Deletion of tetraspanin Cd151 results in decreased pathologic angiogenesis in vivo and in vitro

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Tetraspanin protein CD151 is abundant on endothelial cells. To determine whether CD151 affects angiogenesis, Cd151-null mice were prepared. Cd151-null mice showed no vascular defects during normal development or during neonatal oxygen-induced retinopathy. However, Cd151-null mice showed impaired pathologic angiogenesis in other in vivo assays (Matrigel plug, corneal micropocket, tumor implantation) and in the ex vivo aortic ring assay. Cd151-null mouse lung endothelial cells (MLECs) showed normal adhesion and proliferation, but marked alterations in vitro, in assays relevant to angiogenesis (migration, spreading, invasion, Matrigel contraction, tube and cable formation, spheroid sprouting). Consistent with these functional impairments, and with the close, preferential association of CD151 with laminin-binding integrins, Cd151-null MLECs also showed selective signaling defects, particularly on laminin substrate. Adhesion-dependent activation of PKB/c-Akt, e-NOS, Rac, and Cdc42 was diminished, but Raf, ERK, p38 MAP kinase, FAK, and Src were unaltered. In Cd151-null MLECs, connections were disrupted between laminin-binding integrins and at least 5 other proteins. In conclusion, CD151 modulates molecular organization of laminin-binding integrins, thereby supporting secondary (ie, after cell adhesion) functions of endothelial cells, which are needed for some types of pathologic angiogenesis in vivo. Selective effects of CD151 on pathologic angiogenesis make it a potentially useful target for anticancer therapy. (Blood. 2007;109:1524-1532)

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Introduction

Angiogenesis, the formation of new blood vessels from preexisting vasculature, is crucial for many physiologic and pathologic situations. It involves coordinated endothelial cell proliferation, migration, and tube formation, with integrin-type adhesion receptors playing major roles. Although vitronectin-, fibronectin-, and collagen-binding integrins have been most extensively studied, laminin-binding integrins (α3β1, α6β1, α6β4) are also involved. For example, during the later stages of angiogenesis, α3β1 and α6β1 integrins may retard morphogenic and proliferative events, while promoting basement membrane assembly, pericyte association, and tube stabilization. By contrast, α6β4 may regulate the onset of pathologic angiogenesis in mature blood vessels.

CD151, a tetraspanin protein family member, associates closely with laminin-binding integrins, thereby modulating α3β1-, α6β1-, and α6β4-dependent neurite outgrowth, cell migration, cell morphology, and adhesion strengthening. Mutation of Cd151 in humans yielded kidney and skin disorders, but no obvious impairments in blood vessel formation or function were noted.14 Mice in which Cd151 was deleted were surprisingly normal, while showing only ex vivo deficiencies in platelet aggregation, keratinocyte migration, and T-cell proliferation.15,16 Again, there were no obvious blood vessel deficiencies. CD151 is present on many cell types, including endothelial cells, where it may either promote or inhibit angiogenesis, and between CD151 and laminin-binding integrins.

We hypothesized that CD151 might possibly support or suppress pathologic angiogenesis, even though it was apparently not needed for normal developmental angiogenesis. To test this hypothesis, we first generated Cd151-null mice. As seen elsewhere15, our Cd151-null mice showed normal viability, fertility, laminin-binding integrin expression, tissue organization, and organ function, under normal physiologic conditions. Also, developmental angiogenesis appeared normal. With respect to in vivo angiogenesis assays, Cd151-null mice showed surprisingly selective defects. Whereas neovascularization was unaffected in an oxygen-induced retinopathy assay, it was markedly deficient in other in vivo assays (Matrigel plug, corneal micropocket, tumor implantation). Furthermore, Cd151-null endothelial cells showed marked in vitro alterations in cell spreading, motility, 3-dimensional morphology, and tissue remodeling assays, as well as selective signaling defects. Defects were particularly evident for cells plated on laminin substrate and were accompanied by changes in molecular organization of laminin-binding integrin protein complexes.
Cd151-coding exons and part of the fifth (of 6), and includes the first 168 amino acids (of 253). Cd151 deletion was verified by Southern analysis of embryonic stem (ES) cells and reverse transcription–polymerase chain reaction (RT-PCR) analysis of embryo fibroblasts, as will be described in detail elsewhere. Cd151-null mice were backcrossed more than 7 generations into the C57BL/6j mouse strain, and genotyping of breeding pairs was confirmed by PCR. In all studies, Cd151-null mice, 7 to 12 weeks old and pathogen-free, were compared with littersmates of the same age and sex. Animal studies were performed with approval from the relevant institutional committees.

