CD82 Restraints Pathological Angiogenesis by Altering Lipid Raft Clustering and CD44 Trafficking in Endothelial Cells

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Background—Angiogenesis is crucial for many pathological processes and becomes a therapeutic strategy against diseases ranging from inflammation to cancer. The regulatory mechanism of angiogenesis remains unclear. Although tetraspanin CD82 is widely expressed in various endothelial cells (ECs), its vascular function is unknown.

Methods and Results—Angiogenesis was examined in Cdh2-null mice with in vivo and ex vivo morphogenesis assays. Cellular functions, molecular interactions, and signaling were analyzed in Cdh2-null ECs. Angiogenic responses to various stimuli became markedly increased upon Cdh2 ablation. Major changes in Cdh2-null ECs were enhanced migration and invasion, likely resulting from the upregulated expression of cell adhesion molecules such as CD44 and integrins at the cell surface and subsequently elevated outside-in signaling. Gangliosides, lipid raft clustering, and CD44-membrane microdomain interactions were increased in the plasma membrane of Cdh2-null ECs, leading to less clathrin-independent endocytosis and then more surface presence of CD44.

Conclusions—Our study reveals that CD82 restrains pathological angiogenesis by inhibiting EC movement, that lipid raft clustering and cell adhesion molecule trafficking modulate angiogenic potential, that transmembrane protein modulates lipid rafts, and that the perturbation of CD82-ganglioside-CD44 signaling attenuates pathological angiogenesis. (Circulation. 2014;130:1493-1504.)

Key Words: cell movement • endocytosis • membrane microdomains • neovascularization, pathological

Vascular morphogenesis includes vasculogenesis and angiogenesis. Both involve coordinated endothelial cell (EC) proliferation, EC migration, branching, and tube formation. Deregulated vascular morphogenesis and pathological angiogenesis contribute to the pathogenesis and progression of diseases ranging from cancer and macular degeneration to chronic inflammation. Growth factors promote neovascularization, whereas cell adhesion molecules (CAMs) are also crucial for vascular morphogenesis. More important, signals from growth factors and CAMs cross-talk during vascular morphogenesis. For example, integrin αvβ3 interacts with growth factor receptors and plays complex roles in angiogenesis.

Clinical Perspective on p 1504

Tetraspanins regulate cell adhesion, migration, fusion, and proliferation. Tetraspanin CD82 modulates immune cell activation and viral infection and suppresses tumor progression. In migrating cells, CD82 overexpression inhibits both protrusive and retractive cellular processes by disrupting actin reorganization. CD82 overexpression also alters cell adhesions. At the plasma membrane, CD82 interacts with membrane lipids such as GM2 and modulates membrane lipid composition. CD82 inhibits integrins, EGFR, c-Met, and urokinase-type plasminogen activator receptor and reduces downstream signaling of Src, p130Cas/Crk, Rho small GTPases, and β-catenin. However, how CD82 regulates cytoskeletal organization and membrane protein activities is still unclear.

CD82 is expressed in ECs and arteriolar smooth muscle cells, but whether it regulates vascular function remains
unknown. We found that pathological angiogenesis and EC movement were increased in Cd82 knockout (KO) mice, likely resulting from the upregulations of CAMs and their initiated signaling. Such upregulations were caused by sequential changes in gangliosides, lipid rafts, CAM-membrane microdomain interactions, and then CAM endocytosis. Hence, CD82 modulates CAM trafficking by preventing lipid raft aggregation and dissociating CAMs from lipid rafts, and CD82-ganglioside-CD44 signaling restrains angiogenesis by inhibiting EC adhesiveenes and motility.

Methods
Reagents, Polymerase Chain Reaction, and Cellular Function Assays
Detailed descriptions of reagents, polymerase chain reaction (PCR) analyses, and cell migration, invasion, sprouting, adhesion, proliferation, and survival assays are given in the online-only Data Supplement.

Mice and Cells
The establishment of the CD82-null mouse line, mouse genotyping strategy, and isolation of primary ECs are described in the online-only Data Supplement. Animal studies were performed with approval from the institutional animal care and use committees.

Angiogenesis Assays
Detailed descriptions of the in vivo Matrigel plug, tumor, retina, myocardial infarction (MI) angiogenesis, and ex vivo aortic ring angiogenesis are given in the online-only Data Supplement.

Confocal Microscopy, Fluorescence Resonance Energy Transfer, and Total Internal Reflection Fluorescence Microscopy
See the online-only Data Supplement for details.

Stochastic Optical Reconstruction Microscopy
ECs were plated on fibronectin-coated MatTek dishes for 24 hours and then incubated with Alexa 647-conjugated cholera toxin subunit B for 20 minutes on ice, washed, and fixed. Stochastic optical reconstruction microscopy imaging was performed as described previously. Briefly, image acquisition was performed on a Nikon Eclipse Ti microscope with a 150-mW, 647-nm laser in total internal reflection fluorescence mode on continuous illumination. Thirty thousand frames per image were collected at a rate of 50 Hz with a ×100 PlanApo 1.45NA Nikon objective projected on an Andor iXon DU897 electron-multiplying charge-coupled device camera. Single-molecule fitting and image rendering were performed with N-STORM software within NIS Elements (version AR 4.13.04) with a localization precision of ±40 nm.

Endocytosis Assay
See the online-only Data Supplement for details.

Statistical Analyses
Data are presented as mean±SEM or mean±SD and were analyzed with JMP pro 11 software (SAS Institute Inc, Cary, NC). The normality of data was examined before any test. For 2-group comparisons, 2-tailed, unpaired Student t tests were performed if samples exhibited normal distribution, and nonparametric Wilcoxon rank-sum tests were performed if samples were not normally distributed. For multiple-group comparison, Kruskal-Wallis tests followed by Dunn tests were performed if the Kruskal-Wallis test was significant. Differences are considered significant for values of P<0.05.

Results
Endothelial CD82 Regulates Vascular Morphogenesis
To confirm Cd82 expression in endothelium, we examined CD82 mRNA in murine primary ECs isolated from lung and liver by droplet digital PCR and found that Cd82 was expressed in these ECs. The level of CD82 mRNA was almost 3 times higher than the level of hypoxanthine-guanine phosphoribosyltransferase, a housekeeping gene (Figure IA in the online-only Data Supplement). In addition, we used flow cytometry to detect CD82 proteins at the surfaces of human dermal microvascular ECs, human umbilical vein ECs, and human retinal capillary ECs. We found that CD82 was expressed in these human ECs (Figure IB in the online-only Data Supplement).

Then, we examined CD82 vascular function using a gene ablation approach. The Cd82 KO mouse line was generated by the use of homologous recombination and Cre-LoxP deletion strategies (Figure IIA in the online-only Data Supplement). Because Cre is driven by human cytomegalovirus promoter and gene deletion occurs at all cells, the deletion of Cd82 gene was expected to be ubiquitous and was confirmed by Southern blotting and PCR at the DNA level and quantitative real-time PCR at the mRNA level in various tissues (Figure IIB and IIC in the online-only Data Supplement). For example, using quantitative real-time PCR, we found that the levels of the truncated CD82 mRNA in mouse lung or liver ECs (MLECs) from Cd82-null mice varied from 3% to 6% of the levels of the full-length CD82 mRNA from WT mice (Figure IID in the online-only Data Supplement). The Cd82-null mice are viable and fertile in C57BL/6 and FVB genetic backgrounds.

To determine the role of CD82 in vascular morphogenesis, we first performed Matrigel plug angiogenesis assay. The Matrigel plugs excised from Cd82-null mice exhibited a marked increase in neovascularization, as determined by immunofluorescent and immunohistochemical analyses of CD31 expression (Figure 1A). Vascular area and microvesSEL density increased by ≈100% and 130%, respectively, upon Cd82 ablation. More blood perfusion, which is correlated with functional vessel formation and evidenced by color and hemoglobin content of the Matrigel plugs, was found in Cd82-null mice compared with wild-type (WT) mice. Increased lumen formation was proportional to more vasculature in the Cd82-null group, suggesting that CD82 is dispensable for proper tubulogenesis. CD31 staining was always surrounded by and partially colocalized with the staining of the pericyte marker NG2, confirming that CD31 labels vasculature. Second, we examined tumor angiogenesis by subcutaneously implanting syngenic Lewis lung carcinoma cells. In the implanted tumor, angiogenesis was dramatically greater in Cd82-null mice than in WT mice (Figure 1B), as was tumor size.

For retinal angiogenesis at P18, by which active angiogenesis is largely completed, WT and Cd82-null mice displayed no obvious difference in forming superficial radial and collateral vessels, suggesting that CD82 is not required for the ultimate development of retina vessels (Figure IIIA in the online-only Data Supplement). For hyperoxia-induced ablation of retinal
blood vessels, which leads to an avascular area, no difference was observed between the WT and KO groups (Figure IIIB in the online-only Data Supplement). After mice are returned to normoxia, the surge of neovascularization resulting from the vessel ablation forms vessel tufts to alleviate ischemia. The tuft formation was profoundly higher in both number and area in \( \text{Cd82} \)-null mice than in WT mice (Figure 1C), further supporting that CD82 preferentially inhibits pathological retinal angiogenesis.

MI induces active angiogenesis during the acute phase. Angiogenesis in the MI regions of \( \text{Cd82} \)-null mice at 1 week after MI was apparently more pronounced than in the WT mice (Figure 1D). Importantly, the recovery of cardiac function, reflected by the ejection fraction of left ventricle, was also significantly better in \( \text{Cd82} \)-null than the WT group.

To substantiate these findings, we examined the ex vivo ability of the aortic artery to undergo angiogenesis. \( \text{Cd82} \)-null
aortic rings had greater microvascular sprouting, with the length and area of sprouted vessels increasing by ≈58% and 64%, respectively (Figure 1E). BS1-lectin staining confirmed that the sprouts were endothelial.

ECs assemble into capillary networks in 3-dimensional extracellular matrices, which mimics vasculogenesis. We found that the cable network formation was also markedly enhanced in Cd82-null MLECs after ECs were seeded in fibrin gel for 2, 8, and 24 hours (Figure 1F and Figure IV in the online-only Data Supplement). Time-lapse videomicroscopy revealed that EC migration leading to cable formation was increased in Cd82-null ECs compared with WT ECs (data not shown).

