TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Role of Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ in Wound and Tumor Angiogenesis in Mice

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Integrins are transmembrane receptors composed of one α subunit and one β subunit and are involved in cellular growth, differentiation, and apoptosis. The collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ have been shown to regulate wound and tumor vascularization by different mechanisms. In this study, we assessed wound and tumor vascularization in mice with genetic ablation of both integrin subunits $\alpha 1$ and $\alpha 2$, which resulted in loss of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$. Wound angiogenesis was investigated in excisional wounds that were inflicted on the back skin of control and mice lacking integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$. Mutant mice displayed reduced wound angiogenesis, which correlated with decreased macrophage numbers at 3 and 7 days after injury, and showed significantly attenuated vascularization of sponge implants. Angiogenesis induced by tumors arising from intradermal injection of B16 F1 melanoma cells was also reduced in comparison to controls 7 days after injection. This reduction in angiogenesis correlated with increased levels and activity of circulating matrix metalloproteinase 9 and elevated angiostatin levels in plasma of mutant mice, which reduced endothelial cell proliferation. *Ex vivo* mutant aortic ring explants developed significantly fewer and thinner aortic sprouts with fewer branch points than controls because of impaired endothelial cell proliferation. In conclusion, the loss of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ in mice results in reduced wound and tumor angiogenesis by cell-autonomous and extrinsic mechanisms. (*Am J Pathol* 2016, 186: 3011–3027; <http://dx.doi.org/10.1016/j.ajpath.2016.06.021>)

Integrins are heterodimeric transmembrane receptors consisting of one α subunit in noncovalent association with one β subunit that are linked to the actin cytoskeleton and mediate central functions, such as cell migration, growth, apoptosis, and differentiation.^{1–3} Eighteen α subunits can assort with eight β subunits to form 24 distinct integrin heterodimers.⁴ The $\beta 1$ family of integrins stands out by binding predominantly to extracellular matrix ligands and includes the integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$, and $\alpha 11\beta 1$ as the major receptors that mediate cell attachment to collagens.^{4–8} They recognize the triple helical GFOGER (O = hydroxyproline) sequence within collagens as well as variations of this sequence.^{3,7,9–14}

$\alpha 1\beta 1$ integrins are predominantly present in the mesenchyme and are most abundant in vascular and visceral smooth muscle.¹⁵ They bind with highest affinity to

collagen type IV, but also to collagen type I, IX, XIII, and XVI, laminin-111, matrilin-1, galectins, and other ligands.^{16–24} $\alpha 1\beta 1$ integrin-mediated contact with collagen regulates the cell cycle via activating the mitogen-activated protein—extracellular signal—regulated kinase 1/2 pathway through the adaptor proteins Fyn and Shc.^{25,26} Consequently, $\alpha 1\beta 1$ -null fibroblasts showed reduced proliferation in response to adhesion to collagen.^{25,27} Mice lacking $\alpha 1\beta 1$ have been characterized in detail, which are viable and fertile with normal smooth muscle and liver function, normal rates of wound healing, displaying no overt

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immunodeficiency in the laboratory environment.²⁷ Detailed immunohistochemical analysis of murine and human tissues demonstrated presence of $\alpha 1\beta 1$ on microvascular endothelia and that its expression is induced by vascular endothelial growth factor (VEGF).^{28–30} Characterization of $\alpha 1$ -null mice therefore focused on investigating angiogenesis, using different models. Loss of $\alpha 1\beta 1$ –extracellular signal–regulated kinase 1/2 signaling in $\alpha 1$ -null mice resulted in elevated production and activity of matrix metalloproteinase-9 (MMP-9), leading to increased generation of angiostatin from circulating plasminogen, a potent inhibitor of endothelial cell proliferation.^{31,32} Because of elevated plasma MMP-9 and angiostatin levels, tumors in $\alpha 1$ -null mice were less well vascularized and smaller than wild-type (WT) tumors. These effects were reversed by oral treatment with the MMP inhibitor doxycycline, leading to the reduction of angiostatin levels.^{32–34} However, vasculogenesis and angiogenesis in $\alpha 1$ -null mice in the steady state appeared normal.¹⁵

By contrast, the integrin $\alpha 2\beta 1$ is primarily expressed by epithelial and endothelial cells, platelets/megakaryocytes, and fibroblasts.³⁵ It is also expressed on T cells, depending on their differentiation and state of activation.^{36–39} $\alpha 2\beta 1$ integrin preferentially binds to fibrillar collagens (type I to III, V, and XI) with highest affinity to collagen type I.^{5,40} Other collagens recognized by $\alpha 2\beta 1$ integrin include the network-forming basement membrane collagen IV,^{41,42} the beaded-filament forming collagen VI, and the transmembrane collagen XXIII.⁴³ Adhesion of epidermal keratinocytes to collagens was fully abrogated on ablation of $\alpha 2\beta 1$, and its abundant presence on epidermal keratinocytes suggested an important adhesive function, especially during wound reepithelialization.⁴⁰ The finding that skin wounds healed normally in the absence of $\alpha 2\beta 1$ then revealed alternative mechanisms.⁴⁴ The $\alpha 2\beta 1$ integrin contributes to the regulation of cell migration, proliferation, and survival. The cytoplasmic domain of the $\alpha 2$ subunit, but not that of $\alpha 1$, mediates activation of the p38 mitogen-activated protein kinase pathway and induces a migratory phenotype.^{45,46} It also mediates activation of the cyclin E/cdk2 complex, which allows insulin-dependent entry into S-phase. $\alpha 2$ -Null mice are viable and fertile and do not display an overt phenotype under homeostatic conditions,^{47,48} but reveal significant alterations under stress conditions.^{48–54}

In vivo, expression of $\alpha 2\beta 1$ integrin is up-regulated during VEGF-induced angiogenesis, and $\alpha 2\beta 1$ integrin expression has been observed on the sprouting tips of neonatal blood vessels.^{29,55} Wound healing studies on $\alpha 2$ -null mice have demonstrated increased neoangiogenesis in the wound microenvironment, providing *in vivo* evidence of an antiangiogenic function for $\alpha 2\beta 1$.^{50,54} By contrast, *in vitro* experiments using neutralizing antibodies against $\alpha 2\beta 1$ integrin reduced endothelial cell proliferation on collagen substrates as well as tube and lumen formation by human umbilical vein endothelial cells (HUVECs) in a three-dimensional collagen matrix, all pointing to a

proangiogenic function for $\alpha 2\beta 1$.^{56–58} Genetic ablation of $\alpha 2\beta 1$ integrin led to increased tumor angiogenesis in a growth factor–dependent manner via modulation of VEGF receptor-2 signaling^{59,60} by a mechanism that has been delineated in detail. The $\alpha 2\beta 1$ was shown to be a specific receptor for endorepellin, and $\alpha 2\beta 1$ –endorepellin binding activates the Src homology-2 protein phosphatase-1 that in turn dephosphorylates and inactivates VEGF receptor-2, thereby mediating the angiostatic effects of $\alpha 2\beta 1$.^{59,61}

