the β4 substrate domain interferes with the sprouting of $\beta4^+$ small-size vessels into $\beta4^-$ microvessels.

We next evaluated endothelial cell survival and proliferation in Matrigel plugs from wild-type and mutant mice. Anti-BrdU staining revealed that the number of endothelial cells in S phase was significantly reduced in the plugs from mutant mice. However, the number of $BrdU^+$ nuclei per PECAM-1^+ vessel was similar in wild-type and mutant plugs, suggesting that the overall reduction of BrdU staining in the plugs of mutant mice was secondary to reduced sprouting, and it was not due to an intrinsic proliferative defect (Figure 4B). In addition, the small-size vessels, which express β4, displayed very few $BrdU^+$ nuclei as compared to the smaller PECAM-1^+ $\beta4^-$ capillaries, indicating that α6β4 is expressed in quiescent vessels (Figure 4C). These observations suggest that signaling by the β4 substrate domain is not required for endothelial proliferation during angiogenesis. TUNEL staining did not reveal endothelial cell apoptosis in either wild-type or mutant plugs (data not shown), suggesting that β4 signaling is not required for endothelial cell survival during angiogenesis. Together with the pattern of expression of α6β4 during angiogenesis, these results suggest that α6β4 signaling promotes the onset of the invasive phase of angiogenesis. These observations are consistent with the hypothesis that α6β4 functions at a step of angiogenesis that precedes overt endothelial cell proliferation and migration in the interstitial matrix.

The β4 substrate domain promotes endothelial migration and invasion

To examine the effect of α6β4 signaling on endothelial migration and invasion, we isolated endothelial cells from the lungs of wild-type and mutant mice. However, both types of cells lost expression of α6β4 upon plating in culture (data not shown). We note that endothelial cells migrating out of human saphenous vein explants also lose expression of α6β4 (Hiran et al., 2003). We thus used transient transfection to introduce wild-type or mutant α6β4 in human umbilical vein endothelial cells (HUVECs), as reported previously (Dans et al., 2001). HUVECs, which express almost undetectable levels of endogenous α6β4, were electroporated with plasmids encoding α6 and either wild-type β4 or mutant β4-1355T and panned on anti-β4 coated plates to isolate cells expressing comparable levels of recombinant wild-type or mutant α6β4, respectively (Figure 5A).

To examine the effect of α6β4 signaling on endothelial cell migration, parental HUVECs and their derivatives expressing

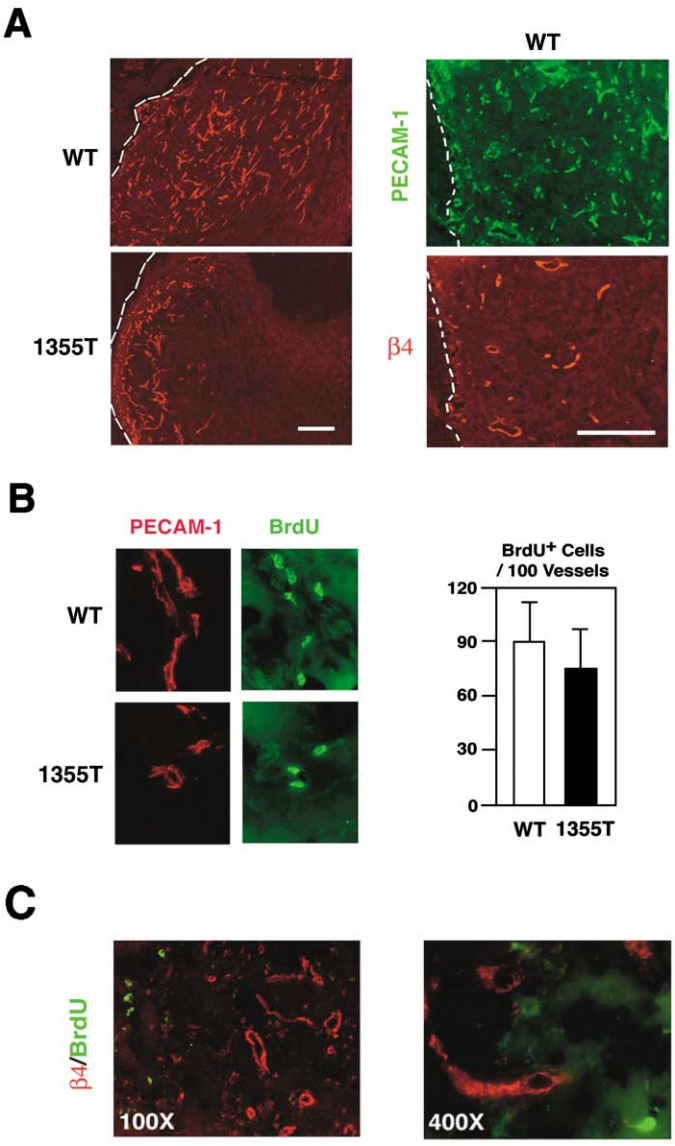

Figure 4. The β4 substrate domain promotes branching of quiescent small-size vessels into proliferative microvessels without exerting a direct effect on endothelial cell proliferation

A: Sections of bFGF-containing plugs from wild-type and mutant mice were stained with rat anti-PECAM-1 (left panels). Sections of bFGF-containing plugs from wild-type mice were subjected to double staining with goat anti-PECAM-1 (green) and rat anti-β4 (red) (right panels). The margins of the plugs are marked with a dotted white line. The endothelial cells of small vessels express α6β4 while those of capillaries do not. Scale bar, 100 μm.
B: Sections of bFGF-containing plugs from wild-type and mutant mice injected with BrdU were subjected to double staining with anti-PECAM-1 (red) and anti-BrdU (green) (left). The graph shows the mean (±SD) number of $BrdU^+$ cells per 100 vessel cross-sections examined (right).
C: Sections of bFGF-containing plugs from wild-type mice injected with BrdU were subjected to double staining with anti-β4 (red) and anti-BrdU (green).