**CD82 Mainly Alters EC Migration and Invasion**

To address the cellular mechanism by which CD82 restrains pathological angiogenesis, we assessed the roles of CD82 in EC proliferation and survival. Cd82 removal slightly promoted EC proliferation and survival (Figure V in the online-only Data Supplement).

Because CD82 inhibits cell movement in cancer cells, we also examined cell movement. First, significantly greater migration was detected for Cd82-null ECs onto fibronectin or laminin 111, toward chemoattractants, than for WT ECs (Figure 2A). Second, in vitro invasion of ECs through Matrigel was also markedly greater in the Cd82-null than the WT group (Figure 2B). More important, the depth that newly formed vessels penetrate into Matrigel gel plug was much greater in Cd82-null than in the WT group (Figure 2B), suggesting that Cd82-null ECs also exhibited higher invasiveness in vivo. Third, the penetration distance of ECs from their coated beads in fibrin gel was largely enhanced on Cd82 silencing (Figure 2B). Because mouse ECs hardly attach to the beads, we performed this experiment with human umbilical vein ECs. Furthermore, markedly more Cd82-silenced ECs were found at the tips of endothelial sprouts originating from the beads coated with equal numbers of Cd82-silenced and nonsilenced ECs (Figure 2C), indicating that ECs gained invasiveness on CD82 reduction. Notably, changes in cell

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**Figure 2.** Motility and adhesiveness of wild-type (WT) and Cd82-null ECs. A, Cell migration. Mouse lung and liver endothelial cell (MLEC) migration onto fibronectin (FN; 10 μg/mL), laminin (LN) 111 (10 μg/mL), or hyaluronan (HA; 200 μg/mL) was assayed for 6 hours; the cells that migrated onto the bottom side of the filter were counted; and the data are reported as relative migration units. B, Cell invasion. For solitary invasion, MLECs that invaded through Matrigel (0.33 mg/mL) in Transwell Inserts were quantified. EC invasion in vivo was evaluated in Matrigel plug angiogenesis assay, and the distance of CD31 staining between the plug edge and vessel frontier was measured and presented as relative units. Scale bar, 0.1 mm. For collective invasion, human umbilical vein ECs (HUVECs) were coated on beads, placed in fibrin gel, and allowed to sprout in the presence of mitomycin for 48 hours. The average lengths of EC outgrowth from beads into fibrin gel were quantified. C, ECs at sprout tips. The siRNA-transfected, PKH26 dye–or PKH67 dye–labeled HUVECs were coated on beads and cultured in fibrin gel for 48 hours. Green or red ECs at sprout tips were quantified. Arrows indicate tip cells. D, Microextrusion morphogenesis. MLECs were immunofluorescently stained for CD31 or CD44 and photographed by confocal microscopy or total internal reflection fluorescence microscopy, respectively. The numbers of microextrusions per cell were counted, and the lengths of microextrusions were measured with Image J software. In each experiment, 10 ECs per group were quantified. Scale bars, 10 μmol/L. E, Cell-matrix adhesion. MLECs were seeded in the wells coated with FN (10 μg/mL), LN111 (20 μg/mL), or HA (200 μg/mL) in triplicate and incubated at 37°C in 5% CO₂ for 35 minutes. Nonadherent ECs were then removed by gentle washes, and the adhered ECs were counted and presented as relative adhesion units. Quantitative results are presented as means±SEM (n=3–5 independent experiments; *P<0.05; **P<0.01). KO indicates knockout.
migration and invasion are apparently much larger than those in cell proliferation and survival.

Because tetraspanins regulate microextrusion morphogenesis and microextrusions may modulate cell movement, we examined microextrusion in ECs. Compared with WT ECs, Cd82-null ECs formed more and developed longer CD31- and CD44-containing microextrusions (Figure 2D), supporting that CD82 inhibits microextrusion morphogenesis.

During retinal angiogenesis, tip cells and their filopodia were markedly increased at P5 in Cd82-null compared with WT retinas (Figure IIIC in the online-only Data Supplement), strongly suggesting that more invasive and robust vessels are developed during angiogenesis in Cd82-ablated mice.

Because CD82 associates with CAMs, we examined cell-matrix adhesion. Cd82-null MLECs showed marked increases in adhesion onto hyaluronan and laminin 111 but no change onto fibronectin (Figure 2E).

**CD82 Inhibits the Cell Surface Presence of Endothelial CAMs**

To determine how CD82 regulates EC motility and adhesiveness, we examined the effect of Cd82 ablation on EC surface expression of tetraspanins and CAMs by using flow cytometry. Tetraspanin CD9, integrin α6, integrin αV, and CD44 were upregulated in Cd82-null ECs, whereas others remained equivalent between 2 groups (Figure 3A). Because CD44 associates with tetraspanins and plays roles in EC motility and angiogenesis, we compared CD44 protein and mRNA levels in WT and Cd82-null ECs and found that total CD44 proteins became increased upon Cd82 ablation (Figure 3B) but

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**Figure 3.** CD82 regulates CD44 level and functions. A, Flow cytometric analysis of the surface levels of various cell adhesion molecules on mouse lung and liver endothelial cells (MLECs). B, Cell surface and total cellular CD44 proteins from MLECs were measured by flow cytometry and Western blot with CD44 monoclonal antibody (mAb) KM114, respectively. CD44 bands were quantified by densitometry and normalized to β-actin signals. C, CD44 and integrin α6 mRNAs were measured by quantitative real-time polymerase chain reaction. D, CD44 expression during angiogenesis. Cryosections of implanted Matrigel plugs from angiogenesis assay were costained with CD44 (red) and CD31 (green) mAbs. Scale bar, 100 μm. Aortic ring angiogenesis (E) and MLEC cable network formation in 3-dimensional matrix (F) were assayed with CD44 mAb KM114 or control IgG (20 μg/mL).  
EC networks were imaged at 24 hours after seeding. Scale bars, 0.2 mm. G, The effect of CD44 mAb KM114 (20 μg/mL) on MLEC migration onto fibronectin (FN; 10 μg/mL), laminin (LN) 111 (10 μg/mL), or hyaluronan (HA; 200 μg/mL). All quantitative data are presented as mean±SEM (n=4 independent experiments; *P<0.05; **P<0.01). KO indicates knockout; and WT, wild-type.
CD44 mRNA remained unchanged (Figure 3C). Similarly, the upregulated surface level of integrin α6 was not accompanied by an increase in integrin α6 mRNA level (Figure 3C). These observations suggest that the increases in CD44 and integrin α6 protein levels on Cd82 ablation were not a result of altered gene transcription but were due to changes in protein turnover. Such a conclusion is consistent with no correlations in gene expression between CD82 and CD44 or integrin α6 in endothelia, although a reverse correlation exists between CD82 and integrin αV genes (Figure VI in the online-only Data Supplement).

EC adhesion onto hyaluronan, a matrix ligand of CD44, was enhanced without CD82 (Figure 2E), reflecting an increase in functional CD44 proteins. In addition, CD44 was upregulated in the vessels of the Matrigel plug implanted in Cd82-null mice (Figure 3D). Moreover, CD44 monoclonal antibody reduced aortic ring angiogenesis and endothelial network formation of Cd82-null ECs to the WT level (Figure 3E and 3F and Figure VII in the online-only Data Supplement). Furthermore, CD44 monoclonal antibody inhibited EC migration and reduced the migration of Cd82-null ECs to the level of WT ECs (Figure 3G).

To substantiate this finding, we examined CD44 expression in MI-induced angiogenesis (Figure VIII A in the online-only Data Supplement). In heart tissue from normal rats, CD44 expression was negligible in capillary within myocardium, indicating that CD44 is minimally expressed in quiescent microvessels. At weeks 1 and 2 after MI, massive angiogenesis was accompanied by a drastic increase in endothelial CD44. At week 4 after MI, CD44 expression was significantly reduced in ECs within the MI areas that underwent fibrosis. Thus, CD44 expression in ECs is correlated with angiogenesis. The same conclusion can be reached during the angiogenesis after MI in WT and Cd82-null mice (Figure VIII B in the online-only Data Supplement).

CD82 Restrains Cytoskeletal Connection and Signaling of CAMs

The changes in CAMs drove us to investigate their cytoskeletal connection. In Cd82-null ECs, tetraspanins CD9 and CD81 were localized in focal complex–like structures at the cell periphery (Figure 4A). Integrin β1 exhibited unaltered distribution but formed more focal adhesions in CD82-null ECs (Figure 4B). Higher staining intensities of integrin α6 and CD44 were found in CD82-null ECs, but their global cellular distributions appeared to be unchanged (Figure 4B and 4C).

Focal adhesion formation and development were markedly enhanced in Cd82-null ECs on the basis of the staining...
of talin, a marker of nascent focal adhesion, and paxillin and vinculin, constituents of focal adhesion (Figure 4D).

FAK/Src-p130CAS/Crk signaling is regulated by CAMs and tetraspanins and controls cell movement. The protein level and autoactivation (pY397) of FAK remained unchanged, but full activation of FAK (pY577) was elevated in Cd82-null ECs (Figure 4E). Tyr410-phosphorylated p130CAS and Tyr106-phosphorylated c-Src were markedly greater on Cd82-null ECs, indicating an upregulated FAK/Src-p130CAS signaling axis.

PI3K-Akt signaling, denoted by Ser473-phosphorylated Akt, was upregulated in Cd82-null ECs (Figure 4F). ERM proteins (Figure 4G), which link tetraspanins and CD44 to actin cytoskeleton, and vascular endothelial growth factor receptor-2 (Figure 4H), which triggers angiogenic signaling, remained unaltered.