Mouse lung endothelial cells (MLECs) were isolated as described. Briefly, cells were selected from collagenase-digested lung tissue (using anti–mouse ICAM-2) and enriched (using anti–mouse ICAM-2) to more than 90% purity (positively stained for von Willebrand factor).

Aortic ring assay
Thoracic aortas were isolated from wild-type (WT) and Cd151-null mice under a dissecting microscope, cut into 1-mm sections, and embedded in 24-well Matrigel-coated plates. Medium containing 20% fetal bovine serum, 10 U/mL heparin, 50 μg/mL endothelial cell mitogen, and 20 ng/mL bFGF was added to each well of gelled Matrigel. Microvessel-like properties of sprouting structures was confirmed by their incorporation of bFGF was added to each well of gelled Matrigel. Microvessel-like structures (positively stained for von Willebrand factor).

Matrigel plug assay
As described, 8-week-old mice were given subcutaneous injections at the abdominal midline with 0.4 mL Matrigel supplemented with 400 ng bFGF. After 7 days, mice were killed and vessels penetrating into the Matrigel were visualized using anti-CD34 antibody (Abcam, Cambridge, MA) and quantified using Scion Image software. To test hemoglobin content, Matrigel plugs were excised, weighed, homogenized in Drabkin reagent (Sigma, St Louis, MO), and centrifuged, and then supernatants were measured at OD 540 nm, according to the manufacturer’s instructions.

Tumor transplantation into mice
Mice were injected subcutaneously with Lewis lung carcinoma (LLC) cells (1 × 10⁶ cells/mouse). Tumors were measured every 5 days using Vernier calipers and volume calculated (length × width² × 0.52). After 15 days, tumors were collected and weighed. Tumor fragments were either frozen in OCT compound and then snap-frozen in liquid nitrogen or fixed in 10% formalin for histologic analysis.

Conical angiogenesis assay
Conical angiogenesis was assessed as described. Briefly, corneal microvessels (KMC8), CD81 (Eat1), and integrins α2 (HMα2), α4 (R1-2), α5 (5H10/27), α6 (GoH3), αV (H9.2B8), β1 (KMC-6), and β4 (346-11A), all from BD Biosciences. Prior to immunoprecipitation, MLECs were washed twice in PBS, serum-starved for 3 hours, labeled for 2 hours in medium containing 0.2 to 0.3 mCi/mL (7.4-11.1 MBq) [³H]–palmitic acid with 5% dialyzed FCS, then lysed in 1% Brij-96 for 1 hour at 4°C. Immunoprecipitation and [³H]-palmitate detection was as described, using anti-CD9 mAb KMC8, and mixed anti-β1 antibodies (HMB1, 9EG7).

Cell spreading and motility assays
To assess cell spreading, MLECs were suspended in DMEM containing 2% FCS and seeded onto plates coated with fibronectin (10 μg/mL), gelatin (0.1%), or Matrigel (1:30 dilution). We counted spread cells and nonspread cells in 3 high-power fields 30 minutes after plating (in duplicate wells for each group). Cells defined as spread showed a flattened morphology and were no longer phase-bright by light microscopy.

Chemotactic migration was performed using polycarbonate filter wells (Transwell, 8-μm pores; Costar, Corning, NY) coated with fibronectin, gelatin, or Matrigel (as described). MLECs were plated (7 × 10⁵ cells/mL, in 200 μL DMEM containing 5% FCS) in the upper chamber. The bottom chamber contained 5% FCS and 20 ng/mL bFGF as chemoattractants in 600 μL DMEM. After 7 hours at 37°C, filters were stained with Diff-Quick (Merz-Dade, Dudingen, Switzerland) and migrated cells, in duplicate wells, were counted each in 4 randomly chosen microscopic fields using a ×20 objective.

For Matrigel invasion assays, MLECs (1 × 10⁶) were resuspended in DMEM supplemented with 5% FCS, in the upper compartment of Matrigel invasion chambers (Becton Dickinson, Bedford, MA). The lower compartment contained DMEM supplemented with 5% FCS and bFGF (20 ng/mL) chemoattractants. After 16 hours at 37°C, filters were stained with Diff-Quick and invaded cells, in triplicate wells, were counted each in 4 randomly chosen microscopic fields using a ×20 objective.