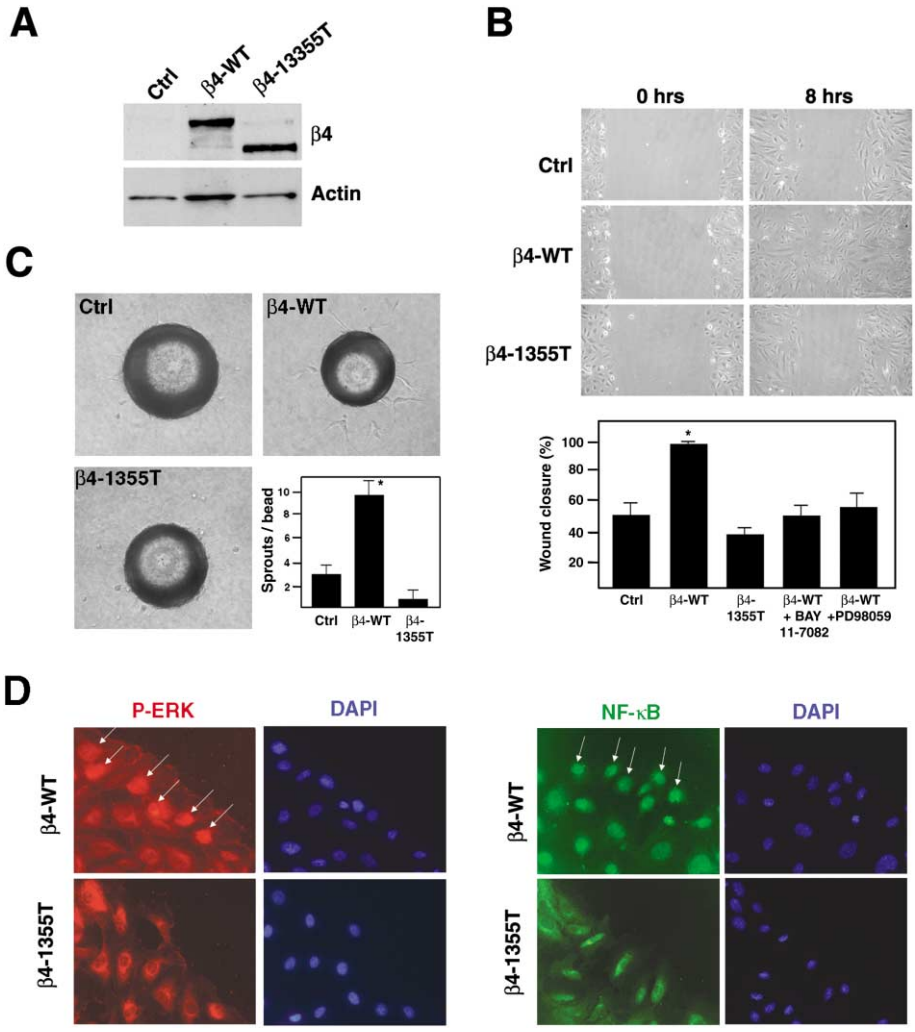

Figure 5. The β4 substrate domain promotes endothelial cell migration and invasion in vitro

A: HUVECs (Ctrl) and HUVECs transfected with α6 in combination with either wild-type β4 or β4-1355T were subjected to immunoblotting with anti-β4-exo and anti-β-actin.

B: HUVECs (Ctrl) and HUVECs transfected with α6 in combination with either wild-type β4 or β4-1355T were plated on laminin-5 and induced to migrate across an artificial wound in response to bFGF. When indicated, migrating cells were treated with the NF-κB inhibitor BAY 11-7082 (12.5 μM) or the MAPK inhibitor PD98059 (50 μM). The graph shows the mean percentage of wound closure at 8 hr (±SD) from three experiments (*$p < 0.001$ versus β4-1355T; $p < 0.002$ versus Ctrl or BAY 11-7082 inhibitor; and $p < 0.003$ versus PD98059 inhibitor).

C: HUVECs and the indicated derivatives were grown on Cytodex-3 beads and placed in collagen gels containing bFGF for 72 hr. The graph indicates the average number (±SD) of cord-like structures emanating from each bead (*$p < 0.002$ versus Ctrl, and $p < 0.001$ versus β4-1355).

D: HUVECs transfected with α6 in combination with either wild-type β4 or β4-1355T were induced to migrate across an artificial wound in response to bFGF for 30 min, fixed, and stained with antibodies to p65 or P-ERK. Nuclei were stained with DAPI. Arrows point to nuclei showing significant nuclear accumulation of P-ERK or NF-κB.

either wild-type or mutant α6β4 were plated on laminin-5 at confluency and subjected to in vitro wound assay. Expression of wild-type α6β4 increased endothelial cell migration in response to bFGF. By contrast, the mutant integrin did not cause this effect (Figure 5B), indicating that the β4 substrate domain promotes endothelial cell migration.