**CD82 Modulates CD44 Endocytosis and Lipid Raft Clustering**

Because tetraspanins regulate endocytosis, more CAMs at the Cd82-null EC surface may result from less endocytosis. Indeed, absolute amounts of the internalized CD9 and CD44 were lower in Cd82-null MLECs than in WT MLECs after 60 minutes of endocytosis (Figure 5A and Figure IXA in the online-only Data Supplement). After normalization to their levels at the cell surface, CD9 and CD44 internalization dropped further in the KO group. In contrast, the endocytosis of CD81 and integrin α5, the levels of which were not altered on Cd82 ablation, was equivalent between the 2 groups. Earlier studies showed that CD44 is internalized through clathrin-independent endocytosis pathway. Using GM1, a cargo of clathrin-independent endocytosis pathway route, as tracer, we found that most internalized CD44 proteins colocalized with GM1 in both groups after 2 and 5 minutes of endocytosis (Figure 5B and Figure IXB in the online-only Data Supplement), suggesting that Cd82 ablation did not alter the endocytosis route of CD44. However, CD44 and GM1 internalizations were substantially reduced after 5 minutes of endocytosis in Cd82-null ECs (Figure 5B and 5C), suggesting that clathrin-independent endocytosis of CD44 and this endocytic pathway per se were both inhibited without CD82.

Because CD82 overexpression alters the interaction between tetraspanin-enriched microdomains (TEMs) and lipid rafts, we analyzed the distributions of CD44, the TEM marker CD9, and the lipid raft marker GM1 at the EC basal surface with total internal reflection fluorescence microscopy. CD44 distributions were similar in a majority of ECs (Figure 6A) but displayed a clustered pattern in approximately one third of Cd82-null ECs (Figure 6A). CD9 exhibited similar staining characteristics at the basal surface.
between the 2 groups. Notably, GM1-positive lipid rafts were evenly distributed to a large extent in fixed WT cells but became clustered much more often to form small patches in fixed KO cells. Using super-resolution imaging, we confirmed that GM1 frequently formed clusters with sizes of \( \approx 30 \) to 50 nm at the basal surface of \( \text{Cd82} \)-null ECs. Normalized Ripley K function \([L(r)-r]\) determines whether a given pattern is clustered, random, or dispersed.\(^{25}\) Positive humps in the normalized curves indicated clustering over those distances. \( \text{Cd82} \)-null ECs displayed significant clustering of GM1 over short distances compared with WT cells. Latrunculin dispersed CD44 and GM1 clusters (Figure X in the online-only Data Supplement), suggesting actin involvement in clustering and excluding GM1 clusters as postfixation artifacts.

We then examined the colocalizations of CD44 with TEM and lipid raft markers at the basal section (an \( \approx 230 \)-nm thick focal plane) of fixed MLECs with confocal microscopy.\(^{26}\) We found that the fluorescence resonance energy transfer signal from C16Dil and C16DiO (Figure XII in the online-only Data Supplement) was drastically elevated in \( \text{Cd82} \)-null ECs and that such an elevation could be abrogated by the raft disupter

**Figure 6.** CD82 modulates cell adhesion molecule distribution at the endothelial cell (EC)–matrix interface and membrane microdomain organization. A, The distributions of CD44, CD9, and GM1 at the EC–matrix interface. Total internal reflection fluorescence microscopy analyses were performed on the fixed mouse lung and liver ECs (MLECs), which were stained with CD44 monoclonal antibody (mAb; KM114) and then FITC-conjugated secondary Ab, Alexa488-conjugated CD9 mAb (KMC8), or Alexa 488–conjugated cholera toxin subunit B (CTxB). Bar, 10 \( \mu \)m. Stochastic optical reconstruction microscopy analyses were performed on the fixed MLECs stained with Alexa 647–conjugated CTxB. Bar, 85 nm. \( L(r)-r \) curves for wild-type (WT; blue) and \( \text{Cd82} \)-null (green) GM1 staining were generated from the analysis of 14 fields for WT cells and 13 for \( \text{Cd82} \)-null cells, respectively. B, Colocalizations of CD44, CD9, and GM1 at the EC basal surface. MLECs were cultured in complete medium on fibronectin-coated coverslips for 2 days, fixed, permeabilized, and incubated with fluorochrome-conjugated CTxB, CD9 mAb, or CD44 mAb. Images of EC basal focal plane (\( \approx 230 \)-nm thickness) were captured with confocal microscopy. Scale bar, 10 \( \mu \)m. C, Fluorescence resonance energy transfer (FRET) analysis was performed in ECs, and relative FRET intensity was calculated as described in Methods. Some \( \text{Cd82} \)-null ECs were treated with filipin (5 \( \mu \)g/mL) at 37°C for 30 minutes before FRET analysis. D, Flow cytometry analyses of GMs, ceramide, and cholesterol at the MLEC surface using GM Abs, ceramide Ab, and filipin, respectively. E, MLECs were labeled with Sulfo-NHS-LC-Biotin and lysed in 1% Brijii-97. Immunoprecipitations (IPs) were performed with CD44 mAb (IM7), \( \beta_1 \) integrin mAb (9EG7), or CD9 mAb (KMC8). Identities of the cell surface proteins associated with integrin \( \beta_1 \) or CD9, marked as X or Y, remain unknown. F and G, In the presence or absence of filipin (10 \( \mu \)g/mL) or PDMP (50 \( \mu \)mol/L), aortic ring angiogenesis assay was performed in Matrigel (F), and Transwell cell migration was assayed onto fibronectin (G). Outgrowth lengths of the vascular sprouts from thoracic aortic rings were quantified, and the ECs migrated onto the bottom side were counted. Scale bar, 0.2 mm. Quantitative data are presented as means±SEM (n=3–5 independent experiments; *P<0.05; **P<0.01; ***P<0.001). KO indicates knockout.
filipin (Figure 6C), suggesting a higher order of lipid rafts in Cd82-null EC membrane. Consistently, the surface levels of ganglioside GM1, GM2, and GM3 were upregulated in Cd82-null ECs (Figure 6D). Ceramide and cholesterol levels remained unaltered.

Detergent solubility of CAMs and tetraspanin was reduced on Cd82 ablation even after cytoskeletal disruption (Figure XIII in the online-only Data Supplement), suggesting that CD82 mainly modulates membrane compartmentalization of these proteins (see the Results section in the online-only Data Supplement). Because in TEMs CD82 associates with tetraspanins and CAMs,9 we analyzed TEMs through immunoprecipitation profiles (Figure 6E). CD9 and CD9-associated unknown surface proteins (X1 and X2), not CD9-associated integrins and CD81, were increased in Cd82-null ECs, suggesting that the extra CD9 proteins at the EC surface are integrin and CD81 free. Levels and species of the surface proteins associated with β1 integrins and CD44 were generally unchanged on Cd82 ablation, despite CD44 upregulation. Thus, integrin-CD9 association and CD44 complex, not CD9-containing TEM, remain unaltered without CD82.

To confirm the roles of lipid raft clustering and ganglioside increase in angiogenesis, we examined the effects of filipin and PDMP, a glucosylceramide synthase inhibitor that blocks ganglioside formation, on aortic ring angiogenesis and EC motility. Filipin and PDMP diminished the difference in angiogenesis between the WT and KO groups and reduced Cd82-null EC cell motility to the WT level (Figure 6F and 6G).

CD82-Ganglioside-CD44 Signaling and Pathological Angiogenesis

Increased angiogenic potential in Cd82-null mice makes us question whether CD82 is downregulated during pathological neovascularization. Indeed, vascular expression of CD82 was largely diminished in proliferative diabetic retinas, which is caused by profound angiogenesis, compared with CD82 expression in the vessels of normal retina (Figure XIV in the online-only Data Supplement). Moreover, we compared vascular expressions of CD82 in human normal breast and breast cancer tissues using tissue microarray and found that CD82 proteins in the blood vessels within invasive breast cancer were reduced compared with those in normal breast tissue (Figure 7A, arrowheads). However, vascular CD44 exhibited a converse pattern, indicating that reduced CD82 and enhanced CD44 coexist in human tumor angiogenesis.

The same changes in CD82 and CD44 expression were also observed from epithelial to tumor cells.

Our study reveals that CD82-ganglioside-CD44 signaling is connected to angiogenic potential (Figure 7B). We perturbed this signaling in vivo to confirm its importance. With

### Table. Colocalization of CD44, CD9, and GM1 at and Near the EC Basal Surface

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<th>Pearson Correlation Coefficient</th>
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Values are mean±SD; n=3–4. EC indicates endothelial cell; KO, knockout; and WT, wild-type.

*P<0.05; †: P<0.01.
administration of PDMP or CD44 shRNA in Matrigel plug angiogenesis assay, the increased angiogenesis in Cdh2-null group was reduced to the levels of WT group (Figure 7B), indicating that increased gangliosides and CD44 are essential for the enhanced angiogenesis in vivo on Cdh2 ablation.

**Discussion**

Here, we identified the confinements of EC movement and angiogenic potential as novel functions for CD82, demonstrated that lipid raft clustering and CAM trafficking modulate angiogenic potential, and revealed a novel mechanistic paradigm that membrane glycosphingolipids tune angiogenic potential through altering CAMs at the plasma membrane. Because no gross vascular abnormality was observed after development, CD82 likely restrains EC movement to suppress angiogenesis under pathological conditions and prevents excessive vascular morphogenesis under physiological conditions. In addition, our study conceptualizes the schemes that membrane protein regulates lipid rafts and that membrane compartmentalization of CAMs modulates EC movement. To inhibit EC movement, CD82 likely enhances CAM endocytosis by changing CAM-membrane microdomain interactions. Without CD82, higher magnitudes of CAMs and CAM-initiated signaling at the EC surface more efficiently induce focal adhesive structures and microextrusions, drive EC movement, and subsequently facilitate pathological angiogenesis (Figure XV in the online-only Data Supplement).

