Endothelial $\alpha 3 \beta 1$ -Integrin Represses Pathological Angiogenesis and Sustains Endothelial-VEGF

Rita Graça da Silva,* Bernardo Tavora,*
Stephen D. Robinson,* Louise E. Reynolds,*
Charles Szekeres,[†] John Lamar,[‡] Sílvia Batista,*
Vassiliki Kostourou,* Mitchel A. Germain,*
Andrew R. Reynolds,* Dylan T. Jones,*
Alan R. Watson,* Janet L. Jones,[§] Adrian Harris,[¶]
Ian R. Hart,[§] M. Luisa Iruela-Arispe,^{||}
C. Michael DiPersio,[‡] Jordan A. Kreidberg,[†]
and Kairbaan M. Hodivala-Dilke*

From the Adhesion and Angiogenesis Laboratory,* and the Centre of Tumor Biology,[§] Barts Institute of Cancer, Queen Mary University of London, London, United Kingdom; the Department of Medicine, Children's Hospital and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts; and the Harvard Stem Cell Institute,[†] Cambridge, Massachusetts; the Center for Cell Biology and Cancer Research,[‡] Albany Medical College, Albany, New York; the Weatherall Institute of Molecular Medicine,[¶] University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom; and the Department of Molecular, Cellular, and Developmental Biology,^{||} University of California Los Angeles, Los Angeles, California

Integrin $\alpha 3 \beta 1$ is a major receptor for laminin. The expression levels of laminins-8 and -10 in the basement membrane surrounding blood vessels are known to change during tumor angiogenesis. Although some studies have suggested that certain ligands of $\alpha 3 \beta 1$ can affect angiogenesis either positively or negatively, either a direct *in vivo* role for $\alpha 3 \beta 1$ in this process or its mechanism of action in endothelial cells during angiogenesis is still unknown. Because the global genetic ablation of $\alpha 3$ -integrin results in an early lethal phenotype, we have generated conditional-knockout mice where $\alpha 3$ is deleted specifically in endothelial cells (*ec- $\alpha 3$ -/-*). Here we show that *ec- $\alpha 3$ -/-* mice are viable, fertile, and display enhanced tumor growth, elevated tumor angiogenesis, augmented hypoxia-induced retinal angiogenesis, and increased vascular endothelial growth factor (VEGF)-mediated neovascularization *ex vivo* and *in vivo*. Furthermore, our data provide a novel method by which an integrin may regulate angiogenesis. We show that $\alpha 3 \beta 1$ is a positive regulator of endothelial-VEGF and that, surprisingly, the VEGF produced

by endothelial cells can actually repress VEGF-receptor 2 (Flk-1) expression. These data, therefore, identify directly that endothelial $\alpha 3 \beta 1$ negatively regulates pathological angiogenesis and implicate an unexpected role for low levels of endothelial-VEGF as an activator of neovascularization. (Am J Pathol 2010, 177:1534–1548; DOI: 10.2353/ajpath.2010.100043)

Angiogenesis, the formation of new blood vessels from pre-existing ones, is enhanced in various pathological conditions including rheumatoid arthritis, diabetic retinopathy, and cancer development. Angiogenic processes are regulated both by growth factors, such as vascular endothelial growth factor (VEGF), and adhesion molecules, such as integrins.^{1–3} Inhibitors of VEGF signaling can extend progression-free survival in colorectal, lung, and breast cancers when used in combination with chemotherapy^{4,5} and in renal cancer when used as a monotherapy.⁶ However, recent data suggest that complications with acquired resistance to these agents and how they affect metastasis may compromise their efficacy.^{7,8} Thus, understanding the molecular mechanisms that underlie the regulation of angiogenesis, and especially VEGF function, is essential for the development of safe and effective antiangiogenic therapies.

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Current address of R.G.d.S.: Toronto Medical Discovery Tower, Hospital for Sick Children, Toronto, Ontario, Canada; of V.K.: Vascular Adhesion Lab, Biomedical Research Center, Alexander Fleming, Athens, Greece; of M.A.G.: Novartis Horsham Research Centre, GB-Horsham West Sussex, United Kingdom; current address for A.R.R.: The Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, London, United Kingdom and of C.S.: College of Medicine, University of South Florida, Tampa, FL.

Address reprint requests to Dr. Kairbaan M. Hodivala-Dilke, the Adhesion and Angiogenesis Laboratory, Centre of Tumor Biology, Barts Institute of Cancer, Queen Mary University of London, Charterhouse Square, London, EC1M 6BQ, UK. E-mail: k.hodivala-dilke@qmul.ac.uk.

Endothelial integrins include the fibronectin receptor $\alpha 5\beta 1$, collagen receptors $\alpha 2\beta 1$ and $\alpha 1\beta 1$, vitronectin receptors $\alpha v\beta 3$ and $\alpha v\beta 5$, and laminin receptors $\alpha 6\beta 1$, $\alpha 6\beta 4$, and $\alpha 3\beta 1$. Although many integrins have been shown to be involved in tumor angiogenesis,¹ the role of endothelial- $\alpha 3\beta 1$ in this process is unknown. In addition, integrin $\alpha 3\beta 1$ is a binding partner for several other ligands, and together these have been implicated in either promoting or inhibiting angiogenesis. For example, deletion of CD151 or embryonic laminin $\alpha 4$ -subunit, molecules that can both bind $\alpha 3\beta 1$, results in impaired angiogenesis,^{9–11} whereas adult laminin-8-deficient mice display increased tumor vascularization.¹² In addition, it has also been proposed that the anti-tumorigenic effects of recombinant $\alpha 3(\text{IV})\text{NC1}$ can, in part, be attributed to its binding to $\alpha v\beta 3$ and $\alpha 3\beta 1$ -integrin,^{13,14} whereas the antiangiogenic functions of thrombospondin-1 are reversed by binding $\alpha v\beta 3$ and $\alpha 3\beta 1$.^{15,16} And finally, the binding of VEGF to $\alpha 3\beta 1$ *in vitro* is thought to enhance angiogenic responses,¹⁷ whereas the interaction of TIMP-2 with $\alpha 3\beta 1$ -integrin has been reported to inhibit VEGF receptor-2 function.¹⁸ Taken together, these contradictory results indicate that a precise and, more importantly, direct role of $\alpha 3\beta 1$ -integrin in pathological angiogenesis is not well understood. Accordingly, because $\alpha 3\beta 1$ -directed inhibitors are being designed to either block tumor cell growth or angiogenesis,^{19,20} the requirement to test directly the role of $\alpha 3\beta 1$ in pathological angiogenesis becomes a priority.

Genetic ablation of the $\alpha 3$ -integrin-subunit in mice results in a lethal phenotype where mice die within hours after birth,²¹ rendering them inappropriate for pathological angiogenesis studies. We therefore have generated mice where the $\alpha 3$ -integrin-subunit is deleted in endothelial cells (*ec- $\alpha 3$ -/-*). These mice are viable and fertile, and here we report that mice deficient in endothelial- $\alpha 3\beta 1$ -integrin display enhanced tumor growth and elevated tumor angiogenesis. In addition we show that the deletion of $\alpha 3$ -integrin in endothelial cells results in enhanced VEGF-mediated angiogenic responses both *ex vivo* and *in vivo*. Moreover, we present evidence for a novel and unexpected mechanism by which deficiency in $\alpha 3\beta 1$ regulates angiogenesis by the regulation of endothelial VEGF. Although autocrine signaling by endothelial VEGF has been shown to regulate endothelial cell survival,²² its role in angiogenesis or a mechanism by which it is regulated has not been demonstrated. We show that $\alpha 3\beta 1$ reduces the expression of endothelial VEGF, which surprisingly, at low levels, elevates VEGFR2 expression and thus enhances VEGF-induced angiogenic responses.

