Deleted in Malignant Brain Tumors 1 is Present in the Vascular Extracellular Matrix and Promotes Angiogenesis

Hanna Müller, Jiong Hu, Rüdiger Popp, Mirko H.H. Schmidt, Karin Müller-Decker, Jan Mollenhauer, Beate Fisslthaler, Johannes A. Eble, Ingrid Fleming

Objective—Deleted in malignant brain tumors 1 (DMBT1) belongs to the scavenger receptor cysteine-rich superfamily of proteins and is implicated in innate immunity, cell polarity, and differentiation. Here we studied the role of DMBT1 in endothelial cells.

Methods and Results—DMBT1 was secreted into the extracellular matrix (ECM) by endothelial cells in vitro and in situ and the presence of DMBT1 in the ECM increased endothelial cell adherence. Endothelial cell-derived DMBT1 associated with galectin-3 (coprecipitation), and human recombinant DMBT1 bound EGF, vascular endothelial growth factor and Delta-like (Dll) 4 (specific ELISAs). Compared to cells from wild-type mice, endothelial cells from DMBT1$^{-/-}$ mice demonstrated reduced migration, proliferation, and tube formation. In vivo recovery from hindlimb ischemia was attenuated in DMBT1$^{-/-}$ animals as was vascular endothelial growth factor -induced endothelial sprouting from isolated aortic rings; the latter response could be rescued by the addition of recombinant DMBT1. The Notch pathway is involved in multiple aspects of vascular development, including arterial-venous differentiation and we found that endothelial cells from DMBT1$^{-/-}$ mice expressed more EphrinB2 than cells from wild-type mice. Levels of Dll1, Dll4, Hes1, Hey1, and EphB4, on the other hand, were decreased.

Conclusion—Taken together, the results of this study indicate that DMBT1 functions as an important endothelium-derived ECM protein that is able to bind angiogenic factors and promote adhesion, migration, proliferation, and angiogenesis as well as vascular repair. Mechanistically, DMBT1 interacts with galectin-3 and modulates the Notch signaling pathway as well as the differential expression of ephrin-B2 and EphB4. (***Arterioscler Thromb Vasc Biol*. 2012;32:442-448.)**

Key Words: angiogenesis ■ endothelium ■ extracellular matrix ■ matrix

The mechanisms that regulate angiogenesis and vascular differentiation are complex but include interactions between the endothelium and the extracellular matrix (ECM).[1] Human deleted in malignant brain tumors 1 (DMBT1; also known as lung glycoprotein-340) is a secreted high molecular weight pattern recognition molecule belonging to the scavenger receptor cysteine-rich superfamily that binds polyphosphorylated and -polysulfated structures found on bacterial and viral surfaces as well as in the ECM. As its name suggests the DMBT1 gene was presumed to play a role in tumor suppression,[2] however it now seems that DMBT1 may play a more complex role in tumorigenesis than originally thought and that its actions differ from that of a conventional tumor suppressor gene.[3]

DMBT1 is expressed by several cell types and the polymerization of hensin, the rabbit orthologue of DMBT1, and its interaction in the ECM with galectin-3 reportedly modulates the polarity of epithelial cells.[4] The molecular mechanisms involved are unclear but the activation of integrin $\alpha v\beta 1$ triggers the secretion of hensin into the ECM where it polymerizes, binds integrin $\alpha 6$, and stimulates cell differentiation.[5] Integrins with a $\beta 1$ subunit are receptors for ECM proteins, such as the laminins and fibronectin, and play an important role in angiogenesis.[6] Given that DMBT1 has been detected in/on endothelial cells in samples from patients,[7] it is tempting to suggest a link between integrin-induced DMBT1 deposition and angiogenesis. The aim of the present study was therefore to assess the expression of DMBT1 in the endothelium and to determine its role in angiogenesis and vascular repair.

Materials and Methods

A detailed description of all of the materials, antibodies and standard methods used appear in Supplemental Materials and Methods, available online at http://atvb.ahajournals.org.

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From the Institute for Vascular Signalling (H.M., J.H., R.P., B.F., I.F.) and Institute for Vascular Matrix Biology (J.A.E.), Centre for Molecular Medicine and Institute of Neurology (M.H.H.S.), Goethe University, Frankfurt, Germany; Core Facility Tumor Models (K.M.-D.), German Cancer Research Centre, Heidelberg, Germany; Lundbeckfonden Center of Excellence NanoCAN and Molecular Oncology (J.M.), Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark.

Correspondence to Ingrid Fleming, Institute for Vascular Signalling, Centre for Molecular Medicine, Johann Wolfgang Goethe University, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany. E-mail fleming@em.uni-frankfurt.de

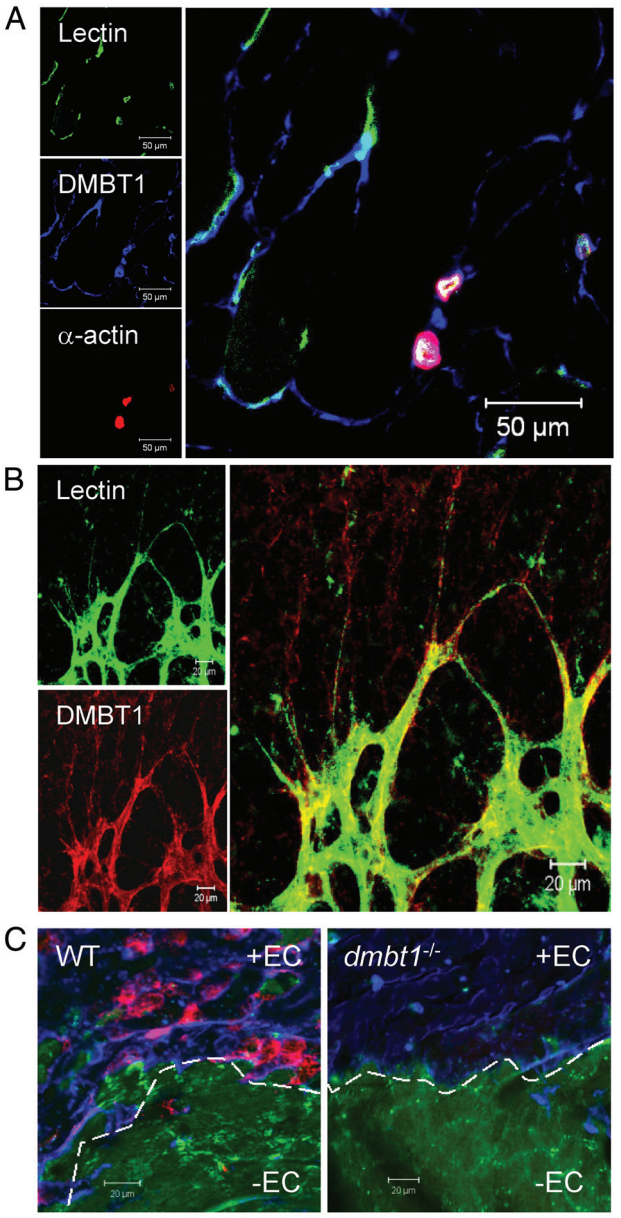

Figure 1. Deleted in malignant brain tumors 1 (DMBT1) is present in the ECM surrounding endothelial cells in vivo. **A**, DMBT1 (blue) in capillaries (lectin=green) and small arteries (α-actin=red) in skeletal muscle. **B**, Localization of DMBT1 (red) with the filipodia extended by tip cells (green) in the developing retina (P5). **C**, En face preparations of partially endothelium-intact (+E) and -denuded (−E) aorta from a wild-type (WT) and DMBT1$^{-/-}$ mouse. CD31=blue, DMBT1=red, α-actin=green. The bars represent (**A**) 50 and (**B**, **C**) 20 μm, respectively.

