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Cytoplasmic LIM domain only 2 enhances tumor endothelial cell migration through integrin β 1-mediated focal adhesion signaling

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Abstract

The LIM domain only 2 (LMO2) is a nuclear transcriptional regulator that influences cell fate in hematopoiesis and vasculogenesis. Recent evidence indicates that LMO2 also functions as a signaling molecule in the cytoplasm of cancer cells. However, its role in tumor endothelial cells (ECs) and angiogenesis remains unclear. In this study, we explored the cytoplasmic role of LMO2 in tumor angiogenesis using a three-dimensional microfluidic sprouting model. LMO2 was found to be enriched in the cytoplasm of migrating ECs, along with increased expression of vascular endothelial growth factor (VEGF) receptor 2. VEGF stimulation elevated cytoplasmic LMO2 levels, and LMO2 was enriched at tumor margins in vivo. Proteomic screening identified integrin β 1 (ITGB1) as a potential interactor with LMO2, and their interaction was confirmed through co-immunoprecipitation and proximity ligation assays. Overexpressing LMO2 enhanced focal adhesion signaling, as indicated by increased activation of FAK, Src, and ERK, and promoted EC migration without altering total ITGB1 levels. Knocking down ITGB1 reversed these effects, suggesting that the LMO2–ITGB1 interaction is essential for focal adhesion signaling and EC migration. Importantly, these effects depended on cytoplasmic, not nuclear, LMO2. Our findings reveal a previously unknown cytoplasmic role of LMO2 in regulating focal adhesion dynamics and endothelial behavior. Targeting the LMO2–ITGB1 pathway could be a promising strategy for anti-angiogenic therapy.

Introduction

Tumor angiogenesis, the formation of new blood vessels from existing vasculature, is a hallmark of cancer progression [1, 2]. This process is crucial for supplying rapidly dividing tumor cells with oxygen and nutrients and enables metastatic spread to distant organs. Consequently, targeting angiogenesis has become a key strategy for treating cancer. Anti-angiogenic agents, especially those targeting the vascular endothelial growth factor (VEGF) and its receptors, have demonstrated clinical benefits in various cancers [3]. Nevertheless, the development of resistance and the limited long-term effectiveness underscore the need to comprehensively clarify the molecular mechanisms underlying tumor angiogenesis,

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as well as identify new therapeutic targets within endothelial signaling pathways [4].

LIM domain only 2 (LMO2) is a small, cysteine-rich adaptor protein traditionally recognized for its nuclear role in transcriptional complexes involved in hematopoietic development [5]. More recently, LMO2 has been linked to diverse cytoplasmic functions, particularly in oncogenic signaling pathways [6, 7]. In glioma stem-like cells, cytoplasmic LMO2 was shown to form a non-transcriptional complex with LIM domain-binding protein 1 (LDB1), glycoprotein 130, and Janus kinases, leading to the activation of STAT3 and upregulation of inhibitor of differentiation 1, a critical mediator of cancer stemness [8]. These findings highlight the context-dependent, non-nuclear role of LMO2 in maintaining the tumor-initiating potential.

In addition to its role in glioblastoma (GBM), LMO2 reportedly influences the cytoskeletal dynamics in basal-type breast cancer [9]. LMO2 promotes cell migration and invasion by binding to cofilin 1 and preventing its inactivation by LIM kinase 1, thereby facilitating actin filament remodeling. These findings highlight the capacity of cytoplasmic LMO2 to interact with signaling and structural proteins, thereby driving tumor aggressiveness.

Although LMO2 is expressed in the vascular endothelial cells (ECs), its role within this context remains elusive. LMO2 has been shown to promote endothelial migration by regulating sphingosine kinase 1 activity, indicating its pro-angiogenic role [10]. However, the potential interactions between cytoplasmic LMO2 and integrins, known focal adhesion signaling molecules, have not been examined in ECs. Given the pivotal role of focal adhesion pathways in EC movement and angiogenic sprouting, it is plausible that cytoplasmic LMO2 functions as an adaptor linking VEGF signaling to integrin-mediated adhesion dynamics [11].

In the current study, we investigated the role of cytoplasmic LMO2 in tumor angiogenesis, focusing on its interaction with integrin $\beta 1$ (ITGB1) in ECs. Using a three-dimensional (3D) microchip-based sprouting model, we tracked endothelial migration in a relevant spatial context, enabling a detailed analysis of protein localization and signaling responses. Utilizing this approach, we aimed to clarify the mechanistic contribution of cytoplasmic LMO2 to focal adhesion signaling and endothelial behavior and assess its potential as a novel target for anti-angiogenic cancer therapy.

Results

LMO2 is highly expressed in the tumor vasculature

To characterize LMO2 expression in ECs, we analyzed single-cell RNA sequencing (scRNA-seq) data from GBM samples [12]. LMO2 was not only detected in tumor-associated compartments, including differentiated-like

and proliferating stem-like populations, but was also prominently expressed in ECs, a pattern not shared by its canonical cofactor LDB1 (Fig. 1A, B and Fig. S1A) [13, 14]. Analysis of the Ivy Glioblastoma Atlas Project (IvyGAP), a transcriptomic atlas, further revealed that among the LMO family members, LMO2 was uniquely enriched in regions of microvascular proliferation and hyperplastic blood vessels, which are histological features characteristic of aggressive GBM vasculature (Fig. 1C, D) [15]. This spatial expression pattern was recapitulated in orthotopic xenograft models, where CD31-positive tumor vessels displayed elevated LMO2 levels compared with the normal brain vasculature, particularly at the tumor margin (Fig. 1E–G). Furthermore, scRNA-seq analyses across multiple cancer types revealed that ECs exhibited greater LMO2 expression than other cell populations (Fig. S1B) [16]. In contrast, LDB2, a structurally related LMO2-binding partner, exhibited an EC-restricted expression pattern, similar to that of LMO2 (Fig. S1C, D) [17]. These findings indicated that LMO2 is not only present in tumor cells but is also especially abundant in tumor-associated ECs.

Cytoplasmic LMO2 is enriched in migrating ECs and upregulated by VEGF stimulation

To mimic the high LMO2 expression observed in tumor-associated vasculature, we overexpressed LMO2 in human umbilical vein endothelial cells (HUVECs) and examined its functional and spatial characteristics. Specifically, we evaluated the subcellular distribution of LMO2 in ECs under both two-dimensional (2D) and 3D culture conditions (Fig. 2A, B) [18, 19]. In the 3D microfluidic systems, cytoplasmic LMO2 localization closely resembled that of VEGF receptor 2 (VEGFR2) in migrating ECs (Fig. 2B and Fig. S2A) [20]. Functionally, ECs overexpressing LMO2 showed increased migratory capacity compared with control cells (Fig. 2C and Fig. S2B–F), along with higher VEGFR2 signal intensity normalized to the sprouting area (Fig. 2D) [20, 21]. To determine whether VEGF signaling influenced cytoplasmic LMO2 expression, we performed subcellular fractionation after VEGF treatment under 2D culture conditions. VEGF stimulation resulted in a significant increase in cytoplasmic LMO2 levels, with a concurrent increase in nuclear LMO2 expression (Fig. 2E–H) [22, 23]. Consistently, in vivo tumor models exhibited higher LMO2 expression at the tumor margin, a region enriched in VEGF and other pro-angiogenic cytokines (Fig. 2I, J) [24–26]. These observations suggested that VEGF signaling induces both nuclear and cytoplasmic LMO2 accumulation in ECs, potentially contributing to the migratory behavior of tumor-associated vasculature.