Materials and Methods

Antibodies

Rabbit antisera to the $\alpha 3$ - (clone 8–4) and $\beta 1$ - (clone 210-H) integrins were prepared as described.^{23–25} The human $\alpha 3$ -integrin antibodies were purchased from Millipore (Watford, UK). Antibodies to $\alpha 1$, $\alpha 2$, and $\alpha 6$ -integrin, biotinylated $\beta 3$ -integrin, Flk-1, phospho-Flk-1, anti-mouse Platelet endothelial cell adhesion molecule (PECAM-1), anti-human PECAM-1, VE-cadherin, and endoglin antibodies

were purchased from BD Biosciences (Oxford, UK). von Willebrand Factor (vWF) antibody was purchased from DAKO (Cambridge, UK). Endomucin and HSC-70 antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Ki-67 and VEGF-A antibodies were purchased from Abcam Ltd (Cambridge, UK). The $\beta 5$ -integrin antibody was a gift from L. Reichardt (University of California, San Francisco, CA). Antibodies against $\alpha 1$, $\alpha 2$, $\alpha 5$, and $\beta 1$ -integrin subunits for flow-cytometric analysis were a kind gift from B. Chan (University of Western Ontario, Canada). Antibody against laminin-1 was purchased from Sigma (Poole, UK). DC101 rat monoclonal anti-Flk-1 antibody and rat IgG control antibody were gifts from Dan Hicklin, ImClone Systems, Inc. (New York, NY). All ERK antibodies were purchased from Cell Signaling Technology (Hitchin, UK). FITC-conjugated anti-rabbit, anti-rat, and anti-mouse antibodies all were purchased from Jackson Immuno Research Labs (Baltimore Pike, PA) or from Biosource (Nivelles, Belgium). Alexa-conjugated antibodies were purchased from Invitrogen (Paisley, UK). Magnetic beads coated with sheep anti-rat antibody were from Invitrogen. Function blocking anti- $\alpha 6$ -integrin and IgG control antibodies for *in vivo* sponge assays and Fc γ III/II receptor and ICAM-2 antibodies were purchased from AbD Serotec (Kidlington, UK). The Flt1 antibody was purchased from R&D Systems (Abingdon, UK).

Extracellular Matrix Reagents

Human plasma fibronectin, for cell culture and adhesion assays, was purchased from Millipore. Human vitronectin was purchased from BD Biosciences. Mouse EHS laminin-1 was purchased from Sigma.

Creation of Endothelial-Specific $\alpha 3$ -Integrin-Deficient Mice

$\alpha 3$ -integrin floxed mice were developed and provided by Jordan Kreidberg (Harvard University, Boston, MA).²⁶ $\alpha 3$ -integrin-floxed mice ($\alpha 3\text{fl/fl}$) were bred with mice expressing Cre-recombinase under the Tie1 promoter, Tie1Cre²⁷ (kindly donated by Dr. Fässler, Department of Molecular Medicine, Max Planck Institute of Biochemistry, Martinsried, Germany). The progeny from this cross, $\alpha 3\text{floxed}/\alpha 3\text{-wt}/\text{Tie1Cre}+$, were bred with $\alpha 3$ -integrin-heterozygous mice²¹ to introduce an $\alpha 3$ -null allele and generated $\alpha 3\text{floxed}/\alpha 3\text{-null}/\text{Tie1Cre}+$ mice (*ec- $\alpha 3$ -/-*) where $\alpha 3$ -integrin was deleted in endothelial cells. As controls, we also generated $\alpha 3\text{fl/wt}/\text{Tie1Cre}-$ mice (*ec- $\alpha 3$ +/+*), which have wild-type levels of $\alpha 3$ -integrin in endothelial cells, and $\alpha 3\text{fl/null}/\text{Tie1Cre}-$ mice (*ec- $\alpha 3$ +/-*), which express less than the normal levels of $\alpha 3$ -integrin in endothelial cells. All of the mice generated are viable and fertile with no obvious defects.

Mouse Genotyping

The PCR reaction for $\alpha 3$ -integrin was set up using the following: wild-type primer P1-5'-CCGTCTATGTCTCA-

TGAACC-3'; neomycin-resistance gene primer P2-5'-GGGGAATTCCTGACTAG-3'; common primer P3-5'-GGAATCCATCCTGGTTGATGTC-3'. The wild-type and common primers amplified a 130-bp fragment from the wild-type $\alpha 3$ -integrin gene; the neomycin resistance and common primers amplified a 285-bp fragment from the targeted allele.

The PCR reaction for Cre was set up using the following: the forward primer 5'-GCCGCATTACCGGTCGATGCAAGA-3'; the reverse primer 5'-GTGGCAGATGGCGCGCAACACCAT-3'. The reaction generates a fragment of approximately 600-bp.

The PCR reaction for the $\alpha 3$ -floxed genotyping was set up using the following: the forward primer 5'-TGATGAC-TATACCAACCGGAC-3'; the reverse primer 5'-ACTCCAAGCCACATATCCTC-3'. The PCR reaction generates a 623-bp fragment for the $\alpha 3$ -floxed allele.

All PCR reactions were performed using MegaMix (Cambio Ltd, Cambridge, UK).

Primary Lung Endothelial Cell Isolation

Mouse endothelial cells were prepared as described previously.²⁸ Briefly, lungs were minced, digested with 0.1% collagenase type I (Invitrogen) in PBS for 30 minutes, passed through a 70- μ m pore size cell strainer (BD Biosciences), resuspended in mouse lung endothelial cell medium containing 20% fetal calf serum and endothelial mitogen (AbD Serotech), and plated onto tissue-culture flasks precoated with a mixture of PureCol (Nutacon, Leimuiden, The Netherlands), human plasma fibronectin (Millipore), and 0.1% porcine skin gelatin (Sigma). Endothelial cells were cultured and purified over a two- to three-week period by a series of magnetic immunosorting, including a single negative sort using antibodies to the Fc γ III/II receptor, to remove macrophages, followed by at least two positive sorts using antibodies to intracellular adhesion molecule (ICAM)-2, to enrich for endothelial cells. All samples were tested for endothelial purity by flow cytometric analysis for PECAM-1, VE-cadherin, endoglin, and ICAM-2 and only used after purity was confirmed. Also, all samples were tested for $\alpha 3$ -integrin levels by Western blot analysis and $\alpha 3$ -deficiency confirmed by the lack of $\alpha 3$ -protein expression before use in experiments.

Transduction of $\alpha 3$ -Integrin into Endothelial Cells

Endothelial cells were grown to 60% confluency and transduced with retrovirus expressing either the human $\alpha 3$ -integrin or an empty expression cassette, for 24 hours at 37°C. After 24 hours incubation, the viral medium was removed and replaced with mouse lung endothelial cell medium followed by another 48 hours incubation. Endothelial cells infected with $\alpha 3$ -integrin were selected from the noninfected cells by magnetic bead sorting (as described above) using an antibody directed against $\alpha 3$ -integrin (Millipore). Cells were lysed, and Western blot

analysis of $\alpha 3$ -integrin and Flk-1 was performed (see below).

Immunohistochemical Analyses

Immunostaining of blood vessels in mouse tumor sections and in organs was performed as described.²⁹ To quantitate endothelial Flk-1 or VEGFA levels *in vivo*, B16F0 tumor sections were double-immunostained for endomucin and either FLK-1 or VEGF-A as described.^{22,30} *In vivo* levels of Flk-1 and VEGF-A were assessed by measuring average pixel intensity per unit area of vessel using image J software.

Immunohistochemistry of $\alpha 3$ -integrin and endomucin in 5- μ m cryosections was performed as follows: sections were fixed in cold methanol for 10 minutes at 4°C and blocked in 5% BSA, 5% normal goat serum in PSB for 1 hour at room temperature. The rabbit-anti- $\alpha 3$ antibody 8-4 was diluted 1:50 in blocking solution and precleared over $\alpha 3$ -null newborn tissue sections, previously fixed in cold methanol for 10 minutes, for 1 hour at room temperature. Rat-anti-endomucin was diluted 1:200 in blocking solution. Sections were incubated for 1 hour at room temperature with the primary antibodies, washed 3 times in PBS 0.02% Triton-X100, and incubated with biotinylated anti-Rabbit IgG (1:200) in blocking solution for 40 minutes at room temperature, washed 3 times in PBS 0.02% Triton-X100 followed by incubation with the secondary antibodies, Alexa-fluor-546-conjugated goat anti-rat and Alexa-fluor-488-conjugated-streptavidin (both at 1:200) in blocking solution for 40 minutes at room temperature. Finally, sections were washed three times in PBS and incubated with 0.1% Sudan Black in 70% ethanol for 30 minutes at room temperature, washed four times in PBS and once in distilled water, and mounted in Prolong Gold antifade mounting medium with DAPI (Invitrogen). Representative fields were photographed using a Hamamatsu Digital Camera (Improvision, London, UK) on a Zeiss Axioplan microscope (Zeiss, HERTS, UK). Identical settings were used for all of the tissue samples.

Immunostaining of endothelial cells *in vitro* was performed as follows: subconfluent cultures of endothelial cells were fixed in 4% paraformaldehyde, blocked with 100 mmol/L glycine/PBS, and permeabilized with 0.5% NP-40/PBS. This was followed by a further blocking step using 1% BSA and normal goat serum. Cells were incubated with primary antibody overnight at 4°C followed by the conjugated secondary antibody for 1 hour at room temperature. For $\alpha 3$ -integrin immunostaining, endothelial cells were crosslinked with fresh BS3X solution (Perbio Science, Northumberland, UK) for 10 minutes at room temperature, after which the reaction was quenched. Coverslips were washed three times followed by addition of 0.5% NP-40, 2 mmol/L phenylmethylsulfonyl fluoride in PBS for 10 minutes at room temperature to lyse the cells. After three further washes, coverslips were blocked with 10% normal goat serum and incubated with primary and secondary antibodies as described above. Cells or coverslips were washed three times in PBS, rinsed in distilled

water, and mounted with Prolong Gold antifade mounting medium.