To evaluate the effect of α6β4 signaling on endothelial cell invasion, HUVECs expressing wild-type or mutant β4 were grown on Cytodex-3 beads and then incubated in collagen gels containing bFGF. Over a 3 day period, the endothelial cells expressing wild-type β4 migrated radially out of the beads and assembled into cords invading the collagen gel. By contrast, both control cells and cells expressing mutant β4 invaded the collagen gel only to a limited extent (Figure 5C). Taken together, these observations suggest that signaling by the β4 substrate domain promotes endothelial cell migration and invasion, in accordance with the hypothesis that it controls the onset of the invasive phase of angiogenesis.

The β4 substrate domain induces nuclear accumulation of ERK and NF-κB during endothelial cell migration in vitro and angiogenesis in vivo

Prior studies had provided evidence that α6β4 controls ERK and NF-κB signaling (Mainiero et al., 1997; Santoro et al., 2003; Zahir et al., 2003). To examine the mechanism by which α6β4 promotes endothelial cell migration, we compared ERK and NF-κB signaling in HUVECs expressing wild-type or mutant α6β4 during migration on laminin-5. The cells were plated at confluency on laminin-5 and, 30 min after wounding, were subjected to immunofluorescent staining with antibodies to P-ERK and the p65 subunit of NF-κB. The cells expressing wild-type α6β4 displayed significant nuclear accumulation of P-ERK and NF-κB as they entered into the wound. In contrast, those expressing mutant α6β4 did not show significant nuclear accumulation of P-ERK or NF-κB under the same conditions (Figure 5D). These results suggest that α6β4 signaling promotes both ERK and NF-κB signaling in migrating endothelial cells.

To examine the role of ERK and NF-κB signaling in endothelial cell migration, HUVECs expressing wild-type α6β4 were subjected to in vitro wound closure assay in the presence of the MEK inhibitor PD98059 or the NF-κB inhibitor BAY11-072. These compounds reduced the migration of α6β4-expressing HUVECs to levels similar to those displayed by control HUVECs or HUVECs expressing mutant α6β4 (Figure 5B), suggesting that the β4 substrate domain promotes endothelial cell migration by inducing NF-κB and ERK signaling.

To examine if deletion of the β4 substrate domain impairs

signaling in endothelial cells in vivo, sections of Matrigel plugs from wild-type and mutant mice were subjected to double staining with antibodies to PECAM-1 and to P-ERK or the p65 subunit of NF-κB. We observed significant levels of P-ERK and p65 in the nuclei of many endothelial cells of small- and intermediate-size vessels from wild-type plugs. By contrast, both signaling molecules were predominantly confined to the cytoplasm in endothelial cells of similar vessels from mutant plugs (Figure 6A). To confirm this observation, the samples were subjected to double staining with antibodies to β4 and to P-ERK or p65 and to counterstaining with DAPI. Both P-ERK and p65 accumulated in the nuclei of endothelial cells expressing wild-type β4 but remained largely confined to the cytoplasm in endothelial cells expressing the mutant integrin (Figure 6B). Taken together, these results suggest that the β4 substrate domain promotes nuclear translocation of ERK and NF-κB during angiogenesis.

The β4 substrate domain promotes tumor angiogenesis

To test the role of the β4 substrate domain in tumor angiogenesis, we injected B16F0 melanoma cells, LLC1 Lewis lung carcinoma cells, B6RV2 lymphoma cells, and 60.5 fibrosarcoma cells s.c. in wild-type and mutant mice. The B16F0, LLC1, and B6RV2 tumors grew in mutant mice to a size significantly smaller than they did in wild-type mice. Although the 60.5 tumors also expanded less rapidly in mutant mice, the reduction in tumor growth was in this case smaller (Figure 7A). To compare the density of microvessels in the tumors grown in wild-type and mutant mice, we used anti-PECAM-1 staining. The density of microvessels in each of the four tumors grown subcutaneously in mutant mice was significantly reduced as compared to that of tumors grown under identical conditions in wild-type mice (Figure 7B). This was also true for the 60.5 tumors, which grew relatively well in mutant mice, suggesting that these tumors are somewhat less dependent on angiogenesis for growth. Finally, in the context of other studies, we also examined the effect of loss of β4 signaling on angiogenesis in an orthotopic model of mammary carcinogenesis. In this case, the tumors became vascularized and grew to a similar extent in wild-type and mutant mice (Figures 7A and 7B), suggesting that α6β4 signaling does not contribute to tumor angiogenesis in this specific system. Four major parameters—tumor cell type, transformation mechanism, injection protocol, and specific genetic background of mice—may have influenced the outcome of this specific experiment. Since loss of β4 signaling inhibited tumor angiogenesis to a significant extent in four out of five xenotransplantation models tested, we concluded that α6β4 signaling plays a significant and broad, but perhaps not universal, role in tumor angiogenesis.