The data obtained from mice with targeted inactivation of $\alpha 1\beta 1$ and $\alpha 2\beta 1$ support that these two integrins exert opposing roles in the regulation of angiogenesis. In agreement with $\alpha 1\beta 1$ playing a proangiogenic role, mechanistically by inhibiting MMP-9, tumor-bearing $\alpha 1$ -null mice showed decreased tumor angiogenesis.^{31,32} By contrast, $\alpha 2\beta 1$ exerts antiangiogenic activities, mechanistically by inhibition of Src homology-2 protein phosphatase-1, and $\alpha 2$ -null mice consequently showed increased wound and tumor angiogenesis.^{50,54,59–61} These studies emphasize that the interplay between $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins has significant consequences in neoangiogenesis. Thus, during vascular remodeling, up-regulation of endothelial $\alpha 1\beta 1$ and $\alpha 2\beta 1$ occurs, and a balanced activity between these two integrins regulates MMP release and ultimately vessel number.¹⁵

Previously, the interaction of both integrins has been discussed, and experiments were designed to reveal compensatory mechanisms operating between them by applying neutralizing antibodies to the respective $\alpha 1$ and $\alpha 2$ subunits. These demonstrated a reduction of angiogenesis induced by s.c. implanted gels containing VEGF or by tumor xenografts.^{29,30} Reduced angiogenesis was also the result of several studies using neutralizing antibodies directed to integrin $\alpha 2\beta 1$,^{56–58} which were clearly in contrast to the studies based on genetic ablation of $\alpha 2\beta 1$ integrin showing increased wound and tumor angiogenesis.^{50,54,60} Because acute loss of function, as induced by the application of neutralizing antibodies, can elicit different effects than the germline deletion of the corresponding gene, the antibody approach needs to be carefully interpreted and may reflect that some neutralizing antibodies bivalently cross-link integrins and thereby simultaneously reduce cell adhesion and act as agonists for integrin signaling.^{8,62,63}

In this study, we therefore investigated vascularization by angiogenesis in the context of wound repair and tumor development in mice deficient for both integrin receptors, $\alpha 1\beta 1$ and $\alpha 2\beta 1$, by a genetic approach ablating the $\alpha 1$ and $\alpha 2$ integrin subunits.

Materials and Methods

Generation of Mice Deficient in Integrin Subunits $\alpha 1$ and $\alpha 2$

To generate a mouse line double deficient for the integrin subunits $\alpha 1$ and $\alpha 2$, a strategy was developed aimed to

target the corresponding genes in two separated targeting experiments. The cloning of the appropriate targeting vectors, subsequent electroporation of ES cells, and all following steps to generate a double-deficient mouse line were performed by a contracted transgenic service (Artemis Pharmaceuticals, Cologne, Germany). As a result, the *Itga1* gene (National Center for Biotechnology Information gene ID 109700) was modified in C57BL/6 ES cells by replacing exons 3 to 6 with an F3—puromycin resistance—F3 cassette, similar to the strategy used to generate $\alpha 1$ -null mice.²⁷ The removal of four exons from the *Itga1* allele generated a frameshift from exon 2 to exon 7, thereby ensuring a null allele. ES cell clones with confirmed homologous integration of the targeting construct into the *Itga1* locus were then targeted to inactivate the *Itga2* gene (National Center for Biotechnology Information gene ID 16398). In the corresponding targeting construct, exon 3 was flanked by loxP sequences and an FRT—neomycin resistance—FRT cassette inserted 5' of the exon sequence. Electroporation of this targeting vector resulted in homologously recombined ES cell clones, which, after subsequent Flp-mediated removal of the selection marker, produced a conditional *Itga2* allele. The resulting ES cell clones harbored a constitutively inactivated *Itga1* allele and a conditional *Itga2* allele. To ensure that both gene loci were targeted on the same chromosome, a PhiC31-mediated approach was applied.⁶⁴ attP and attB sequences were inserted in the corresponding targeting constructs upstream of the F3-flanked puromycin resistance cassette in *Itga1* and downstream of exon 3 in *Itga2*. PhiC31-mediated recombination between these sites in double-positive ES cell clones identified clones in which the entire region (approximately 150 kb) was removed. Using these clones, C57BL/6 mice were generated by blastocyst injections, and subsequent breeding of germline transmitting chimeras produced heterozygous mice harboring a constitutively inactivated *Itga1* gene and a conditional *Itga2* allele. A constitutively inactivated *Itga2* gene was produced by breeding these mice with K14-Cre female mice in which Cre recombinase is active in zygotes and induces deletion at an early preimplantation stage (deleter Cre⁶⁵). Removal of exon 3 from *Itga2* resulted in loss of function by generating a frameshift in downstream exons. The strategy is illustrated in Figure 1A. Mice with a constitutive ablation of both integrin subunits are termed double knockout (dKO). Genotype analysis by PCR using DNA from tail tip biopsy specimens was performed according to standard procedures with the following primers: ID 1767_9 (5'-GTTGCTTGACGTTGATATGG-3') and ID 1767_10 (5'-CTTGCTTCCTGTCATCATAGC-3') produce an amplicon of 345 bp, indicating the unmodified WT locus. ID 1766_5 (5'-CTTAGGGGTGCTTAGTGTGG-3') and 1766_6 (5'-CAAGTGACACTGGATACAATGC-3') produce an amplicon of 283 bp, indicating the recombined *Itga1* allele. ID 2127_9 (5'-GTTGCTTGACGTTGATATGG-3') and 1683_3 (5'-CAATATCCAGGTACACATCAACAC-3') produce an amplicon of 328 bp,

indicating the recombined (following deleter Cre recombinase) *Itga2* allele.

Mice were generated on a pure C57BL/6 background and housed in a specific pathogen-free unit with ad libitum access to food and water. All animal procedures were performed in compliance with the German Law for Welfare of Laboratory Animals and were approved by the local veterinary authority (North Rhine-Westphalia State Office for Nature, Environment and Consumer Protection, LANUV NRW 84-02.04.2013.A030 and 84-02.04.2013.A069).

Wounding and Harvesting of Wound Samples

Full-thickness excisional wounds were inflicted on the shaved backs of 10-week-old female mice, as described.^{50,66,67} Briefly, mice were anesthetized by an i.p. injection of a solution containing ketamine (10 g/L) and xylazine (8 g/L) (10 μ L/g body weight) (Park Davis, Karlsruhe and Erlangen, Germany, respectively). Two full-thickness excisional wounds were generated next to the spine at the scapular region using standard biopsy punches (6 mm; Stiefel, Offenbach, Germany). Wounds were left uncovered and harvested 3 and 7 days after injury. For histological analysis, wounds were bisected in the cranio-caudal direction at the indicated time points. Tissue was either fixed for 2 hours in 4% paraformaldehyde [in phosphate-buffered saline (PBS)] or frozen unfixed in Tissue Tek OCT Compound (Sakura, Staufen, Germany). Histological analyses were performed on serial sections from the central part of the wounds.

Vascularization of Sponge Implants

The s.c. sponge model was applied to determine *in vivo* angiogenesis, as described.^{50,68} Two sterile polyvinyl acetate CF-50 round sponges (6 mm in diameter, soaked in sterile PBS for 24 hours before use; Medtronic Xomed, Jacksonville, FL) were implanted s.c. under the upper thoracic skin of female mice. The incision was then sutured. Implants were removed after 16 days and processed for cryopreservation and staining of vascular structures.