Quantification of Blood Vessel Density

Blood vessel density was quantified by counting the total number of PECAM/laminin-1 or von Willebrand or endomucin positive blood vessels in typical midline sections of tumors or sponges from growth factor-induced angiogenesis assays, as described.²⁹ All blood vessels were quantified in a double-blind fashion. Blood vessel density was presented as number of blood vessels per field of midline sections from age-matched size-matched tumors.

Mouse Tumor Transplantation Assays

Age- and sex-matched $ec-\alpha 3^{-/-}$, $ec-\alpha 3^{+/-}$, and $ec-\alpha 3^{+/+}$ mice on a mixed genetic background (129/C57BL6) were given a single subcutaneous injection in the scruff of the neck of either 1×10^6 B16F0 or CMT19T cells. Ten days after injection, mice were sacrificed, tumors were measured, and samples were either snap-frozen or fixed in 4% paraformaldehyde for histological analysis.

Endothelial Proliferation *in Vivo*

To stain for proliferation *in vivo*, paraffin-embedded CMT19T tumor sections were deparaffinized to H_2O . Slides were rinsed in PBS, and antigen retrieval was performed by microwaving the slides for 10 minutes on full power (950 W) in sodium citrate buffer pH 6.0. Slides were allowed to cool for 20 minutes at room temperature and rinsed in PBS. Nonspecific binding of antibodies to sections was blocked by incubating in PBS containing 10% normal goat serum and 1% bovine serum albumin for 30 minutes at room temperature. Subsequently, sections were incubated with rat anti-endomucin (1:500) and rabbit anti-Ki-67 (1:100), both diluted in blocking buffer and incubated overnight at 4°C. Slides were washed in PBS/0.1% Triton X-100 and incubated with Alexa-fluor 488-conjugated donkey anti-rat IgG and Alexa fluor 555-conjugated donkey anti-rabbit IgG (both at 1:200) in blocking buffer. Slides were washed as above and mounted in Prolong Gold antifade mounting media containing DAPI. The number of Ki-67 positive cells per mm of endomucin stained tumor blood vessels from both $ec-\alpha 3^{+/+}$ and $ec-\alpha 3^{-/-}$ animals were then quantified.

Hypoxia-Induced Retinal Angiogenesis

This assay was performed as described.³¹ The retinas were fixed in 4% formaldehyde and either flat-mounted or paraffin-embedded for histochemical analysis. The numbers of vascular glomeruli were quantified across the whole retinas, in a double blind fashion, using a Zeiss Axioplan microscope.

Subcutaneous Growth Factor-Induced Angiogenesis *in Vivo*

This assay was performed as described.³² Briefly, two sterile sponges were implanted subcutaneously into the flanks of $ec-\alpha 3^{+/+}$ and $ec-\alpha 3^{-/-}$ mice. Every two days sponges were injected *in situ* with 100 μ l of 10 ng/ml VEGF or PBS (VEGF_{A164} was made in house according to the method of Krilleke et al³³). After 14 days the mice were sacrificed and sponges were excised and fixed for paraffin embedding. To test the effect of the VEGFR-inhibitor DC101 and the $\alpha 6$ -integrin inhibitor GoH3 on VEGF-induced angiogenesis, mice were administered with endotoxin-free DC101 (800 μ g in 100 μ l of PBS), GoH3 (250 μ g in 100 μ l of PBS), or the appropriate IgG control on the day of sponge implantation and then every three days thereafter. Concentrations used were based on previously published doses that showed inhibitory effects *in vivo*.^{34,35} For vWF immunostaining, paraffin-embedded samples were deparaffinized to H_2O . For antigen retrieval, sections were incubated with proteinase K solution, for 15 minutes at 37°C in a humidified chamber. Sections were cooled for 10 minutes at room temperature, blocked with 2% normal goat serum, 1% BSA, and 0.1% Triton-X100, and incubated with primary antibody (1:200) overnight at 4°C, followed by a FITC-conjugated secondary antibody (1:100) for 1 hour at room temperature. Samples were washed three times with PBS, rinsed in distilled water, and mounted in Prolong Gold antifade mounting media containing DAPI. Blood vessel infiltration was quantified in a double-blind fashion by counting numbers of vWF-positive blood vessels per unit area of sponge section across entire sections using a Zeiss Axioplan microscope.

Surface Iodination and Immunoprecipitation

These experiments were performed as described.^{25,36} Briefly, endothelial cells were trypsinized and equal numbers of cells surface labeled with 0.5 mCi $Na[^{125}I]$ (Amersham Pharmacia, Amersham, UK) using lactoperoxidase and glucose oxidase. 1×10^6 TCA-precipitable counts were immunoprecipitated with each anti-integrin antibody and precipitated integrins resolved by non-reducing SDS-PAGE using 6% resolving and 3% stacking gels.

Flow Cytometric Analysis

Single cell suspensions of wild-type and $\alpha 3$ -null endothelial cells were incubated with either anti- $\alpha 1$, - $\alpha 2$, - $\alpha 5$, - $\alpha 6$, - $\beta 1$, - $\beta 3$, or -Flk-1 antibodies followed by incubation with a FITC-conjugated secondary antibody. Antibodies for flow cytometric analysis of mouse $\alpha 3$ were not available. Cells were then washed and resuspended in FACS buffer (0.1% BSA in PBS) and analyzed for fluorescence using a Becton Dickinson FACSCalibur flow cytometer. For negative controls, IgG matched isotype controls were used.

Adhesion Assays

These assays were performed as described.³⁶ The numbers of adherent cells were counted directly from the plate using a Zeiss timelapse microscope. Adhesion assays were performed in triplicate within each test and the experiment repeated 5–7 times. Relative adhesion is presented as adhesion of cells relative to adhesion of $\alpha 3$ -null endothelial cells on LM-1.

Ex Vivo Aortic Ring Assay

Mouse aortic ring assays were performed essentially as described previously.³⁷ Briefly, thoracic aortic rings were transferred into a bed of polymerized rat tail collagen I (Millipore) and overlaid with 150 μ l of DMEM, 2.5% FBS, and VEGF (30 ng/ml) with or without the blocking antibody against $\alpha 6$ -integrin, GoH3 (1:100), the Flk-1 specific inhibitor, DC101 (20 ng/ml), or rat IgG as a negative control. Aortic rings were cultured at 37°C for 3–6 days and the medium replaced every 2 days. Aortic rings were fixed and stained with FITC-conjugated BS1-Lectin (Sigma), and the number of microvessels sprouting from each aortic ring was counted in a double blind fashion. Microvessel outgrowth was visualized by confocal microscopy (LSM510META, Carl Zeiss Inc).

Real-Time Quantitative PCR

Total RNA was extracted using Trizol (Invitrogen) and subjected to reverse transcription using the High Capacity cDNA archive kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. cDNA was synthesized from 1 μ g purified RNA. Real-time quantitative PCR (qPCR) reactions were performed in triplicate. Each 25- μ l reaction contained 2–5 ng cDNA, 12.5 μ l Platinum SYBR Green qPCR SuperMix-UDG with Rox (Invitrogen), 300 nmol/L (VEGF or Flk1), or 50 nmol/L (Actin) forward and reverse primers and nuclease free H₂O in an individual well of a 96-well plate. VEGF primers recognize all VEGF-A isoforms. The following conditions were used to run the PCR amplification process: 50°C for 2 minutes, denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 1 minute annealing/extension at 60°C. Data analyses were accomplished using the ABI PRISM 7700 Sequence Detection Software (Applied Biosystems). Data were normalized to endogenous actin and fold changes in gene expression were calculated using the comparative CT method.

qPCR Primers were as follows: VEGFA164: forward 5'-TGACCTCCACCATGCCAAGT-3', reverse 5'-CGCTGTAGACGTCCATGAA-3'. Flk-1: forward 5'-CAGTGGTACTGGCAGCTAGAAG-3', reverse 5'-CAGTGGTACTGGCAGCTAGAAG-3'. Actin: forward 5'-AAGGCCAACCGTGAAAAGAT-3', reverse 5'-GTGGTACGACCA-GAGGCATAC-3' reverse.

Western Blot Analysis

Endothelial cells were grown to 70 to 80% confluency and lysed using RIPA buffer. Equal amounts of protein from

each genotype (20–400 μ g) were analyzed by separation on SDS-PAGE gels. Gels were transferred to nitrocellulose and incubated for 1 hour in 5% milk-TBS plus 0.1% Tween-20 (TBST). This was followed with an overnight incubation with the appropriate primary antibody in 5% BSA-TBST at 4°C. Chemiluminescence was detected by exposure to high performance chemiluminescence film (Amersham). Densitometric readings were obtained using image J software.