To visualize the effect of loss of β4 signaling on tumor vasculature, we injected wild-type and mutant mice bearing B16F0 xenografts with FITC-Lectin. Confocal analysis and 3D reconstruction confirmed that the defective angiogenic response of mutant mice to tumors was due to reduced branching (Figure 7C). Two arguments rule out the possibility that immunological factors contribute to the tumor angiogenesis defect of mutant mice. First, α6β4 is not expressed in the immune system. Second, the 60.5 fibrosarcoma, which are derived from 129 Sv mice, were injected in wild-type and mutant mice of pure syngeneic background, making an immunological response unlikely. In addition, the reduced angiogenesis in tumors of mutant mice

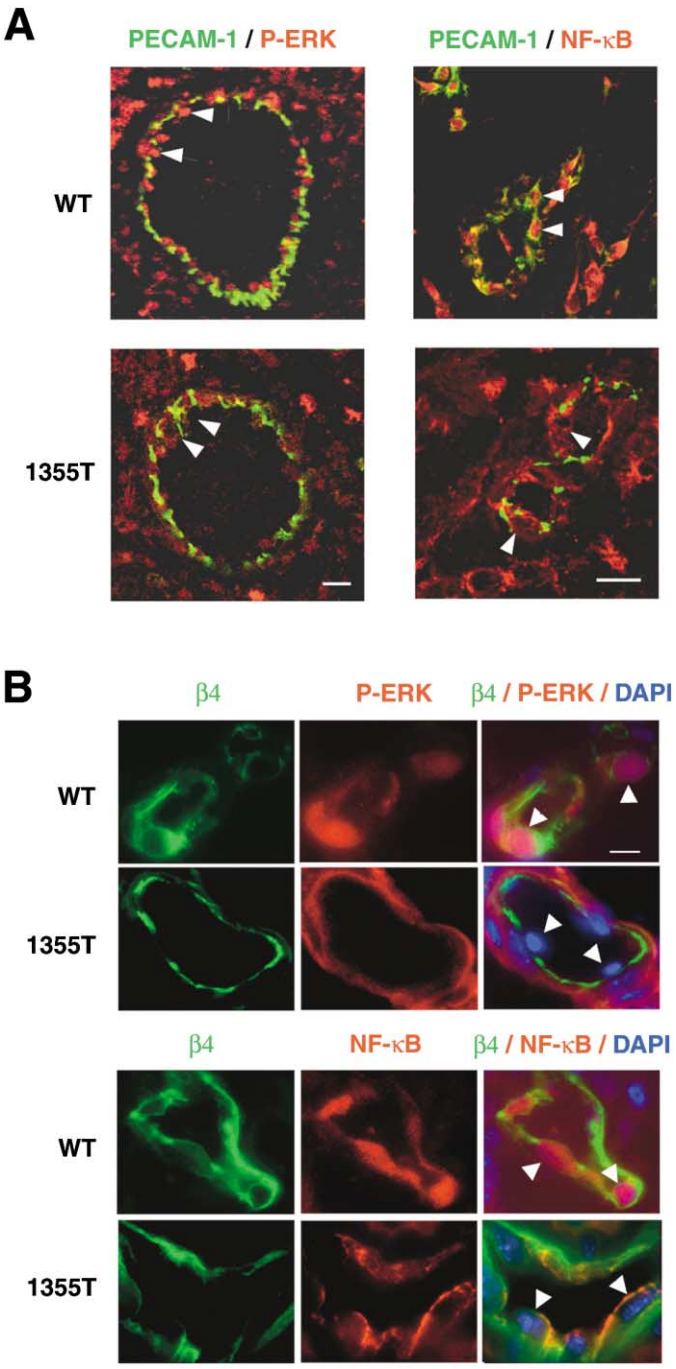

Figure 6. The β4 substrate domain promotes nuclear translocation of ERK and NF-κB in endothelial cells

A: Confocal analysis of nuclear translocation of P-ERK and NF-κB in vivo. Sections of bFGF-containing plugs from wild-type and mutant mice were stained with anti-PECAM-1 (green) and anti-P-ERK (red) (left) or anti-PECAM-1 (green) and anti-p65 (red) (right). Nuclei were stained with DAPI (blue). Arrowheads point to endothelial cells of wild-type vessels containing nuclear P-ERK or NF-κB or to endothelial cells of mutant vessels containing cytoplasmic P-ERK or NF-κB. Scale bar, 4 μm.

B: Sections from the same plugs were subjected to double staining with anti-β4 (green) and anti-P-ERK (red) or anti-p65 (red) and counterstaining with DAPI. Arrowheads are as in **A**. Scale bar, 10 μm.