**CD82 Confines Angiogenesis Mainly by Restraining EC Movement**

Although CD82 is expressed in ECs and Cdh2-null mice exhibit elevated vascular morphogenic potential, these mice do not display obvious vascular defects, suggesting that CD82 is not essential for physiological vessel development. However, it is unlikely to have developmental vascular defects when vascular morphogenic potential is above normal. In addition, increased vascular morphogenesis is not needed for normal development of animals. Increased pathological angiogenesis in Cdh2-null mice underlines that loss of CD82 function cannot be mitigated or compensated for after development and that CD82 inhibits molecular and cellular events unique to or critical for pathological angiogenesis, which is distinct from physiological angiogenesis.28 Our observations highlight that pathological angiogenesis lacks an efficient regulatory mechanism for EC movement, which is the main cellular event that CD82 controls during angiogenesis, and suggest that downregulating EC movement serves a therapeutic strategy selectively against pathological angiogenesis.

Pathological angiogenesis is characterized by its morphogenic simplicity.29 In Cdh2-null mice, the increased angiogenic potential is associated with more rapid and efficient formation of capillaries, driven mainly by enhanced EC movement. In other words, the relative simplistic morphogenic program of pathological angiogenesis depends more on EC movement than significantly more complex physiological angiogenesis does. Notably, angiogenesis without CD82 is functional, as evidenced by better blood-perfused Matrigel plug, larger tumor, more perfused vascular tufts, and stronger cardiac function after MI in Cdh2-null mice.

**CD82 Restrains EC Movement by Inhibiting CAMs**

CD82-dependent alteration in cell adhesion is likely to be directly responsible for the change in movement. For example, enhanced adhesion onto hyaluronan promotes EC infiltration in interstitial tissue, whereas upregulated FAK/Src-p130CAS and Akt activities serve as promigratory signaling in Cdh2-null ECs.

Tetraspanins preferentially associate with laminin-binding integrins like α6 integrins. The upregulated α6 integrins correlate with the enhanced Cdh2-null EC adhesion onto laminin 111. Interestingly, the upregulated αv integrins and unchanged α5β1 integrin, both RGD-binding integrins, did not enhance Cdh2-null EC adhesion onto fibronectin. Hence, whether CD82 affects integrin activation remains to be determined. Alternatively, the upregulated α6 and αv integrins may contribute to other activities such as Notch and Netrin signaling.30,31 To visualize how CD82, CD44, and integrin are related at the molecular level, we cross-referenced the top 20 genes most frequently cotranscribed with CD82 with their known protein-protein interactions (Figure XVI in the online-only Data Supplement). Most of the 4 known protein-protein interactions shared by CD82 and CD44 are associated with cell migration and angiogenesis, suggesting potential genetic partners by which the 2 may exert their phenotype-altering influence.

CD44 and tetraspanins activate multiple signaling pathways.8,32 FAK/Src-p130CAS and Akt signaling affects cell adhesion and movement and is altered on CD82 overexpression.8,9 CD44 partitions to lipid rafts and associates with Src through its cytoplasmic domain.33,34 The increased FAK/Src-p130CAS activity in Cdh2-null ECs likely results from the elevated levels or altered microdomain coexistences of CAMs like CD44 at the plasma membrane. Higher Akt activity could also be caused by more surface CAMs because hyaluronan-CD44 binding activates PI-3 kinase–Akt signaling.32 Src is likely situated between CD44 and Akt because Src indirectly activates PI-3 kinase and Akt during angiogenesis,35 and Src maximally activates FAK through FAK-Y577 phosphorylation. We propose that CD82 transdominantly inhibits CD44 and its downstream signaling by modulating the membrane microdomain coexistence of CD44 to confine EC-hyaluronan adhesion. Consistently, CD44 has proangiogenic properties, evidenced by the observations that vascular morphogenesis becomes attenuated in Cdh4-null mice, CD44 antibodies inhibit EC proliferation and vascular morphogenesis, and CD44v6 serves as a coreceptor for c-Met and vascular endothelial growth factor receptor-2 in ECs during angiogenesis.19,22,36–38 Our study further revealed how CD44 promotes angiogenesis at the molecular level.

**CD82 Inhibits CAMs by Altering the Microdomain Coalescence and Then Endocytosis of CAMs**

CD44 perturbation reduced the enhanced migration and angiogenesis of the Cdh2-null group to the levels of WT, supporting the notion that CD44 has immediate and major functional connections to CD82. Reductions were also found in the WT group, suggesting that CD44 also controls these events at physiological conditions.

The increased expression of CD44, integrin α6, CD9, and gangliosides at Cdh2-null EC surface likely results from their
decreased turnover at the plasma membrane. Less endocytosis of CD44 apparently causes more CD44 at the CD82-null EC surface. Because cholesterol is important for clathrin-independent/CLIC endocytic pathway,39,40 CD82 modulates this pathway probably by reorganizing saturated lipids into or between membrane microdomains.13 Increased gangliosides in CD82-null EC plasma membrane likely facilitate lipid raft clustering in a cholesterol-dependent manner, as found in the model membrane,41 and coalescence of CD44 to TEMs and clustering in a cholesterol-dependent manner, as found in the CD44 and gangliosides.11,42 We predict that microdomain reorganization leads to less endocytosis of microdomain residents such as CD44, CD9, and gangliosides.

Stronger cell-matrix adhesiveness and greater focal adhesive structures in Cd82-null ECs reflect a robust membrane-cytoskeleton connection, which may reduce CAM endocytosis. However, increased interactions of CD44 with lipid rafts and TEMs likely play more dominant roles in its trafficking because CD44 is internalized through a clathrin-independent, raft-dependent pathway.23,24

**CD82 Is a Lipid Raft Organizer**

Robust microextrusions in CD82-null ECs likely result from the reorganized membrane microdomains and contribute to active motile behaviors and strong adhesiveness during angiogenesis, given that microextrusions may modulate cell adhesion and movement.18

Tetraspanins had not been found in focal complex and adhesion,1 the membrane microdomains of cytoskeleton-connected CAM clusters. Upon Cd82 ablation, redistribution of tetraspanins/TEMs to focal complexes further corroborates CD82 as a membrane domain organizer. Because TEMs contain various CAMs, such redistribution suggests greater clustering of CAMs and stronger EC adhesion strengthening.

**CD82-Ganglioside-CD44 Signaling in Angiogenesis**

Ganglioside build up on Cd82 removal and subsequent lipid raft clustering upregulate CD44 in pathological angiogenesis, which can be attenuated by inhibiting CD82-ganglioside-CD44 signaling. Although filipin could exert a broader effect on cells, the notion that filipin inhibits angiogenesis by disrupting lipid rafts is supported by the effects of ganglioside reduction and CD44 blockade.

Our study revealed that the membrane microdomain landscape plays a key role in pathological angiogenesis and delineated that CD82 modulates CAM trafficking and then surface expression by altering lipid rafts clustering (Figure XIV in the online-only Data Supplement). Importantly, we first demonstrated that CD82 protein drives lipid raft reorganization. Because angiogenesis is linked to many diseases, our observations have far-reaching implications. Future studies will determine how CD82 alters membrane lipids and evaluate the therapeutic potentials of CD82-ganglioside-CD44 signaling against pathological angiogenesis. Studies on CD82 and tumor progression have so far focused on the metastasis-suppressive effect that CD82 exerts directly on tumor cells.9 Given that endothelial CD82 inhibits tumor angiogenesis, CD82 can be a drug candidate with dual benefits against tumor progression.

**Acknowledgments**

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**Disclosures**

None.

**References**

Angiogenesis is fundamentally important for the pathogenesis and progression of various diseases, including cardiovascular diseases such as myocardial infarction and stroke. Selective, efficient, and persistent perturbation of angiogenesis for the purposes of disease treatment is still beyond our reach. The limited efficacy of the angiogenesis therapy based on vascular endothelial growth factor and fibroblast growth factor antagonism highlights that the mechanism of pathological angiogenesis is unique. In addition to unveiling the inhibitory roles of tetraspanin CD82 in endothelial cell movement and pathological angiogenesis, our study reveals a novel angiogenesis-regulatory mechanism by which membrane glycosphingolipids and their derived lipid rafts in endothelial cells modulate angiogenic potential. Our study also presents CD82-ganglioside-CD44 signaling as a potential therapeutic target against angiogenesis. More important, our findings provide a novel strategy to intervene angiogenesis under pathological conditions, that is, the reorganization of membrane microdomains at the endothelial cell surface. Together with earlier observations of tetraspanin CD151, this study of tetraspanin CD82 supports an emerging notion that tetraspanins could be clinically beneficial through upregulation or downregulation of vascular functions such as endothelial cell movement and angiogenesis.
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CD82 Restrains Angiogenesis by Altering Lipid Raft Clustering and CD44 Trafficking in Endothelial Cells

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Supplemental Inventory

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Detergent solubility of CD44, integrin β1, and CD9 in WT and *Cd82-null* ECs.

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2. Supplemental Methods

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1. Supplemental Figures

Figure S1 CD82 expression in ECs. (A) CD82 mRNA expression in murine ECs was examined by droplet digital PCR. The mRNA levels of CD82 and house-keeping gene *HGPRT* are presented as the mRNA copy numbers in 20-µl of droplet digital PCR reaction mixture. (B) The cell surface expression of CD82 proteins in various human ECs was examined by flow cytometry. Red curve: mouse IgG as negative control, blue curve: human CD82 mAb TS82b, and orange curve: human CD44 mAb G44-26 as positive control.

Figure S2 Establishment of *CD82-null* mice. (A) Generation of KO allele by homologous recombination and Cre-Lox P deletion. The exon/intron structure of *Cd82* gene is shown at the top. The floxed allele was generated with the targeting vector that contains a *Neo* cassette, exons 5 and 6, and two flanked *Lox* P sites. The KO allele resulted from the removal of exons 5 and 6 from the floxed allele by Cre recombinase. The localizations of the genotyping primers P1, P2, and P3 at genomic DNA are also shown. (B) PCR-based genotyping. The results from a PCR experiment are shown after agarose electrophoresis. These primers amplify only a 673 bp-segment in the WT allele, only a 502 bp-segment in the KO allele, and both segments in the heterozygous allele. (C) Southern blot. The DNA extracted from mouse spleen, T cells, B cells, and dendritic cells were digested and then hybridized with a CD82 probe that covers exons 5 and 6 and a β2-microglobulin probe. β2-microglobulin DNAs are served as an internal control. (D) The levels of CD82 mRNA sequence in MLECs from WT and *Cd82-null* mice were examined by qRT-PCR. The first pair of primers is located at the region encoded by exons 2 and 3, and the second pair of primers is located at the region corresponding to exons 7 and 8. Shown are the averages of the results from both qRT-PCRs.