Random migration was assessed using acid-washed glass coverslips spanning a 12-mm hole drilled in a 60-mm dish, which had been coated overnight with Matrigel and blocked with 5 mg/mL cell culture-grade bovine serum albumin (ICN Pharmaceuticals, Santa Ana, CA). For image acquisition, MLECs were detached and plated at 10⁵ cells/dish in medium containing 5% FCS. Images were acquired every minute for 30 minutes using a Zeiss Axiouvert 135 microscope (Zeiss, Thornwood, NY). Using Scion Image freeware tools, x and y centers were calculated, and the distance moved was determined.

Three-dimensional assays
To assess spheroid formation, endothelial cells (1 × 10⁶ in 200 μL DMEM plus 5% FCS) were placed on Matrigel, in 48-well plates (in duplicate for each group), then cables were quantified from high-power fields (×200) and at least 3 random fields were photographed. For Matrigel contraction assays, DMEM containing 1% agarose (250 μL) was added to 24 well-plates. After cooling, 50 μL Matrigel was added and gelled at 37°C for 30 minutes, then MLECs (2 × 10⁵/well in DMEM with 5% FCS) were added. After 18 hours, images were digitally recorded and Matrigel diameter was measured.

To assess tube formation in collagen, MLECs (1 × 10⁵ in 200 μL DMEM plus 5% FCS) were placed on Matrigel, in 48-well plates (in duplicate for each group), then cables were quantified from high-power fields (×200) and at least 3 random fields were photographed. For Matrigel contraction assays, DMEM containing 1% agarose (250 μL) was added to 24 well-plates. After cooling, 50 μL Matrigel was added and gelled at 37°C for 30 minutes, then MLECs (2 × 10⁵/well in DMEM with 5% FCS) were added. After 18 hours, images were digitally recorded and Matrigel diameter was measured.

To assess tube formation in collagen, MLECs (1 × 10⁵) were suspended in medium containing 0.25% (wt/vol) carboxymethylcellulose, seeded in nonadherent round-bottom 96-well plates, and cultured at 37°C for 24 hours. Spheroids were collected by centrifugation at 500g for 3 minutes. A collagen spheroid solution, prepared as above, was mixed 1:1 with DMEM containing 20% FCS and 0.5% carboxymethylcellulose. This spheroid-containing gel was transferred into 24-well plates and polymerized for
30 minutes. Then, 400 μL complete medium containing bFGF (20 ng/mL), PMA (10 nM), and VEGF (20 ng/mL) was overlaid on the collagen gel. After 24 and 48 hours, cumulative lengths of all capillary-like sprouts from individual spheroids were measured using Scion Image software. For each data point, at least 8 randomly selected spheroids were analyzed. All statistical analyses used 2-tailed Student t test. Results showing P values less than .05 were regarded as significant. For images acquired using an Axiovert 135 inverted microscope (Zeiss, Thornwood, NY), we used objective lenses of 5×/0.15 (Figures 1A and 5A-C) and 10×/0.25 (Figures 1B and 5D). Photos were acquired using Scion Image software and an RT Monochrome SPOT camera (Diagnostic Instruments, Sterling Heights, MI). Fluorescent images in Figures 2B and 3 were obtained using a Nikon Eclipse TE300 inverted microscope (Nikon, Melville, NY) equipped with an objective lens of 10×/0.3 and an RT SE SPOT camera (Diagnostic Instruments), and were acquired with Scion Image software. Images in Figures 2A and 5B were acquired using a digital camera. All photos were processed (cropped, labeled, organized) for publication using Canvas 9.0 (ACD Systems, Miami, FL).

**Signaling assays**

Serum-starved MLECs plated on diluted Matrigel were lysed in buffer containing 1% NP-40. Equal amounts of proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, then immunoblotted. Anti–phospho-Akt (Ser473), anti-Akt, anti–phospho-Raf (Ser259), anti–phospho-p38, anti–phospho-FAK (Tyr925), anti–phospho-eNOS (Ser1177), anti–phospho-Erk (Thr202/Tyr204), all were from Cell Signaling Technology (Beverly, MA), anti–c-Raf was from Santa Cruz Biotechnology (Santa Cruz, CA). To measure Rac1 activity (using Rac1 Activation Assay Kit, Upstate Biotechnology, Lake Placid, NY), cell supernatants were incubated with glutathione S-transferase (GST)–PBD agarose beads (1 hour, 4°C), washed, then bound material and total cell lysates were analyzed by Western blotting with anti-Rac antibody (Upstate Biotechnology) and anti-Cdc42 antibody (Santa Cruz Biotechnology).