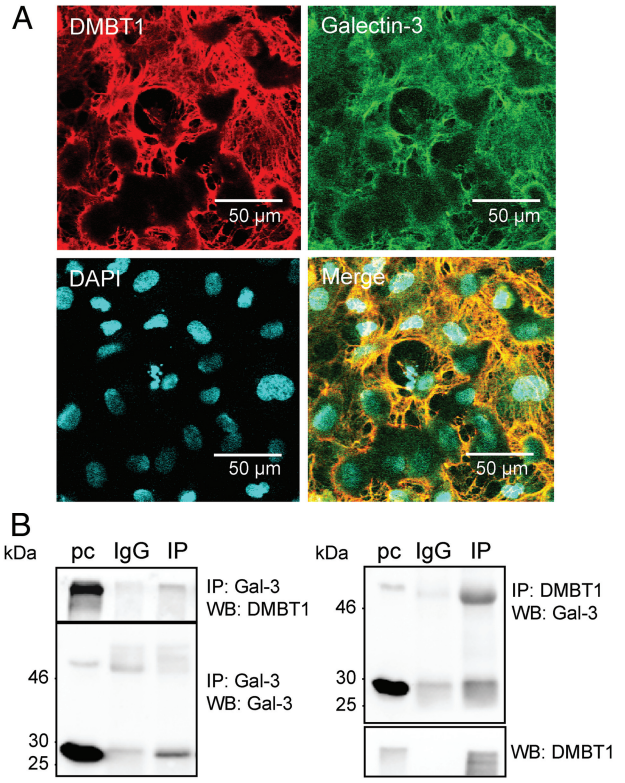

Figure 2. Expression of deleted in malignant brain tumors 1 (DMBT1) in cultured endothelial cells and interaction with galectin-3. **A**, DMBT-1 expression in cultured human umbilical vein endothelial cells. DMBT1=red, galectin-3=green, DAPI=blue. **B**, Coimmunoprecipitation (IP) of DMBT1 and galectin-3 from human endothelial cells, pc indicates positive control. Identical results were obtained in 2 additional experiments each using a different cell batch.

Animals

Male wild-type and DMBT1$^{-/-}$ mice (C57BL/6 background),[8] were bred at the central animal facility at the German Cancer Research Center Heidelberg. All animal experiments were approved by the Regierungspräsidium Karlsruhe (license G172-09).

Hindlimb Ischemia

Hindlimb ischemia was induced as outlined.[9] Limb perfusion was measured with a laser Doppler imager every three to four days (Moor Instruments Ltd, Millwey, England). After 14 days the mice were killed and the M. adductor and M. semimembranosus were removed for analysis.

Aortic Ring Assay

Aortae from wild-type and DMBT1$^{-/-}$ mice were removed, cleaned, and embedded in a collagen gel (collagen type I, BD Biosciences, Heidelberg, Germany) in a 48-well plate containing MCDB 131 medium (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with murine serum (2.5%) from the donor animals. After 24 hours, murine vascular endothelial growth factor (VEGF) (30 ng/mL) and/or human recombinant DMBT1 (100 ng/mL) were added and the tube-like structures were allowed to develop over 7 days. Thereafter, the samples were fixed (4% paraformaldehyde) and endothelial cells were visualized using antibodies against CD31.

Cell Culture

Murine endothelial cells from wild-type and DMBT1$^{-/-}$ mice and human umbilical vein endothelial cells were isolated and cultured as described previously.[10] Murine endothelial cells were used up to passage 9 while first passage human endothelial cells were used throughout. The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki and the isolation of endothelial cells was approved by the ethic committee at the Goethe University.

Statistics

Data are expressed as the mean±SEM, and statistical evaluation was performed using Student *t*-test for unpaired data, 1-way ANOVA followed by a Bonferroni *t*-test, or ANOVA for repeated measures

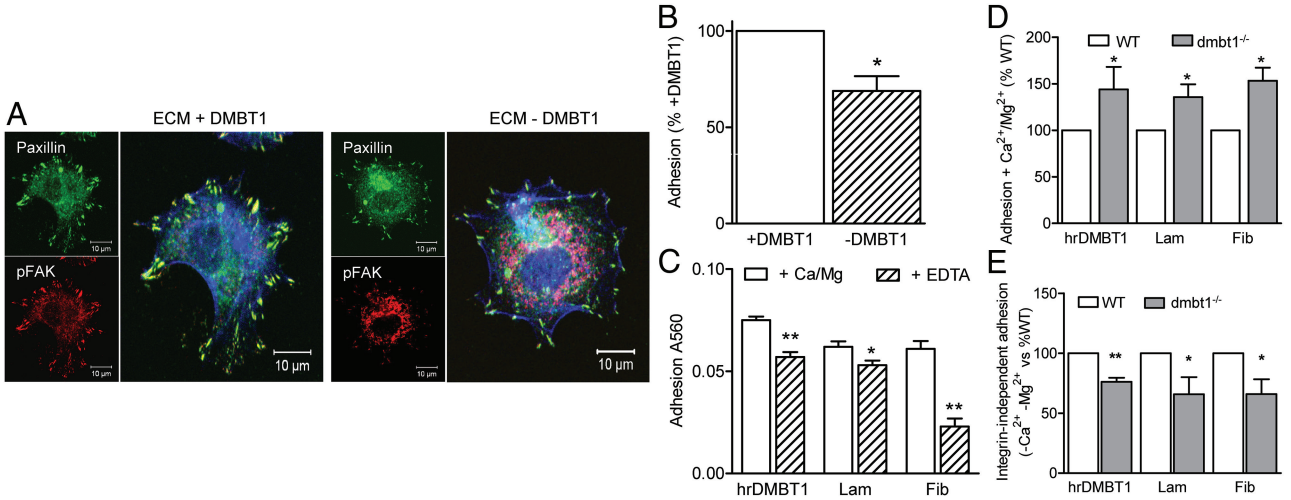

Figure 3. Role of deleted in malignant brain tumors 1 (DMBT1) in endothelial cell adhesion. **A**, Intracellular localization of paxillin and phosphorylated FAK (pFAK) in wild-type endothelial cells adhering to ECM generated by wild-type (WT) (+DMBT1) and DMBT1$^{-/-}$ cells (−DMBT1). Paxillin=green, pFAK=red, phalloidin=blue. **B**, Adhesion of wild-type endothelial cells to the +DMBT1 or −DMBT1 ECM. **C**, Adhesion of wild-type endothelial cells to human recombinant DMBT1 (hrDMBT1), laminin (Lam), and fibronectin (Fib) in the presence of Ca^{2+}/Mg^{2+} or EDTA. **D**, **E**, Adhesion of WT and DMBT1$^{-/-}$ endothelial cells to ECM proteins; (**D**) in the presence of Ca^{2+}/Mg^{2+} and (**E**) in presence of EDTA. The bar graphs represent data obtained using 3 different cell batches; $^{*}P<0.05$, $^{**}P<0.01$.