Induction of Melanoma and Harvesting of Tumor Samples

Tumors were generated by intradermal injection of 1×10^6 B16F1 cells in 100 μ L PBS into the flank of 9-week-old dKO and WT littermate mice, as described.⁶⁹ Tumor size was measured as a function of time using a precision caliper. Tumor volume was calculated and expressed as average $\pm$ SD. Tumor tissues and blood (described below) were harvested at day 4, 7, or 11, by bisecting the tumors in transverse direction, and tissue samples were processed either for paraffin embedding or frozen unfixed, as described above.

Figure 1 Generation and characterization of mice with constitutive ablation of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$. **A:** Targeting strategy. The $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins were inactivated by targeting the respective *Itga1* and *Itga2* genes located on chromosome 13 by homologous recombination, as described in [Materials and Methods](#). Exons 3 to 6 in *Itga1* were replaced by an F3—puromycin resistance—F3 cassette. In *Itga2*, exon 3 was flanked by loxP sequences containing an FRT—neomycin resistance—FRT cassette. Fip-mediated recombination removed both selection cassettes, and Cre-mediated recombination removed exon 3 in *Itga2*. Deletions in both loci generated a frameshift in downstream exons. **Black boxes** represent functional exons, and **gray boxes** indicate nonfunctional exons induced by recombination. The region is not drawn to scale. **B:** Semiquantitative RT-PCR showing the presence of *Itga1* and *Itga2* transcript levels in the indicated organs of wild-type (WT) but absence in double-knockout (dKO) mice. S26 was used as a control. **C:** Levels of integrin $\alpha 1$ subunit expressed at the surface of fibroblasts isolated from dKO and WT mice were assessed by flow cytometry using hamster anti-rat/mouse Ha31/8 antibody, followed by R-phycoerythrin—conjugated anti-hamster antibodies and expressed as percentage of live cells. Quantification confirmed virtual absence of the $\alpha 1$ subunit on dKO fibroblasts. **D:** Immunoblotting of dermal fibroblast membranes from WT and dKO mice showed presence of the integrin $\alpha 2$ subunit in WT mice but not in the dKO samples. Na/K ATPase was used to indicate comparable loading. $n = 4$ (C). * $P < 0.05$. FN, fibronectin; FSC, forward scatter.

Histology and Immunohistochemistry

Paraffin sections (6 μ m thick) were stained with hematoxylin and eosin, according to standard procedures for histological analyses. For immunohistochemical stainings, cryosections (6 μ m thick) were fixed in either 4% paraformaldehyde or acetone, blocked with 10% normal goat serum or 5% bovine serum albumin (in PBS), and incubated overnight at 4°C with primary antibodies diluted in 10% normal goat serum or 1% bovine serum albumin. Bound primary antibodies were detected by incubation with Alexa-conjugated secondary antibodies for 1 hour at room temperature (Life Technologies, Darmstadt, Germany). Nuclei were stained with DAPI

(1:1000, D9542; Sigma-Aldrich, Munich, Germany). Specimens were mounted in Immu-Mount (9990402; Thermo Scientific, Braunschweig, Germany). The following primary antibodies were used: CD31 (1:1000, clone MEC13.3, 557355; BD Biosciences, Heidelberg, Germany); desmin (1:100, clone D33, M0760; Dako, Hamburg, Germany); F4/80 (1:200, T-2028; Dianova GmbH, Hamburg, Germany); Ki-67 (1:500, ab15580; Abcam, Cambridge, UK).

Semiquantitative RT-PCR

Total RNA was isolated from lungs, intestine, kidney, spleen, skin, and heart using an RNeasy RNA extraction kit,

according to manufacturer's instructions (74104; Qiagen, Hilden, Germany). mRNA was reverse transcribed (K1612; Thermo Scientific) and amplified using a Biometra T 3000 thermocycler system. Itga1 forward primer (5'-TCACCAATCCGAAGGGAGGAT-3') and reverse primer (5'-GCCACCAGGACCTCTTCTGTG-3') amplified a 384-bp fragment. Itga2 forward primer (5'-GCTAGTTCAAGGTATTTTGA-3') and reverse primer (5'-AACATTTGATGCTTGCT-3') amplified a 261-bp fragment.

S26 mRNA was used as an internal control and was amplified by a forward primer (5'-AATGTGCAGCCCATTCGCTG-3') and reverse primer (5'-CTTCCGTCTTACAAAACGG-3'), resulting in a 324-bp fragment.

Aortic Ring Assay

Three-week-old mice were sacrificed by cervical dislocation. Blood was collected by heart puncture, coagulated, centrifuged ($3000 \times g$, 10 minutes), and the supernatant was heat inactivated for 45 minutes at 56°C and sterilized by filtration (0.2 μm). The descending thoracic aorta was explanted and transferred into ice-cold MCDB131 cell culture medium (P04-80057; PAN-Biotech, Aidenbach, Germany) supplemented with 2 mmol/L glutamine (M11-004; PAA Laboratories, Cölbe, Germany) and 10,000 U/mL penicillin, 10 $\mu\text{g}/\text{mL}$ streptomycin, 25 $\mu\text{g}/\text{mL}$ amphotericin B (A5955-100ML; Sigma-Aldrich), and kept cold. After careful removal of fibroadipose tissue under binoculars (Zeiss, Oberkochen, Germany) and several washing steps with cold medium, the aortas were cut into approximately 0.75-mm rings. Rat tail tendon collagen type I (3 mg/mL, A10483-01; Thermo Scientific) was neutralized with sodium hydroxide (A1551.1000; AppliChem, Darmstadt, Germany) on ice and diluted to 1.25 mg/mL collagen in $1 \times \text{PBS}$ and 20 mmol/L HEPES, pH 7.4 (H4034-500G; Sigma-Aldrich). Aortic rings were embedded individually in 25- μL droplets of this collagen solution in 48-well plates (677-180; Greiner Bio-One, Frickenhausen, Germany). After gelation, gels were overlaid with medium containing antibiotics, supplemented additionally with 2% heat-inactivated mouse serum, and incubated overnight at 37°C and 5% CO_2 in a humidified incubator. Angiogenic sprouting was then stimulated over 6 days, with medium exchange every other day, by 25 ng/mL murine VEGF-A, 30 ng/mL murine fibroblast growth factor-2 (450-32 and 450-33; PeproTech, Hamburg, Germany), and 2% heat-inactivated mouse serum.

Cell outgrowth from collagen-embedded aortic rings at 37°C , 5% CO_2 , and 100% humidity was monitored in an Incucyte ZOOM microscope (Essen Bioscience, Ann Arbor, MI) at $\times 10$ magnification over 6 days with one frame per hour. For each aortic ring, four nonoverlapping image collections were analyzed for cell confluence over time with the basic analyzer module (Graphical User Interface version 2015A Rev2; Essen Bioscience, Ann Arbor, MI).