Figure S3 Retinal angiogenesis. Fluorescence angiography was performed as described in “Materials and Methods”. Angiography images were captured with fluorescent microscopy. (A) The retinas at P18 were separated and flat-mounted. Shown are the representative photos of the
retinal angiogenesis under normoxia. Scale bar, 250 µm. (B) Quantification of the avascular areas of retinas, caused by hyperoxia, in oxygen-induced retinopathy assay (mean±SEM; n=4 experiments, 3 retinas from each group were examined in each individual experiments). (C) Angiography images of retinas at P5 were captured with fluorescent microscopy. The number of tip cells per mm of vascular periphery and the number of filopodia per cell were counted. For tip cells, ten mice from each group were examined, and for filopodia, two dozens of cells were quantified in each group. Scale bar, 50 µm.

**Figure S4** *In vitro vasculogenesis*. MLECs were seeded in fibrin gel and photographed at 2 h and 8 h. Scale bar, 0.2 mm. Images show the representative experiment derived from the results of 3~5 independent experiments.

**Figure S5** The effect of *Cd82*-ablation on EC proliferation and survival. (A) Cell proliferation. MLECs were seeded onto the 96-well plates coated with gelatin at 3×10³ cells per 200 µl medium per well for 24, 48, and 72 h. Cell proliferation was measured with the MTT colorimetric assay by using the absorbance at 490 nm as the readout. (B) Cell survival. MLECs were seeded and cultured as described above, viable cells were determined using the trypan blue staining assay, and the results were processed and presented as described above. The quantitative data in this Figure are presented as mean±SEM and were obtained from 5 independent experiments. *: P<0.05.

**Figure S6** Gene expression correlation in ECs between CD82 and CD9, CD44, integrin α6, and integrin αV. A total of 139 publicly available microarray experiments for endothelium were obtained from NCBI’s GEO database. Experiments were included only if both probes being compared were present. To enable direct comparison of cross-platform values, data were quantile normalized to range from 1-10,000 as described previouslyżą.
Figure S7  The early phase effect of CD44 Ab on EC cable network formation. MLECs were seeded on Matrigel at the concentration of $7.5 \times 10^4$ cells per well, together with CD44 mAb KM114 or control Ig (20 ug/ml). The images were acquired at 2 and 8 h after seeding. Scale bar, 0.2 mm.

Figure S8  Changes of CD44 expression in the angiogenesis induced by myocardial infarction. Heart tissue sections from normal and myocardial infarcted rats (A) or from WT and Cd82-null mice (B) were stained immunofluorescently for CD31 and CD44, and the images were acquired by confocal microscopy. Scale bars 50µm

Figure S9  Endocytosis of CD44 and CD9 (A) CD44 and CD9 endocytosis were examined for 1 h by Ab uptake assay using CD44 mAb(KM114) and CD9 mAb(KMC8), respectively. Unlike the endocytosis was quantified as the number of fluorescent intracellular vesicle in Figure 5A, the endocytosis presented herein was quantified as the internalized fluorescent intensity. (B) Clathrin-independent endocytosis of CD44 was examined in MLECs for 2 and 5 min with Alexa594-conjugated CD44 mAb. Images of internalized CD44 were captured by confocal microscopy. Scale bar, 10µm.

Figure S10  The effect of latrunculin on CD44 and GM1 distribution at the EC basal surface. ECs were treated with latrunculin B (1 µM) at 37°C for 30 min, stained for CD44 and GM1, and analyzed with TIRFM. Scale bar, 10µm.

Figure S11  The role of CD82 in flotillin distribution at the EC basal surface. (A) The CD44-flotillin co-localization at the EC basal surface. MLECs were cultured in complete medium on FN-coated coverslips for 2 days, fixed, permeabilized, and incubated with the fluorochrome-conjugated CD44 and flotillin Abs. The images of EC basal focal plane (~230 nm thickness) were captured with confocal microscopy. Bar, 10 µm. (B) The distribution of flotillin at the EC–matrix interface. TIRFM
analysis was performed on the fixed MLECs, which were stained with flotillin Ab and then FITC-conjugated 2nd Ab. Bar, 10 µm.

**Figure S12** The C$_{16}$DiO:C$_{16}$DiL FRET analysis was performed in ECs as described in “Materials and Methods” section. Images were acquired by using a confocal microscope. Bar, 10µm.

**Figure S13** Detergent solubility of CD44, integrin β1, and CD9 in WT and **Cd82-null** ECs. MLEC cells were lysed with 0.03% Triton X-100 lysis buffer in cell culture dishes. The soluble and insoluble fractions were separated by SDS-PAGE, followed by immunoblotting with CD44 mAb (KM114), β1 integrin mAb(9EG7), or CD9 mAb(KMC8). β-actin served as protein loading control. Band densities were quantified by densitometry and normalized with the ones of β-actin. *, P<0.05. To disrupt actin polymerization, cells were treated with latrunculin B (1 µM) at 37°C for 30 min before cell lysis.

**Figure S14** Immunohistochemical analysis of CD82 expression in the blood vessels of human normal and proliferative diabetic retinas. Retina tissue sections were stained with CD82 mAb (TS82b) (brown) and hematoxylin (blue). Light microscopic images show the GCL (ganglion cell layer), IPL (inner plexiform layer), and INL (inner nuclear layer) of retinas. The retina sections were obtained from nondiabetic human donors and type 2 diabetic donors with proliferative retinopathy. Profound neovascularization exists in the GCL and IPL layers of proliferative diabetic retina. Arrows indicate the examples of vessels. Scale bar, 50µm

**Figure S15** The schematic diagram of the molecular and cellular mechanisms by which CD82 trans-dominantly inhibits angiogenic potential.

**Figure S16** A molecular perspective on the relations between CD82, CD44 and integrin α6 (yellow nodes). A transcriptional network was constructed around CD82 as described previously$^2$ to
identify genes highly correlated in their expression (green) with CD82. Known protein-protein interactions (gray) connecting at least two correlated genes were identified and extracted from HPRD\textsuperscript{3}, and visualized in this network. CD82 and CD44 are known to interact physically with four other proteins, and share a number of indirect connections to several of Src kinases.
2. Supplemental Methods

Reagents

Matrigel, murine CD9, CD44, integrin α5, integrin β1, integrin αV, CD31, and ICAM-2 monoclonal antibodies (mAbs), ERK, FAK, and p130<sup>CAS</sup> mAbs, and PE-conjugated murine CD44, PE-conjugated rat CD31, and FITC-conjugated rat CD44 mAbs were purchased from BD Biosciences (San Diego, CA). Abs against eNOS, p-eNOS, p-ERK, and p-SRC were obtained from Cell Signaling Technology (Beverly, MA). Murine CD81 (EAT-2) mAb was kindly provided from Dr. Shoshana Levy. FAK phosphotyrosine (pY<sup>397</sup> and pY<sup>577</sup>) Abs were from Biosource International (Camarillo, CA). Integrin α6, phosphotyrosine, c-Src, β-actin, and β-tubulin Abs and CD44 and control shRNA lentiviruses were from Santa Cruz Biotechnology (Santa Cruz, CA). NG2 Ab was from Millipore (Billerica, MA). Murine integrin α3 Ab, recombinant human basic FGF (bFGF), and mouse VEGF164 were from R&D systems (Minneapolis, MN). Alexa Fluor 488-conjugated mouse CD9 mAb was from Serotec (Raleigh, NC). Anti-mouse and -rabbit IgG secondary Abs, Alexa Fluor 488- or 594-conjugated phalloidin, cholera toxin B subunit, or isolectin Griffonia simplicifolia (GS)-IB<sub>4</sub>, anti-rat IgG conjugated Dynabeads, human plasma fibronectin (FN), and mouse laminin (LN) 111 were obtained from Invitrogen (Carlsbad, CA). FITC-conjugated anti-rat IgG or BS1-lectin, fibrinogen, latrunculin, filipin, thrombin, aprotinin, and dextran-coated Cytodex 3 microcarriers were from Sigma-Aldrich (St. Louis, MO), Horseradish peroxidase (HRP)-conjugated anti-hamster, anti-mouse, and anti-rabbit IgG were obtained from GE Healthcare (Uppsala, Sweden). GM3 mAb (Clone: M2590) was purchased from Nippon Biotest Laboratories, Inc. (Tokyo, Japan). Type I collagenase was from Worthington Biochemical Corp. (Lakewood, NJ). EC mitogen was from Biomedical Technologies (Stoughton, MD). PDMP (D-threo-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol·HCl) was obtained from Matreya LLC (Pleasant Gap, PA). Tissue microarrays of human breast cancer were purchased from US Biomax, Inc. (Rockville, MD). Hyaluronic acid sodium salt from bovine vitreous humor, heparin, methyl thiazolyl tetrazolium (MTT), and other chemicals and reagents were obtained from Sigma-Aldrich unless otherwise indicated.
Experimental animals and cells

By the homologous recombination-based gene targeting, Cd82 was partially deleted from mice after crossing the Lox P-bearing mice (Figure S2) with the CMV promoter-driven Cre deleter mice. The deletion includes the fifth and sixth exons of 10 exons of Cd82, which encode 67 amino acid residues (from 46th to 112th) of the entire 266-residue CD82 peptide sequence (Figure S2). These residues cover the C-terminal 9 residues of the small or 1st extracellular loop, 2nd transmembrane domain, intracellular loop, 3rd transmembrane domain, and N-terminal 4 residues of the large or 2nd extracellular loop. Cd82 deletion at the genomic level was verified by Southern blot and PCR analyses.