where appropriate. Values of $P<0.05$ were considered statistically significant.

Results

Localization and Regulation of DMBT1 in Endothelial Cells

To determine the vascular localization of DMBT1 we compared its expression with that of endothelial (FITC-lectin) and smooth muscle cell (α-actin) markers in murine tissues. In the hindlimb adductor muscle; DMBT1 surrounded muscle fibers and was detected in close proximity to capillary endothelial cells as well as small arteries (Figure 1A). In the developing retina (postnatal day 5) DMBT1 was detected at the leading edge of the capillary network and was closely associated with the filipodia that extended from tip cells. DMBT1 was also evident in the stalk cells of the retina where it seemed to be adjacent to, rather than within, the endothelium (Figure 1B), a finding consistent with the fact that DMBT1 is a secreted protein. In en face preparations of the murine aorta, DMBT1 was detected when the endothelium was intact but not once the endothelial layer was removed mechanically, indicating the endothelial association of the protein (Figure 1C). No DMBT1 was detected in samples from DMBT1$^{-/-}$ mice (Figure 1C and Supplementary Figure I).

Primary cultures of human umbilical vein endothelial cells expressed DMBT1; low levels were detected in the cell cytosol with the majority of the protein being in the ECM (Figure 2A). Consistent with the fact that it is highly glycosylated, DMBT1 was detected by Western blotting as a diffuse 340 kDa band. Galectin-3, which interacts with DMBT1 in other tissues to promote DMBT1 polymerization,[11] was also expressed by the endothelium,[12] and could be coprecipitated with DMBT1 from confluent cultures of human endothelial cells and vice versa (Figure 2B).

Effect of DMBT1 on Endothelial Cell Adhesion

The effect of DMBT1 on endothelial cell adhesion and migration was studied using DMBT1-containing and -depleted ECM generated by wild-type or DMBT1$^{-/-}$ lung endothelial cells. This procedure was chosen in preference to plating cells on human recombinant DMBT1 (hrDMBT1) as the native protein is highly glycosylated, a modification that affects its function including its association with galectin-3.[13] In the endothelial cells plated onto DMBT1-containing ECM, paxillin and phosphorylated focal adhesion kinase (FAK) colocalized in clusters at the cell membrane. However, in cells plated onto DMBT1-deficient ECM, phosphorylated FAK was only detected close to the nucleus (Figure 3A). Moreover, fewer endothelial cells adhered to the DMBT1-depleted ECM (Figure 3B).

The function of DMBT1 was further addressed by determining the attachment of cells to culture dishes coated with hrDMBT1. Endothelial cells adhered to hrDMBT1-coated surfaces to a similar degree as to laminin- and fibronectin-coated surfaces (Figure 3C). The dependency of hrDMBT1-mediated cell adhesion on divalent cations (an index of integrin-mediated cell adhesion) was similar to that of laminin but less than that observed when cells were plated onto fibronectin. Interestingly, endothelial cells from DMBT1$^{-/-}$ mice displayed a significantly stronger adhesion to hrDMBT1, laminin, and fibronectin in the presence but not in the absence of divalent cations (Figure 3D, 3E). We found no significant difference in the expression of αv or β1 integrins in wild-type versus DMBT1$^{-/-}$ endothelial cells; however, the expression of β3 integrin was significantly lower in endothelial cells from DMBT1$^{-/-}$ mice (Supplementary Figure II).

Binding of hrDMBT1 to EGF, VEGF, and Dll4

Several ECM constituents are able to bind growth factors and thus modulate angiogenesis. Because DMBT1 has a repetitive structure with multiple binding sites we hypothesized that it may also be able to sequester growth factors. Indeed, hrDMBT1 was able to bind to EGF (Supplementary Figure IIIA) and VEGF (Supplementary Figure IIIB). Moreover, we also found that hrDMBT1 bound the Notch ligand Delta-like

4 (Dll4; Supplementary Figure IIIC). Because all of these factors are involved in angiogenesis it is feasible that their association with DMBT1 can effectively increase their local concentrations thus promoting the vascular response. The latter interactions were disturbed by heparan sulfate (Supplementary Figure IIID).

Role of DMBT1 in Endothelial Cell Migration and Angiogenesis In Vitro

We next compared the ability of endothelial cells from wild-type and DMBT1$^{-/-}$ mice to migrate in a scratch-wound assay as well as to proliferate and form tube-like structures. Although wild-type endothelial cells migrated to cover approximately 50% of the wound area after 22 hours, the migration of endothelial cells from DMBT1$^{-/-}$ mice was markedly delayed (Supplementary Figure IVA). Moreover, although endothelial cells from wild-type mice proliferated well in the presence of 20% FCS, responses were attenuated in cells from Dmbt1$^{-/-}$ mice (Supplementary Figure IVB) without any detectable increase in apoptosis.

The attenuated endothelial cell proliferation and migration were also reflected in an impaired ability to form tube-like structures on Matrigel in response to VEGF (Figure 4A). This phenomenon was not restricted to endothelial cells in culture as the formation of endothelial cell (CD-31 positive) capillary sprouts was also attenuated in aortic rings from DMBT1$^{-/-}$ mice following either incubation with solvent or VEGF (Figure 4B). In a rescue experiment we next determined whether or not the addition of hrDMBT1 could restore responsiveness to VEGF. Recombinant DMBT1, either alone or in combination with VEGF, slightly enhanced sprouting in rings from wild-type mice (Figure 4B). In aortic rings from DMBT1$^{-/-}$ mice, hrDMBT1 alone was without significant effect but it did restore responsiveness to VEGF to levels similar to that observed in wild-type tissue.