**Results**

**CD151 deletion selectively impairs pathologic angiogenesis**

Mouse “aortic ring” explants embedded in Matrigel give rise to capillary-like structures. Aortic rings from Cd151-null mice showed impaired microvascular sprouting (representative photographs are shown in Figure 1A). Mean length of newly sprouted vessels was reduced by about 35% after 4 days in this ex vivo assay (Figure 1A). To assess angiogenic capacity in vivo, we used an assay in which implanted Matrigel plugs are invaded by host endothelial cells to form a capillary network. Matrigel plugs excised from Cd151-null mice, 7 days after implantation, showed diminished vascularization by 2 different staining methods (Figure 1B left panels), and vascular area and hemoglobin content were both decreased by about 60% (right panels).

Alterations in aortic ring outgrowth or Matrigel plug infiltration is typically accompanied by altered tumor angiogenesis. Hence, we implanted LLC cells into our mice. Representative photos (day 15) show smaller solid tumors in CD151-null mice compared to WT mice, with marked reduction in tumor volume and weight (Figure 2A). Furthermore, tumors from CD151-null mice showed significantly decreased microvessel density, as assessed by anti–CD31 staining (Figure 2B). Hence, CD151 may play a key role during tumor angiogenesis, in host mice, and possibly also in humans. In this regard, CD151 is present on both human tumor cells and tumor vasculature. CD151 showed especially high levels during tumor angiogenesis, in host mice, and possibly also in humans. In this regard, CD151 is present on both human tumor cells and tumor vasculature. CD151 showed especially high levels on colon cancer vessels and some increase also on tumor vessels in lung, breast, and prostate cancer compared to normal vessels in the same organs (Figure S1, available on the Blood website; see the Supplemental Figures link at the top of the online article).

To gain mechanistic insight into the role of CD151 during angiogenesis in vivo, we used a cornal micropocket assay. After 5 days of exposure to an implanted pellet containing a low dose of bFGF (20 ng), corneas of WT mice showed limbic vessel looping, dilation, and sprouting (Figure 3A). In sharp contrast, corneal vessels from Cd151-null mice showed minimal bFGF-stimulated sprouting, as evidenced by a greater than 95% reduction in clock-hour length (Figure 3A). At a higher bFGF dose (40 ng), neovascularization in CD151-deficient corneas was again markedly diminished (Figure 3B), resulting in reduced vascular area.

Cd151-null mice developed normally in different mouse genetic backgrounds (129Sv and C57BL/6) without obvious vascular deficiencies, in any tissues or organs analyzed, including skin,
lungs, kidneys, liver, and heart (not shown). For example, in retina, in which vascularization is readily visualized, WT and Cd151-null mice showed no difference in the development of superficial radial and collateral vessels (Figure S2). Furthermore, Cd151-null mice showed no difference in oxygen-induced retinal neovascularization, in multiple experiments, as seen either by lectin staining or by histologic examination (Figure S3). These results indicate that CD151 is not needed for normal vascular development but supports pathologic angiogenesis in some, but not all, circumstances.

**CD151 minimally affects endothelial cell proliferation, adhesion, and integrin expression**

To address mechanisms whereby CD151 might affect pathologic angiogenesis, we isolated MLECs from WT and CD151-null mice. MLECs, enriched to more than 90% purity, showed minimal differences in proliferation (Figure S4A) or in static cell adhesion to 5 different ECM substrates, including purified laminin-1 and laminin-rich Matrigel (Figure S4B). Apoptosis, assessed by a DNA/histone complex assay, was unaltered in cells plated on either 2-dimensional Matrigel or gelatin (not shown). Also, cells were plated within 3-dimensional collagen or Matrigel for 2 days, with or without 10% serum, and then thin sections were analyzed by TUNEL staining. In the presence of serum, 10% to 14% of cells showed apoptosis. In the absence of serum apoptosis was elevated (34%-38% in collagen; 21%-24% in Matrigel). In no condition was there a significant difference between Cd151 WT and null MLECs. Furthermore, WT and knockout MLECs expressed other tetraspanins (CD9 and CD81) and integrins (α2, α4, α5, α6, αV, β1, β4) at similar levels on the cell surface, except for a possible slight increase in α6 in Cd151-null cells (Figure S4C, and not shown).