To visualize angiogenic sprouts, embedded aortic rings were fixed with 2% paraformaldehyde in PBS for 30 minutes, blocked, and permeabilized overnight at 4°C with 2%

horse serum (12499C-500ML; Sigma-Aldrich), 0.1% bovine serum albumin (A1391.0500; AppliChem), 0.5% saponin (S-4521; Sigma-Aldrich) in PBS, and immunostained with CD31-specific antibody from rat (1:300, 550274; BD Pharmingen, Heidelberg, Germany), followed by rat-specific Alexa Fluor-488-labeled secondary antibody from goat (1:1000, A11006; Life Technologies/Thermo Scientific) overnight at 4°C , respectively. Angiogenic sprouts were imaged at $\times 4$ magnification with an Olympus IX-71 microscope and measured with Metamorph software version 7.6 (Molecular Devices, Sunnyvale, CA).

Proliferation of collagen I-embedded aortic rings was determined by 5-bromo-2'-deoxyuridine (BrdU; Calbiochem/Merck, Darmstadt, Germany) incorporation. Rings were allowed to form angiogenic sprouts for 6 days in complete medium and received a pulse of 10 mmol/L BrdU for 3 hours. Subsequently, samples were washed with PBS, fixed, and blocked as above. DNA was denatured with 2N HCl for 10 minutes at 20°C . Subsequently, the samples were neutralized and washed with 100 mmol/L sodium tetraborate, pH 7.4, and PBS, pH 7.4, and then blocked with 5% normal horse serum for 1 hour at 20°C . Incorporated BrdU was labeled overnight at 4°C with 1:200 diluted BrdU-specific rabbit antibody (250563; ABBOTEC, San Diego, CA), and overnight with an Alexa Fluor-568-labeled secondary antibody, as mentioned above. Endothelial cells were identified by CD31 staining. Images were acquired at $\times 20$ magnification.

Cell Culture

Primary Dermal Fibroblasts

Mouse dermal fibroblasts were isolated, as previously described, and used in experiments up to passage 6.^{50,67} Briefly, the entire trunk skin of postnatal day 3 C57BL/6N mice and dKO mice was removed and incubated overnight at 37°C with 0.05% trypsin (15090-046; Life Technologies)-EDTA (0.02%) in PBS. On manual detachment from the epidermis, the dermis was minced and cells were liberated by incubation with Dulbecco's modified essential medium (41965; Life Technologies) containing collagenase I (400 U/mL; LS004196; Worthington, Lakewood, NJ) for 1 hour at 37°C . Debris was removed by straining through 70- μm filters (352350; BD Falcon, Heidelberg, Germany), and the cells isolated from one mouse were plated on a 10-cm dish in Dulbecco's modified essential medium supplemented with 10% fetal calf serum (10270; Life Technologies), 2 mmol/L L-glutamine (K0283; Biochrom, Berlin, Germany), and 10,000 U/mL penicillin and 10,000 $\mu\text{g}/\text{mL}$ streptomycin (A2213; Biochrom).

HUVECs

HUVECs (5×10^3 , C2519A; AMP, Clonetics, Lonza, Cologne, Germany) were plated on uncoated 96-well plates in the presence of basal endothelial growth medium (CC-3156 EA, EBM-2 basal medium; Clonetics) containing 2% fetal calf serum and plasma from tumor-bearing mice (0.4

mg/mL final concentration of mouse plasma proteins). After 2 days in culture, cells were pulsed for an additional 48 hours with BrdU (10 μ mol/L), and then subjected to BrdU enzyme-linked immunosorbent assay (11647229001; Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions.

Flow Cytometry

For fluorescence-activated cell sorter (FACS) analyses, mouse dermal fibroblasts were cultured for 6 hours on rat tail collagen type I-coated dishes (30 μ g/mL, 354236; BD Biosciences, Heidelberg, Germany) and harvested by trypsin/EDTA. Cells were resuspended at 2×10^5 cells in 100 μ L FACS buffer (5% fetal calf serum/PBS) and incubated with unlabeled primary antibody against integrin α 1 (hamster anti-rat/mouse, Ha31/8, 1:10, 555001; BD Biosciences) or isotype control (anti-hamster IgG2, λ 1 isotype, Ha4/8, 1:10, 553962; BD Biosciences) for 30 minutes on ice. Cells were washed and resuspended in 100 μ L FACS buffer containing secondary antibody (R-phycoerythrin-conjugated goat anti-hamster IgG, 1:100, HA6004; Thermo Scientific) and incubated for 30 minutes. To discriminate live from dead cells, fibroblasts were resuspended in 100 μ L FACS buffer containing 7-aminoactinomycin D (1:100, 51-2359KC; BD Biosciences). Samples were analyzed using a FACS Calibur (BD Biosciences), and analysis of processed samples was performed using FlowJo software version 7.6.1 (Tree Star, Ashland, OR).

Immunoblotting

Mouse dermal fibroblasts were cultured on dishes coated (for 1 hour at 37°C) with monomeric or polymeric rat tail collagen type I (30 μ g/mL in 0.2 mol/L acetic acid or in PBS, respectively, 5061002; BD Biosciences) or fibronectin (10 μ g/mL in PBS, 11080938001; Roche). After 6 hours of culture, cells were washed, scraped into 1 mL of PBS, and centrifuged (1200 $\times$ g for 5 minutes). The cell pellet was resuspended in sucrose lysis buffer (1.59 mol/L sucrose, 48.25 mmol/L HEPES, 100 μ mol/L EDTA, 5 mmol/L EGTA) and disrupted by three cycles of freezing (liquid nitrogen) and thawing (37°C). Intact cells were removed by centrifugation (1000 $\times$ g, 4°C, 10 minutes), and the supernatants were centrifuged (16,000 $\times$ g, 4°C, 10 minutes) to sediment membranes. This pellet was resuspended in PBS containing protease inhibitors (1:20, P2714; Sigma-Aldrich) and subjected to immunoblotting, as described.⁷⁰ Briefly, protein concentration was determined using the BCA method (23227; Pierce Biotechnology, Rockford, IL). Total protein lysate (10 μ g) was resolved by SDS-PAGE using 10% polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (10600003; Amersham Protran, GE Health Care, Freiburg, Germany). Membranes were blocked in 2% bovine serum albumin, 5% nonfat milk in 10 mmol/L Tris-HCl/150 mmol/L NaCl/0.05% Tween, followed by incubation with primary antibodies directed against integrin α 2.^{40,50} Na/K ATPase (1:500, 185065; Abcam, Cambridge, UK) was used as loading control. After

incubation with horseradish peroxidase-conjugated secondary antibodies (1:10,000 anti-rat horseradish peroxidase, P0450, 1:1000 anti-rabbit, P0399; Dako), membranes were developed using an enhanced chemiluminescence detection system (SuperSignal West Pico, 34080, and Femto substrate 34095; Thermo Scientific).