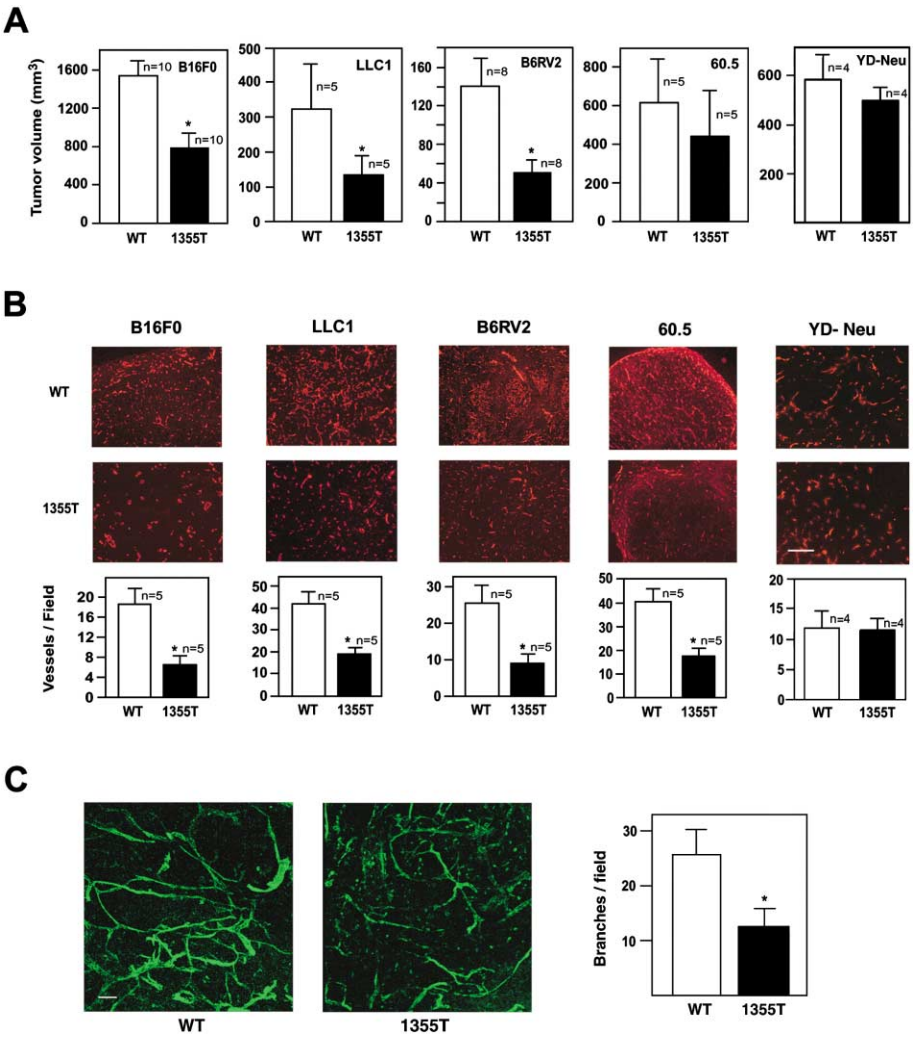

Figure 7. The β4 substrate domain promotes tumor angiogenesis

A: The B16F0 melanoma, LLC1 Lewis Lung Carcinoma, B6RV2 lymphoma, and 60.5 fibrosarcoma cells were injected s.c. in wild-type or β4 mutant mice. The YD-Neu mammary carcinoma cells were injected orthotopically in MMTV-Neu mice expressing wild-type or mutant β4 to avoid an immune response to rat Neu. The graphs show mean tumor volumes (±SD) after 10 days (60.5), 12 days (B16F0 and LLC1), 13 days (B6RV2), or 20 days (YD-Neu) (*$p < 0.004$ in B16F0; $p < 0.09$ in LLC1; $p < 0.01$ in B6RV2).

B: Sections of the indicated tumor xenografts from wild-type and mutant mice were stained with anti-PECAM-1 antibodies. The graphs show the average microvessel densities (±SD) in each tumor. Ten random high-power fields per tumor section were evaluated. Scale bar, 200 μm (*$p < 0.01$ in B16F0; $p < 0.004$ in LLC1; $p < 0.02$ in B6RV2; and $p < 0.005$ in 60.5).

C: Confocal images of B16F0 melanoma tumors excised from FITC-Lectin-injected wild-type and mutant mice. The graph shows the average number of branches (±SD) per high-power field for each tumor (*$p < 0.02$). Scale bar, 100 μm.

does not appear to be a consequence of reduced tumor growth, because the 60.5 tumors grew relatively well but evoked reduced angiogenesis in mutant mice. Taken together, these results identify a role for α6β4 signaling in tumor angiogenesis.

Discussion

Our results provide genetic evidence that the α6β4 integrin promotes tumor angiogenesis—and, presumably, other forms of pathological angiogenesis—by a signaling mechanism. Immunohistochemical and cell biological experiments suggest that α6β4 promotes nuclear translocation of P-ERK and NF-κB and acquisition of an invasive phenotype at the onset of the invasive phase of angiogenesis. These results suggest the intriguing possibility that α6β4 performs a similar signaling function in cancer cells and in angiogenic endothelial cells.

In order to design effective anti-integrin drugs for antiangiogenesis, it is important to understand the mechanisms by which specific integrins participate in this process. Prior studies with adhesion-blocking antibodies and RGD-containing peptides have led to the hypothesis that αvβ3 and avβ5 promote tumor angiogenesis by a signaling mechanism (Eliceiri and Cheresh, 1999). However, genetic studies suggest more complex roles (Hynes, 2002). In particular, it is possible that the αv integrins may have both positive and negative signaling roles during tumor angiogenesis. Perhaps they stimulate endothelial cell proliferation and migration by binding to components of the interstitial matrix during the invasive phase of tumor angiogenesis, but they induce an active, negative signal at the end of the process, either upon becoming unligated or upon binding to a known negative regulator of angiogenesis, such as Thrombospondin, Tumstatin (a fragment of the α3 chain of type IV collagen), or PEX (a fragment of MMP2) (Sheppard, 2002). In this model, the blocking agents interfere with positive signaling while allowing negative signaling to occur. Among other integrins involved in angiogenesis, α5β1 has attracted considerable interest. Both knockout studies and antibody-blocking experiments have indicated that α5β1 and its ligand, fibronectin, are required for developmental and pathological angiogenesis (Hynes, 2002). However, it is not known at what step of angiogenesis α5β1 functions and whether it acts by an adhesive or signaling mechanism. Our results indicate that α6β4 signaling specifically controls the invasive phase of pathological angiogenesis. In addition to adding to our understanding of integrin function during angio-

genesis, these results provide a novel potential target for therapeutic intervention.