Across all datasets analyzed, LMO2 expression was predominantly enriched in endothelial cells compared to

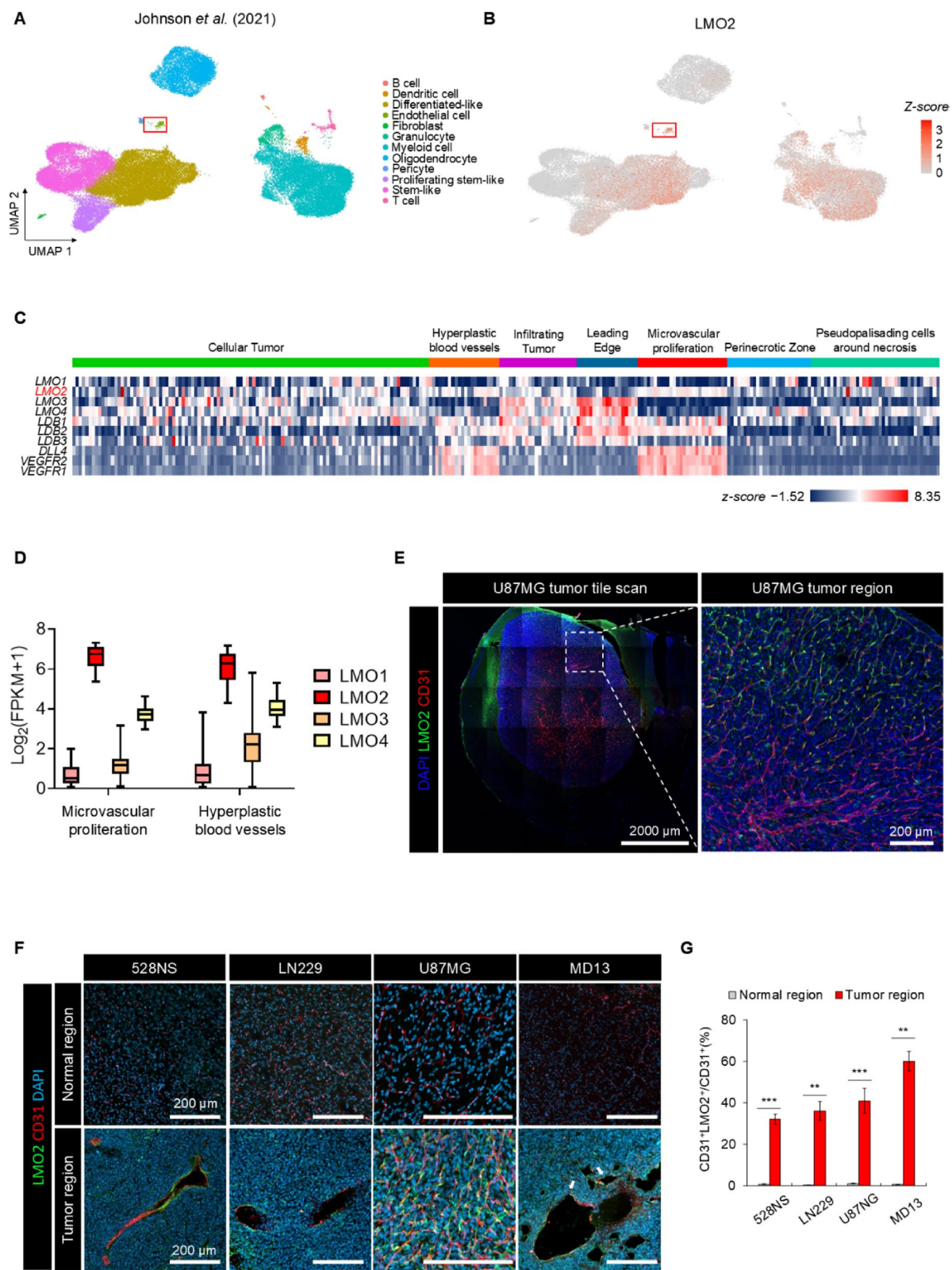


Fig. 1 (See legend on next page.)

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Fig. 1 LMO2 is highly expressed in the tumor vasculature. UMAP visualization of single-cell transcriptomic data from patients with glioblastoma ($n = 55,284$ cells from 11 individual datasets), annotated by cell type using the Johnson et al. dataset [12], colored, and grouped by cell type. **A** UMAP highlighting the distribution of LMO2-positive cells across the annotated cell populations. **B** Heatmap showing the histological localization of mRNA expression of LMO family members (*LMO1–4*), generated from the Ivy Glioblastoma Atlas Project (IvyGAP, <https://glioblastoma.alleninstitute.org/>). *VEGFR1*, *VEGFR2*, and *DLL4* were used as positive controls for tumor vasculature-associated histological regions. The relative expression levels are normalized, converted to z-scores, and visualized as a heatmap. **C** mRNA expression levels of LMO family members (*LMO1*, *LMO2*, *LMO3*, and *LMO4*) specifically in the microvascular proliferation and hyperplastic blood vessels region within the brain tumor histological subtype, determined using data from the Ivy Glioblastoma Atlas Project (<https://glioblastoma.alleninstitute.org/>). Expression values are normalized and presented as $\log_2(\text{FPKM} + 1)$. Statistical significance was assessed using ordinary one-way ANOVA ($****P < 0.0001$). Sample size: *LMO1* ($n = 17$), *LMO2* ($n = 25$), *LMO3* ($n = 24$) and *LMO4* ($n = 25$) for microvascular proliferation; *LMO1* ($n = 17$), *LMO2* ($n = 25$), *LMO3* ($n = 24$) and *LMO4* ($n = 25$) for hyperplastic blood vessels. Data are presented as mean $\pm$ SEM. **D** Immunofluorescence imaging of U87MG xenograft sections showing CD31**LMO2** vessels (DAPI, blue; *LMO2*, green; CD31, red). Tile scan images are shown at low (left, scale bar = 2000 μm) and high (right, scale bar = 200 μm) magnifications. $n = 2$ biological replicates. **E** Immunofluorescence analysis of CD31**LMO2** vessels in multiple glioma xenografts, including 528NS, LN229, U87MG, and MD13 tumors (DAPI, blue; *LMO2*, green; CD31, red). Scale bar = 200 μm . $n = 3$ biological replicates per model. **F** Quantification of CD31**LMO2** vessel proportion among total CD31* vasculature in tumor versus adjacent normal brain regions. Data are presented as mean $\pm$ SEM. Statistical significance was assessed using two-sided unpaired t-tests ($**P < 0.01$, $***P < 0.001$). $n = 3$ biological replicates

other tumor microenvironment populations and showed a positive correlation with KDR (*VEGFR2*) expression (Fig. S2G). This relationship was consistent across multiple independent cohorts [27–29] and was especially evident in endothelial subsets with high VEGF responsiveness. Overall, these findings suggest that LMO2 expression reflects VEGF pathway activity within the brain tumor endothelial compartment, regardless of the specific in vitro or in vivo system studied.

In addition, to directly assess whether hypoxia regulates LMO2, we exposed HUVECs to the hypoxia mimetic CoCl_2 (100 μM) and monitored the HIF–VEGF axis over time. CoCl_2 treatment induced robust accumulation of HIF1 α protein, increased phosphorylation of *VEGFR2*, and a modest but reproducible increase in LMO2 protein levels, whereas total *VEGFR2* changed only slightly (Fig. S2H). In parallel, qRT–PCR analysis showed time-dependent upregulation of *VEGF*, *VEGFR2*, and LMO2 transcripts under the same conditions (Fig. S2I). Confocal imaging further revealed that CoCl_2 stimulation markedly enhanced cytoplasmic LMO2 signal, with a shift away from the predominantly nuclear pattern observed in control cells (Fig. S2J,K). Together with previous reports that hypoxic endothelial or tumor cells can establish VEGF-dependent autocrine loops to sustain *VEGFR* signaling in the tumor vasculature, these findings indicate that hypoxia/HIF-driven activation of the VEGF–*VEGFR2* pathway increases both the abundance and cytoplasmic localization of LMO2, thereby functionally linking the hypoxic microenvironment to LMO2-dependent pro-angiogenic programs in tumor ECs [30–32].

Integrin $\beta 1$ is a putative LMO2 interactor enriched in tumor-associated ECs

Given the enrichment of cytoplasmic LMO2 in migrating ECs, we explored its potential role by analyzing LMO2-positive clusters within tumor-derived CD31-positive EC populations (Fig. S3A) [33]. Gene Ontology (GO) term analysis of this cluster revealed significant enrichment in

extracellular matrix and cell adhesion molecule-binding functions (Fig. 3A). To identify putative LMO2-interacting partners, we integrated the results from LMO2 pull-down proteomics with protein interaction data curated from the Human Protein Atlas (Fig. 3B) [8, 34]. Among these candidate proteins, ITGB1 and actin $\alpha 1$ (*ACTN1*) emerged as potential interactors. We focused on ITGB1 because of its relatively high expression in both tumor vascular histological regions and tumor-associated EC populations, unlike *ACTN1* and other integrin- β family members (Fig. 3C–G and Fig. S3B) [15, 16, 28, 29, 33]. These findings suggested that LMO2 and ITGB1 are co-expressed in ECs, with higher LMO2 expression correlating with increased adhesion-related gene expression and potential cytoplasmic interactions with ITGB1.