Analysis of Plasma Proteins

Equal amounts of plasma proteins were analyzed by gelatin zymography, as previously described.⁷¹ Plasma from tumor-bearing mice was obtained by collection of blood using heparin microvettes (20.1282.100; Sarstedt, Nümbrecht, Germany), followed by centrifugation for 20 minutes at 1000 $\times$ g. Plasma was precleared with protein G sepharose (17-0618-01; Amersham, GE Health Care), and unbound proteins were collected after centrifugation (1000 $\times$ g for 10 minutes). Protein concentration was determined using the BCA assay, as described above. Total plasma proteins (20 μ g) were resolved by SDS-PAGE on 12% polyacrylamide gels under reducing conditions and blotted, as described above. After blockage of non-specific binding sites with 5% skimmed milk (w/v) in PBS containing 0.5% Tween (v/v), the blots were incubated with the primary antibody against angiostatin (ab2904, 1:500; Abcam). Angiostatin bands (42- and 38-kDa fragments) were quantified by densitometry.

Microscopy

Photomicrographs were acquired using a Leica DM4000B microscope (Leica Microsystems, Wetzlar, Germany) with Diskus software version 4.50.1638-#393 (Hilger, Königswinter, Germany). Immunofluorescence microscopy was conducted using an Eclipse 800E (Nikon, Düsseldorf, Germany) and a Keyence BZ 9000E microscope (Keyence, Neu Isenburg, Germany).

Morphometric Analyses

The macroscopic wound area was quantified by processing of images taken at various time points and was calculated as the percentage of the wound area immediately after surgery (day 0). The distance between the edges of the *panniculus carnosus* muscle was determined as a measure of wound contraction. For quantitative analysis of CD31 and desmin expression, the percentage of the area in sponge implants, granulation, or tumor tissue, which stained positive for CD31 or desmin, was calculated. F4/80 analyses in wounds were performed similar to the CD31 analyses. F4/80 abundance in tumor tissues is provided as number of F4/80-positive cells per visual field. All image analyses were performed with ImageJ software version 1.49 (NIH, Bethesda, MD; <http://rsb.info.nih.gov/ij>). Analysis of colocalization of Ki-67 and CD31 in wounds and tumors used an additional plugin (JACOP)⁷² (ImageJ software version 1.49; NIH, Bethesda, MD; http://imagejdocu.tudor.lu/doku.php?id=plugin:analysis:jacop_2.0:just_another_colocalization_plugin:start).

Statistical Analysis

Image analyses were conducted in a blinded manner (S.G., S.N., J.-N.S., and P.Z.). Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Statistical significance was determined by Mann-Whitney *U* test or unpaired *t*-test. Normal distribution was confirmed with the Kolmogorov-Smirnov test ($\alpha = 0.05$). Data are presented as means $\pm$ SD. Experiments were repeated at least three times with three or more mice per genotype.

Results

Generation of Mice with Combined Loss of Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$

The strategy to generate mice with simultaneous loss of both integrin receptors involved targeting the *Itga1* and *Itga2* loci on chromosome 13 (Figure 1A). Ablation of the α subunits encoded by these loci results in failure to assemble the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrin receptors. As previous work had shown that absence of either receptor affected the vasculature and could potentially negatively affect developmental angiogenesis,^{30,32,50,54} thereby leading to early lethality, a strategy was adopted that generates mice with a constitutive *Itga1* and a conditional *Itga2* gene. This strategy permits ablation of the $\alpha 2$ integrin subunit by Cre-mediated recombination *in vivo* at postnatal time points. The $\alpha 1$ integrin subunit was ablated using the strategy reported by Gardner et al²⁷ to generate $\alpha 1$ -null mice by replacing exons 3 to 6 and inducing a frame shift to downstream exons. The conditional ablation of the $\alpha 2$ integrin subunit was achieved by flanking exon 3 in the *Itga2* gene with loxP sequences. A PhiC31 integrase-mediated approach⁶⁴ was used to confirm that both genes were targeted on the same chromosome. However, crossing mice harboring these modified gene loci with a ubiquitously expressed Cre recombinase produced offspring with constitutive ablation of both $\alpha 1$ and $\alpha 2$ integrin subunits, which were fully viable and fertile without any obvious phenotypic alterations when compared to double-heterozygous or WT C57BL/6 mice. All further work was therefore performed using mice with constitutive inactivation of integrin $\alpha 1$ and $\alpha 2$ subunits, termed herein dKO. Because these were generated on a pure C57BL/6 background, they were compared to age- and sex-matched C57BL/6 WT mice.

Inspection of internal organs from dKO mice confirmed unaltered anatomy and histology (data not shown), and demonstrated complete absence of *Itga1* and *Itga2* transcripts (Figure 1B). Absence of the $\alpha 1$ integrin subunit was confirmed by flow cytometry of dermal fibroblasts cultured for 6 hours on monomeric collagen I substrates that induced $\alpha 1$ expression in WT controls but not in dKO fibroblasts (Figure 1C). Absence of the integrin $\alpha 2$ subunit was confirmed from plasma membrane lysates of dermal fibroblasts from controls and dKO mice. Fibroblasts were

cultured for 6 hours on either monomeric or polymeric collagen I substrates or on fibronectin, which is not a ligand for these two integrins. Immunoblotting confirmed the loss of $\alpha 2$ integrin subunit from dKO fibroblasts as compared to controls (Figure 1D).

Lack of Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ Impairs Angiogenic Sprouting from Mouse Aortic Explants

The opposing effects of integrin $\alpha 1$ and $\alpha 2$ in vascularization and divergent data obtained using antibodies directed against the $\alpha 1$ and $\alpha 2$ subunits^{56–58} prompted us to investigate the effect of genetic ablation of both collagen-binding integrin subunits in angiogenesis. To this end, we first analyzed blood vessel formation in an *ex vivo* aortic ring angiogenesis model.⁷³ Aortic explants from WT and dKO mice were embedded in collagen I gels, and angiogenesis was stimulated with VEGF-A, fibroblast growth factor-2, and serum. Significantly fewer endothelial cell sprouts emerged from dKO in comparison to WT explants (Figure 2A). Within 6 days of angiogenic sprouting, a significant reduction in sprout numbers was observed in the dKO explants compared to controls (Figure 2B). Markedly reduced sprouting from dKO aortic rings correlated with significantly fewer branching points in the emerging dKO sprouts (Figure 2C). Both these observations suggest a migratory impairment of tip cells, which is essential for angiogenic sprouting.⁷⁴ In addition, the overall length of angiogenic endothelial cell sprouts was significantly decreased in dKO explants (Figure 2D), and their diameters were strikingly smaller than in controls (Figure 2E). BrdU incorporation into nuclei of proliferating cells showed a significantly reduced proliferation of dKO endothelial stalk cells (Figure 2F) in addition to reduced migratory potential of dKO tip cells.

To quantitatively compare the migratory behavior of dKO endothelial tip cells with WT cells, the outgrowth of these cells was measured from collagen I–embedded aortic rings over a period of 6 days by phase-contrast time-lapse video microscopy. These results showed reduced outgrowth of cells, indicating that the migratory behavior was significantly impaired by the lack of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (Figure 2G).