Role of DMBT1 in Angiogenesis In Vivo

Next we determined the ability of DMBT1$^{-/-}$ mice to recover from hindlimb ischemia. This model seemed particularly appropriate because DMBT1 was detected in skeletal muscle capillaries. In wild-type animals, femoral artery ligation compromised hindlimb blood flow (laser Doppler measurements), which recovered to approximately 75% of preligation values within 14 days. Although limb perfusion was comparable in wild-type and DMBT1$^{-/-}$ mice prior to ligation, blood flow in the ischemic limb recovered more slowly with a maximal recovery of only 25% after 2 weeks (Figure 5A). This correlated with the impaired vascularization (arterial and capillary numbers) of the M. adductor in hindlimbs from dmbt1$^{-/-}$ mice (Figure 5B).

DMBT1 and Notch Signaling

Notch signaling plays a crucial role in reparative angiogenesis and changes in the expression of the Notch ligands, Dll1 and Dll4, have been reported to occur during postischemic angiogenesis.[14,15] Given that hrDMBT1 was able to bind Dll4, we assessed the consequences of DMBT1 deletion on the expression of components of the Notch signaling cascade. Although Notch1 expression was slightly increased in

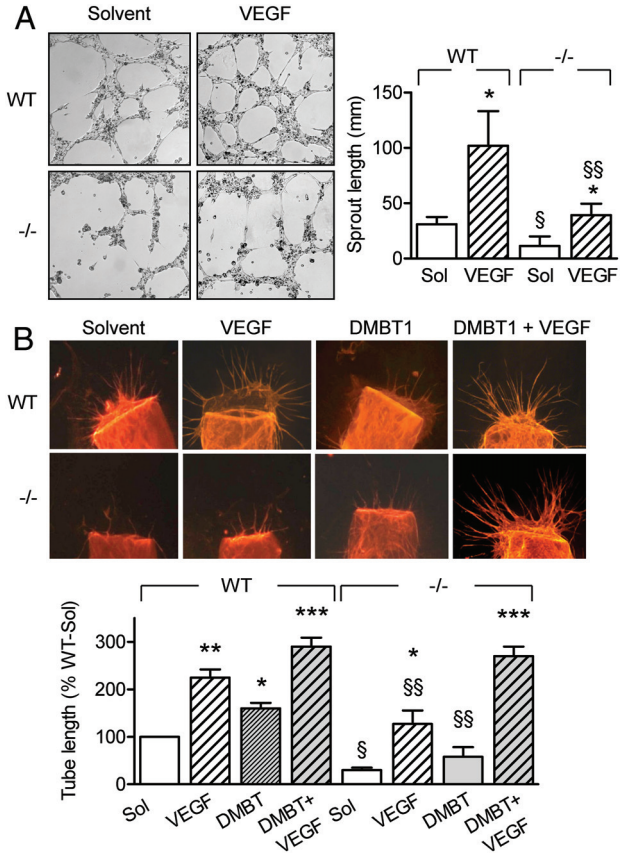

Figure 4. Defective angiogenesis in endothelial cells from deleted in malignant brain tumors 1 (DMBT1)$^{-/-}$ mice. **A**, In vitro tube formation on Matrigel by lung endothelial cells from wild-type (WT) and DMBT1$^{-/-}$ mice in the absence or presence of murine vascular endothelial growth factor (VEGF) (50 ng/mL). **B**, Endothelial cell sprouting (visualized using antibodies against CD31) from aortic rings from wild-type and DMBT1$^{-/-}$ mice embedded in a collagen gel containing either solvent or murine VEGF (30 ng/mL) in the absence and presence of human recombinant DMBT1 (100 ng/mL). The bar graphs represent data obtained using 3 independent cell batches (each in duplicate) or 4 animals per group; *$P<0.05$, **$P<0.01$ vs solvent, §$P<0.05$, §§$P<0.01$ vs WT.

DMBT1$^{-/-}$ endothelial cells (Figure 6A), the expression of the ligands Dll1 and Dll4 mRNA was attenuated as was the expression of Hes1 and Hey1, 2 downstream effectors of the Notch signaling cascade.

The Notch pathway is involved in multiple aspects of vascular development, including arterial-venous differentiation, therefore we determined the consequences of DMBT1-deletion on the expression of ephrin-B2 and EphB4, 2 proteins differentially expressed in arterial and venous endothelium, respectively.[16] Murine lung microvascular endothelial cells expressed both ephrin-B2 and EphB4 but whereas EphB4 levels were consistently higher in wild-type than in DMBT1$^{-/-}$ cells, the reverse was true for ephrin-B2 (Figure 6B). Similarly, decreased levels of EphB4 were detected in vivo in skeletal muscle capillaries (Figure 6C). Moreover, while coating mouse lung endothelial cells onto hrDMBT1 had no effect on EphB4 levels, it increased EphB4 levels in DMBT1$^{-/-}$ cells (Figure 6D).

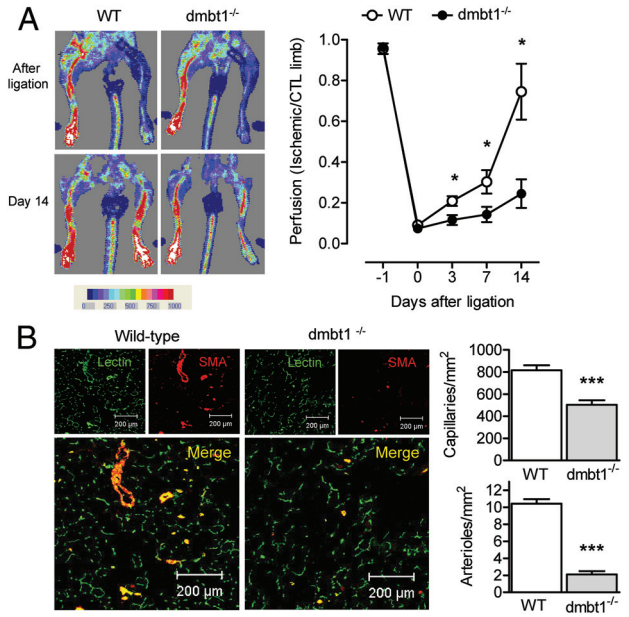

Figure 5. Delayed recovery from hindlimb ischemia in deleted in malignant brain tumors 1 (DMBT1) $^{-/-}$ mice. **A**, Hindlimb perfusion in wild-type (WT) and dmbt1$^{-/-}$ mice immediately and 14 days after ischemia. **B**, Capillary and arteriole density assessed measured by immunofluorescence staining of the muscles in the affected limb 14 days after ischemia induction. The bar graphs represent data obtained using 8 animals per group; *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs WT.