The role of α6β4 in angiogenesis described here is unexpected. Prior studies have shown that neither α6β4 nor its signaling functions are required during developmental angiogenesis (Dowling et al., 1996; Murgia et al., 1998; van der Neut et al., 1996). In addition, based on the observation that α6β4 levels increase during vessel maturation, La Flamme and colleagues have proposed that α6β4 limits angiogenesis (Hiran et al., 2003). In retrospect, it is not surprising that α6β4 does not play a role during developmental angiogenesis, as it is expressed in endothelial cells only after completion of this process (Hiran et al., 2003). In addition, our studies do not rule out the possibility that α6β4 also contributes to the maturation of adult vessels. They simply show that that its signaling function contributes to initiate the invasive phase of angiogenesis. We have demonstrated this role of α6β4 signaling in several systems: the Matrigel plug assay, the retinal neovascularization model, and four xenograft models of tumor angiogenesis. This said, increasing evidence indicates that angiogenesis is driven by different growth factors and cytokines and, hence, proceeds by partially distinct mechanisms, depending on developmental stage, tissue, and disease state (LeCouter et al., 2002; Risau, 1997). In particular, the two major angiogenic growth factors, bFGF and VEGF, cooperate with distinct αv integrins to induce angiogenesis (Friedlander et al., 1995; Hood et al., 2003). We have observed that loss of β4 signaling does not suppress angiogenesis in a specific orthotopic model of mammary carcinogenesis. Thus, although our results suggest that α6β4 signaling participates in both bFGF- and VEGF-induced angiogenesis, future studies will be necessary to examine how general the requirement for α6β4 signaling is during tumor angiogenesis. Since the angiogenic Id transcription factors induce expression of the genes encoding α6β4 and its ligand, laminin-5 (Ruzinova et al., 2003), it is possible that α6β4 signaling is especially important when angiogenesis is driven by Id.

What is the mechanism by which α6β4 signaling controls angiogenesis? α6β4 is expressed during angiogenesis in relatively mature vessels. The endothelial cells of $\beta4^+$ vessels display a very low proliferative index, making it unlikely that α6β4 signaling promotes endothelial proliferation. In addition, the angiogenic endothelium of mutant mice does not display evidence of increased apoptosis, excluding the hypothesis that α6β4 signaling plays a necessary role in endothelial cell survival. The severe reduction of $PECAM^+$ $\beta4^-$ capillaries observed in mutant plugs suggests that α6β4 signaling is necessary for the generation of $\beta4^-$ sprouts from $\beta4^+$ vessels, i.e., during the initial step of the invasive phase of angiogenesis. As expression of wild-type, but not mutant, α6β4 promotes endothelial cell migration and invasion in vitro, we propose that α6β4 plays a similar role in vivo (Figure 8). In fact, α6β4 may play a general role during branching morphogenesis, as it has been shown that anti-α6β4 antibodies suppress branching of the ureteric bud in the developing kidney (Zent et al., 2001) and the formation of epithelial cords by breast epithelial cells embedded in Matrigel (Stahl et al., 1997).

Sprouting angiogenesis is thought to commence with the acquisition of an invasive phenotype by specific endothelial cells. The basement membrane underlying these cells is degraded as they migrate into the underlying interstitial matrix. We have observed a defect in nuclear accumulation of ERK and NF-κB in the endothelial cells of small vessels in mutant plugs. This finding suggests that the β4 substrate domain regulates sprouting angiogenesis by promoting nuclear translocation of key transcription factors and that this event precedes and may indeed be necessary for the acquisition of an invasive phenotype by sprouting endothelial cells. In agreement with this model, prior studies have shown that angiogenesis requires integrin signaling to both ERK and NF-κB (Hood et al., 2003; Klein et al., 2002). Although these transcriptional regulators may play multiple distinct roles in angiogenesis, our observations suggest that they play specific roles in the acquisition of the invasive phenotype. In particular, we have shown that β4 signaling promotes nuclear translocation of P-ERK and NF-κB as endothelial cells commence to migrate on laminin-5 and that these signals are necessary to promote endothelial cell migration in vitro. In agreement with this model, it is known that AP-1 and NF-κB coordinately control the expression of genes involved in cell migration and invasion (Vincenti and Brinckerhoff, 2002).