Reduced Vascularization of Sponge Implants on Loss of Integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ in Mice

The *ex vivo* sprouting assays confirmed an angiogenic defect in the dKO aortas. To test whether similar defects would arise from ablation of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ *in vivo*, scaffolds of inert material were implanted s.c. under the thoracic skin of mice and left for 16 days to be vascularized by angiogenesis.^{50,68} Macroscopically, sponges from dKO mice appeared strikingly less well vascularized than those from control mice (Figure 3A). Microscopically, a significant decrease in vascularity was confirmed by morphometric quantification of expression of the endothelial cell marker

Figure 2 Impaired sprouting and endothelial cell proliferation in aortic ring explants of double-knockout (dKO) mice. **A:** Representative immunofluorescence micrographs showing that ablation of integrin subunits $\alpha 1$ and $\alpha 2$ impairs angiogenic sprouting from collagen I-embedded aortic rings. CD31-expressing endothelial cells are labeled in green. Morphometric image analysis of angiogenic sprouting indicated a significant reduction in sprout initiation (**B**), sprout branching (**C**), overall sprout length (**D**), and average sprout thickness (**E**) in dKO explants compared to wild type (WT). **F:** Proliferation of endothelial cells was quantified by incorporation of 5-bromo-2'-deoxyuridine (BrdU; red) into CD31-positive cells (green). **G:** Representative phase-contrast images of collagen I-embedded dKO aortic rings showing reduced outgrowth of cells, compared to WT. Cell-covered area is expressed as % confluency. Data of four image collections are provided from five WT and six dKO aortic rings. Data are expressed as medians and interquartile ranges (**B–F**) or means $\pm$ SD (**G**). $n = 5$ or 6 aortas per genotype (**A–F**); $n = 2$ aortas per genotype (**G**); $n = 3$ mice per genotype (**A–G**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: $500 \mu\text{m}$ (**A**); $100 \mu\text{m}$ (**F**).

CD31 and the type III intermediate filament protein desmin that marks the smooth muscle layer in blood vessels (Figure 3, B and C). These results confirmed our observations that combined loss of both collagen binding integrins causes reduction in angiogenesis also *in vivo*.

Reduced Wound Angiogenesis and Delayed Wound Closure in dKO Mice

To confirm reduced angiogenesis *in vivo*, we studied angiogenesis in two further well-established models, which are

formation of granulation tissue in healing skin wounds and tumor growth. Full-thickness wounds were inflicted on the shaved backs of female dKO and C57BL/6N mice. At day 3 and 7 after injury, the extent of reepithelialization, extent of wound contraction, and area of deposited granulation tissue were determined from hematoxylin and eosin-stained mid-wound sections. Macroscopic inspection of the scabs revealed delayed wound closure at 7 days after wounding in dKO mice compared to controls. The dKO wounds were still covered by scabs, whereas the control wounds had already lost them (Figure 4A). Quantification of granulation tissue

Figure 3 Decreased vascularization of implanted sponges in double-knockout (dKO) mice. **A:** Macroscopic aspect of sponges implanted for 16 days into controls and dKO mice. **B:** Sections from sponges were stained for CD31 and desmin. Nuclei were stained with DAPI and appear blue. **C:** Positively stained areas were quantified by image analysis from immunofluorescence images shown in **B**. Data are expressed as means $\pm$ SD (**C**). $n = 5$ sponges per genotype (**A–C**). * $P < 0.05$. Scale bar = 100 μ m (**B**). WT, wild type.

area in wound sections at day 7, expressed as percentage of initial wound area at day 0, confirmed that wounds in dKO mice were larger than those in controls (Figure 4B). Distance between epithelial tips, which serves as an indicator of wound epithelialization, and distance between the cut edges of the *panniculus carnosus* muscle, serving as an indicator of wound contraction, were also significantly larger in dKO wounds as compared to controls at 3 days after wounding

(Figure 4, C and E). Dermal repair, as reflected by the development of a cell-rich granulation tissue, showed a consistent trend toward larger wounds in dKO mice at 7 days after wounding, which, however, did not reach statistical significance (Figure 4D). Moreover, wound contraction did not differ at 7 days after wounding (Figure 4E). These results suggest that, although a delay in wound healing was observed macroscopically at 7 days after wounding in the dKO mice as compared to controls, histological analysis revealed only a transient impairment in wound reepithelialization and contraction at day 3 after wounding.

Wound angiogenesis was evaluated in midwound sections as in sponges by morphometric quantification of CD31 and desmin expression (Figure 4, F and G, and Supplemental Figure S1A). These analyses revealed that the vascular density marked by CD31- and desmin-positive area was decreased in the granulation tissue of the dKO mice as compared to controls both at 3 (Supplemental Figure S1A) and 7 days after wounding (Figure 4, F and G). Because wound vascularization was shown to be controlled by macrophage infiltration during the early (day 0 to day 4) inflammatory phase of wound repair,⁷⁵ we asked if reduced macrophage numbers in the dKO wounds might contribute to reduced wound angiogenesis. Immunostaining of the macrophages using F4/80 as marker revealed significantly reduced density of macrophages in the granulation tissue at both 3 and 7 days after wounding (Figure 4, F and G, and Supplemental Figure S1B), suggesting their involvement in formation of a vascularized granulation tissue.

Transient Reduction in Tumor Angiogenesis in dKO Mice

To address the role of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ in tumor angiogenesis, we grafted B16F1 melanoma cells intradermally into the flanks of WT and dKO mice and analyzed tumor growth as a function of time.⁶⁹ Macroscopic analyses of tumor volume through day 4, when tumors were first visible, until day 11, when tumors reached the ethically permissible maximal tumor volume and mice needed to be sacrificed, revealed no significant differences between WT controls and dKO mice (Figure 5A). No overt differences were detected between control and dKO tumors at days 4, 7, or 11 by histological analysis (Figure 5B).

To test whether loss of integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ might affect tumor angiogenesis, the density of vascular structures present in the peritumoral and intratumoral (tumor center) areas was analyzed by immunostaining for CD31 and desmin. Although no differences were detected at day 4 (Supplemental Figure S2A) or day 11 (Figure 6, A–C), there was a significant decrease in CD31- and desmin-positive vasculature in tumors at day 7 (Figure 6, A–C).

A similar decrease in tumor angiogenesis had been reported in the $\alpha 1\beta 1$ -deficient tumor-bearing mice and was attributed to altered levels of MMP-7 and MMP-9, leading to enhanced proteolytic release of angiostatin from

Figure 4 Delayed wound closure, reduced angiogenesis, and number of macrophages in double-knockout (dKO) mice at 7 days after wounding. **A:** Macroscopic appearance of wounds after injury at day 0 and day 7 after wounding. **B–E:** Using hematoxylin and eosin–stained sections from wounds of dKO and wild type (WT), the following parameters were determined. **B:** Wound area at day 7, expressed as % of initial area at day 0. **C:** Distance between the migrating epithelial tips at day 3 after wounding. **D:** Area of granulation tissue formed at day 7. **E:** Distance between the cut edges of the *panniculus carnosus* muscle (p.c.). **F:** Sections from day 7 wounds were stained for CD31, desmin, and F4/80. **G:** Positively stained areas were quantified by image analysis from immunofluorescence images shown in **F**. Nuclei were stained with DAPI and appear blue. Each data point represents one wound. Three to five sections per wound were analyzed. Data are expressed as means $\pm$ SD (**B–E** and **G**). ** $P < 0.01$, *** $P < 0.001$. Scale bars: 100 μ m (**F**, CD31 and desmin); 300 μ m (**F**, F4/80).

circulating plasminogen.^{31,32} Analysis of angiostatin in plasma from melanoma-bearing mice at day 7 after injection revealed that dKO plasma contained significantly (37%) higher levels of angiostatin fragments (containing Kringle 1-3 and 1-4 of plasminogen; approximately 38 and 42 kDa) compared to control plasma (Figure 6, D and E). In parallel, the plasma of tumor-bearing dKO mice showed increased

levels of MMP-9 as compared to WT mice (Figure 6D). MMP-2 levels were comparable in all analyzed samples.