Knockdown for $\alpha 6$ -Integrin

A smart pool of four siRNAs targeting $\alpha 6$ -integrin was purchased from Dharmacon (Chicago, IL). A nonsilencing control pool (cp) was used as a control. Mouse ECs at ~50% confluency were transfected in OPTIMEM medium (Invitrogen) using Oligofectamine (Invitrogen) following the manufacturer's instructions. The final concentration of siRNA duplexes was 100 nmol/L. After 4 hours, OPTIMEM was replaced with EC medium and cultures were maintained for up to 48 hours before gene expression analysis.

Transwell Migration Assay

The underside of 8 mm transwells (BD Biosciences) were coated with 0.1% gelatin containing 30 μ g/ml collagen and 10 μ g/ml fibronectin for 1 hour at room temperature, after which they were rinsed once with PBS. Each transwell was seeded with 2.5×10^4 endothelial cells in serum-free medium and then placed in 0.5 ml serum-free medium with or without VEGF (30 ng/ml) in 24-well plates. Cells were allowed to migrate through the transwell for 24 hours at 37°C. The total number of cells that migrated through each transwell into the lower chamber medium and after trypsinization of the underside of each transwell was measured using a Casy counter.

Trypan Blue Survival Assay

Endothelial cells (2.5×10^3) were plated into a 96-well plate in medium with or without VEGF (30 ng/ml), and the numbers of viable cells was quantified every 24 hours up to 96 hours. Briefly, after each 24-hour time point the cells were trypsinized, washed once with PBS, and centrifuged. The cell pellets were resuspended in PBS containing 0.4% trypan blue and incubated at room temperature for approximately 3 minutes. Ten microliters of cell suspension was placed in a hemocytometer, and the total number of cells was counted as well as the number of trypan blue-positive cells. The percentage of viable cells was then calculated.

VEGFA164 and Flk1 RNAi Nucleofection

Cells (5×10^5) were electroporated with 0.5–1.5 μ g of pCEP-VEGF₁₆₄ DNA (a gift from Dr Christiana Ruhrberg, UCL, London, UK), or 0.5–1.5 μ g of pCEP-GFP plasmid as a mock controls, or with either Flk-1 smart pool or

control pool siRNAs (Dharmacon) using the primary endothelial cell Amaxa nucleofactor kit (Lonza, Cologne, Germany) according to the manufacturer's protocol. Endothelial cells (2.5×10^5) were plated per 6-cm dish and allowed to adhere. After cell attachment the medium was replaced by OPTIMEM. After a 48-hour incubation period, the conditioned medium was collected for subsequent analysis of VEGF-secreted protein levels by ELISA, and the adherent cells were washed three times with PBS before RNA or protein extraction.

Detection of VEGF-A Protein

After a 48-hour incubation period in OPTIMEM, the conditioned medium from endothelial cells was collected and concentrated 10 $\times$ using Amicon Ultracentrifuge filters (Millipore). To detect cellular-VEGF protein levels, adherent endothelial cells were washed three times with PBS and washing media completely aspirated off. Cells were scraped off the dishes with 50 μ l PBS, transferred to microcentrifuge tubes, and cell pellets subjected to three freeze-thaw cycles. Samples were either immediately used or stored at -20°C for future use. VEGF protein levels were measured using the mouse VEGF immunoassay ELISA Quantikine kit from R&D Systems following the manufacturer's instructions. VEGF protein values obtained from the ELISA were adjusted for total protein concentration and cell number. The antibodies used in this ELISA recognize both VEGFA₁₆₄ and VEGFA₁₂₀.

Statistical Analysis

Statistical significance was calculated using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Ethical Regulations

All animals were used in accordance with United Kingdom Home Office regulations.

Results

Integrin $\alpha 3\beta 1$ is expressed by several cell types, including endothelial cells, but its *in vivo* function during pathological angiogenesis is unclear. We generated conditional knockout mice to study specific inactivation of $\alpha 3$ -integrin in endothelial cells with a focus on its effect on tumor growth and pathological angiogenesis in adult mice. Specifically, we produced $\alpha 3^{\text{flox}}/\text{null}/\text{Tie1Cre}+$ mice ($\text{ec-}\alpha 3^{-/-}$) where $\alpha 3$ -integrin was deleted in endothelial cells. As controls, we also generated $\alpha 3^{\text{fl}}/\text{wt}/\text{Tie1Cre}^-$ mice ($\text{ec-}\alpha 3^{+/+}$), which have wild-type levels of $\alpha 3$ -integrin in endothelial cells, and $\alpha 3^{\text{fl}}/\text{wt}/\text{Tie1Cre}+$ mice ($\text{ec-}\alpha 3^{+/-}$), which express less than the normal levels of $\alpha 3$ -integrin in endothelial cells. Approximately 580 mice were analyzed and genotyped by PCR. All of the progeny generated, including $\text{ec-}\alpha 3^{-/-}$ mice, were viable and fertile (Figure 1, A and B). Western blot analysis of lung-derived endothelial cells isolated from adult

$\text{ec-}\alpha 3^{-/-}$ mice showed a loss of the endothelial- $\alpha 3$ -integrin subunit expression when compared with control endothelial cells isolated from $\text{ec-}\alpha 3^{+/+}$ mice (Figure 1C). Histological analysis of H&E-stained sections of kidney, spleen, lung, liver, and heart from $\text{ec-}\alpha 3^{+/+}$ and $\text{ec-}\alpha 3^{-/-}$ adult mice showed no apparent tissue defects or gross vascular abnormalities (see Supplemental Figure 1A at <http://ajp.amjpathol.org>). In $\text{ec-}\alpha 3^{-/-}$ adult mouse tissue high-power observations of H&E stained sections did not reveal any apparent or consistent morphological abnormalities in blood vessels (see Supplemental Figure 1B at <http://ajp.amjpathol.org>). Additionally, blood vessel density, quantified on endomucin-stained tissue sections, showed no apparent differences between genotypes (see Supplemental Figure 1C at <http://ajp.amjpathol.org>).

To test the effect of endothelial $\alpha 3$ -deficiency on tumor growth and angiogenesis, adult $\text{ec-}\alpha 3^{-/-}$ and control mice were injected subcutaneously with murine tumor cells; melanoma (B16F0) or lung carcinoma (CMT19T). Tumors from both cell lines grew significantly larger in $\text{ec-}\alpha 3^{-/-}$ mice when compared with $\text{ec-}\alpha 3^{+/+}$ mice ($P < 0.001$ for B16F0 and CMT19T) indicating that $\alpha 3$ -deficiency in endothelial cells is sufficient to enhance tumor size. In $\text{ec-}\alpha 3^{+/-}$ mice, tumors grew to an intermediate size, demonstrating that even partial loss of $\alpha 3$ -integrin in endothelial cells enhanced tumor growth ($P < 0.05$ for B16F0 and CMT19T) (Figure 1D).

Double-immunostaining for laminin and PECAM in sections of tumors grown in $\text{ec-}\alpha 3^{+/+}$, $\text{ec-}\alpha 3^{+/-}$, and $\text{ec-}\alpha 3^{-/-}$ mice was used to analyze the effect of $\alpha 3$ -integrin-deficiency on tumor angiogenesis (Figure 1E). Blood vessel density was quantified by counting the number of blood vessels across entire tumor sections from age- and size-matched tumors. The density of blood vessels in tumors grown in $\text{ec-}\alpha 3^{-/-}$ mice was significantly higher than those from $\text{ec-}\alpha 3^{+/-}$ ($P < 0.05$), and $\text{ec-}\alpha 3^{+/+}$ mice ($P < 0.001$) (Figure 1E). These data suggested that a decrease in endothelial $\alpha 3\beta 1$ -integrin is sufficient to enhance tumor angiogenesis. Double immunostaining for endomucin and Ki-67 in sections of tumors grown in both genotypes showed that endothelial proliferation was enhanced in tumor blood vessels of $\text{ec-}\alpha 3^{-/-}$ mice (see Supplemental Figure 2 at <http://ajp.amjpathol.org>).