**CD151 affects MLEC spreading, chemotaxis, invasion, and intrinsic migration**

Although absence of CD151 did not affect parameters measured in Figure S4, it did alter several cell functions (spreading, migration, invasion, chemotaxis) that are relevant to angiogenesis initiation and progression. First, the number of Cd151-null cells spread on Matrigel (for 30 minutes) was decreased by about 50%, whereas spreading on fibronectin and gelatin was decreased to a much lesser extent (Figure 4A). Second, Transwell migration of Cd151-null MLECs, toward bFGF chemoattractant, was significantly reduced when wells were coated with Matrigel or gelatin, but not fibronectin (Figure 4B). Third, invasion through Matrigel was more than 50% diminished for CD151 knockout MLECs compared to WT cells (Figure 4C).

 Whereas cell spreading, chemotaxis, and invasion were decreased for Cd151-null cells, intrinsic 2-dimensional migration was increased, most notably for cells plated on Matrigel, and to a lesser extent on gelatin (Figure 4D). In addition, Cd151-null cells displayed increased directional persistence, as evidenced by a higher D/T ratio, where D is the distance between starting and ending point, and T is the total distance traveled.

**CD151-null MLECs show attenuated 3-dimensional functions in vitro**

Formation of a network of cellular cables on Matrigel is a property of endothelial cells that models formation of new vasculature. Figure 3. Corneal angiogenesis is deficient in Cd151-null mice. (A) Five days after implantation of pellets containing 20 ng bFGF, vessels were stained with PE-conjugated anti-CD31. Clock-hour length of sprouting vessels was quantitated (**P < .005). (B) Corneal pellets contained 40 ng bFGF. After 5 days, vessels were stained with anti-CD31, and vascular area was quantitated (*P < .05). Scale bars represent 100 μm in panels A-B.

Formation of a network of cellular cables on Matrigel is a property of endothelial cells that models formation of new vasculature.
Cellular cables formed by Cd151-null MLECs were markedly diminished (Figure 5A), as seen in representative photos (after 8 and 24 hours), and by quantitation (38% decrease after 8 hours). Another assay involving laminin-binding integrins (and therefore possibly CD151) is the Matrigel contraction assay, which assesses mechanical forces relevant to angiogenesis.27 MLECs from WT mice contracted Matrigel as it detached from the underlying agarose support, whereas contraction by Cd151-null cells was about 60% impaired (Figure 5B). As expected, Matrigel contraction was completely inhibited by anti-α6 antibody (GoH3, 20 μg/mL; not shown).

Although the Matrigel cable assay is instructive (Figure 5A), cells form few if any lumens. However, endothelial cells grown within type I collagen gels form branching networks that do contain lumens.28 In this in vitro model for angiogenesis, Cd151-null MLECs again were deficient because tube formation was decreased by approximately 48% (Figure 5C).

Sprouting from preexisting blood vessels can be modeled in a 3-dimensional assay, in which capillary-like structures, containing lumens, sprout from spheroids embedded in collagen gel together with growth factors.34 To evaluate the role CD151, spheroids from WT and Cd151-null MLECs were grown in collagen gel containing bFGF and VEGF. After 24 and 48 hours, the cumulative length of all capillary-like sprouts from Cd151-null spheroids was significantly shorter than that of Cd151 WT spheroids (Figure 5D). Furthermore, the mean number of sprouts from each spheroid, after 2 days, was also significantly decreased (4.1 ± 2.1 versus 10.5 ± 3.0; P < .001).

Cd151-null MLECs show altered signaling

Many signaling pathways (eg, involving Akt, eNOS, Rac, Cdc42, ERK, Raf, p38 MAPK, FAK, Src) contribute to endothelial cell angiogenesis.35-37 Here we determined which are affected by
absence of CD151. Phosphorylation of Akt was markedly attenuated in Cd151-null MLECs compared to WT MLECs, when plated for 30 or 60 minutes on Matrigel (Figure 6A). No difference was seen for cells plated on gelatin (not shown). Up-regulation of phosphorylated eNOS was also somewhat attenuated in Cd151-null MLECs (Figure 6A), consistent with endothelial nitric oxide synthase (eNOS) being activated in an Akt-dependent manner.  By contrast, phosphorylation of components in, or upstream of, the MAPK pathway, such as Raf (Figure 6A), p38 MAPK, ERK, Src, and FAK (not shown), were not affected by the absence of CD151. Rac and Cdc42 are molecules frequently linked to endothelial cell spreading, migration, and 3-dimensional morphology.  After 3 hours on Matrigel (when cable formation begins to become obvious), Cd151-null MLECs, compared to WT MLECs, showed markedly diminished activation of Rac and Cdc42 (Figure 6B).