Immune cells infiltrating the tumor microenvironment, in particular macrophages, are known to affect the tumor microenvironment and the angiogenesis/angiostasis balance by secretion of growth factors, chemokines, and cytokines.^{76,77} Thus, tumor-associated macrophages were shown

Figure 5 No difference in tumor volume in wild-type (WT) and double-knockout (dKO) mice. A total of 10^6 B16F1 melanoma cells were injected intradermally into the flanks of WT and dKO mice. **A:** Tumor volume was determined at the indicated days by caliper measurement. **B:** Shown are representative hematoxylin and eosin–stained sections from tumors of mice sacrificed at day 4, 7, or 11 after injection. Tumor outlines are indicated by dashed white lines. Histologically, no significant differences were observed in dKO tumors as compared to controls. The images displayed for day 11 have been assembled from overlapping single images by ImageJ software version 1.49 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). Each data point represents one tumor (one mouse). Data are expressed as means $\pm$ SD (**A**). Scale bar = 500 μ m (**B**).

to promote tumor growth, angiogenesis, metastasis, and immunosuppression.^{78–80} Therefore, we asked if macrophages are involved in the regulation of angiogenesis in melanoma formed in dKO mice and whether they correlate with the transiently reduced tumor angiogenesis at day 7. Numbers of F4/80-positive macrophages in the tumor center (intratumoral) and at the tumor edges (peritumoral) at day 4 (Supplemental Figure S2B) and day 7 (Supplemental Figure S3, A and B) revealed no significant differences in the dKO mice from controls. In summary, these data revealed that angiogenesis in these tumors is controlled independently of tumor-invading macrophages.

Suppression of Proliferation of HUVECs by dKO Plasma

Because angiostatin is an inhibitor of endothelial cell proliferation,³² we tested whether the increased amount of angiostatin in the plasma of tumor-bearing dKO mice would reduce endothelial cell proliferation *in vitro*. To this end, we cultured HUVECs with plasma from tumor-bearing control as well as dKO mice. Plasma from tumor-bearing dKO mice showed a tendency in the reduction of HUVEC proliferation as compared to plasma derived from controls (Figure 6F). This indicates that the increased amounts of angiostatin in plasma from tumor-bearing dKO mice inhibit endothelial cell proliferation and that reduced angiogenesis in dKO tumors results from impaired endothelial cell proliferation and also from elevated angiostatin.

Reduced Proliferation of Endothelial Cells in dKO Wounds and Tumors

Impaired sprout formation from explanted dKO aortas was attributed to a cell-autonomous reduction in endothelial cell

proliferation, and reduced proliferation of HUVECs was conferred by plasma from dKO mice. These findings prompted us to hypothesize that the impaired wound and tumor angiogenesis in the dKO mice could also result from reduced endothelial proliferation. Staining of sections from wounds and tumors of controls and dKO mice for the proliferation marker Ki-67 and its colocalization with CD31-positive endothelial structures revealed that the dKO mice not only showed reduced areas staining positive for CD31 and Ki-67 (Figure 7, A–D), but also a reduction in the CD31–Ki-67 colocalized signal (Figure 7, A–D) in both wounds (Figure 7, A and B) and tumors (Figure 7, C and D). These results confirm that the reduction in wound and tumor angiogenesis in the dKO mice is caused by impaired endothelial cell proliferation.

Discussion

The $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins are expressed on a variety of different cell types, including endothelial and epithelial cells, fibroblasts, platelets, and immunocompetent cells.^{15,25,27,35,36,41,48} They play an important role in the control of tissue homeostasis. Their role in angiogenesis has been addressed in mice with genetic ablation of each individual integrin. These studies showed that absence of $\alpha 1\beta 1$ resulted in reduced angiogenesis of tumors because of elevated circulating angiostatin levels and clearly established an angiogenic function for this receptor.^{31,32} By contrast, genetic inactivation of $\alpha 2\beta 1$ resulted in strongly enhanced angiogenesis in wounds and implanted sponges^{50,54} as well as in tumors because of failure of the angiostatic endorepellin signaling via $\alpha 2\beta 1$.^{59,61} These studies clearly established an anti-angiogenic function for

Figure 6 Transient decrease in tumor angiogenesis in double-knockout (dKO) mice at day 7. **A:** Tumor sections at day 7 and 11 after injection were stained for CD31 and desmin. Single images were assembled with BZ-II Analyzer software (Keyence, Neu Isenburg, Germany). Nuclei were stained by DAPI and appear blue. **B** and **C:** Positively stained areas were quantified from images shown in **A**. **D: Top panel:** Immunoblot displaying increased angiostatin levels (38- and 42-kDa fragments) in plasma (20 μ g plasma proteins/lane) obtained from tumor-bearing dKO and wild-type (WT) mice at day 7 after injection. **Bottom panel:** Gelatin zymography of plasma from tumor-bearing dKO and WT mice. Increased levels of matrix metalloproteinase-9 (MMP-9; 92 and 82 kDa) but not of pro-MMP-2 (72 kDa) were detected in dKO as compared to WT. **E:** Densitometric quantification revealed significantly higher angiostatin levels in dKO than in control plasma. **F:** Plasma from tumor-bearing dKO mice reduces human umbilical vein endothelial cell (HUVEC) proliferation. The y axis indicates absolute values of 5-bromo-2'-deoxyuridine (BrdU) incorporation normalized to untreated HUVECs. Each data point represents one tumor (one mouse). Data are expressed as means $\pm$ SD (**B**, **C**, **E**, and **F**). $n = 5$ mice per genotype (**E** and **F**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bar = 300 μ m (**A**). AU, arbitrary unit.

$\alpha 2\beta 1$. The opposing functions in angiogenesis mediated by integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ implicated that there was no functional compensation by one for the loss of the other. In the current study, we aimed to address the question of whether combined loss of both receptors would result in balanced angiogenesis or how the interplay of the two different signaling cascades induced by loss of these integrins would affect different functions involved in angiogenesis.

The double deficiency was not achievable by breeding the fertile $\alpha 1$ -null with $\alpha 2$ -null mice, because the *Itga1* and *Itga2* gene loci are separated by only 26 kb on chromosome 13. This proximity precludes chromosomal crossing over and genetic recombination, and generation of the dKO mice therefore required a complex strategy involving sequential targeting of both loci with two separate targeting vectors in mouse ES cells. Successful modification of the two adjacent gene loci by homologous integration of both targeting vectors on the same chromosome was achieved and confirmed by PhiC31-mediated deletion between the *attP* and *attB* sites inserted in the modified *Itga1* and *Itga2* loci,

respectively. Mice generated with this strategy and bred with a ubiquitously deleting Cre line produced normally appearing and fertile offspring that were full body knockout for both integrins.