Contrary to the increased tumor blood vessel density, blood vessel density in unchallenged skin from $\text{ec-}\alpha 3^{+/+}$, $\text{ec-}\alpha 3^{+/-}$, and $\text{ec-}\alpha 3^{-/-}$ mice showed no significant differences (Figure 1F). Previous work has shown that Cre activity can be patchy in quiescent blood vessels of adult Tie1-Cre mice²⁷ and that Tie1 expression is low in nonangiogenic blood vessels in adult mice but up-regulated in tumor blood vessels.^{38,39} Our results raised the possibility that $\alpha 3$ deletion may not be the same in tumor blood vessels and quiescent blood vessels of the skin. Indeed, double immunostaining for $\alpha 3$ -integrin and endomucin showed that the number of blood vessels with detectable $\alpha 3$ -integrin staining was reduced significantly in tumors grown in $\text{ec-}\alpha 3^{-/-}$ mice when compared with tumor blood vessels in $\text{ec-}\alpha 3^{+/+}$ control mice (see Supplemental Figure 3 at <http://ajp.amjpathol.org>). In contrast, the number of blood vessels with detectable $\alpha 3$ -

Figure 1. Tumor angiogenesis is enhanced in *ec-α3*^{-/-} mice. **A:** Photographs of *ec-α3*^{+/+}, *ec-α3*^{+/-}, and *ec-α3*^{-/-} adult mice show no obvious gross phenotype. **B:** *α3*-integrin, Cre-recombinase, and *α3*-floxed PCR analysis of *ec-α3*^{+/+}, *ec-α3*^{+/-}, *α3*^{+/-}, and *ec-α3*^{-/-} mice. The *α3*-PCR reaction generates a 130-bp fragment for the wild-type *α3*-gene and a 285-bp fragment for the targeted knockout allele (*α3*-null); the Cre PCR generates a 600-bp fragment; the *α3*-floxed PCR reaction generates a 623-bp fragment. **C:** Western blot analysis of lysates prepared from lung endothelial cells isolated from *ec-α3*^{+/+}, *ec-α3*^{+/-}, and *ec-α3*^{-/-} mice show that *α3*-integrin is not detectable in *ec-α3*^{-/-} endothelial cells. High levels of *α3*-integrin were found in endothelial cells from *ec-α3*^{+/+} mice and lower levels found in endothelial cells from *ec-α3*^{+/-} mice. HSC-70 provides loading control. *n* > 20 Western blots. **D:** Macroscopic appearance and quantification of volumes of murine 10-day-old B16F0 and CMT19T subcutaneous tumors, grown in *ec-α3*^{+/+}, *ec-α3*^{+/-}, and *ec-α3*^{-/-} mice. Tumor volume was increased significantly in *ec-α3*^{-/-} mice compared with other genotypes. Results are given as mean tumor volume in mm³ ± SEM. *n* = 10–12 mice per genotype, one tumor per mouse. **E:** Double-immunofluorescence staining of microvessels for laminin (red) and PECAM (green) in tumor sections from *ec-α3*^{+/+}, *ec-α3*^{+/-}, and *ec-α3*^{-/-} mice. **Arrows** indicate representative microvessels. Quantification of tumor microvessel density was enhanced significantly in *ec-α3*^{-/-} mice in both B16F0 and CMT19T tumors. *n* = 10–12 mice per genotype, one tumor per mouse. **F:** No differences in microvessel density were observed in unchallenged skin from *ec-α3*^{+/+}, *ec-α3*^{+/-}, and *ec-α3*^{-/-} mice. *n* = 7–10 mice per genotype, one section from each mouse. Results are given as the mean number of vessels per area ± SEM. Scale bar: 1 cm (**D**); 200 μm (**E**). **P* < 0.05; ***P* < 0.001.

integrin staining was indistinguishable between skin samples of *ec-α3*^{-/-} mice and *ec-α3*^{+/+} control mice (see Supplemental Figure 3 at <http://ajp.amjpathol.org>). These results indicate that a substantial loss of *α3β1*-integrin in tumor endothelial cells is sufficient to enhance tumor angiogenesis and tumor growth, and that the lack of any changes in blood vessel density in unchallenged skin are most likely due to persistent expression of endothelial-*α3β1* in adult blood vessels that are not undergoing active remodeling.

Because *ec-α3*^{-/-} mice displayed enhanced tumor angiogenesis, we decided to test the effect of endothelial *α3*-integrin deletion in another adult neovascularization assay, the hypoxia-induced retinopathy assay. Hypoxia-induced neovascularization in neonatal mice is a model for the ischemic disease retinopathy of prematurity, pro-

vides a useful method to assay angiogenesis, and is predominantly a VEGF-driven response.⁴⁰ The vascular glomeruli that develop in the retina after hypoxia exposure are highly proliferative clusters of tortuous vessels and act as an indicator of the degree of pathological angiogenesis.⁴⁰ Retinas from hypoxia exposed *ec-α3*^{-/-} mice had significantly higher numbers of vascular glomeruli when compared with hypoxia exposed *ec-α3*^{+/+} mice (*P* < 0.001) (Figure 2A, upper panels). However, P17 *ec-α3*^{-/-} and *ec-α3*^{+/+} mice maintained in normoxia throughout the experiment revealed no detectable differences in vascular patterning between genotypes (Figure 2A, lower panels). Therefore, the deficiency of *α3*-integrin in endothelial cells is sufficient to enhance not only tumor angiogenesis but also hypoxia-induced retinal angiogenesis.

Figure 2. Hypoxia-induced retinal angiogenesis and VEGF-induced angiogenesis are both enhanced in $ec-\alpha 3^{-/-}$ mice *in vivo*. **A:** Flat-mounted retinas from P17 $ec-\alpha 3^{+/+}$ and $ec-\alpha 3^{-/-}$ mice that had undergone exposure to hypoxia (upper panels) or were maintained under normoxic conditions (lower panels). The number of vascular glomeruli was enhanced significantly in retinas of hypoxia exposed $ec-\alpha 3^{-/-}$ when compared with $ec-\alpha 3^{+/+}$ controls. Vascular pattern appeared normal in retinas of mice maintained in normoxia. Results are given as mean number of glomeruli per retina $\pm$ SEM. Arrowheads indicate vascular glomeruli. OS indicates optical stalk; av, avascular areas. For these experiments, separate neonatal litters were exposed to oxygen to induce retinopathy. Experiment 1 had four $ec-\alpha 3^{+/+}$ mice and three $ec-\alpha 3^{-/-}$ mice; experiment 2 had two $ec-\alpha 3^{+/+}$ mice and three $ec-\alpha 3^{-/-}$ mice; experiment 3 had five $ec-\alpha 3^{+/+}$ mice and two $ec-\alpha 3^{-/-}$ mice; experiment 4 had one $ec-\alpha 3^{+/+}$ mouse and four $ec-\alpha 3^{-/-}$ mice giving a total of 12 mice per genotype. **B:** VEGF-induced angiogenesis *in vivo* was assessed by counting the numbers of vWF-positive blood vessels in VEGF-impregnated sponge sections from $ec-\alpha 3^{+/+}$ and $ec-\alpha 3^{-/-}$ mice. Quantification showed that VEGF stimulated microvessel density was enhanced significantly in $ec-\alpha 3^{-/-}$ mice. Results are given as the mean number of vessels per area $\pm$ SEM relative to $ec-\alpha 3^{+/+}$ + VEGF. $n = 7$ –10 mice per genotype. Scale bars: 500 μ m (A); 100 μ m (B). * $P < 0.05$; ** $P < 0.001$.

Angiogenesis during hypoxia-induced retinopathy and in several tumor types is, predominantly, a VEGF-driven response.⁴⁰ Given the enhanced retinal and tumor angiogenic responses in $ec-\alpha 3^{-/-}$ mice, we tested the specific effect of $\alpha 3$ -integrin deficiency in endothelial cells on VEGF-mediated adult angiogenesis *in vivo*. VEGF-impregnated sponges were implanted subcutaneously into $ec-\alpha 3^{-/-}$ and $ec-\alpha 3^{+/+}$ mice, and the degree of neovascularization was assessed 14 days postimplantation. VEGF-induced angiogenic responses were increased significantly in sponges implanted in $ec-\alpha 3^{-/-}$ mice when compared with controls ($P < 0.005$) (Figure 2B) indicating that, *in vivo*, VEGF-induced angiogenesis is enhanced significantly when $\alpha 3\beta 1$ is lacking in endothelial cells.

It is conceivable that the enhanced angiogenesis, and specifically VEGF-induced neovascularization, observed in the $ec-\alpha 3^{-/-}$ mice could be due to compensation by other endothelial integrins. As expected, a combination of surface iodination and flow-cytometric and immunofluorescence analysis confirmed that $\alpha 3$ -null endothelial cells did not express $\alpha 3$ -integrin subunits, that $\alpha 6$ - and $\beta 1$ -integrin subunits were more highly expressed, and that $\alpha 1$, $\alpha 2$, $\alpha 5$, αv , $\beta 3$, and $\beta 5$ integrin subunit levels were unchanged between genotypes (see Supplemental Figure 4 at <http://ajp.amjpathol.org>). Note that no antibodies are available for the detection of mouse $\alpha 3$ by flow cytometry.