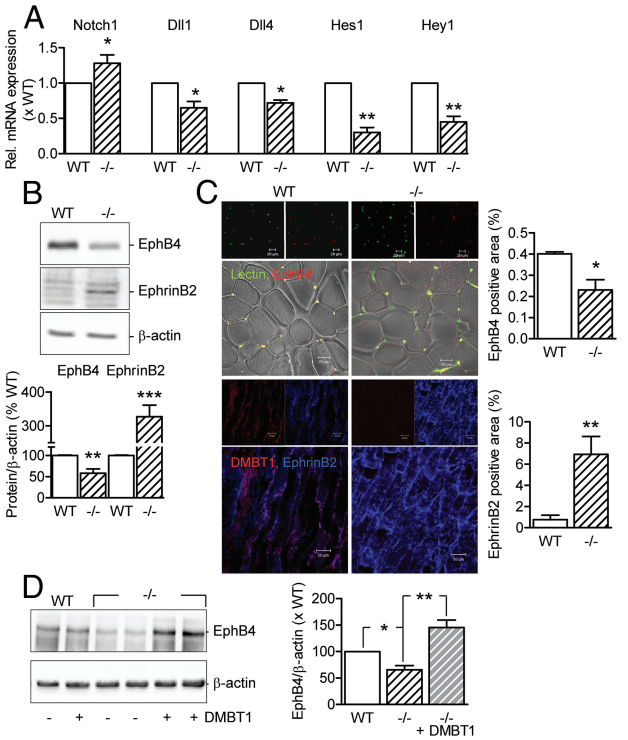

Figure 6. Deleted in malignant brain tumors 1 (DMBT1) and endothelial cell signaling. **A**, Relative expression of mRNA encoding components of the Notch signaling cascade (mRNA/transferrin receptor) in endothelial cells from wild-type (WT) and DMBT1$^{-/-}$ mice. **B**, Expression of ephrin-B2 and EphB4 in WT and DMBT1$^{-/-}$ endothelial cells. **C**, Expression EphB4 (cross-section, red) and EphrinB2 (longitudinal section, blue) in skeletal muscle from WT and DMBT1$^{-/-}$ mice. **D**, Effect of DMBT1 (100 ng/mL, 12 hours) on the expression of EphB4 in cultured lung endothelial cells from WT and DMBT1$^{-/-}$ mice. The bar graphs represent data obtained using 3 independent experiments each using a different cell batch or 4 animals per group; *$P<0.05$, **$P<0.01$ vs control or WT.

Discussion

The results of the present investigation demonstrate that DMBT1 is an important vascular ECM protein, promoting endothelial cell adhesion, migration, proliferation and reparative angiogenesis. To our knowledge, this is the first time that a protein of the scavenger receptor cysteine-rich super family has been linked to these processes. Although the precise molecular mechanisms underlying these effects remain to be determined in detail, we found that DMBT1 interacts with galectin-3, EGF, VEGF, and Dll4. Moreover, the genetic deletion of DMBT1 attenuates Notch signaling and thus differentially affects the expression of ephrin-B2 and EphB4.

Almost nothing is known about the vascular actions of DMBT1. Immunohistochemical analysis of heart tissue previously revealed sporadic DMBT1 expression in/on endothelial cells as well as in the heart valves of patients with bacterial endocarditis[7]; which fits well with reports linking DMBT1 with inflammation and innate immune responses in different organs.[17,18]

Our data indicate that DMBT1 is secreted by endothelial cells to the ECM under normal physiological conditions in vitro and in vivo, and that human DMBT1 is similar to hensin (rabbit DMBT1) with regard to its interaction with galectin-3, a β-galactoside-binding lectin. The interaction of the 2 proteins is likely to be required for their vascular effects as not only does the deletion of DMBT1 attenuate angiogenesis and vascular repair (present study) but galectin 3 inhibition and downregulation also reduce VEGF- and basic fibroblast growth factor-mediated angiogenesis in vitro, and angiogenic responses are reduced in galectin-3$^{-/-}$ animals.[19] Mechanistically, the angiogenic effects of galectin-3 have been attributed to binding; via its carbohydrate recognition domain, to the GnTV synthesized N-glycans of integrin $\alpha v\beta 3$, to induce its clustering.[19] Although we did not address the direct interaction of DMBT1 with integrins we did observe that the expression of $\beta 3$ integrin was attenuated in endothelial cells from DMBT1$^{-/-}$ mice. More recently galectin-3 was also reported to modulate angiogenesis in human endothelial cells by retaining the cell surface expression of the VEGF receptor 2.[20] However, whether or not DMBT1 also affects VEGF receptor cycling remains to be determined.

DMBT1 has a repetitive structure with 14 SRCR domains and multiple binding sites making it likely that 1 molecule DMBT1 binds a number of other molecules.[21] Thus binding to galectin-3 may not account for all of the effects of DMBT1 and its ability to bind EGF, VEGF, and Dll4 and may account for some of its effects on angiogenesis and vascular repair. This is of relevance as the interaction of growth factors with the ECM is one key mechanism thought to regulate the actions of growth factors, including VEGF.[22] It is thus tempting to speculate that interaction with DMBT1 may be a means of increasing the local concentrations of angiogenic growth factors. The results of the aortic ring assays indicate

that DMBT1 is required for normal responsiveness to VEGF as the sprouting response to VEGF could be restored in DMBT1$^{-/-}$ vessels by the addition of VEGF and hrDMBT1 but not by hrDMBT1 alone. Interestingly, all of the growth factors tested are glycosylated and contain disulfide bonds, implicating that these characteristics might be involved in the binding to DMBT1.[18]

The Notch ligand, Dll4, is upregulated in response to hindlimb ischemia in mice and localized at the forefront of sprouting capillaries.[15] Functionally Dll4 is required for angiogenesis and its inhibition by the intramuscular injection of an adenovirus encoding the soluble form of Dll4 extracellular domain precipitates the formation of a disorganized, low-perfused capillary network in ischemic muscles.[15] We found that the deletion of DMBT1 elicited similar effects as those described following Dll4 inhibition, ie, attenuated capillary formation and markedly attenuated recovery of blood flow in ischemic hindlimbs. Moreover, DMBT1 deletion was found to have marked consequences on Notch signaling. Indeed, compared to cells from wild-type mice, endothelial cells from DMBT1$^{-/-}$ mice showed attenuated expression of the Notch regulated genes Hes1 and Hey1. To further assess the link between DMBT1 and Notch signaling, we studied the consequences of DMBT1 deletion on ephrin-B2 and EphB4, 2 proteins that are involved in arteriovenous differentiation and previously reported to be regulated by Notch.[14,16,23,24] Indeed, we observed that the deletion of DMBT1 altered the expression of both markers, ie, decreased EphB4 while increasing ephrin-B2 expression in murine lung endothelial cells.