Consistent with these GO term results (Fig. 3A), region-specific transcriptomic analysis of the IvyGAP dataset revealed that LMO2-high vascular areas, especially hyperplastic vessels and microvascular proliferation zones, co-express several ITGB1-partner integrin α subunits (*ITGA1*, *ITGA2*, *ITGA4*, *ITGA5*, *ITGA8*) along with ECM ligands such as fibronectin (*FN1*), collagen IV (*COL4A1*), laminin chains (*LAMA4*, *LAMC1*), nidogens (*NID1/2*), vitronectin (*VTN*), periostin (*POSTN*), tenascin C (*TNC*), and osteopontin (*SPP1*) (Fig. S3C, D) [15]. These molecules are abundant in the glioblastoma ECM, especially in perivascular regions, indicating that LMO2-positive tumor ECs reside in collagen IV- and fibronectin-rich niches that promote $\beta 1$ -integrin-dependent adhesion [35, 36]. Consistent with this, LMO2 overexpression substantially increased HUVEC adhesion to collagen IV, fibronectin, and laminin (Fig. S3E, F), supporting a functional connection between cytoplasmic LMO2, ECM-engaged ITGB1–integrin α heterodimers, and endothelial attachment.

LMO2–ITGB1 interaction is enhanced in LMO2-overexpressing ECs

To further investigate potential interactions between LMO2 and ITGB1, we analyzed their spatial proximity

using a 3D endothelial migrating assay. LMO2 and ITGB1 exhibited markedly increased co-localization in HUVECs overexpressing LMO2 (HUVEC-LMO2), which models LMO2-high tumor vasculature, compared to control cells (HUVEC-Con) (Fig. 4A, B). In contrast, expression levels of LDB1 and LDB2, known binding partners of LMO2, remained unchanged upon LMO2 overexpression (Fig. S4A, B). Notably, LMO2 overexpression did not impact total ITGB1 expression under both 3D and 2D conditions (Fig. 4C and Fig. S4C–E).

Co-immunoprecipitation (Co-IP) assays further revealed that ITGB1 binding to LMO2 was increased in HUVEC-LMO2 cells compared with control cells, as evidenced by a stronger ITGB1 band upon LMO2 pull-down (Fig. 4D). This pattern was also seen in LMO2-overexpressing human brain microvascular endothelial cells (HBMECs) and HEK293FT cells (Fig. S4F, G). The proximity ligation assay (PLA) confirmed that LMO2-overexpressing ECs exhibited a marked increase in cytoplasmic LMO2–ITGB1 interactions in 3D sprouts, while LMO2–LDB2 interactions were unchanged in 2D and less prominent within 3D sprout areas (Fig. 4E, F and Fig. S4H, I). These results suggested that LMO2 physically associates with ITGB1 in ECs, independent of canonical nuclear partners, such as LDB1 or LDB2, and that this interaction may be particularly relevant in the cytoplasmic compartment of LMO2-overexpressing tumor vasculature.

LMO2–ITGB1 interaction activates focal adhesion signaling and enhances EC migration

Given that VEGF stimulation increased cytoplasmic LMO2 in tumor endothelium (Fig. 2 and Fig. S2), we then explored whether this LMO2 pool could directly interact with the integrin β 1–talin-1 axis to promote inside-out signaling [37, 38]. Guided by our proteomics data, we modeled a ternary complex consisting of the ITGB1 cytoplasmic tail, the TLN1 head domain, and full-length LMO2 using AlphaFold3 (Fig. S5B, C). This model positioned LMO2 at the interface between the β 1-integrin tail and the talin-1 head, and predicted discrete contact sites between LMO2 K44 and ITGB1 T788, and between LMO2 R81 and TLN1 D375 (Fig. S5D, F) [39]. Co-immunoprecipitation experiments in HEK293FT cells confirmed that HA-LMO2 forms a complex with ITGB1-FLAG and endogenous TLN1 (Fig. S5A), supporting a physical link between LMO2 and the β 1–talin-1 machinery. These findings suggest that VEGF-induced cytoplasmic LMO2 acts as an adaptor within ITGB1–TLN1 complexes to facilitate integrin β 1 activation at focal adhesions, rather than merely altering ITGB1 levels.

We next examined integrin α 5 to place this β 1-centered module into an angiogenesis-relevant heterodimer context. α 5 β 1 is a major fibronectin receptor and has been

linked to tumor angiogenesis [40, 41]. This choice also aligned with our functional setting in which fibronectin supported the strongest adhesion phenotype, and the 3D matrix contains fibrin/fibronectin. In line with this, multimer modeling supported a quaternary assembly containing ITGB1, ITGA5, TLN1, and LMO2, and ITGA5 was recovered together with TLN1 in the pull-down assay results (Fig. S5A–F). Together, these data are consistent with a model in which cytoplasmic LMO2 participates in integrin–talin-1 assemblies to potentiate β 1-dependent focal-adhesion signaling [42, 43].

To determine the downstream effects of the LMO2–ITGB1 interaction, we analyzed the activation of the focal adhesion pathway in ECs [44–46]. Western blotting analysis revealed that LMO2 overexpression enhanced the expression and phosphorylation of key effectors involved in focal adhesion and MAPK signaling, including focal adhesion kinase (FAK), Src, and ERK (Fig. 5A and Fig. S5G). Likewise, immunofluorescence staining of the 3D sprouting model demonstrated that LMO2-overexpressing HUVECs exhibited higher levels of phosphorylated FAK (p-FAK), total FAK, and p-Src (Fig. 5B, C). These markers were selected as core indicators of focal adhesion dynamics owing to their spatial and functional relevance [47, 48]. In contrast, LMO2 overexpression did not alter the expression of LDB2 (Fig. S5H, I).

To determine whether these signaling changes depend on ITGB1, we performed ITGB1 knockdown using small interfering RNA (siRNA). In HUVEC-LMO2 cells, silencing ITGB1 markedly reduced expression levels of p-FAK, total FAK, and p-Src, restoring them to baseline levels (Fig. 5D, E and Fig. S5J). Consistently, the LMO2-driven increase in EC migration was reversed (Fig. 5F). ITGB1 knockdown also reduced the viability of HUVEC-LMO2 cells, possibly owing to impaired FAK-dependent survival signaling [49, 50] (Fig. S5K). These findings suggest that the LMO2–ITGB1 interaction may promote focal adhesion signaling, migration, and survival of ECs.

Cytoplasmic, but not nuclear, LMO2 mediates ITGB1-dependent phenotypes in ECs

To determine whether the observed phenotypic effects were specifically linked to cytoplasmic rather than nuclear LMO2, we measured the subcellular distribution of LMO2 in each endothelial sprout using a 3D microchip assay. Immunofluorescence staining was performed to detect LMO2, along with either LDB1 or LDB2, in the endothelial sprouting model (Fig. 6A, B). According to the quantitative fluorescence analysis, we observed that nuclear LMO2 levels remained relatively constant across all siRNA-treated control EC groups. In contrast, the intensity of cytoplasmic LMO2 was significantly increased in LMO2-overexpressing ECs and was influenced by ITGB1 knockdown, while its baseline