$\alpha 3$ -deficiency had no effect on endothelial cell adhesion to fibronectin and vitronectin (Figure 3A). Although $\alpha 3\beta 1$ -integrin has been reported to act as a *trans*-dominant inhibitor of $\alpha v\beta 3$ in tumor cells, it does not in human umbilical vein endothelial cells.¹³ We also did not find any evidence for such *trans*-dominant inhibition in mouse mi-

crovascular endothelial cells because $\alpha 3$ -null and wild-type endothelial cells did not show any difference in binding to the predominant $\alpha v\beta 3$ ligand vitronectin (Figure 3A). In contrast, $\alpha 3$ -null cells adhered significantly better than wild-type cells on LM-1, a ligand shared by $\alpha 3\beta 1$ and $\alpha 6\beta 1$. The addition of GoH3, an antibody that specifically blocks the function of $\alpha 6$ -integrin⁴¹ reduced significantly $\alpha 3$ -null endothelial cell adhesion (Figure 3A). However, although the enhanced VEGF-mediated angiogenic responses observed in $ec-\alpha 3^{-/-}$ mice *in vivo* were not affected by blocking $\alpha 6\beta 1$ function with GoH3, they were reduced significantly by treatment with DC101, an antibody that specifically blocks the major VEGF-receptor, VEGF-receptor 2 (Flk-1)⁴² (Figure 3B). The involvement of Flk-1 in the enhanced angiogenic responses was further demonstrated in a second angiogenesis model, the *ex vivo* aortic ring angiogenesis assay.³⁷ The enhanced VEGF-mediated responses of aortic rings from $ec-\alpha 3^{-/-}$ mice were not affected by inhibition of $\alpha 6\beta 1$ but were significantly reduced by treatment with DC101⁴² (Figure 3C). Together, these *in vivo* and *ex vivo* data indicate that Flk-1, but not $\alpha 6\beta 1$, is involved in the angiogenic responses observed in the $ec-\alpha 3^{-/-}$ mice.

Because inhibition of Flk-1 with DC101 abrogated VEGF-mediated angiogenic responses in $ec-\alpha 3^{-/-}$ mice we tested whether $\alpha 3$ -integrin affected Flk-1 expression levels and downstream signaling. Flow cytometric analysis, real-time quantitative PCR (qPCR), and Western blot analysis showed significantly elevated surface, mRNA, and total protein Flk-1 expression levels in $\alpha 3$ -null endothelial cells (Figure 4, A–C). Moreover, transduction of $\alpha 3$ -null endothelial cells with human $\alpha 3$ -integrin not only induced $\alpha 3$ -integrin expression in these cells but also reduced Flk-1 back to wild-type levels

Figure 3. Flk-1 is responsible for enhanced VEGF-mediated angiogenesis in $\alpha 3$ -integrin-null mice. **A:** Quantification of relative adhesion of wild-type (+/+) and $\alpha 3$ -null (-/-) endothelial cells on fibronectin, vitronectin, LM-1, and LM-1+GoH3. $\alpha 3$ -null endothelial cells displayed enhanced adhesion to LM-1. Addition of an $\alpha 6$ -integrin-specific functional blocking antibody (GoH3) significantly reduced LM-1-mediated adhesion in $\alpha 3$ -null, but not wild-type endothelial cells. Values for the adhesion assay are given as mean percentages relative to adhesion of $\alpha 3$ -null endothelial cells to LM-1 $\pm$ SEM. White bars, wild-type; black bars, $\alpha 3$ -null. $n = 3$ –5 experiments performed in triplicate. **B:** The roles of $\alpha 6$ -integrin and Flk-1 in VEGF-induced angiogenesis in ec- $\alpha 3$ +/+ and ec- $\alpha 3$ -/- mice were tested by analyzing the number of blood vessels in subcutaneously-implanted sponges that were impregnated with either VEGF, VEGF+GoH3 ($\alpha 6$ -integrin inhibitor), or VEGF+DC101 (Flk-1 inhibitor). The graph represents mean blood vessel numbers, relative to ec- $\alpha 3$ +/+ responses to VEGF alone. White bars, ec- $\alpha 3$ +/+ mice; black bars, ec- $\alpha 3$ -/- mice. The enhanced angiogenic responses to VEGF in ec- $\alpha 3$ -/- mice were inhibited by DC101 but not by GoH3. $n = 4$ –5 individual mice, two sponges per mouse. n.s., not significant. **C:** *Ex vivo* VEGF-mediated microvessel sprouting. Aortic rings from ec- $\alpha 3$ +/+ and ec- $\alpha 3$ -/- mice, grown in the presence of either PBS, VEGF, VEGF+GoH3, or VEGF+DC101, were stained with BS-1 lectin to identify microvessels. Representative rings are shown for each genotype and condition. Scale bar: 500 μ m. **Arrows** indicate microvessels. Quantification of microvessels shows enhanced VEGF-induced angiogenesis in ec- $\alpha 3$ -/- aortic rings. Graph represents the mean numbers of sprouting microvessels per condition $\pm$ SEM. The enhanced angiogenic responses to VEGF were inhibited by DC101 but not GoH3. White bars, ec- $\alpha 3$ +/+; black bars, ec- $\alpha 3$ -/. $n = 25$ –40 rings per genotype, three individual experiments. n.s., not statistically different. * $P < 0.05$; ** $P < 0.001$.

(Figure 4C, upper blots). On VEGF stimulation phosphorylated-Flk1 levels were also elevated in $\alpha 3$ -null endothelial cells when compared with wild-type cells (Figure 4C, lower blots). Finally, we showed that Flk1 levels were elevated significantly in endomucin-positive blood vessels from tumors grown in ec- $\alpha 3$ -/- animals as compared with those grown in ec- $\alpha 3$ +/+ animals (Figure 4D), suggesting that this up-regulation of Flk-1 is evident *in vivo*. In contrast, Flt-1 levels were apparently reduced in $\alpha 3$ -null endothelial cells when compared with wild-type cells suggesting that $\alpha 3$ -integrin may differentially regulate these two VEGF-receptors (see Supplemental Figure 5 at <http://ajp.amjpathol.org>). However, because the current view is that Flk-1 is a major receptor transducing the effects of VEGF into endothelial cells we focused our attentions to how $\alpha 3$ -integrin regulates Flk-1.

The control of Flk-1-downstream signaling by $\alpha 3$ -integrin was investigated by testing the consequence of $\alpha 3$ -integrin deficiency on VEGF-induced ERK1/2 phosphorylation in the presence or absence of DC101. VEGF-treatment induced a significant increase in ERK1/2 phosphorylation in mock-transduced $\alpha 3$ -null endothelial cells when compared with mock transduced wild-type controls and this response was abrogated by the addition of DC101 (Figure 4E). Transduction of human $\alpha 3$ -integrin into $\alpha 3$ -null endothelial cells reduced VEGF-mediated

ERK1/2 phosphorylation back to levels similar to mock transduced wild-type endothelial cells ($P < 0.05$) (Figure 4E). In contrast, although depletion of $\alpha 6$ -integrin in $\alpha 3$ -null cells reduced $\alpha 6$ -integrin expression levels by approximately 80%, it had no effect on Flk-1 expression levels or VEGF-mediated ERK1/2 phosphorylation (see Supplemental Figure 6 at <http://ajp.amjpathol.org>). Finally, VEGF-induced endothelial cell migration and long-term cell survival were also enhanced significantly in $\alpha 3$ -null endothelial cells (Figure 4, F and G). These results implicate $\alpha 3$ -integrin as a key regulator of VEGF-mediated responses.