The fact that DMBT1 promotes angiogenesis and vascular repair is at odds with the assumption—based on the function of hensin and its presumed role in tumor suppression—that DMBT1 might inhibit cell proliferation and initiate cell differentiation. However, as mentioned previously, DMBT1 may play a more complex role in tumorigenesis than originally thought and that its actions differ from that of a conventional tumor suppressor gene.[3] It is also important to remember that the vascular repair processes studied in the hindlimb are not solely dependent on angiogenesis but also on different subsets of cells of the hematopoietic lineage. The latter promote the vascularization of matrix material and tumors by acting as accessory cells secreting a number of proangiogenic factors and differentiating to form new blood vessels.[25,26] This is of relevance as DMBT1 is upregulated in stem/progenitor cells during regeneration[4] and was proposed as a bone marrow hepatic stem cell primer in rats subjected to common bile duct ligation.[27] It will be interesting to assess whether or not DMBT1 is expressed in circulating progenitor cells with angiogenic potential (previously referred to as endothelial progenitor cells), or whether its expression affects the recruitment of such cells to sites of injury. To date studies on the role of DMBT1 on tumor development have focused on nonvascular cells, but it remains to be determined to what extent the lack of endothelial cell-derived DMBT1 (and eventually decreased Notch signaling) can contribute to the abnormal organization, structure and altered function (eg, hyper-permeability and heterogeneous blood flow) of tumor vasculature and eventually metastasis.

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Disclosures

None.

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Arteriosclerosis, Thrombosis, and Vascular Biology

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Deleted in Malignant Brain Tumors 1 is Present in the Vascular Extracellular Matrix and Promotes Angiogenesis

Hanna Müller, Jiong Hu, Rüdiger Popp, Mirko H.H. Schmidt, Karin Müller-Decker, Jan Mollenhauer, Beate Fisslthaler, Johannes A. Eble and Ingrid Fleming

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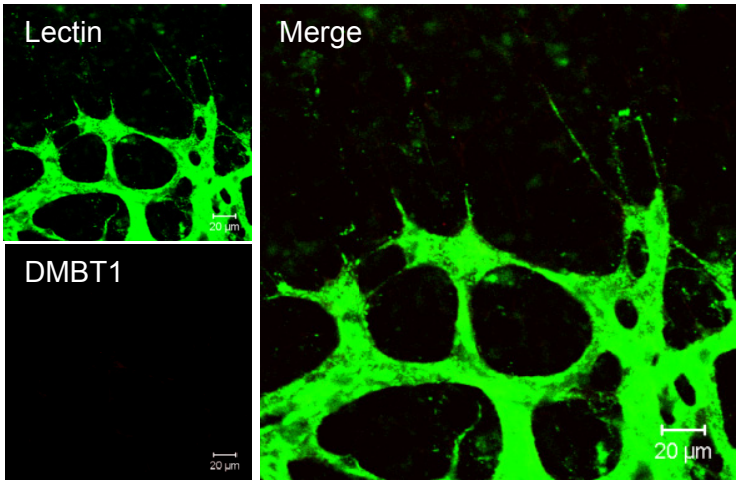
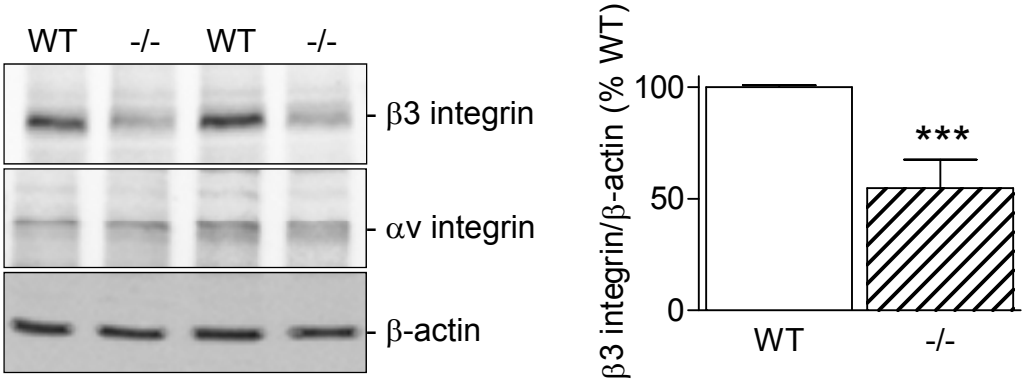
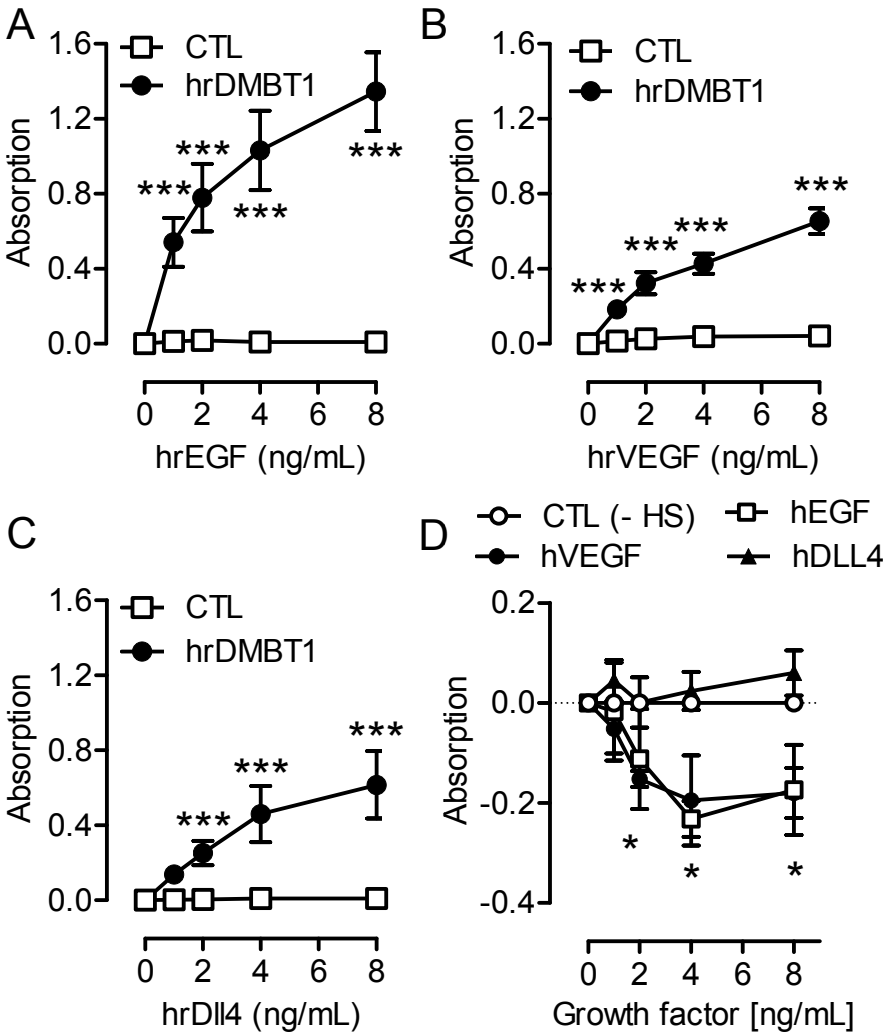