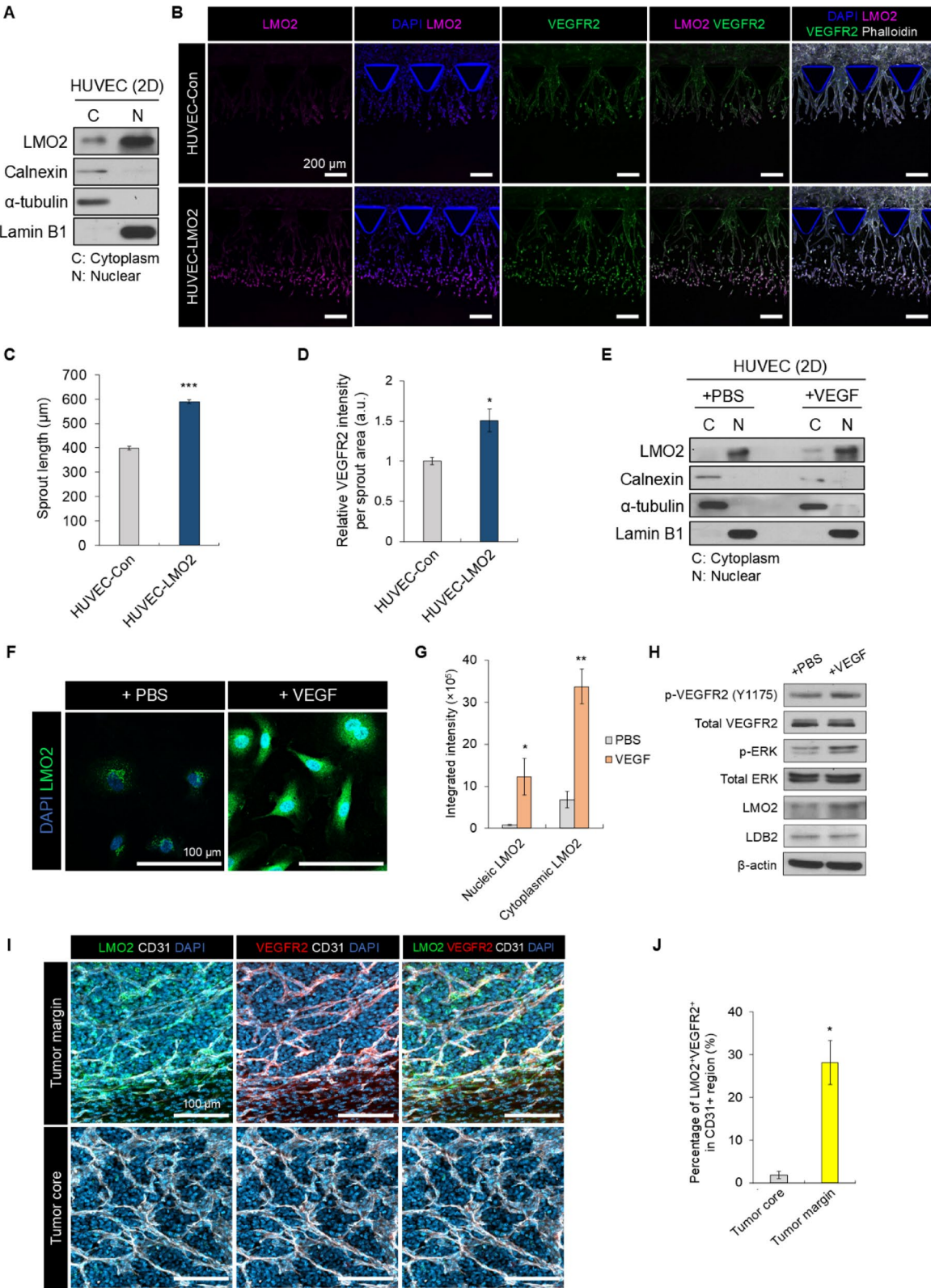


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Fig. 2 Cytoplasmic LMO2 is enriched in migrating endothelial cells and upregulated by VEGF stimulation. **A** Western blot analysis showing subcellular localization of LIM domain only 2 (LMO2) in two-dimensional (2D)-cultured human umbilical vein endothelial cells (HUVECs). Calnexin and α -tubulin serve as cytoplasmic (C) markers, whereas Lamin B1 serves as a nuclear (N) marker. $n=2$ biological replicates. **B** Immunofluorescence imaging of endothelial sprouts in a 3D microfluidic angiogenesis model showing co-expression of LMO2 and vascular endothelial growth factor receptor 2 (VEGFR2) (DAPI, blue; LMO2, magenta; VEGFR2, green; phalloidin, white). Scale bar = 200 μ m. $n=2$ biological replicates. **C** Quantification of endothelial sprout length (μ m) in the 3D angiogenesis model. Data are presented as mean $\pm$ SEM. Statistical significance was determined using a two-sided unpaired t-test ($***P < 0.001$). $n=3$ biological replicates. **D** Quantification of VEGFR2 fluorescence intensity (arbitrary units [a.u.]) per endothelial sprout area in the three-dimensional (3D) angiogenesis model. Data are presented as mean $\pm$ SEM. Two-sided unpaired t-tests were used for statistical analyses ($*P < 0.05$). $n=3$ biological replicates. **E** Western blotting analysis of LMO2 subcellular distribution in 2D-cultured HUVECs treated with or without VEGF (100 ng/mL, 24 h). Cytoplasmic (C) and nuclear (N) markers include Calnexin, α -tubulin, and Lamin B1, respectively. $n=2$ biological replicates. **F** Immunofluorescence analysis of LMO2 localization in 2D-cultured HUVECs treated with or without VEGF (100 ng/mL, 24 h) (DAPI, blue; LMO2, green; phalloidin, white). Scale bar = 100 μ m. $n=2$ biological replicates. **G** Quantification of nuclear and cytoplasmic LMO2 fluorescence intensity ($\times 10^5$) per cell area in 2D-cultured HUVECs $\pm$ VEGF. Data are presented as mean $\pm$ SEM. Two-sided unpaired t-tests were performed ($**P < 0.01$). $n=3$ biological replicates. **H** Western blot analysis of LMO2 and VEGF signaling-related proteins in 2D-cultured HUVECs treated with or without VEGF (100 ng/mL for 24 h). β -actin was used as a loading control. $n=2$ biological replicates. **I** Immunofluorescence staining of CD31**LMO2***VEGFR2** vessels in U87MG xenografts comparing the tumor core and margin (DAPI, blue; CD31, white; LMO2, green; VEGFR2, red). Scale bar = 100 μ m. $n=3$ biological replicates. **J** Quantification of LMO2 (VEGFR2) vessel proportions within the total CD31 + vasculature in the tumor core versus the margin. Data are presented as mean $\pm$ SEM. Statistical analyses were performed using two-sided unpaired t-tests ($*P < 0.05$). $n=3$ biological replicates

level was similar to that in the control group (Fig. 6C). To further confirm the cytoplasmic specificity of this regulation, we examined whether ITGB1 knockdown could alter the expression of LDB1 and LDB2, which are well-known nuclear partners of LMO2. Expression levels of LDB1 and LDB2 remained largely unchanged across all conditions regardless of LMO2 overexpression or ITGB1 silencing (Fig. 6D). Overall, these findings suggested that the phenotypic effects observed in LMO2-overexpressing ECs are primarily driven by cytoplasmic LMO2 rather than by nuclear LMO2 or its interaction with LDB family members.

Discussion

In the current study, we identified the cytoplasmic role of LMO2 in ECs, which contributes to tumor angiogenesis. Specifically, we demonstrated that cytoplasmic LMO2 physically interacts with ITGB1, promoting the activation of focal adhesion signaling pathways, including FAK, Src, and ERK, and enhancing EC migration and sprouting behavior [11, 44, 47]. These findings expand the functional repertoire of LMO2 beyond its well-established nuclear roles in hematopoiesis and transcriptional regulation, highlighting the cytoplasmic adhesion-related axis of LMO2 activity in tumor vasculature [5, 13, 21]. Our structural modeling and biochemical data further indicate that cytoplasmic LMO2 is incorporated into ITGB1–talin-1 complexes at the integrin β 1 cytoplasmic tail, and that ITGB1 knockdown alone is sufficient to abolish LMO2-driven focal-adhesion activation, migration, and survival, highlighting a direct, ITGB1-dependent mechanism rather than an indirect transcriptional effect.