We next studied the mechanism by which $\alpha 3\beta 1$ might regulate Flk-1 expression. The potent proangiogenic function of exogenous VEGF in neovascularization is unquestionable.⁴³ However, recent reports have shown that endothelial cells can produce their own VEGF,^{22,44} and although endothelial-VEGF is important in the survival of these cells, relatively little is known about its molecular regulation or angiogenic function. Because we show that specifically deleting $\alpha 3\beta 1$ in endothelial cells leads to enhanced endothelial cell survival, we examined the possibility of cross-regulation between $\alpha 3\beta 1$, endothelial-VEGF, and Flk-1 in endothelial cells. First, we demonstrated that endothelial-VEGF mRNA, cellular, and secreted protein levels were, surprisingly, reduced significantly in $\alpha 3$ -null endothelial cells when compared with wild-type controls (Figure

Figure 4. $\alpha 3$ -integrin regulates Flk-1 expression and signaling. **A:** Flow cytometric analysis of IgG control and Flk-1 in wild-type (gray lines) and $\alpha 3$ -null (black lines) endothelial cells. Bar chart shows mean surface Flk-1 expression $\pm$ SEM in wild-type (+/+) and $\alpha 3$ -null (-/-) endothelial cells. Surface Flk-1 levels were elevated significantly in $\alpha 3$ -null (-/-) endothelial cells. $n = 6$ experiments. **B:** Real-time qPCR showed a significant increase in Flk-1 mRNA in $\alpha 3$ -null endothelial cells. Results are relative to wild-type controls. $n = 3$ experiments. **C:** Western blot analysis of $\alpha 3$ -integrin and Flk-1 levels in wild-type endothelial cells mock infected (+/+ mock), $\alpha 3$ -null endothelial cells mock infected (-/- mock), and $\alpha 3$ -null endothelial cells infected with viral supernatant containing human $\alpha 3$ -integrin cDNA (-/- Hu $\alpha 3$). Infection with viral supernatant induced $\alpha 3$ -integrin expression in $\alpha 3$ -null endothelial cells. Transduction of $\alpha 3$ -integrin reduced Flk-1 levels. Bar charts represent densitometric results of mean relative values of $\alpha 3$ -integrin and Flk-1 levels $\pm$ SEM. HSC-70 provided loading control. $n = 3$ experiments. The lower Western blot shows analysis of phospho-Flk1 levels in endothelial cells under basal (-) and VEGF-stimulated (+) conditions. Levels of VEGF-stimulated phosphorylated Flk-1 are elevated in $\alpha 3$ -/- endothelial cells. HSC-70 provided loading control. **D:** Flk-1 expression levels are enhanced in tumor blood vessels of ec- $\alpha 3$ -/- mice. Double-immunofluorescence staining of blood vessels for Flk1 (green) and endomucin (red) in tumor sections from ec- $\alpha 3$ +/+ and ec- $\alpha 3$ -/- mice. Bar chart represents mean endothelial Flk-1 intensity/ μm^2 of endomucin-positive blood vessel *in vivo*. Scale bar = 50 μm . **E:** Western blot analysis of ERK1/2 phosphorylation in mock transfected wild-type (+/+ mock), $\alpha 3$ -null (-/- mock), or $\alpha 3$ -null cells transduced with human $\alpha 3$ -integrin cDNA (-/- Hu $\alpha 3$). Cells were stimulated (+) or not (-) with VEGF, in combination with (+) or without (-) DC101. Western blotting for total ERK1/2 provided the loading control. Bar charts represent mean densitometric results of phospho-ERK2 relative to total ERK2 levels $\pm$ SEM. $n = 3$ experiments. **F:** VEGF-stimulated chemotactic migration of $\alpha 3$ -null (-/-) endothelial cells was significantly higher than that of wild-type control (+/+) cells. $n = 3$ experiments performed in triplicate. **G:** Endothelial cell survival, as assayed by trypan blue exclusion, was significantly higher for $\alpha 3$ -null (-/-) endothelial cells than for wild-type control (+/+) cells at 96 hours in culture. $n = 6$ samples per treatment/genotype performed in duplicate. * $P < 0.05$; ** $P < 0.001$.

5A, left panel) suggesting that $\alpha 3\beta 1$ positively regulates endothelial-VEGF expression. Indeed, endothelial VEGF-A levels also tended to be reduced in endomucin-positive tumor blood vessels of ec- $\alpha 3$ -/- mice *in vivo* (Figure 5A, right panel). To test whether the reduced expression of endothelial-VEGF was a result of the enhanced expression of Flk-1 in $\alpha 3$ -null endothelial cells, we next depleted Flk-1 in these cells and examined whether this affected endothelial-VEGF levels. Although siRNA-depletion of Flk-1 in $\alpha 3$ -null endothelial cells inhibited significantly Flk-1 expression,

both at the mRNA and protein levels (Figure 5B, left panel), it had no effect on endothelial-VEGF expression at either level (Figure 5B, right panel) suggesting that Flk-1 was not capable of affecting endothelial-VEGF expression. In contrast, electroporation of $\alpha 3$ -null endothelial cells with plasmids to overexpress VEGF_{A164} led to a significant up-regulation of endothelial-VEGF_{A164} both at the mRNA and protein levels (Figure 5C, left panel) and, importantly, reduced significantly Flk-1 mRNA and protein levels (Figure 5C, right panel). These effects on Flk-1 levels were likely

Figure 5. $\alpha 3\beta 1$ -integrin regulates endothelial-VEGF, which in turn controls Flk-1 expression. **A:** Real-time qPCR of endothelial-VEGF mRNA, cellular and secreted levels from wild-type (+/+) and $\alpha 3$ -null (-/-) endothelial cells *in vitro*. Results show a significant decrease in endothelial-VEGF mRNA secreted and cellular protein levels in $\alpha 3$ -null endothelial cells when compared with wild-type controls. $n = 3$ experiments per genotype per test. Double-immunofluorescence staining of blood vessels for VEGFA (red) and endomucin (green) in tumor sections from $\alpha 3^{+/+}$ and $\alpha 3^{-/-}$ mice. Bar chart represents mean endothelial VEGFA intensity per μm^2 of endomucin-positive blood vessel *in vivo*. Endothelial VEGFA expression is also enhanced in tumor blood vessels of $\alpha 3^{-/-}$ mice. Scale bar = 50 μm . **B:** Flk-1-depletion does not affect endothelial-VEGF levels. Transfection of $\alpha 3$ -null endothelial cells with Flk-1-specific siRNA (Flk-1) inhibited significantly Flk-1 mRNA levels as detected by real-time qPCR, and Flk-1-protein levels, as detected by Western blot analysis (left panel). Flk-1 depletion had no effect on endothelial-VEGF mRNA or secreted VEGF protein levels as detected by real-time qPCR and ELISA respectively (right panel). Transfection with control pool (CP) acted as a negative control. $n = 3$ experiments per genotype per test. **C:** Overexpression of VEGF₁₆₄ in $\alpha 3$ -null endothelial cells induced a significant increase in both VEGF mRNA and secreted protein levels as detected by real-time qPCR and ELISA respectively (left panel). In contrast, the overexpression of endothelial-VEGF₁₆₄ decreased significantly Flk-1 mRNA and protein levels, as detected by real-time qPCR and Western blot analysis respectively (right panel). $n = 3$ experiments per genotype per test. **D:** The treatment of $\alpha 3$ -null (-/-) endothelial cells with either 500 pg/ml or 30 ng/ml exogenous VEGF for 48 hours had no effect on Flk-1 at either the mRNA or protein levels as detected by real-time qPCR and Western blot analysis respectively. Densitometric readings in **B–D** are from three independent experiments relative to CP, mock electroporation, or untreated null endothelial cells. HSC-70 provides the loading control. **E:** Microvessel outgrowth of VEGF^{ECWT}, VEGF^{ECHEt}, and VEGF^{ECOK} aortic rings stimulated with 30 ng/ml VEGF show that although there was no significant difference between VEGF^{ECOK} and VEGF^{ECWT} outgrowth. VEGF^{ECHEt} aortic rings displayed significantly enhanced VEGF-mediated microvessel sprouting. Micrographs of representative aortic rings are provided. Graph represents mean numbers of microvessel sprouts $\pm$ SEM. $n = 10$ –23 aortic rings per test. These results suggest that endothelial-VEGF levels have a biphasic effect on microvessel outgrowth. n.s., not significant. * $P < 0.03$; ** $P < 0.006$; *** $P < 0.08$. Bar = 1 mm.

due to the autocrine effect of endothelial-VEGF because exogenous application of VEGF_{A164} for 48 hours, the same treatment time used for the electroporated VEGF_{A164} $\alpha 3$ -null endothelial cells, had no effect on Flk-1 levels (Figure 5D).

Because deletion of endothelial-VEGF has been reported to compromise blood vessel homeostasis without any apparent defect in Matrigel plug angiogenesis assays or Flk-1 levels,²² we hypothesized that low levels of endothelial-VEGF may have different biological consequences to the total deletion, or normal levels, of endothelial-VEGF. Indeed we showed that VEGF-mediated aortic ring sprouting from mice heterozygous for endothelial-VEGF (VEGF^{ECHEt}) showed significantly en-

hanced microvessel sprouting when compared with wild-type (VEGF^{ECWT}) control or endothelial-VEGF-deleted (VEGF^{ECOK}) rings (Figure 5E) suggesting that low levels of endothelial VEGF elevate Flk-1 expression and angiogenic responses.