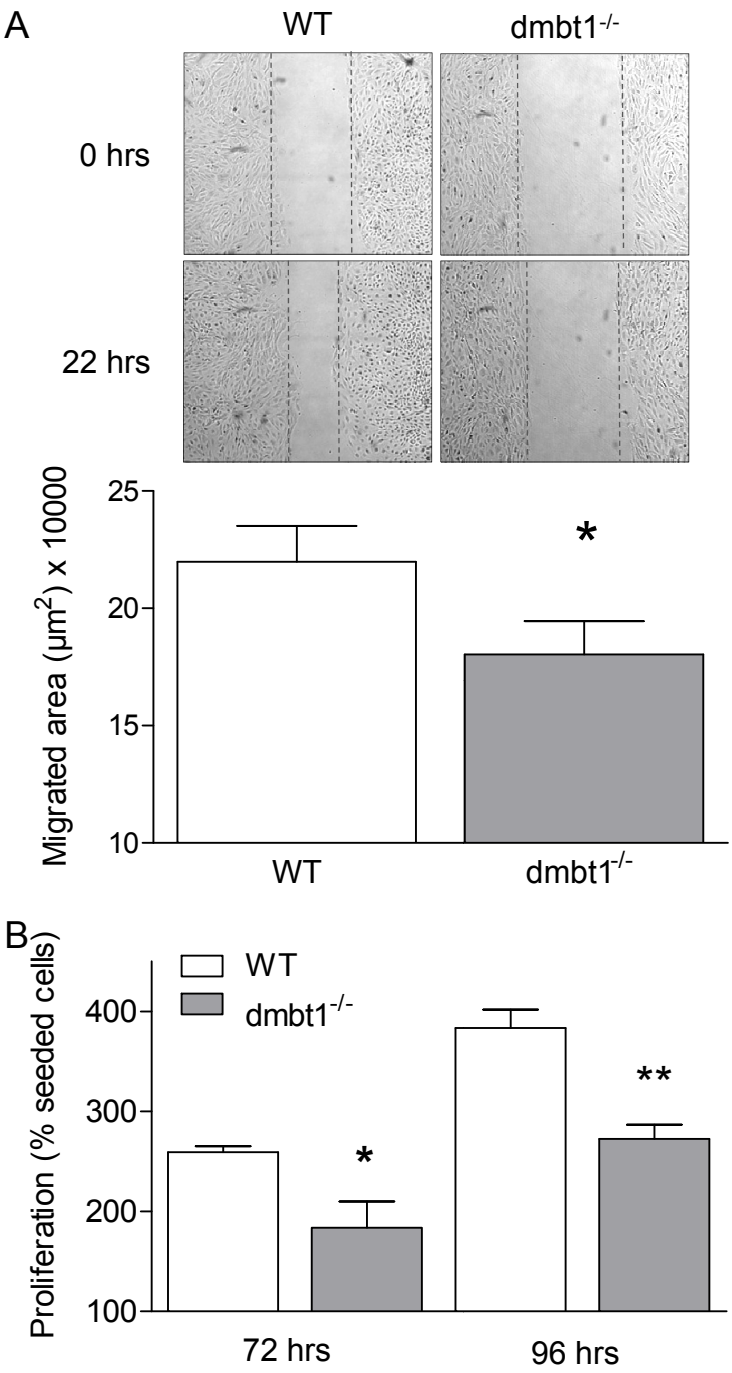
Supplementary Figure I. Lack of expression of DMBT1 (red) in *dmbt1*$^{-/-}$ mice. Endothelial cells/tip cells in the developing retina (P5) were visualized using lectin (green).



Supplementary Figure II. Expression of β3- and αv integrin in lung endothelial cells (passage 4) from wild-type (WT) and *dmbt1*$^{-/-}$ mice. The bar graph represents data obtained using 3 independent cell batches, each in duplicate; ***P<0.001.



Supplementary Figure III. Binding of DMBT1 to EGF, VEGF and Dll4. Binding of hrDMBT1 to (A) EGF, (B) VEGF and (C) DLL4. (D) Effect of heparan sulfate on the binding of human recombinant DMBT1 to hVEGF, hEGF and hDLL4. Culture wells were coated with hVEGF or hEGF or hDLL4 followed by hrDMBT1 in the absence and presence of heparan sulfate (HS, 50 µg/mL). Bound hrDMBT1 was analyzed by ELISA using specific antibodies. The graphs represent data obtained using 3 independent experiments; *P<0.05, **P<0.01 versus control (CTL).



Supplementary Figure IV. Effect of DMBT1 on endothelial cell migration and proliferation. (A) Migration of wild-type (WT) and *dmbt1*$^{-/-}$ lung endothelial cells in the presence of 5% FCS. (B) Proliferation of WT and *dmbt1*$^{-/-}$ endothelial cells in the presence of 20% FCS. The graphs summarize data obtained using 3-4 different cell batches; *P<0.05, **P<0.01 versus WT.

It should be noted that the migration of murine lung endothelial cells was much slower than that of the human umbilical vein endothelial cells generally used in such assays.

Supplement Material

Deleted in malignant brain tumors 1 (DMBT1) is present in the vascular extracellular matrix and promotes angiogenesis

Materials and Methods

Materials. Purified human recombinant DMBT1 was generated as described.[1] The polyclonal anti-DMBT1p84 antibody used was raised against human DMBT1,[2] the antibodies against galectin-3 and CD31 were from BD Biosciences (Heidelberg, Germany) the β3 integrin antibody from Epitomics (Burlingame, CA), and the αv integrin antibody from Chemicon (Temecula,CA). The polyclonal anti-mouse EphB4 and ephrinB2 antibodies were from R&D Systems (Wiesbaden, Germany), the paxillin antibody from abcam (Cambridge, UK), the phospho-FAK (Y397) antibody and the Alexa Fluor 546 and Alexa Fluor 647 secondary antibodies from Invitrogen (Karlsruhe; Germany). EGF, VEGF and interferon-γ were from PeproTech (Hamburg, Germany) and hrDLL4 from R&D Systems (Wiesbaden, Germany). All other substances were from Sigma-Aldrich (Munich, Germany).

Animals. Male wild-type and *dmbt1*$^{-/-}$ mice (C57BL/6 background),[2] were bred at the central animal facility at the German Cancer Research Center Heidelberg. All animal experiments were approved by the Regierungspräsidium Karlsruhe (license G172-09).

Cell Culture. Murine endothelial cells from wild-type and *dmbt1*$^{-/-}$ mice and human umbilical vein endothelial cells were isolated and cultured as described previously.[3] Murine endothelial cells were used up to passage 9 while first passage human endothelial cells were used throughout. The use of human material in this study conforms to the principles outlined in the Declaration of Helsinki and the isolation of endothelial cells was approved by the ethic committee at the Goethe University.