The tumor microenvironment plays a crucial role in guiding angiogenic responses through paracrine signaling [51, 52]. Among these, VEGF, secreted by reactive astrocytes and other stromal components, is a key

regulator of new blood vessel formation in GBMs, as well as other aggressive cancers [25]. Herein, we found that VEGF stimulation induced cytoplasmic accumulation of LMO2 in ECs. Additionally, LMO2 overexpression led to its co-localization with VEGFR2 in migrating tip-like cells and resembled the LMO2-enriched phenotype observed in tumor-associated vasculature. This indicates that VEGF not only promotes traditional angiogenic signaling but also directs the spatial relocation of LMO2 to support pro-migratory endothelial programs. Consistent with this, cross-cohort transcriptomic analyses showed that LMO2 is preferentially enriched in endothelial populations and positively correlates with VEGFR2 expression across multiple brain tumor datasets and in an EC-enriched subset of the TCGA-GBMLGG cohort. This implies that LMO2 functions as a VEGF-responsive effector that tracks the strength of angiogenic signaling in tumor endothelium.

To identify the potential molecular effectors downstream of LMO2, we performed pull-down proteomics and combined these findings with vascular protein expression profiles. ITGB1 was identified as a potential binding partner, as confirmed by Co-IP and PLAs. Notably, LMO2 overexpression did not alter the expression of ITGB1, indicating that LMO2 influences ITGB1-associated signaling through direct interactions rather than transcriptional regulation. ITGB1 knockdown abolished focal adhesion activation and the migratory phenotype induced by LMO2, supporting a functional LMO2–ITGB1 axis in endothelial dynamics. Supporting this, anatomical transcriptomics from the Ivy Glioblastoma Atlas showed that LMO2-high vascular regions are enriched for ITGB1 partner integrin α subunits (ITGA1/2/4/5/8) and their ECM ligands, including collagen IV, fibronectin, laminins, and nidogens, which are abundant in the GBM vascular basement membrane. Among these, ITGA5 is particularly interesting because

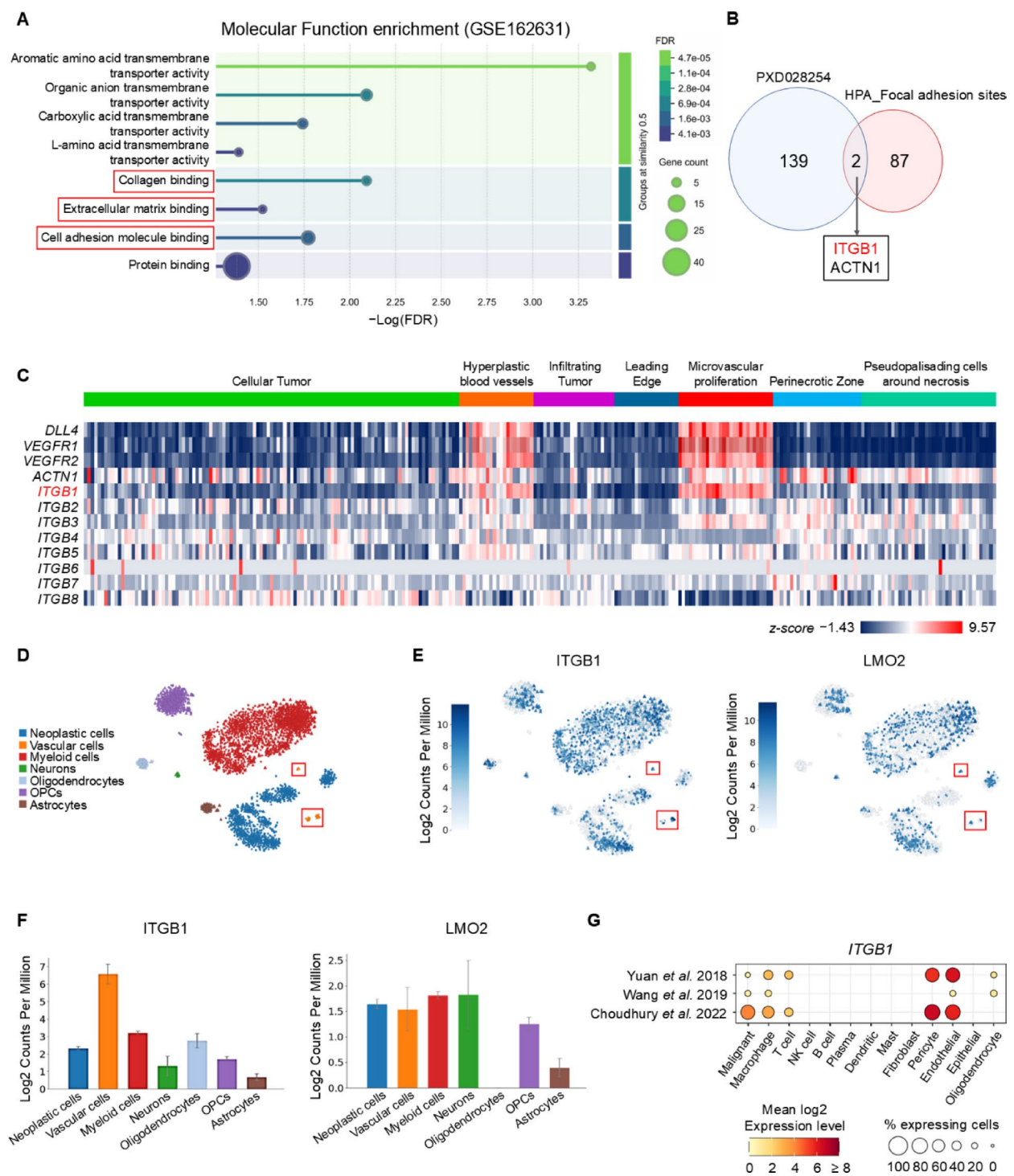


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it forms the canonical $\alpha 5 \beta 1$ integrin with ITGB1 and acts as a main fibronectin receptor in endothelial cells. This provides a direct mechanistic link between the LMO2–ITGB1 complex and the engagement of the fibronectin-rich vascular basement membrane. Functionally, overexpression of LMO2 increased EC adhesion to these

matrices, linking the LMO2–ITGB1 axis to ECM engagement in situ. Overall, the simultaneous enrichment of ITGA5 and fibronectin-related signatures in LMO2-high vascular regions supports a model in which cytoplasmic LMO2 enhances $\beta 1$ -integrin–dependent adhesion programs, likely including $\alpha 5 \beta 1$ –fibronectin interactions, to

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Fig. 3 Integrin $\beta 1$ is a putative LMO2 interactor enriched in tumor-associated endothelial cells. **A** Gene Ontology (GO) enrichment analysis of molecular function terms in the LMO2-positive endothelial cell (EC) cluster derived from the GSE162631 single-cell RNA sequencing dataset. Significantly enriched GO terms were selected using a false discovery rate cutoff of <0.05 . An enrichment graph was generated using the STRING (<https://string-db.org/>). $n=3$ biological replicates. **B** Venn diagram illustrating the overlap of LMO2-associated proteins identified by proteomics (ProteomeXchange: PXD028254) and the “focal adhesion”-annotated protein subset from the Human Protein Atlas. Two shared candidates were identified: integrin $\beta 1$ (ITGB1) and α -actinin 1 (ACTN1). **C** Heatmap illustrating the spatial transcriptomic distribution of *ITGB1*, *ACTN1*, and other integrin- β family members within glioblastoma histological subregions, as analyzed using the Ivy Glioblastoma Atlas Project (IvyGAP, <http://glioblastoma.alleninstitute.org/>). *VEGFR1*, *VEGFR2*, and *DLL4* served as positive controls for tumor vasculature-specific gene expression. Relative mRNA expression values were converted to z-scores and visualized in a heatmap format. **D–F** t-SNE plots visualizing single-cell RNA sequencing data ($n=3$ 589 cells) from the tumors of patients with glioblastoma obtained from the GBMseq database (www.gbmseq.org; GSE84465). **D** Global distribution of all the annotated single cells. **E** Subpopulations of ITGB1- and LMO2-expressing cells were mapped onto the same t-SNE projection. **F** ITGB1 and LMO2 expression levels across distinct cell types, including tumor, endothelial, immune, and stromal compartments. **G** Dot plots showing the average expression ($\log_2(\text{TPM}/10)$) and the percentage of cells expressing ITGB1 across several brain tumor scRNA-seq datasets (Yuan et al., 2018 [33], Wang et al., 2019 [28], Choudhury et al., 2022 [29]). Expression levels and percentages were calculated for each cell type in each dataset and then averaged across each cohort

promote pro-migratory endothelial behavior within the tumor microenvironment.