**CD151 deletion alters laminin-binding integrin complexes**

CD151 may link laminin-binding integrins to multiple other proteins, within tetraspanin-enriched microdomains (TEMs), to affect integrin-dependent functions.  Laminin-binding integrins, nearly all tetraspanins, and various tetraspanin-associated proteins readily incorporate [3H]-palmitate. Thus, metabolic labeling with [3H]-palmitate is a useful and selective method for analyzing integrin-tetraspanin complexes. As indicated (Figure 7A lanes 1 and 2), anti-beta1 antibodies immunoprecipitated similar levels of [3H]-palmitate–labeled laminin-binding integrins (alpha3beta1, alpha6beta1) from WT and null MLECs. As expected, Cd151-null cells yielded no CD151 (Figure 7A lane 2). In parallel, amounts of 5 other proteins (labeled d1-d5) were markedly diminished when CD151 was absent (lane 2). The decrease in proteins d3 (CD9) and d4 (CD81) was confirmed by immunoblotting with anti-CD9 and anti-CD81 antibodies (not shown). Identities of d1, d2, and d5 are unknown. In control experiments, amounts of palmitoylated proteins associated with CD9 (Figure 7A lanes 3 and 4) were relatively unchanged by the absence of CD151, except, of course, for CD151 itself. In conclusion, structural links between specific laminin-binding integrins (alpha3beta1, alpha6beta1) and other proteins (eg, d1-d5) are disrupted when CD151 is absent (shown schematically in Figure 7B).

**Discussion**

Although CD151 is abundant on endothelial cells, anti-CD151 antibodies showed conflicting or marginal functional effects in vitro.  Furthermore, disruption of the CD151 gene in humans caused no changes in normal physiology suggestive of vascular deficiencies. Indeed, our Cd151-null mice developed without overt vascular defects in any normal tissues or organs analyzed, including the retina, in which vessels are readily visualized. Nonetheless, we now provide strong evidence for CD151 playing a critical role during angiogenesis under selective pathologic conditions in vivo (Matrigel plug, tumor implantation,
and corneal micropocket assays) and ex vivo (aortic ring sprouting assay). At present we do not understand why CD151 is apparently not needed during normal vascular development. We speculate that other tetranspins, such as TSPAN11 (which shows the most sequence homology), could compensate for the absence of CD151 under normal conditions, but not in selected pathologic conditions.

With few exceptions, genetic alterations that affect Matrigel plug invasion, tumor angiogenesis, or corneal angiogenesis result in parallel effects on oxygen-induced retinal angiogenesis.\(^1\) Amplification of the gene encoding LGALS1, a member of the galectin family, has been associated with increased microvessel density in breast cancer.\(^2\) However, some contributions of laminin-binding integrins to angiogenesis may be independent of CD151. For example, deletion of Cd151-null MLECs (this study) and Cd151-null platelets\(^6\) did not show reduced static cell adhesion, consistent with CD151 not affecting initial ligand binding by laminin-binding integrins. Nonetheless, Cd151-null endothelial cells showed reduced cell spreading, invasion, chemotactic migration, Matrigel contraction, Matrigel cable formation, tube formation in collagen, and spheroid sprouting. A common feature of each of these assays is that tensional forces play a critical role, and thus a deficit in adhesion strengthening, such as can be caused by the absence of CD151, would have a major impact. Importantly, these in vitro functional assays recapitulate the invasion, sprouting, migration, and lumeno-forming steps that occur in vivo, during angiogenesis.\(^3,7\)