The mouse genotyping analyses were performed by PCR with the strategy exhibited in Figure S2. A common reverse primer (primer 3) has the sequence 5′-TAACTAGCATTGCTTTCCCCTGT-3′ and is homologous to a far downstream sequence of exon 5 (Figure S2). This primer was used in conjunction with two different forward primers designed to distinguish WT and Cd82-null alleles (Figure S2). The forward primers include primer 1 (5′-GAGAGAGGGCTAGAGCTTGAGATG-3′), which is homologous to a sequence immediate downstream of exon 5 and was used to detect WT alleles, and primer 2 (5′-CTGCAGGAATTCGATATCAAGC-3′), which is homologous to a sequence within Lox P and was used to detect Cd82-null alleles.

Cd82-null mice were backcrossed more than 6 generations into the C57BL/6 NCI mouse strain. In all experiments, 8~12 week old Cd82-null mice were compared with littermates of the same sex. Animal studies were performed with the approval from the institutional animal care and use committee.

Female rats of SD strain were obtained from Harlan (Indianapolis, IN).

All procedures involving animals were performed according to protocols approved by our Institutional Committees for Use and Care of Laboratory Animals.
Mouse lung or liver endothelial cells (MLECs) were isolated as described. Briefly, ECs were collected from collagenase-digested lung or liver tissue by murine CD31 mAb-coated Dynabeads and enriched by murine ICAM-2 mAb to more than 90% of purity. The isolated MLECs were cultured in MDCB131 media (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml EC mitogen, and 10 units/ml heparin and used between passages one and three.

Human umbilical vein cells (HUVECs) were cultured in DMEM/F12K media (Invitrogen) supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 1mM sodium pyruvate, 10 units/ml heparin, and 10 μg/ml bovine brain extract. CD82 was silenced by a pool of 3 siRNAs (Santa Cruz Biotechnology), which include siRNA 1, sense: GGGCCCUCUUCUACUCAAtt, anti-sense: UUGAAGUAGAAGAGGGCCCtt; siRNA 2, sense: GCCCUCUUCUACUUCACAtt, anti-sense: UGUUGAAGUAGAAGGGCtt; and siRNA 3, sense: CAAGGGUGUAGUUAUGUAtt, anti-sense: UACAAUAUACACACCCUUGtt. HUVECs were transfected by either CD82 siRNAs or control siRNA with Lipofectamine RNAiMax (Invitrogen) and used for experiments at 48 hr after transfection.

Quantitative real-time PCR (qRT-PCR) and droplet digital PCR (dd-PCR)

For qRT-PCR, total RNA was extracted from MLEC cells in triplicates using RNase Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The RNA concentration was determined by measuring the absorbance at 280 nm using a NanoDrop ND-1000 spectrophotometer. Total mRNA was reverse-transcribed into cDNA using a Taqman Reverse Transcription Reagent Kit (Applied Biosystems, Carlsbad, CA). RT-PCR was performed on a cDNA template with a Roche Light Cycler instrument according to the manufacturer’s instructions. Briefly, each 10μl reaction contained 2ng cDNA, 5μl Light Cycler Probe Master (Roche), 100 nmol/L forward and reverse primers, 100nmol/L probe, and nuclease free H₂O in an individual well of a 96-well plate. The following conditions were used to run the PCR amplification process: denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 10 seconds, 30 seconds for annealing at 60°C and 10
seconds for extension at 72°C. Data analyses were performed using the Roche 480 Sequence Detection Software (Applied Biosystems). Data were normalized to endogenous actin and fold changes in gene expression were calculated using the comparative CT ($2^{-\Delta\Delta Ct}$) method.

For dd-PCR, total RNA preparation and reverse-transcription were performed as described above. The dd-PCR reaction mixture contained 2×ddPCR Supermix (Bio-Rad), primers (100nM), probe (100nM), and cDNA. Each 20 µL ddPCR reaction mixture was loaded into a sample well of an eight-channel droplet generator cartridge (Bio-Rad). Sixty µL of droplet generation oil (Bio-Rad) were loaded into each oil well through each channel of the cartridge. The cartridge was then placed into QX100 Droplet Generator (Bio-Rad) to generate an emulsion of monodispersed droplets, which were transferred to a 96-well PCR plate by using a multichannel pipet. The plate was heat-sealed with a foil seal and placed on a thermal cycler for PCR reaction in total of 40 cycles. After the PCR reaction, the fluorescence intensity of the droplets was measured by QX100 Droplet Reader (Bio-Rad), which automatically calculates absolute copy number from each well of the 96-well PCR plate.

**In vivo Matrigel plug angiogenesis assay**

Eight-week-old sex-matched WT and Cd82-null mice were injected subcutaneously at the abdominal midline with 0.5 mL Matrigel (10 mg/mL) containing 150 ng basic fibroblast growth factor (bFGF)\(^5\). After 7 days, the mice were sacrificed, the Matrigel plugs were isolated, and the ECs of blood vessels in the Matrigel were detected by using immunohistochemistry and immunofluorescence with the murine CD31 monoclonal Ab (mAb). For the immunohistochemistry experiments, the extent of angiogenesis was quantified as the blood vessel density by visually counting the total number of CD31-positive vessels per microscopic field. For the immunofluorescence experiments, the angiogenesis level was quantified as the blood vessel area by using ImageJ software to measure the total area of CD31-positive staining per microscopic field\(^6\) and was represented as a ratio relative to the total area of CD31-positive cells in the WT group. The quantification was performed in a double-blinded fashion.
In vivo tumor angiogenesis assay

Eight-week old, sex-matched WT and Cd82 KO mice were injected subcutaneously with Lewis lung carcinoma (LLC) cells (2×10^6 cells/mouse). Tumors were measured every 6 days by using Vernier calipers, and tumor volume was calculated (length × width^2 × 0.50). After 16 days, tumors were harvested, weighed, and frozen. The tissues were cryosectioned and stained with CD31 Ab for immunofluorescence analysis as described above.

In vivo retinal angiogenesis assay

The ischemia-induced retinal neovascularization⁷ was examined using C57BL/6 NCI mouse littermates at postnatal day 7 (P7). The pups with their mothers were exposed to 75% ± 2% oxygen (hyperoxia) for 5 days and then returned to room air (normoxia) for 5 days. The mice produce retinal ischemia and undergo neovascularization by P17. The same sex littermates kept in room air were used as normal control subjects.

Fluorescence angiography was performed at P18. Fluorescein isothiocyanate (FITC)-conjugated high molecular weight dextran (2%; 10 mL/kg) was intracardially injected into the anesthetized mice. Eyes were dissected and fixed with 4% paraformaldehyde in Hanks’ balanced saline prepared immediately before use for overnight at 4°C. The retinas were separated and flat-mounted. Angiography images were visualized using a fluorescent laser microscope (FV1000; Olympus, Tokyo, Japan).

For the analysis of retina vascular sprouting at P5, mouse eyes were collected and fixed in 4% paraformaldehyde at 4°C overnight. After the eyes were washed with PBS, the retinas were dissected and partially cut in four quadrants. After blocking and permeabilization in 1%BSA/0.3% Triton at 4°C overnight, the retinas were washed three times in Pblec buffer (1% Triton X-100, 1mM CaCl₂, 1mM MgCl₂, and 1mM MnCl₂ in PBS, pH 6.8) and then further incubated overnight in the Pblec buffer
containing Alexa488-conjugated isolectin GS-IB4, followed by four washes with 1% BSA/0.3% Triton solution and flat-mounting. Photos were captured as described above.

**In vivo myocardial infarction (MI)-induced angiogenesis assay and MI analysis**

For rat MI model, left ventricular anterior transmural MI was created in 8-week-old female SD rats by permanent ligation of the left coronary artery with silk ligature. Rats were anaesthetized, intubated, and ventilated with a rodent mini-respirator (Harvard Apparatus, Hollston, MA). After left thoracotomy, the heart was exposed and 7-0 silk suture placed around the left coronary artery. The vessel was ligated, which resulted in 40–45% left ventricular infarction, the chest closed, and lungs re-inflated using positive-end expiratory pressure. Animals were sacrificed at days 7, 14, and 28 following surgery (n=4/time point).

For mouse MI model, 8~12-week-old male mice were anesthetized by intramuscular injection of ketamine (100 mg/kg) and xylazine (5 mg/kg), placed in a supine position on a heating pad (37°C), intubated with a 19G stump needle, and ventilated with a rodent mini-respirator. Left thoracotomy was performed between the fourth and fifth ribs, and the left anterior descending artery was visualized under a microscope and ligated by using a 6-0 prolene suture. Regional ischemia was confirmed by electrocardiogram. One week after MI surgery, cardiac functions were evaluated by two-dimensional, transthoracic echocardiography with Vevo2100 ultrasound instrument (Visual Sonics, Canada) on conscious mice. M-mode tracings were used to measure anterior and posterior wall thicknesses at end diastole and end systole. Left ventricular internal diameter (LVID) was measured as the largest anteroposterior diameter in either diastole (LVIDd) or systole (LVIDs). Ejection fraction was calculated by (end diastolic volume-end systolic volume)/end diastolic volume. Results were presented as mean±SEM (n=8 mice from each group).

Hearts were removed from euthanized animals, rinsed in cold normal saline, frozen in isopentane with dry ice, and kept at −80 °C until sectioning. Cryostat cardiac sections (6 μm) were prepared for immunofluorescence analysis of CD31 and CD44 as described above.
**Ex vivo aortic ring angiogenesis assay**

Thoracic aortas were isolated from WT and *Cd82* KO mice under a dissecting microscope, cut into 1-mm sections, and embedded in Matrigel-coated plates. MCDB131 medium containing 20% FBS, 10 U/mL heparin, 50 µg/mL EC mitogen, and 20 ng/mL bFGF was added to each well. Aortic rings were cultured at 37°C for 5 days, and the medium was replaced in every 2 days. Microvessel outgrowth was visualized by using light microscopy. The outgrowth distance and area of microvessels sprouting from each aortic were measured in a double-blinded fashion.

**In vitro vasculogenesis assay**

To assess EC cable network formation, fibrin gel was plated in 48-well plates and incubated at 37°C for 1 hour for gelation. ECs (7.5~8.0×10^4 cells) were suspended in 200 µL MCDB131 medium containing 10% FBS and sandwiched in fibrin gel. EC networks were either photographed with an Olympus CK2 inverted microscope under a 4X(N.A.=0.10) objective, connected with a DCM500 microscope digital camera at different time points, or recorded by time-lapse video microscopy. For some experiments, the assay was performed in the presence of various Abs. The numbers of EC cable-enclosed regions per field of view were counted visually, and the lengths of EC cables per field of view were measured by using Image J software.