Despite these insights, our study had some limitations. Although the use of a 3D microfluidic sprouting platform enabled spatially relevant endothelial modeling, it did not allow for the resolution of specific endothelial subtypes, such as tip versus stalk cells, which differ in their VEGF responsiveness and migratory capacity [49, 50]. In this context, HUVECs were used in the microfluidic sprouting assay as a standardized and reproducible measure of morphogenesis to quantify sprouting dynamics. To ensure relevance to the disease context and to support mechanistic interpretation in brain endothelium, we complemented these assays with direct biochemical and signaling validation in HBMECs and with analyses of tumor tissue endothelial cells. Thus, the microfluidic sprouting platform functions as a quantitative system for morphogenesis, while glioma vascular relevance and mechanistic insights are anchored in brain endothelial and tissue-based evidence. Additionally, while we aimed to evaluate VEGFR2 pathway engagement at the receptor activation level in tumor vessels, detecting phospho-VEGFR2 in tumor cryosections was technically challenging, consistent with the known instability of phospho-epitopes in tissue samples. Nevertheless, VEGF stimulation and HIF1A induction in endothelial cells resulted in consistent increases in LMO2 and VEGFR2 signaling markers in our cell-based assays (Fig. 2 and Fig. S2), supporting a VEGF-responsive LMO2 program despite limitations in tissue phospho-epitope detection.

Future studies utilizing single-cell transcriptomics and live imaging approaches may help elucidate how LMO2 functions are distributed among EC subpopulations. Furthermore, although our data suggest a functional interaction between cytoplasmic LMO2 and ITGB1, the precise molecular interface, timing regulation, and involvement of adaptor proteins need to be clarified. In particular, mutational analysis of the predicted LMO2 contact residues within the ITGB1–talin-1 complex, combined with subtype-specific *in vivo* models, will be necessary to understand how LMO2 quantitatively modulates integrin

activation dynamics across different endothelial states and microanatomical niches.

The broad relevance of this mechanism across various cancer types and microenvironmental settings warrants further investigation. Because LMO2 is also present in non-ECs, it is crucial to examine its effects specifically in ECs *in vivo*. Disrupting the LMO2–ITGB1 pathway through genetic or pharmacological means could provide a novel anti-angiogenic approach, particularly for tumors resistant to existing VEGF-targeted therapies [53, 54]. Resistance to anti-VEGF therapies remains a major clinical challenge, underscoring the need to identify downstream or parallel pathways that can be co-inhibited. Our research indicates that targeting the LMO2–ITGB1–focal adhesion signaling pathway may reduce the growth of tumor blood vessels by impairing EC movement and attachment. Since LMO2 links VEGF signaling to $\beta 1$ -integrin–mediated adhesion to GBM-specific ECM components, selectively disrupting cytoplasmic LMO2 function in endothelial cells—either alone or in combination with VEGF/VEGFR2 or integrin inhibitors—may be an effective strategy to overcome adaptive angiogenic escape and enhance the durability of anti-vascular treatments.

In summary, we uncovered a previously unrecognized cytoplasmic function of LMO2 in tumor ECs and identified its interaction with ITGB1 as the mechanistic link between focal adhesion signaling and angiogenic behavior. These findings provide mechanistic insights into the VEGF–LMO2 axis and suggest that cytoplasmic LMO2 may function as a potential therapeutic target for anti-angiogenic cancer therapy.

Materials and methods

Cell culture and culture conditions

Human glioma stem-like cell lines 528NS and MD13 were generously provided by Dr. Ichiro Nakano (Department of Neurosurgery, Ohio State University, USA). Sanger sequencing confirmed that both cell lines possessed wild-type sequences at IDH1 (R132) and IDH2 (R172) mutation hotspots. These cells were cultured

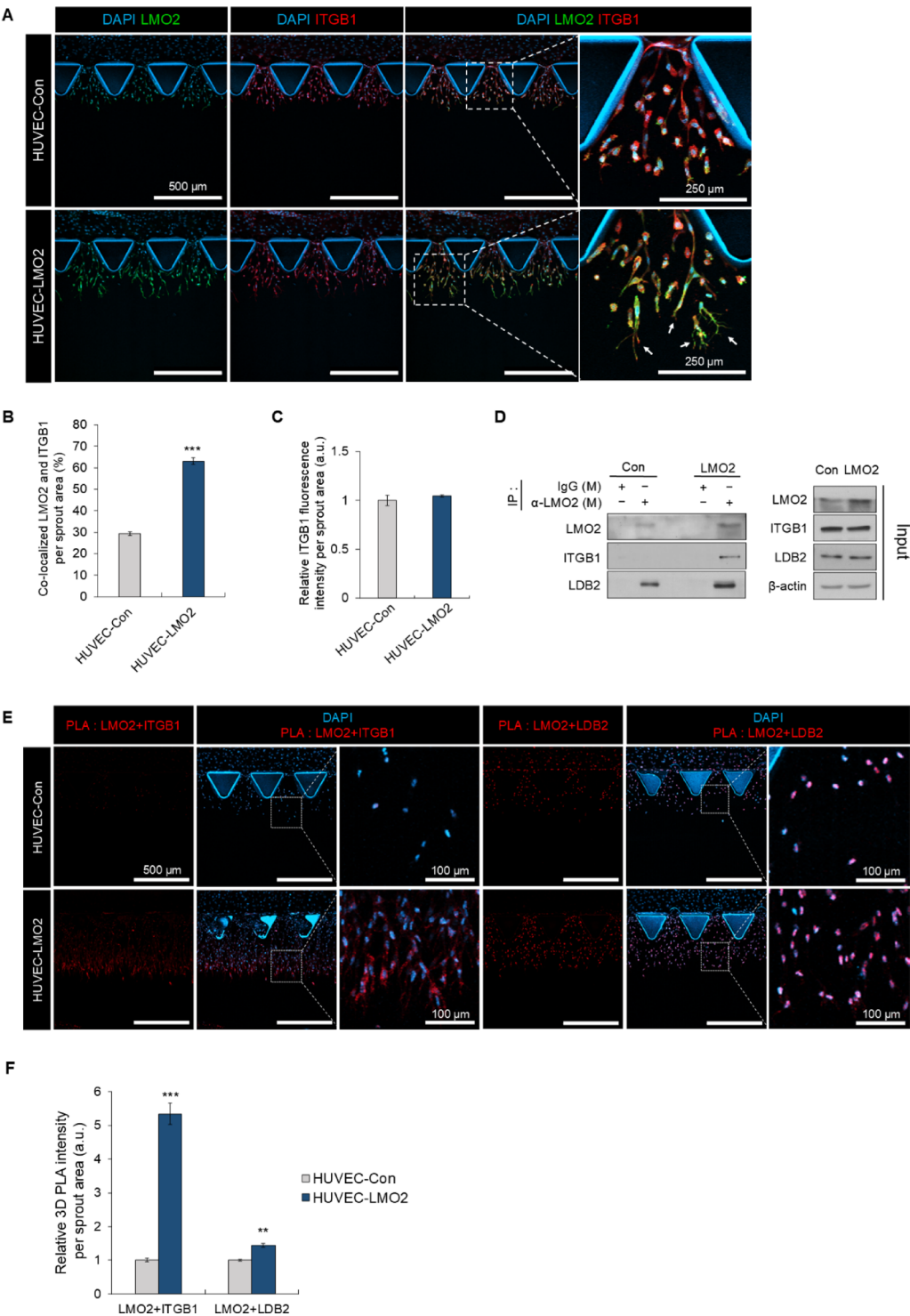


Fig. 4 (See legend on next page.)

in DMEM/F12 medium (SH3002301; HyClone, Logan, UT, USA), supplemented with 0.2% B27 (17,504–044; Invitrogen, Carlsbad, CA, USA), 20 ng/mL epidermal growth factor (236-EG; R&D Systems, Minneapolis, MN, USA), 20 ng/mL basic fibroblast growth factor (4114-TC; R&D Systems), 1% penicillin/streptomycin (SV30010;