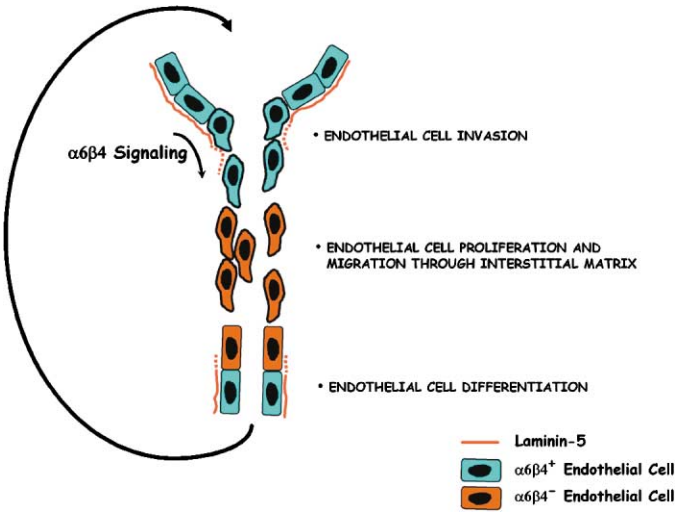

Figure 8. Hypothetical model of α6β4 function in angiogenesis

The possibility of treating chronic diseases, such as diabetic retinopathy, rheumatoid arthritis, and cancer, with antiangiogenic compounds has attracted considerable interest. Because α6β4 signaling is not required during development and normal adult life, compounds blocking α6β4 signaling may curb pathological angiogenesis without exerting significant toxic effects. In addition, it is clear that neoangiogenesis is an integral component of tumor invasion (Hanahan and Folkman, 1996). As cancer cells invade through the extracellular matrix, they are met by cords of angiogenic endothelial cells, bringing them nourishment. Since α6β4 signaling appears to play key roles in both tumor invasion and tumor angiogenesis, its inhibition may be especially beneficial for cancer therapy. If validated, this model would provide a rational basis to future efforts to develop α6β4 inhibitors for cancer therapy.

Experimental procedures

Targeted deletion of the β4 substrate domain

The ClaI/XbaI fragment of mouse *β4* gene was isolated from a 129 Sv library (Murgia et al., 1998) and subcloned in pBluescript to generate pB/S-mβ4-

ClaI/Xba. Site-directed mutagenesis was used to introduce a NheI site within the sequences encoding the transmembrane domain of pB/S-mβ4-ClaI/XbaI, as well as pcDNA3-hβ4 (Dans et al., 2001), without altering their reading frames. PCR was used to introduce a stop codon followed by an XbaI and an EcoRI site in pcDNA3-β4, thereby generating pcDNA3-β4Cyto-1355T. To insert the cDNA fragment encoding the N-terminal portion of the cytoplasmic domain of β4 (amino acids 741–1355) downstream of and in frame with the exon encoding the transmembrane domain of the protein, a NheI/XbaI fragment of pcDNA3-β4Cyto-1355T was subcloned in pB/Smβ4-ClaI/XbaI, and a ClaI/EcoRI fragment of the resulting plasmid was inserted in the targeting vector previously used to delete the entire the cytoplasmic domain of β4 (Murgia et al., 1998). The resulting replacement vector, which carried a left arm of 5 kb and a right arm of 3.8 kb, was linearized and electroporated in ES cells. Positively transfected cells that had undergone homologous recombination were selected in 0.5 mg/ml G418 and 0.2 mM Gancyclovir and identified by Southern blotting. Two distinct ES cell lines were found to carry the expected mutation, and both were injected into blastocyst-stage C57BL/6 mouse embryos. The embryos were then transplanted into the uteri of pseudopregnant C57BL/6 mice. Extensively chimeric mice derived from both lines were crossed to C57BL/6 females. Heterozygous offspring were used to generate mice homozygous for the targeted deletion. Mice were genotyped by PCR using tail genomic DNA. The following primers were used for amplification: 5′-GGAAATAGCAGAGCAGGATAC-3′ (wild-type), 5′-CTCGTGCTTTACGGTATCGC-3′ (recombinant), 5′-CTCGGTTGCAGCA AGGAAG-3′ (common). For Southern blotting, tail genomic DNA was digested with NcoI and, after agarose gel electrophoresis and transfer to a nylon membrane, was hybridized to a 500 bp radioactive cDNA probe complementary to sequences in the extracellular domain of β4, as described previously (Murgia et al., 1998). Except when indicated, the experiments were conducted on mice of mixed genetic background.

Cells, antibodies, and other reagents

Keratinocytes were isolated from the skin of newborn mice and grown on collagen I-coated plates in EMEM.06 with 8% Chelex-treated FBS, 2 ng/ml EGF, and 0.06 mM $CaCl_2$ (Hager et al., 1999). Primary HUVECs were cultured on gelatin-coated dishes (Klein et al., 2002). Rat mAbs to β4 (346-11A), α6 (GoH3), and PECAM-1 (MEC 13.3) were from Pharmingen. Goat anti-PECAM-1 (M-20) and -β4 (C-20) and rabbit anti-VEGF-R2 (C-20) and -NF-κB p65 (C-20) were from Santa Cruz. Rabbit anti-P-ERK and -P-AKT were from Cell Signaling, and anti-keratin-5 (AF 138) was from Babco. Mouse mAbs to smooth muscle α-actin (clone 1A4) and to β-actin (clone AC-74) were from Sigma, and those to NF-κB p65 (clone 2A12A7) were from Zymed. Affinity-purified rabbit antibodies to the LE4-6 modules of the mouse laminin γ2 chain (Sasaki et al., 2001), mouse mAb 3E1 to β4, and rabbit anti-β4-exo serum to a GST fusion protein comprising the N-terminal domain of β4 (Mainiero et al., 1997) were described previously. FITC- and Cy3-conjugated affinity-purified secondary antibodies were from Jackson Laboratories. Laminin-5 matrices were prepared as previously described (Spinardi et al., 1995). Purified laminin-5 was from Chemicon. Human fibronectin and rat tail collagen type I were from Collaborative Research. FITC-Lectin (isolectin B4) was from Vector Laboratories. The MEK inhibitor PD98059 and the NF-κB inhibitor BAY 11-7082 were from Calbiochem.

Keratinocyte studies

For immunoprecipitation and immunoblotting analyses, keratinocytes were lysed in RIPA buffer with 10 mM EDTA and protease inhibitors. Equivalent amounts of total proteins were immunoprecipitated with mAb GoH3 and subjected to immunoblotting with anti-β4-exo or directly subjected to immunoblotting. FACS analysis and adhesion assays were performed as previously described (Murgia et al., 1998). For signaling studies, the keratinocytes were plated on laminin-5 or collagen I for the indicated times, lysed, and subjected to immunoblotting with anti-phospho-ERK and anti-phospho-AKT.