**Flow Cytometry**

Single cell suspensions of WT and *Cd82-null* ECs were blocked and then incubated with primary Abs followed by incubation with an AlexaFluor488- or FITC-conjugated secondary Ab. Cells were then washed and resuspended in PBS and analyzed for fluorescence by using a Becton Dickinson FACS Calibur flow cytometer. Isotype-matched IgGs were used for negative controls.

**Cell proliferation and cell survival assays**
For cell proliferation, MLECs were seeded onto the gelatin-coated 96-well tissue culture plates at the concentration of 3,000 cells/well. Cell proliferation was assessed at 24, 48, and 72 h by labeling cells with methyl thiazolyl tetrazolium (MTT) for 4 h and measuring absorbance at 490 nm.

For cell survival, MLECs (5×10^3 cells) were plated into a 96-well plate in complete medium and the numbers of viable cells was quantified every 24 h up to 72 h. At each time point, the cells were trypsinized, washed once with PBS, and centrifuged. The cell pellets were resuspended in PBS containing 0.4% trypan blue and incubated at RT for approximately 3 minutes. Ten microliters of cell suspension was placed in a hemocytometer, and the total number of cells and the number of trypan blue–positive cells were counted. The percentage of viable cells was then calculated.

**Cell migration, cell invasion, and cell sprouting assays**

Cell migration assay was performed in a chemo-haptotactic manner using the Transwell insert sealed by a polycarbonate filter with 8-µm pores at the bottom (Corning, NY). The inserts were coated with HA, FN, or LN111 on the bottom side of the filters and placed in the wells of 24-well plate. MLECs are detached at 90% confluence with 0.25% Trypsin-EDTA, washed once in PBS, and resuspended in serum-free MCDB131 medium containing 0.1% BSA. A 300-µl cell suspension was loaded to the inserts at a density of 6×10^3 cells/insert. The lower chamber contained 500 µl of MCDB131 media with 1% FBS as chemoattractants. Migration was allowed to proceed at 37°C for 6 h. The filters were then stained with Diff-Quick solutions (Merz-Dade, Dudingen, Switzerland). The cells that migrated onto the bottom side of an insert were counted in 4 randomly chosen microscopic fields using a 20× objective. In each experiment, each group was tested in triplicate.

For the solitary cell invasion assay, MLECs were resuspended in MCDB131 media supplemented with 1% FBS and placed in the Matrigel-coated Transwell inserts at the concentration of 3×10^4 cells per well. The lower compartment contained MCDB131 media, and FN (5 µg/ml) was coated on the bottom side of the inserts. After incubation at 37°C for 16 h, the filters were stained with Diff-Quick solutions. For each chamber, the MLECs that invaded through Matrigel were counted in 4
randomly chosen microscopic fields using a 20 × objective. In each experiment, each group was tested in duplicate.

For the collective cell invasion assay or cell sprouting assay, the plasma membrane of HUVECs was labeled with either PKH26 (red channel) or PKH67 (green channel) dye (Sigma-Aldrich). Equal number of HUVECs from each labeled group and dextran-coated Cytodex 3 microcarrier beads were mixed at a concentration of 400 HUVECs/bead in DMEM/F12K medium with gentle shakes in every 20 min at 37°C and 5% CO2 for 4 h. The beads with cells were further incubated in 5 ml of complete medium at 37°C and 5% CO2 overnight, then washed three times with complete medium, and resuspended in fibrinogen solution (2.5 mg/ml fibrinogen in DPBS with 50 µg/ml aprotinin) at a concentration of 200 cell-coated beads/ml. The fibrinogen/bead solution was treated for clotting with thrombin at room temperature for 5 min and then at 37°C and 5% CO2 for 30 min, followed by the addition of complete medium. Cell sprouting were imaged after HUVECs on beads were cultured in fibrin gel for 2 days.

**Cell-matrix adhesion assay**

Cell-matrix adhesion analysis was performed in 96-well plates. Plates were coated with substrates such as FN, LN 111 overnight. LMECs suspended in serum free media were seeded into wells at a concentration of 10,000 cells/well. After a 35-min incubation at 37°C in 5% CO2, non-adherent cells were removed by three gentle washes with serum free media. Adherent cells were photographed and counted.

For cell adhesion on HA, HA (0.2 mg/ml) in 100 mM NaHCO₃ (pH 9.0) was added into each well of 96-well plate and incubated at 37°C for 4h. After being washed with PBS, the HA-coated wells were used for cell-matrix adhesion experiments as described above.
Endocytosis assay

Adhered ECs were incubated with 0.25 µg/250 µl/well of mAb and/or CTxB on ice for 1 h. The unbound mAbs were removed by 3 washes with ice-cold PBS. The cells were incubated at 37°C in 5%CO₂ for 2, 5, or 60 min. The cell surface-bound mAbs or CTxB were removed by 3 acidic washes with 0.1M glycine/0.1M NaCl solution (pH 2.5). Then, the cells were fixed, permeabilized, and incubated with FITC-conjugated secondary Ab, followed by 3 washes with PBS. The internalized molecules were examined using confocal microscopy. Clathrin-independent endocytosis was assessed by examining the internalized, CTxB-colocalized CD44 after 2- or 5-min endocytosis, as described¹⁰.

Immunoprecipitation and immunoblotting

For immunoprecipitation profile analysis, confluent MLECs were harvested by trypsinization or 2 mM EDTA, washed two times with PBS, labeled with sulfo-NHS-LC-Biotin (Pierce) at RT for 30~60 min, and washed 3 times with 100 mM glycine in PBS. Then the ECs were lysed at 4°C for 30 min by using lysis buffer containing 1% Brij97, 50 mM HEPES (pH 7.2), 150 mM sodium chloride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 2 mM sodium orthovanadate, 2 mM sodium fluoride. The insoluble fraction was removed by centrifugation at 13,000 x g for 15 min, and the lysates were pre-cleared with protein-A and -G Sepharose beads three times with each clearance at 4°C for 1 h and then incubated with a primary mAb (1 µg mAb/500 µg total protein) at 4°C for 1.5 h, followed by another 3-h incubation with protein-A and -G Sepharose beads. After being washed three times in lysis buffer, the precipitates were processed for SDS-PAGE and immunoblotting.

For immunoblotting, the membranes were blocked with 5% nonfat milk at RT for 30 min, probed with a primary Ab at 4°C overnight and then an HRP-conjugated secondary Ab, followed by a chemiluminescence assay. For general protein immunoblotting, Confluent MLECs were lysed on ice for 15 min by using lysis buffer containing 1% NP40, 50 mM HEPES (pH 7.2), 150 mM sodium chloride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF, 2 mM sodium orthovanadate, 2 mM
sodium fluoride, and 2.5 mM sodium pyrophosphate, and 1 mM beta-glycerophosphate. The insoluble fraction was removed by centrifugation at 13,000 x g for 15 min, and the amounts of protein in lysates were quantified and adjusted to equal concentration. Then the proteins were separated in SDS-PAGE and examined in immunoblotting as described above.

For the analysis of the cytoskeletal association of integrin β1, CD44 and CD9, confluent HMECs were lysed with 0.03% Triton X-100 in 50mM HEPES containing 2mM NaCl. After centrifugation at 100,000 x g for 30 min, the supernatants of lysates were labeled as soluble fractions, the pellets of cell lysates were dissolved in 1X Laemmli buffer and labeled as insoluble fractions. The levels of integrin β1, CD44 or CD9 distributed in soluble and insoluble fractions were detected by immunoblotting with specific mAbs.

**Immunohistochemistry**

Immunostaining of blood vessels in Matrigel plug was performed with CD31 mAb in 5-µm cryosections as described. The sections were fixed in cold acetone for 10 min at 4°C, blocked in 5% BSA in PBS for 30 min at RT, and incubated sequentially with CD31 mAb at 4°C for overnight and biotinylated secondary Ab for 45 min at RT. Then the sections were stained by hematoxylin, dehydrated by alcohol, and treated with xylene. Images from representative fields were captured on a Zeiss Axioplan microscope (Zeiss, Heidelberg, Germany).

**Immunofluorescence and confocal microscopy**

Immunofluorescence and confocal microscopic analysis were performed as described in our previous study. Briefly, primary ECs isolated from WT and Cd82-null mice were cultured on FN- or gelatin-coated glass coverslips for 2 days, fixed with 3% paraformaldehyde in PBS, permeabilized with 0.1% Brij 98 in PBS, incubated sequentially with 1st and 2nd Abs, mounted with Vectashield solution (Vector laboratories, Burlingame, CA) with or without DAPI, and examined on LSM 710 (Zeiss) or SP2 MP (Leica, Heidelberg, Germany) confocal microscope.
In the microextrusion experiments, MLECs were spread on FN-coated glass coverslips for 24 h, fixed, permeabilized, incubated sequentially with CD31 or CD44 mAb and FITC-conjugated secondary Ab, and analyzed by confocal microscopy or TIRFM, respectively. To acquire microextrusion images, we photographed 1) CD31-positive microextrusions with confocal microscopy by selecting the focal plane that generally showed the maximal length of microextrusions and 2) CD44-positive microextrusions with TIRF microscopy, which visualizes the microextrusions at the basal periphery of cells.

For immunofluorescence analysis of Matrigel, the sections were incubated sequentially with primary mAb and Alexa-fluor-594-conjugated rat anti-mouse Ab in blocking solution, washed three times in PBS after each incubation, mounted with Vectashield solution with DAPI. Representative fields were photographed with Zeiss LSM710 confocal microscope under identical instrument setting.