Adhesion Assays. Endothelial cells from wild-type and *dmbt1*$^{-/-}$ mice were allowed to deposit their ECM on a 96-well plate while being cultured with DMEM/F12 containing 20% FCS for five to six days. After washing with PBS the adherent cells were removed by washing with Triton X-100 (1% v/v) in PBS containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride and 2 mmol/L *N*-ethylmaleimide) leaving the deposited matrix on the plastic surface. Wells were then washed with PBS and BSA (0.1%) to block non-specific adhesion before endothelial cells from wild-type mice were seeded in the presence of either Ca^{2+}/Mg^{2+} (1 mmol/L each) or EDTA (5 mmol/L). Adherent cells where washed with warm PBS, fixed by the addition of 70% ethanol and incubated with crystal violet (0.2%, 30 minutes). Samples were then incubated in ethanol overnight and adherence determined with an ELISA reader at 560 nm. In separate experiments the wells were coated with hrDMBT1, laminin or fibronectin and the adhesion of endothelial cells from wild-type and *dmbt1*$^{-/-}$ mice was analyzed..

Immunohistochemistry. *Skeletal muscle*: Cryo-sections were stained using muscles imbedded in Tissue Tek (Sakura, Heppenheim, Germany). After fixation in phosphate buffer (100 mmol/L, pH 7.3) containing 4% formalin samples were blocked with 5% goat serum and permeabilized with 0.5% Triton X-100 followed by incubation with lectin-B4-FITC and anti α-smooth muscle actin-Cy3.

Aorta: Perivascular tissue was removed; aortae were cut into rings and pinned with the endothelial cell surface facing upwards. The endothelial cells on one side of each preparation were removed by scraping and then washed carefully with sterile Hank´s solution. Thereafter, samples were fixed, permeabilized and incubated with antibodies as described.[4]

Retina: Frozen eyes from 5 day old mice were fixed in phosphate buffer (100 mmol/L, pH 7.3) containing 4% formalin (overnight 4°C). Thereafter, the retina was isolated, washed and permeabilized as described.[5] Endothelial cells were visualized using lectin-B4-FITC.

Endothelial cells: Human endothelial cells cultured on glass coverslips were fixed in 4% paraformaldehyde, permeabilized with Triton X-100 (0.03%) and incubated with appropriate

antibodies as outlined in the result section. The samples were viewed and images analyzed using a confocal microscope (LSM 510 META, Zeiss) and Axiovision software.

Immunoprecipitation and Immunoblotting. Endothelial cells were lysed and DMBT1 or galectin-3 were immunopreciptated with the appropriate antibodies. Triton X-100-soluble proteins or immunoprecipitates were heated with SDS-PAGE sample buffer, separated by SDS-PAGE and specific proteins were detected as described.[3]

Proliferation, Migration and Tube Formation Assays. Primary cultures of murine endothelial cells were seeded on 24 well plates (40000 cells/well) coated with fibronectin. After 72 and 96 hours culture in DMEM/F12 medium (Gibco/Invitrogen, Karlsruhe, Germany) with 20% FCS and 0.4% ECGS-H (Promocell, Heidelberg, Germany), the cells were trypsinized and counted (Casy 1 TT, Schärfe System, Reutlingen, Germany).

Endothelial cell migration was assessed using culture inserts with a 500 µm cell-free barrier (ibidi, Martinsried, Germany). Once cells reached confluence the FCS and ECGS-H content was reduced to 25% and the culture insert removed. Endothelial cell migration was monitored over 24 hours and the distance migrated was calculated using Axiovision software (Zeiss, Jena, Germany). It should be noted that the migration of murine lung endothelial cells was much slower than that of the human umbilical vein endothelial cells generally used in such assays.

Tube formation was assessed using primary cultures of murine endothelial cells seeded onto microscope slides (µ-angiogenesis; ibidi, Martinsried, Germany) coated with Matrigel. Cells were cultured in DMEM/F12 containing 25% of the usual concentration of FCS and ECGS-H and in the absence and presence of murine VEGF (50 ng/mL). Tube formation was analyzed after four hours.

Aortic Ring Assay. Aortae from wild-type and *dmbt1*$^{-/-}$ mice were removed, cleaned and embedded in a collagen gel (collagen type I, BD Biosciences, Heidelberg, Germany) in a 48 well plate containing MCDB 131 medium (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with murine serum (2.5%) from the donor animals. After 24 hours murine VEGF (30 ng/mL) was added and the tube-like structures allowed to develop over 7 days. Thereafter, the samples were fixed (4% paraformaldehyde) and endothelial cells were visualized using antibodies against CD31.

Hindlimb Ischemia. Hindlimb ischemia was induced as outlined [6]. Limb perfusion was measured with a laser Doppler imager every three to four days (Moor Instruments Ltd, Millwey, England). After 14 days the mice were killed and the M. adductor and M. semimembranosus were removed for analysis.

RT-qPCR. Total RNA was isolated from cultured mouse endothelial cells. Random hexanucleotide primers (Promega, Mannheim, Germany) were used for reverse transcription of the RNA. The cDNA was used for quantitative PCR using following primers: Notch 1 forward: 5´-CCTCAGATGGTGCTCTGATG-3´, reverse: 5´-CTCAGGTCAGGGAGAACTAC-3´; Dll1 forward: 5´-CATCCGATACCCAGGTTGTC-3´, reverse: 5´-ACGGCTTATGGTGAGTACAG-3´; Hes1 forward: 5´-GAGGCGAAGGGCAAGAATAAA-3´, reverse: 5´-GTGGACAGGAAGCGGGTCA-3´; Hey1 forward: 5´-TGAGCTGAGAAGGCTGGTAC-3´, reverse: 5´-ACCCCAAACTCCGATAGTC-3´. The primer for the transferrin receptor (Assay ID: Mm00441941_m1) and for dll4 (Assay ID: Mm00444619_m1) were from Applied Biosystems (Darmstadt, Germany). The transferrin receptor was used as reference gene.

ELISA for Binding of hrDMBT1 to EGF, VEGF and DLL4. Microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were coated with human recombinant EGF, VEGF or DLL4 in serial 1:2 dilutions in carbonate buffer overnight. After washing with Tris-buffered saline including 0.1% (v/v) Tween 20 (TBS-T), the plates were incubated with a 0.4 nmol/L solution of hrDMBT1 and 2 mmol/L Ca^{2+} for 40 minutes at room temperature. After washing with TBS-T the DMBT1-specific monoclonal mouse antibody Hyb213-06 (1:5000; Antibodyshop, Gentofte, Denmark) was added, followed by a horseradish peroxidase-conjugated anti-

mouse antibody (Chemicon International, Temecula, California, USA). The bound enzyme was detected by adding TMB-substrate solution (125 µg/mL 3,3`,5,5`-Tetramethyl-benzidine; 125 µg/mL (Sigma-Aldrich) in 0.1 mol/L citrate buffer pH 4.5 with 0.05 % (v/v) H_2O_2. After incubation for 20 minutes, the reaction was stopped with 2 mol/L HCl and the intensity of the dye reaction at 450 nm was analyzed with an ELISA reader. As control, wells without hrDMBT1 incubation were used.

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