Endothelial cell studies

HUVECs were electroporated with equimolar amounts of plasmids encoding α6 and either β4 or β4-1355T (Dans et al., 2001), deprived of growth factors for 18 hr, and then panned on plates coated with the anti-β4 mAb 3E1. Bound cells were washed with PBS and recovered by trypsin-EDTA treatment. For in vitro wound assays, equal numbers of cells expressing wild-type β4 or mutant β4-1355T were plated on dishes coated with laminin-5, grown until confluent, and starved. Monolayers were scratched with a P200 pipette tip and incubated in the presence of serum and 20 ng/ml bFGF for 18 hr. Wound closure was monitored by digital photography. To monitor ERK and NF-κB signaling during migration, control and transfected HUVECs were subjected to in vitro wounding for 30 min, fixed with 3.7% formaldehyde, and subjected to immunofluorescent staining with anti-P-ERK and -p65, as described (Klein et al., 2002). To examine the effect of β4 signaling on endothelial cell invasion, control and transfected HUVECs were grown on Biosolin Cytodex-3 microcarrier beads (NUNC) until confluent. The beads were then placed in collagen gels (3D Collagen Cell Culture Kit; Chemicon). The gels were overlaid with DMEM with 10% fetal bovine serum, 2 mmol glutamine, and 10 ng/ml bFGF. HUVEC invasion was quantified 72 hr later by counting the average number of capillary-like structures per microcarrier bead.

Immunofluorescence microscopy and immunohistochemistry

Tissues and plugs were embedded in paraffin or snap frozen in OCT compound (Tissue-Tek). Paraffin-embedded sections were stained with hematoxylin and eosin or subjected to immunoperoxidase staining with the indicated antibodies using the ABC Staining Kit (Vector Laboratories). Frozen sections were subjected to immunofluorescent staining with the indicated antibodies. To measure cell proliferation in vivo, mice were injected i.v. with 5 μM BrdU/100 g body weight and sacrificed 1 hr later. Cryostat as well as paraffin-embedded sections of Matrigel or tumors were subjected to immunofluorescent or immunohistochemical staining with anti-BrdU antibodies (BrdU Labeling and Detection Kit I; Roche). To estimate cell death in vivo, TUNEL assays were performed on paraffin-embedded sections (In Situ Cell Death Detection Kit; Roche).

Matrigel plug assay

Eight-week-old mice were injected s.c. with 400 μl of growth factor-depleted Matrigel (BD Biosciences) supplemented with 400 ng/ml bFGF and 1 μg/ml heparin sulfate and sacrificed 7 days later (Passaniti et al., 1992). To visualize angiogenesis, the mice were injected intravenously with 20 μg of FITC-Isolectin B4 (Vector Labs) 30 min before harvesting the plugs. Fluorescently labeled vessels were examined by confocal microscopy. To quantify angiogenesis, FITC-Lectin-containing plugs were homogenized in RIPA buffer containing protease and phosphatase inhibitors and subjected to fluorimetric analysis. Alternatively, plug lysates were immunoprecipitated and subjected to immunoblotting with anti-VEGF-R2 antibodies. Each experimental group consisted of five mice. Each mouse was injected with Matrigel alone or Matrigel supplemented with bFGF and heparin sulfate. Experiments were repeated three times.

Retinal hypoxia model

P7 mice were exposed to 75% oxygen for 5 days and then returned to normoxic conditions for 5 days. Mice of the same age kept in normal air were used as controls. Eyes were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and subjected to staining. Angiogenesis was quantified by counting the number of PECAM-1-positive glomeruli penetrating the inner limiting membrane.

Tumor xenografts

Six-month-old mice were injected s.c. with 10^6 tumor cells per flank. B6RV2 human lymphoma cells, B16F0 mouse melanoma cells, and LLC1 Lewis Lung carcinoma cells were injected in wild-type and mutant mice of mixed genetic background. The 60.5 fibrosarcoma cells, which are derived from 129 Sv mice (Pozzi et al., 2000), were injected in syngeneic wild-type and mutant mice of pure background. The YD-Neu mouse mammary carcinoma cells, which were generated by introducing rat Neu in YD cells (Dankort et al., 2001), were implanted orthotopically at 5×10^6 in Matrigel diluted 1:1 in PBS. To avoid an immune response to rat Neu, the cells were injected in MMTV-Neu transgenic mice expressing either wild-type or mutant β4, as these mice are tolerant to rat Neu. These mice had been backcrossed into an FVB/n background (W.G. and F.G.G., unpublished data). The tumors were excised after the indicated number of days. Final tumor dimensions were measured by caliper.

Acknowledgments

We thank K. Owaribe, R. Timple, and H. Gardner for reagents; the Transgenic and Knockout Mouse Facility and the Molecular Cytology Facility of the Memorial Sloan-Kettering Cancer Center (MSKCC) for help; and M. Dans, S. Klein, D. Lyden, and A. Petridis for help and discussions. This work was supported by NIH awards F32 CA97886 (to S.N.N.), R37 CA58976 (to F.G.G.), and P30 CA08748 (to MSKCC).

Received: April 16, 2004
Revised: July 14, 2004
Accepted: September 10, 2004
Published: November 15, 2004

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