For colocalization, confocal immunofluorescence images were analyzed with Volocity4.4 software, and colocalization was projected as Pearson’s and Mander’s coefficients. Pearson’s correlation coefficient measures any linear dependencies between two color channels and therefore is used for describing the correlation of the intensity distribution between the channels. Mander’s overlap coefficient indicates the overlap of two color channel signals, is presented as the ratio of the two color channels’ overlapping area to the two color channels’ total area, and therefore represents the true degree of colocalization from both channels. Manders’ overlap coefficient M1 or M2 describes the contribution of M1 or M2 channel, respectively, to the scatter gram regions of interest within images.

**Fluorescence resonance energy transfer (FRET)**

After being cultured on FN- or gelatin-coated glass-bottom dishes for 2 days, fixed with 3% paraformaldehyde in PBS for 30min in 37°C, and washed with 10mM glycine in PBS, cells were labeled with C_{16}-DiO and C_{16}-Dil(2µM). FRET was measured by detecting sensitized emission of acceptor following donor excitation. Accordingly, images were acquired in three separate channels:
donor channel (DiO, 488 nm excitation/505 to 550 nm emission), acceptor channel (Dil, 543 nm excitation/560 nm emission), and FRET channel (488 nm excitation/560 nm emission) by using a Zeiss LSM 510 META confocal microscope. FRET efficiency was calculated using images collected in the FRET channel. Images of cells labeled with either C16-DiO or C16-Dil alone were collected in the DiO and Dil channels to determine correction factors necessary to eliminate contributions from donor and acceptor bleed-through to the FRET channel\(^\text{13}\).

**Total internal reflection fluorescence microscopy (TIRFM)**

Cells attached on glass bottom dishes were fixed, labeled with primary mAb followed by FITC-conjugated secondary Ab staining or Alexa488-conjugated GM1 directly. Staining at the cell basal surface was visualized with an Olympus IX71 microscope equipped with a 60X(N.A.=1.45) objective. TIRF illumination was achieved by using a 20 mW 488 nm laser source. Images were acquired with a Quantix57 CCD camera (Photometrics, Tucson, AZ) air-cooled to -25°C and controlled by IPlab 3.9.4. (Scanalytics, Fairfax, VA). Images were processed by using IPlab software.
3. Supplemental Results

Effect of Cd82 ablation on detergent solubility of CD44, CD9, and integrin β1

To further determine membrane compartmentalization of CAMs and tetraspanins, we examined their detergent solubility by lysing ECs with 0.03% Triton X-100. Compared to WT ECs, Cd82-null ECs have relatively more insoluble integrin β1, CD44, and CD9 but less of these proteins in the soluble fraction (Figure S13), suggesting that CD82 prohibits the cytoskeletal connection and/or alters membrane compartmentalization of CAMs and tetraspanins. Latrunculin treatment prior to cell lysis disrupted actin cytoskeleton, evidenced by almost lack of actin in insoluble fraction, but didn’t diminish the difference in integrin β1, CD44, and CD9 between WT and KO groups in both insoluble and soluble fractions, supporting altered membrane compartmentalization of CAMs and tetraspanins is the determining factor for their less detergent solubility upon Cd82-ablation. Latrunculin treatment shifted more proteins to soluble fractions in both WT and KO groups, suggesting actin cytoskeleton also contributes to the insolubility.
4. Supplemental Discussion

The Cd82 ablation strategy and the Cd82-null mouse phenotype

No noticeable vascular phenotype during development but enhanced vascular morphogenic potential in CD82 KO mice was unlikely to be the result of the gene ablation strategy used in this study. Since no murine CD82 Ab is available to us, we could not examine whether the truncated CD82 molecule can actually be expressed in Cd82-null mice. However, even if the mRNA of the Cd82 KO allele is expressed, it is unlikely to be fully translated because the mRNA bears a new stop codon in the exon 7 region due to a reading frame shift. The mRNA could be translated into a peptide containing 45 residues in the N-terminus of CD82, but the peptide is more likely to be degraded because of improper folding. Even if the peptide in KO mice is functional or malfunctional, it only accounts for approximately 3~6% of the CD82 proteins in the WT mice. Thus, the contribution of this putative truncated peptide is negligible.

Why does Cd82 ablation preferentially promote angiogenesis under pathological conditions?

No noticeable vascular phenotype during development but enhanced angiogenesis under pathological conditions drive us to predict the selective regulatory mechanism of CD82. One possibility is that the vascular abnormality caused by Cd82 ablation can be compensated by another tetraspanin or a few other tetraspanins during developmental angiogenesis but not during angiogenesis under pathological conditions.

Alternatively or additionally, if the downstream events within CD82-ganglioside-CD44 signaling axis are less available during development or affect less developmental angiogenesis, it is explainable that Cd82 ablation does not affect developmental angiogenesis. Indeed, Cd44-null mice do not exhibit defects in developmental angiogenesis but do exhibit reduced pathological angiogenesis. The observations with Cd44-null mice are consistent with our finding that Cd82 ablation leads to increases in CD44 level and then pathological angiogenesis.
Moreover, the growth factors, cytokines, and/or chemokines that promote EC movement are different under physiological versus pathological conditions. Because of the difference, the increased EC movement upon \textit{Cd82} ablation could become more potentiated under pathological conditions than under physiological conditions, leading to more angiogenesis under pathological conditions. On the other hand, developmental or physiological angiogenesis is a tightly controlled and well regulated morphogenic event, increased EC movement may not easily result in more angiogenic process. In contrast, angiogenesis under pathological conditions is a relatively simplistic morphogenic process, and increased EC movement probably more easily result in elevated angiogenic process.

It has been documented that the angiogenesis under pathological conditions can be selectively perturbed without affecting angiogenesis under physiological conditions, although the mechanisms responsible for such selective perturbation are largely unclear\textsuperscript{5,17}. Also, developmental angiogenesis is different from pathological angiogenesis in several aspects, such as structure, function, distribution, and maturation process of vessels\textsuperscript{18,19}. These differences in principle could serve as the basis of selective regulation and perturbation. Together, how CD82 selectively regulates angiogenesis under pathological conditions will be determined in coming studies.

On the other hand, it is also possible that \textit{Cd82} ablation slightly affects angiogenesis under physiological conditions, but we haven't systematically assessed this possibility yet. For example, we observed that under physiological conditions \textit{Cd82} ablation affects the angiogenic potential during retina development, with more i) tip cells and ii) filopodia in the tip cells of sprouting vessels (Figure S3 panel C). This observation suggests that vessels are formed in a more robust and efficient manner during physiological angiogenesis upon \textit{Cd82} ablation. However, this increased angiogenic potential did not ultimately result in more vascularization in \textit{Cd82-null} retinas (Figure S3 panel A), probably because an above-normal level of blood supply is not needed for normal retinal development and a surplus of nutrients and oxygen eventually lead to vascular regress at the end of development. In addition, it is difficult to reveal developmental vascular phenotypes and their functional consequences when vascular development is above normal. In addition, we have observed that \textit{Cd82-null} mice are
typically more robust, agile, and aggressive and a bit larger in size than WT mice (unpublished observation). Whether such differences are caused by better vascularization in relevant tissues and organs remains to be investigated in future studies.
5. Supplemental References


A

M Lung EC  M Liver EC

CD82 mRNA expression in murine ECs

B

HUVEC  HMEC  HRCEC

CD82 protein expression in human ECs

Figure S1
Figure S2
Figure S3

A. Normoxia (P18)

B. Avascular area/retina (Relative Unit)

C. Normoxia (P5)
Figure S5
Figure S6
Figure S7
Figure S8
Figure S9

(A) CD44 Endocytosis vs. CD9 Endocytosis

(B) Internalized CD44 images for WT and KO conditions at 2 min and 5 min.
Figure S10
Figure S11
**Figure S12**
Figure S13
Figure S14

CD82

Normal Retina

GCL

IPL

INL

Proliferative Diabetic Retina

GCL

IPL

INL
A

CD82

Fewer Glycosphingolipids at the Plasma Membrane of ECs

Few Clustering of Lipid Rafts and Less Coalescence of CAMs to Microdomains

More Endocytosis of CAMs

Lower Levels of CAMs at the Cell Surface and Less Signaling from the Surface CAMs

Reduced EC-Matrix Adhesion, Fewer and Less Developed Focal Adhesions and Focal Complexes, and Fewer Membrane Microextrusions

Reduced EC Migration and Invasion

Reduced Angiogenic Potential

Restrained Angiogenesis under Pathological Conditions

B

CD82 WT EC

CD82 KO EC

Figure S15
혈관내피세포에 발현하는 CD82는 혈관생성을 억제한다

한 기 혼 교수 서울아산병원 심장내과

Summary

배경
혈관생성은 염증으로부터 염증 반응물에 이르기까지 수많은 병
적인 투쟁에서 중추적인 역할을 하며, 한편으로는 이들에 대한 치
료 전략에 중요한 타겟이기도 하다. Tetraspanin의 구조를 가지
는 CD82는 혈관내피세포에서 중요한 역할을 하지만, 이와
연관된 혈관내피세포의 기능 및 혈관생성의 기전에 대해서는 연구된
바 없다.

방법 및 결과
Cd82-null 마우스에서 혈관생성을 유도하였으며, 관련된 세포
기능, 분자 간 영향 및 신호 전달 기전 등을 연구하였다. Cd82-
null 마우스에서는 다양한 자극에 대한 혈관생성이 급격하게 증
가하였으며, CD44 및 integrin과 같은 cell adhesion molecules
의 발현이 증가하였고, 그 결과 혈관내피세포의 이동 및 침윤
이 활발히 증가하였다. 세포막에서 gangliosides 및 lipid raft
clustering 그리고 CD44 microdomain의 상호작용이 증가하였
으며, 이로 인해 CD44의 endocytosis의 감소 및 세포막에서의
발현 증가가 나타났다.

결론
CD82는 병적인 혈관생성을 억제하며, 이는 혈관내피세포의 혈
관 생성 잠재력을 의미하는 이동 능력을 억제함으로써 기능을
하는 것으로 보인다. 이 과정에는 lipid raft clustering, CD44
microdomain의 상호작용, 그리고 cell adhesion molecules 이
동 잠재 등을 관여하며, 여기에는 CD82-ganglioside-CD44 신
호 전달 체계가 관여하는 것으로 보인다.