HyClone), 2 mM L-glutamine (SV30082.01; HyClone), and 50 μg/mL gentamicin (6442; Tocris, Bristol, UK). Human GBM cell lines LN229 and U87MG were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in high-glucose (4500 mg/L) DMEM (SH30243.01; HyClone),

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Fig. 4 LMO2–ITGB1 interaction is enhanced in LMO2-overexpressing endothelial cells. **A** Immunofluorescence staining of control and LMO2-overexpressing endothelial sprouts cultured on a three-dimensional (3D) microfluidic angiogenesis chip. The samples were stained with DAPI (blue), anti-LMO2 (green), and anti-ITGB1 (red). Scale bars: Low magnification, 500 μ m; High magnification, 250 μ m. $n = 3$ biological replicates. **B** Quantification of LMO2 and ITGB1 co-localization percentage in control and LMO2-overexpressing endothelial sprouts. Statistical significance was determined using a two-sided unpaired *t*-test ($***P < 0.001$). Data are presented as mean $\pm$ SEM. $n = 3$ biological replicates. **C** Quantification of relative ITGB1 fluorescence intensity (arbitrary units [a.u.]) in control and LMO2-overexpressing endothelial sprouts. Statistical significance was determined using two-sided unpaired *t*-tests. Data are presented as mean $\pm$ SEM. $n = 3$ biological replicates. **D** Co-immunoprecipitation of LMO2 from lysates of control and LMO2-overexpressing human umbilical vein endothelial cells (HUVECs) using anti-LMO2 or control IgG. LDB2 was used as a positive control for LMO2. β -actin was used as an input loading control to ensure equal protein loading across samples. $n = 2$ biological replicates. **E** Representative PLA images of the LMO2–ITGB1 and LMO2–LDB2 interaction in 3D-cultured control or LMO2-overexpressing HUVECs. DAPI (blue) and PLA (red) signals. Scale bars: Low magnification, 500 μ m; High magnification, 100 μ m. **F** Quantification of PLA signal intensity (arbitrary units [a.u.]) per field from **(E)**. Statistical significance was determined using two-sided unpaired *t*-tests ($**P < 0.01$, $***P < 0.001$). Data are presented as mean $\pm$ SEM. $n = 3$ biological replicates

supplemented with 10% fetal bovine serum (SH30919.03; HyClone), 1% penicillin/streptomycin, 2 mM L-glutamine, and 50 μ g/mL gentamicin.

Primary HUVECs (CC-2517; Lonza) and HBMECs (ACBRI 376; Cell Systems, Kirkland, USA) were cultured on plates coated with 0.2% gelatin (G1890; Sigma-Aldrich, Missoula, MO, USA). Cells were maintained in endothelial growth medium (EGM-2) (CC-3162; Lonza) and used for experiments at passages 5–6. For VEGF treatment, HUVECs were incubated with 100 ng/mL VEGF in the EGM-2 medium for 24 h. All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

Orthotopic xenograft models

Female BALB/c nude (Nu/Nu) mice (5 weeks old; Orient Bio, South Korea) were anesthetized with a combination of Zoletil (19 mg/kg) and Rompun (4.8 mg/kg) prior to tumor cell implantation. Cells—528NS (5×10^3), MD13 (1×10^3), U87MG, and LN229 (1×10^4)—were collected, washed, and resuspended in 1 $\times$ phosphate-buffered saline (PBS) (D5652; Sigma-Aldrich) following viability confirmation using trypan blue exclusion (T8154; Sigma-Aldrich). Then, cells were stereotactically injected into the caudate putamen (coordinates: 2 mm lateral, 0.5 mm anterior to bregma, 3 mm below the skull surface) using a stereotaxic apparatus (JD-SI-02; Jeung Do Bio & Plant, Seoul, Korea). The animals were randomly divided into experimental groups, with five biologically independent mice per group.

Mice were maintained in a SPF environment under controlled conditions (12-h light/dark cycle, 22 ± 2 °C, and $50 \pm 10\%$ humidity). Only female mice were used to reduce the variability associated with hormonal cycles. General health status and tumor-related symptoms were monitored daily. Humane endpoints were predefined in accordance with institutional animal care policies, including ≥ 10 –20% weight loss, ocular abnormalities (e.g., eyelid closure, periorbital swelling), diminished mobility or alertness, abnormal responses to stimuli, or persistent crouched posture. Animals exhibiting any of these conditions were euthanized promptly. The tumor

burden was restricted to less than 10% of the body weight or 15 mm in diameter.

The mice were euthanized via cardiac perfusion under deep anesthesia with 1 $\times$ PBS, followed by fixation with 4% paraformaldehyde (PFA) (158127; Sigma-Aldrich). The brains were post-fixed in 4% PFA for 24 h, cryoprotected in 30% sucrose for 72 h, and then embedded in OCT compound for histological analysis.

Plasmids

HA-LMO2 and ITGB1-FLAG were inserted into the pcDNA 3.1(+)-puromycin vector. The original ITGB1-FLAG construct (#30153; pCAX-ITGB1-FLAG) was purchased from Addgene (Cambridge, UK).

Lentiviral induction and siRNA transfection

In brief, HUVECs were transduced with lentiviral particles generated in HEK293FT cells (R70007; Life Technologies, USA), which were co-transfected with a lentiviral expression vector (pLL-CMV-puro, pLL-CMV-LMO2-puro, or pLL-CMV-GFP) and third-generation packaging plasmids (pMDLg/pRRE, pRSV-Rev, and pMD2.G).

For siRNA-mediated gene silencing, HUVECs (3.0×10^5) were transfected with 10 nM of commercially available siRNAs targeting human ITGB1, siITGB1-1 (ID: SASI_Hs01_00159474) and siITGB1-2 (ID: SASI_Hs02_00333437) (both from Sigma-Aldrich, Missoula, MO, USA), using ScreenFect A transfection reagent (Wako Pure Chemical Industries, 293-73201, Osaka, Japan) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cultured cells using QIAzol lysis reagent (QIAGEN, Hilden, Germany; Cat. 79306). One microgram of RNA was treated with DNase I (RNase-free, 1 U; Thermo Fisher Scientific, Cat. EN0525) at 37 °C for 30 min to remove genomic DNA, after which the enzyme was inactivated by adding 50 mM EDTA and heating at 65 °C for 10 min. The DNase-treated RNA was then reverse-transcribed into cDNA using the RevertAid First Strand cDNA Synthesis