Laminin-binding integrins can activate PI3K-Akt,\(^5,5,5\) Rac1,\(^5,5,5,5,5,5\) Cdc42,\(^5,5,5,5,5,5,5,5\) ERK,\(^5,5,5,5,5,5,5,5\) p38 MAPK,\(^5,5,5,5,5,5,5,5\) FAK,\(^5,5,5,5,5,5,5,5\) src,\(^5,5,5,5,5,5,5,5\) and Rap\(^5,5,5,5,5,5,5,5\) pathways. However, only a subset of these signaling molecules (Akt, Rac1, and Cdc42) showed markedly reduced laminin-dependent activation in Cd151-null MLECs. These selective signaling deficiencies could help to explain the contribution of CD151 to endothelial cell physiology. The PI3K-Akt pathway regulates multiple steps in angiogenesis including migration, invasion, capillary formation, and permeability.\(^5,5,5,5,5,5,5,5\) Of the 3 major isoforms of Akt, Akt1 is the one most prominent in endothelial cells. Akt1-null ECs showed impaired sprouting from aortic rings and a decrease in directed endothelial cell motility in vitro.\(^5,5,5,5,5,5,5,5\) Hence, reduced aortic ring sprouting, capillary formation, invasion, and chemotactic migration seen in Cd151-null MLECs may be due to impaired Akt signaling. Because deletion of CD151 was accompanied by parallel reductions in Akt signaling and angiogenesis, we can overlook, for the moment, the recent finding that Akt can have both positive and negative effects on angiogenesis in vivo.\(^5,5,5,5,5,5,5,5\) Akt also can activate eNOS, leading to critical changes in vasodilation, vascular permeability, and cell motility needed for angiogenesis.\(^5,5,5,5,5,5,5,5\) Indeed, diminished Akt activation was accompanied by partly decreased eNOS activation in Cd151-null MLECs. Although Akt is also known to regulate endothelial cell survival and proliferation,\(^5,5,5,5,5,5,5,5\) these functions may be less important here, because the absence of Cd151 did not affect survival or proliferation of MLECs. It remains to be seen which of the many downstream targets of Akt\(^5,5,5,5,5,5,5,5\) are most important during Cd151-dependent signaling.

pathways in MLECs, consistent with lack of an effect on endothelial cell proliferation. Furthermore, lack of an effect on ERK signaling may help to explain why CD151 does not affect ERK-dependent, α6β4-dependent functions such as oxygen-induced retinopathy.12 Also essential during angiogenesis is matrix remodeling by metalloproteinases (MMPs).7 Even though CD151 has been linked to MMP-2 production,68 there was no change in MMP-2 or MMP-9 production or activation in Cd151-null MLECs (Figure S5). It remains to be seen whether other relevant MMPs, such as MT1-MMP, are altered by Cd151 deletion. In conclusion, CD151 makes a substantial contribution to a subset of laminin-dependent signaling pathways, thereby supporting selectively those laminin-dependent functions of endothelial cells that are needed for pathologic angiogenesis.

We propose that CD151 affects signaling, and associated downstream functions, by providing a physical link between laminin-binding integrins and key molecules (as yet unidentified) that are upstream of Akt, Rac1, and Cdc42 on endothelial cells. Different laminin-binding integrins and multiple tetraspanins (CD9, CD151) were previously shown to reside in overlapping complexes, known as tetraspanin-enriched microdomains (TEMs)47,69. Here we show that absence of CD151 disrupts these TEMs, leading to reduced association of α3β1 or α6β1 integrins with at least 5 other [3H]-palmitate-labeled proteins (including tetraspanins CD9 and CD81). Within the context of TEMs, CD151, CD9, and CD81 can recruit signaling molecules such as PI 4-kinase70 and conventional PKC isoforms71 into complexes with laminin-binding integrins. This may be relevant to angiogenesis because PI 4-kinase either directly (through PtdIns4P) or indirectly (through PtdIns(4,5)P2) can provide substrates for PI-3 kinase, thus enhancing PI 3-kinase–Akt signaling. In addition, recruitment of PKC could potentially influence PKC-dependent modulation of vascular permeability, endothelial cell sprouting, migration, and vessel formation.72,74

Studies regarding CD151 and tumor progression have so far focused on the promalignancy role of CD151 on tumor cells.75-77 For example, recent RNAi knockdown studies78,79 point to CD151 contributing to migration and spreading of tumor cell lines. Now we provide perhaps the first definitive evidence that CD151 can affect tumor growth in a different way—by contributing to tumor angiogenesis in the host animal. Hence, if appropriate anti-CD151 inhibitors can be designed, they potentially could deliver a double hit—on the tumor as well as on supporting host vessels. Normal vessels would presumably be spared because CD151 did not appear to be essential for vasculogenesis or angiogenesis in the developing or adult mouse.

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Authorship

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