Discussion

Our work is the first to test the effect of the genetic deletion, specifically, of endothelial $\alpha 3\beta 1$ -integrin on angiogenesis *in vivo* and highlights a critical role for this integrin in neovascularization. Our study shows that $\alpha 3\beta 1$ is not required for tumor or VEGF-induced angiogenesis

and that deletion of this integrin in endothelial cells can enhance these responses. In contrast to previous studies on the effect of the global genetic ablation of $\alpha v\beta 3$ or $\alpha 2\beta 1$ on angiogenesis,^{29,30,45–47} which includes the effects of the deletion of these integrins in many cell types on neovascularization, our study is the first to dissect the precise role of $\alpha 3\beta 1$ integrin in endothelial cells *in vivo*. It is noteworthy that although $\alpha v\beta 3$ -integrin deficiency also enhances angiogenic responses by elevating Flk-1 levels,^{29,30,45} and that endothelial $\alpha 6$ integrin deficiency is associated with increased Flk-1 levels,⁴⁸ the regulation of Flk-1 is at the transcriptional level in the $\alpha 3$ -null endothelial cells but at the protein level in $\beta 3$ -null and $\alpha 6$ -null endothelial cells. This difference suggests that, although the regulation of angiogenesis by these integrins converge on Flk-1, the mechanisms by which they do so are different. Previous work has suggested that although $\alpha 3\beta 1$ can act as a transdominant regulator of $\alpha v\beta 3$ in tumor cells it does not in human umbilical vein endothelial cells.¹³ Our data on endothelial cells corroborate such findings and show that loss of endothelial $\alpha 3\beta 1$ does not affect $\alpha v\beta 3$ function. Taken together, these studies demonstrate that the loss of either $\alpha 3\beta 1$ or $\alpha v\beta 3$ is sufficient to enhance pathological angiogenesis and suggest that because the loss of $\alpha 3\beta 1$ does not affect $\alpha v\beta 3$ or *vice versa* that these two mechanisms of angiogenic regulation are not wholly interdependent.

Because $\alpha 3\beta 1$ is not detectable in embryonic endothelial cells^{21,49–51} we focused on defining the requirement of $\alpha 3$ in adult pathological angiogenesis. Conflicting data have suggested both pro- and antiangiogenic properties for the binding partners of $\alpha 3\beta 1$ in cultured cells with no direct evidence for the role of this integrin during neovascularization *in vivo*.^{10–18,52} For example, interactions of $\alpha 3\beta 1$ with thrombospondin-1 have been shown to reverse the antiangiogenic functions of thrombospondin-1 during neovascularization, thus promoting angiogenesis,^{15,16} and suggesting a proangiogenic role for this integrin. In contrast, Boosani et al¹⁴ have demonstrated that the noncollagenous domain of type IV collagen ($\alpha 3(\text{IV})\text{NC1}$) can inhibit angiogenesis via several mechanisms including binding to $\alpha v\beta 3$ and $\alpha 3\beta 1$. In addition, the interaction of TIMP2 with $\alpha 3\beta 1$ -integrin has been reported to inhibit FGF-2 signaling and VEGFR-2 activity without an effect on VEGFR-2 levels.^{18,52} Finally, the deletion of the laminin $\alpha 4$ subunit appears to inhibit angiogenic responses in the developing embryo but increases tumor angiogenesis in the adult.^{11,12} Thus, together these reports suggest different negative roles for the binding partners of $\alpha 3\beta 1$ in angiogenesis, but they do not address a direct requirement for $\alpha 3\beta 1$ in pathological angiogenesis. Although, we cannot rule out the potential that the loss of endothelial- $\alpha 3\beta 1$ in mice may repress the effect of endogenous angiogenesis inhibitors *in vivo*, this would not be enough to explain the enhanced angiogenic responses and elevated Flk-1 levels that we observe in $\alpha 3$ -null aortic rings and endothelial cells *ex vivo* and *in vitro*.

$\alpha 6$ -integrin subunits can heterodimerize with either $\beta 1$ - or $\beta 4$ -integrin subunits, both of which have been implicated in angiogenesis,^{53–55} suggesting that the up-regulation of $\alpha 6$ -integrins could account for the enhanced

Figure 6. Schematic diagram summarizing the effects of $\alpha 3\beta 1$ -integrin deficiency on pathological angiogenesis. The expression of $\alpha 3\beta 1$ in wild-type endothelial cells positively regulates endothelial-VEGF production, which in turn restricts Flk-1 expression and therefore controls exogenous VEGF-induced angiogenesis. In contrast, the absence of $\alpha 3\beta 1$ in $\alpha 3$ -null endothelial cells limits endothelial VEGF production, which promotes Flk-1 expression and therefore enhances exogenous VEGF-stimulated angiogenesis. Thus $\alpha 3\beta 1$ can act as a negative regulator of pathological angiogenesis.

angiogenic responses found in $ec-\alpha 3^{-/-}$ mice. In contrast we have shown recently that $\alpha 6$ integrin deletion can enhance angiogenesis.⁴⁸ We found no evidence the elevated $\alpha 6$ integrin levels in the $\alpha 3$ -null endothelial cells were able to regulate Flk-1 expression or that inhibition of this $\alpha 6$ integrin with GoH3 in $\alpha 3$ -null endothelial cells affected VEGF-mediated angiogenesis, either *ex vivo* or *in vivo*. Thus, our findings rule out the likelihood of compensation by $\alpha 6$ -integrin in the angiogenic responses found in $ec-\alpha 3^{-/-}$ mice *in vivo*.

Our data indicate a novel mechanism by which $\alpha 3\beta 1$ coordinates angiogenesis that involves the regulation of endothelial-VEGFA production and the ultimate control of Flk-1 expression (Figure 6). Previous work has shown that the deletion of endothelial-VEGF, driven by the VE-cadherin promoter, leads to poor blood vessel homeostasis, decreased endothelial cell survival and early lethality without any apparent defect on *in vivo* angiogenesis or Flk-1 levels.²² The lack of a similar phenotype in the $ec-\alpha 3^{-/-}$ mice led us to a hypothesis that low levels of endothelial-VEGF expression may have different effects than its total deletion. Indeed, the importance of ligand dosage in angiogenesis is not unprecedented. For example, a biphasic role for TGF $\beta 1$ has been previously reported: low doses of TGF $\beta 1$ enhance angiogenic processes, while high doses inhibit them by stimulating

different downstream signaling cascades.⁵⁶ We found that endothelial-VEGF levels were reduced in $\alpha 3$ -null endothelial cells and do indeed have a biphasic effect on angiogenesis: low levels of endothelial VEGF elevate Flk-1 expression, whereas deletion does not. In fact, the notion that different levels of endothelial-VEGF can differentially regulate Flk-1 function is also supported in a recent article where incorporation of EGFR loaded microvesicles into cultured human umbilical vein endothelial cells is associated with an overexpression of VEGF (ie, above normal levels of endothelial VEGF) with a corresponding increase in Flk-1-phosphorylation.⁵⁷ We also went on to demonstrate that the low levels of endothelial-VEGF found in $\alpha 3$ -null endothelial cells are able to enhance Flk-1 expression and VEGF-mediated responses. Although beyond the scope of this study, it would be of great interest to understand the mechanism by which endothelial-VEGF regulates Flk-1 expression. Given the recent data suggesting that inhibition of VEGF can ultimately enhance tumor progression,^{7,8} it would be of value to test the effect of low doses of VEGF-inhibitors such as Bevacizumab on human endothelial cells. We speculate that such low doses might reduce but not eliminate endothelial-VEGF to levels that would enhance endothelial-VEGFR2 expression and function thereby counteracting the effects of VEGF-inhibition and enhancing tumor angiogenesis.

Previous work has shown that tumor cell production of VEGF is approximately 1000–10,000 times higher than that of endothelial cells.⁵⁸ Thus, tumor cell VEGF is the major source of VEGF and any changes in endothelial-VEGF levels are unlikely to have a significant additive effect to overall VEGF levels. What is more likely is that a decrease in endothelial-VEGF levels are responsible for the up-regulation of Flk-1 levels, thus sensitizing the cells to VEGF stimulation and enhancing angiogenic responses.

Recent work has indicated that there is an antiangiogenic form of VEGFA, VEGFA164b.⁵⁹ Thus it is tempting to suggest that underexpression of such an inhibitor of angiogenesis might be responsible for the enhanced angiogenic responses. However because this form of VEGF has not been detected in mouse tissue, this is rather an unlikely mechanism by which $\alpha 3$ may regulate angiogenesis in mice.

Given the emerging interest in $\alpha 3\beta 1$ -directed inhibitors designed to either block tumor cell growth, with short chain Fv antibodies,²⁰ or angiogenesis, with drugs developed to suppress the interaction of thrombospondin-1 with $\alpha 3\beta 1$,¹⁹ our findings provide a timely insight into the *in vivo* angiogenic activities of $\alpha 3\beta 1$ -integrin. Although the efficacy of such inhibitor approaches is yet to be established, our data suggest that caution should be exercised when attempting to inhibit $\alpha 3\beta 1$ -integrin function, because this may actually stimulate tumor angiogenesis.

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