These mice were investigated to address the question of balanced or altered angiogenesis using three well-characterized *in vivo* models.^{68,81–83} Our results clearly established that angiogenesis in wounds and sponge implants is reduced and that tumor angiogenesis is transiently impaired in dKO mice. Reduced angiogenesis in these models was in good agreement with results from *ex vivo* aortic sprouting assays. Explanted aortas from dKO mice exhibited significantly fewer primary sprouts that were thinner and shorter and showed significantly fewer branch points as compared to WT controls. These changes are because of reduced endothelial cell proliferation and migration, suggesting that $\alpha 1\beta 1$ and $\alpha 2\beta 1$ support proliferation and migration of endothelial cells during sprouting. Because the major endothelial fibronectin receptors, the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$,^{84,85} are still present on these cells, a basal level of migration of these dKO cells in the sprouting

Figure 7 Reduced proliferation of endothelial cells in wounds and tumors in double-knockout (dKO) mice at day 7. **A:** Sections from day 7 wounds were stained for CD31 and Ki-67. Nuclei were stained with DAPI and appear blue. **B: Top panel:** Areas positively stained for CD31 and desmin were quantified from immunofluorescence images shown in **A**. **Bottom panel:** Colocalized CD31 and Ki-67 signals were quantified and are displayed as Pearson's correlation. Three to five sections per wound were analyzed. **C:** Day 7 tumor sections were stained for CD31 and Ki-67. Nuclei were stained with DAPI and appear blue. **D: Top panel:** Areas positively stained for CD31 and desmin were quantified from immunofluorescence images shown in **C**. **Bottom panel:** Colocalized CD31 and Ki-67 signals were quantified and are displayed as Pearson's correlation. Each data point represents one tumor (one mouse). Data are expressed as means $\pm$ SD (**B** and **D**). * $P < 0.05$, ** $P < 0.01$. Scale bar = 300 μ m (**A** and **C**). WT, wild type.

experiments was retained. This result is consistent with a report stating an important role for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ in endothelial cell migration.⁸⁶

These *in vitro* results highlighted the contribution of both collagen-binding integrins to endothelial cell function, and prompted us to further address their role in angiogenesis *in vivo*, where systemic factors, including other cell types and complex communication of endothelial cells with the microenvironment, contribute to vascularization by angiogenesis.

In agreement with our observation of impaired sprouting from aortic explants, vascularization of sponges implanted

s.c. was strongly reduced in dKO mice, both at macroscopic and microscopic inspection. In addition, a significant reduction in wound contraction and wound closure was noted at day 3, and a tendency toward delayed closure persisted at day 7. Of note, reduced angiogenesis in the dKO mice correlated with reduced numbers of F4/80-positive macrophages at both day 3 and 7 after wounding. Macrophages have been described to play a critical role during all phases of wound healing and are crucial for the formation of a functional granulation tissue.^{75,82} Macrophage numbers were unaltered in the wounds of $\alpha 2$ -null mice, suggesting that $\alpha 2\beta 1$ integrin is not necessary for macrophage

migration into the wound area.⁵⁰ By contrast, lower macrophage numbers⁸⁷ and reduced monocyte activation⁸⁸ were reported in $\alpha 1$ -null mice, pointing to a role for $\alpha 1\beta 1$ integrin in monocyte activation and differentiation into tissue macrophages.^{37,89} Therefore, the overall reduced number of macrophages in the dKO wounds could be attributed to the lack of $\alpha 1\beta 1$. Mechanistically, reduced angiogenesis in dKO wounds could be associated with decreased levels of VEGF released by fewer macrophages.^{75,76} Our results therefore indicate that the overall reduction in wound angiogenesis in dKO mice may be contributed mostly by the absence of $\alpha 1\beta 1$ integrin and cannot be compensated.

Applying the B16F1 melanoma model⁶⁹ to dKO mice failed to induce significant differences in tumor volume as compared to controls at any time point during tumor development (at 4, 7, or 11 days after tumor cell injection). Because tumor volume was reduced in $\alpha 1$ -null^{31,32} but increased in $\alpha 2$ -null mice,⁵⁹ the unchanged tumor volume in the dKO mice could reflect a balanced outcome of the opposing roles of these two integrins.

In contrast to unaltered tumor volume, a clear although transient reduction in tumor angiogenesis was noted in dKO tumors. This coincided with elevated plasma levels and activity of MMP-9 and angiostatin in tumor-bearing dKO mice, reflecting the angiostatic phenotype detected in $\alpha 1$ -null mice.^{31,32} The angiostatic activity of angiostatin has been attributed to its ability to reduce endothelial cell proliferation.³² Accordingly, plasma from tumor-bearing dKO mice caused a reduction in HUVEC proliferation. More important, reduced proliferation in vascular structures was corroborated *in vivo* in sections from wound granulation tissue and melanoma tumors, clearly pointing out reduced endothelial cell proliferation as the mechanism underlying impaired vascularization of these tissues.

Why angiogenesis in tumors of dKO mice was reduced only transiently, accompanied by unaltered macrophage numbers, is not clear. Tumor cells are producers of soluble factors, such as VEGF and monocyte chemoattractant protein-1,^{90,91} and can stimulate matrix synthesis by cells of the peritumoral environment.⁹² These additional matrices may, in turn, activate endothelial cell receptors besides $\alpha 1\beta 1$ and $\alpha 2\beta 1$, such as $\alpha 5\beta 1$ and αv receptors, which may compensate for the lack of the collagen receptors. The function of $\alpha 5\beta 1$ and αv receptors is dispensable for early angiogenic processes but required at later stages, in particular during remodeling, as was shown by the combined ablation of $\alpha 5\beta 1$ and all αv integrins in endothelial cells.⁸⁵

In summary, the current study corroborates important and specific functions of the collagen-binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ in controlling angiogenesis through different mechanisms. Combined loss of both receptors leads to significantly attenuated angiogenesis, shown in an *ex vivo* and three different *in vivo* models, which all clearly displayed impaired endothelial cell proliferation as the underlying cause. This can be explained by endothelial

cell-autonomous defects attributable to lack of adhesive signaling, but also to aberrant responses of dKO endothelial cells to signals from inflammatory and tumor cells. Loss of direct contact of endothelial cells with collagen does not fully abrogate angiogenesis, as other mechanisms maintain basal angiogenesis, probably by engaging the activity of other receptors, including integrins.

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B.E., P.Z., and T.K. conceived and supervised this study; S.G. and J.-N.S. designed and performed most of the experiments and analyzed data; M.L. designed the targeting strategy; S.N., F.W., and P.Z. performed *ex vivo* explant experiments and analysis, flow cytometry, and plasma analysis, respectively; C.M. and J.A.E. provided critical discussion; S.G., T.K., and B.E. wrote the manuscript. All authors commented on and edited the manuscript.

Supplemental Data

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