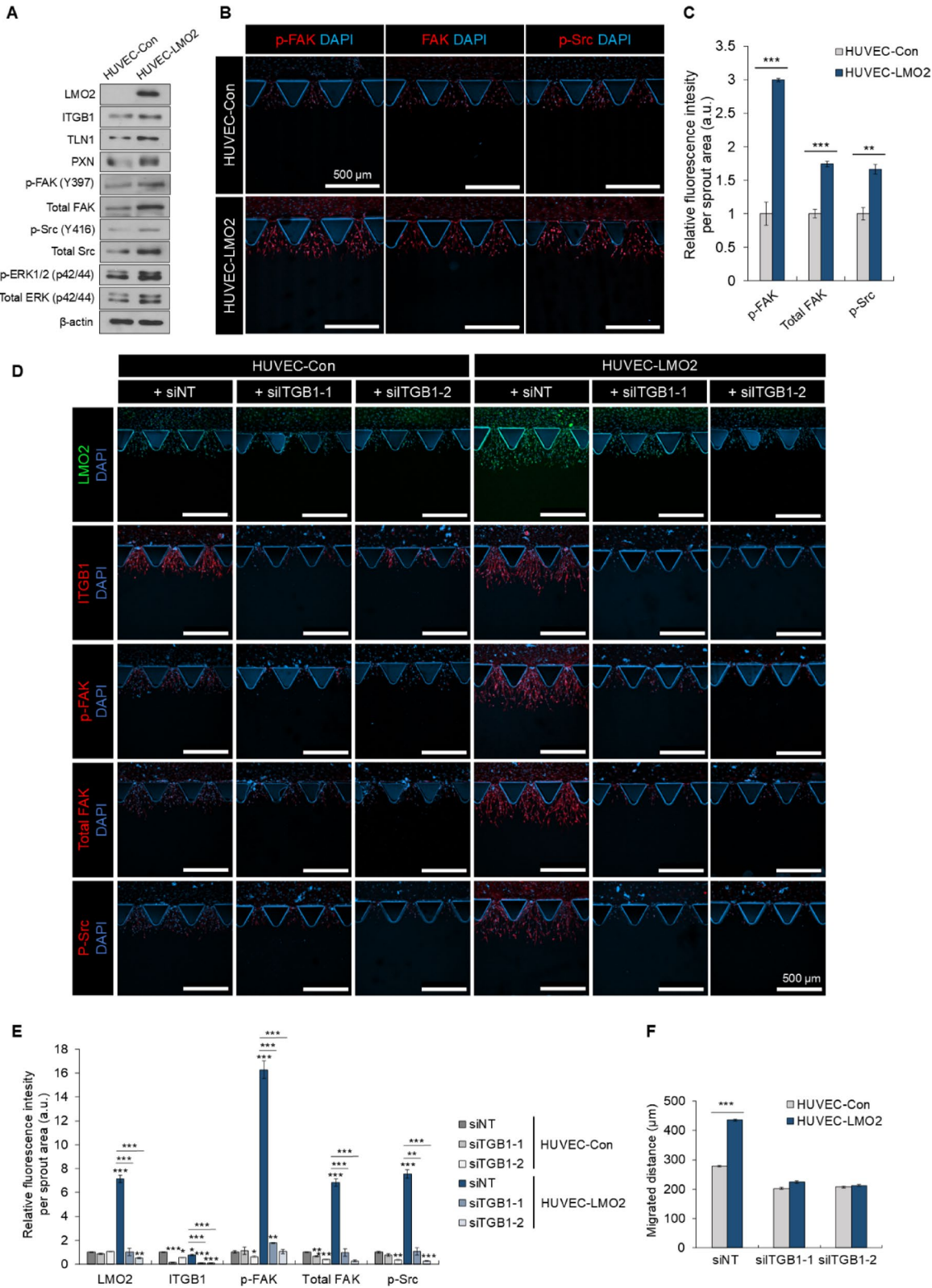


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Fig. 5 LMO2–ITGB1 interaction activates focal adhesion signaling and enhances endothelial cell migration. **A** Western blot analysis of focal adhesion signaling proteins in two-dimensional (2D)-cultured human umbilical vein endothelial cells (HUVECs) transfected with control or LMO2-overexpression constructs. β -actin was used as a sample processing control. $n=2$ biological replicates. **B** Representative immunofluorescence images of control and LMO2-overexpressing endothelial sprouts in a three-dimensional (3D) angiogenesis microchip model. Sprouts were stained for phosphorylated FAK (p-FAK), total FAK, and phosphorylated Src (p-Src) (red). The nuclei were stained with DAPI (blue). Scale bar, 500 μ m. $n=3$ biological replicates. **C** Quantification of the relative fluorescence intensity (arbitrary units [a.u.]) of p-FAK, total FAK, and p-Src in the control and LMO2-overexpressing endothelial sprouts. Data are presented as mean $\pm$ SEM. Statistical significance was determined using two-sided unpaired t -tests ($**P<0.01$, $***P<0.001$). $n=3$ biological replicates. **D** Representative immunofluorescence images of control or LMO2-overexpressing endothelial sprouts after siRNA treatment stained for LMO2 (green), total FAK, p-FAK, and p-Src (red). Nuclei were counterstained with DAPI (Blue). Scale bar, 500 μ m. $n=3$ biological replicates. **E** Quantification of fluorescence intensity (arbitrary units [a.u.]) of LMO2, ITGB1, p-FAK, total FAK, and p-Src in siRNA-treated endothelial sprouts. Data are presented as mean $\pm$ SEM. Statistical significance was determined using two-sided unpaired t -tests ($*P<0.05$, $**P<0.01$, $***P<0.001$). $n=3$ biological replicates. **F** Quantification of endothelial migration distance (μ m) in control or LMO2-overexpressing sprouts following siRNA treatment. Statistical significance was determined using a two-sided unpaired t -test ($***P<0.001$). Data are presented as the mean $\pm$ SEM. $n=3$ biological replicates

Kit (Thermo Fisher Scientific, Cat. K1622) according to the supplier's protocol. Quantitative PCR was carried out on a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA) using TB Green Premix Ex Taq (Takara Bio, Kusatsu, Japan; Cat. RR420A). Reactions were run under the following cycling conditions: an initial denaturation at 95 °C for 30 s, followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s. Gene expression values were normalized to GAPDH, and relative mRNA levels were calculated using the $2^{-\Delta\Delta C_q}$ method. Primer sequences for all targets are listed in Supplemental Table 1.

Western blot analysis

Western blotting was performed to detect protein expression levels. Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris–HCl; pH 7.4) supplemented with phosphatase inhibitors—1 mM β -glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM sodium fluoride, 1 mM sodium orthovanadate—and a protease inhibitor cocktail (11836170001; Roche). Protein concentrations were determined using the Bradford assay (Bio-Rad) following the manufacturer's protocol. Equal amounts of protein were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (BSP0861; Pall Corporation, USA). Membranes were blocked with 5% non-fat dry milk and incubated with primary antibodies at the indicated dilutions: anti-LMO2 (1:500) (NB110-78626; Novus Biologicals), anti- β -actin (1:10 000) (sc-47778; Santa Cruz, USA), anti-ITGB1 (1:2 000) (A19072; ABclonal, South Korea), anti-p-VEGFR2 (Y1175; 1:1 000) (2478S; Cell Signaling, Danvers, MA, USA), anti-VEGFR2 (1:500) (2479S; Cell Signaling Technology, USA), anti-HIF1 α (1:1 000) (ab51608; Abcam, Cambridge, England), anti-ITGA5 (1:1 000) (ab150361; Abcam), anti-Talin (1:1 000) (T3287; Sigma-Aldrich), anti-Paxillin (1:10 000) (ab32084; Abcam), anti-p-FAK (Y397, 1:500) (AP0302; ABclonal), anti-FAK (1:1 000) (A11195; ABclonal), anti-p-Src (Y416; 1:1 000) (2101S; Cell Signaling Technology), anti-Src (1:500) (sc-8056; Santa Cruz), anti-p-ERK1/2 (Thr202/Tyr204; 1:1 000) (9101; Cell Signaling

Technology), total ERK1/2 (1:2 000) (9102; Cell Signaling Technology), anti-LDB1 (1:1 000) (ab96799; Abcam), anti-LDB2 (1:2 000) (A10472; ABclonal), anti-HA (1:1 000) (H9658; Sigma-Aldrich), and anti-FLAG (1:1 000) (F1804; Sigma-Aldrich). Following incubation with horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology, Waltham, Massachusetts, USA), signals were developed using SuperSignal West Pico Chemiluminescent Substrate (34580; Pierce Biotechnology).

Co-IP assay

For Co-IP, HEK293FT cells were co-transfected with plasmids encoding HA-tagged LMO2 and FLAG-tagged ITGB1. After 48 h, the cells were lysed in IP lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) (10837091001; Sigma-Aldrich), a protease inhibitor cocktail (11836 153 001; Roche, Basel, Switzerland), and phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM sodium fluoride, and 1 mM sodium orthovanadate). Lysates were pre-cleared using Protein A/G agarose beads (Thermo Fisher Scientific), and 500–1 000 μ g of total protein was incubated with anti-FLAG (1:100; Sigma-Aldrich) or anti-HA (1:100; Cell Signaling Technology) antibodies along with Protein A/G beads. Following extensive washing with IP buffer, bound proteins were eluted in 1 $\times$ LDS sample buffer (NP0007; Invitrogen) at 95 °C for 5 min. Eluted proteins were separated by SDS-PAGE and transferred onto PVDF membranes (IPVH00010; Millipore, Darmstadt, Germany). After blocking with 5% non-fat dry milk, the membranes were incubated with the relevant primary antibodies and horseradish peroxidase-conjugated secondary antibodies (31460 for anti-rabbit, 31430 for anti-mouse; Thermo Fisher Scientific). Detection was performed using SuperSignal west Pico Plus chemiluminescent substrate (34578; Thermo Fisher Scientific). Primary antibodies used were anti-FLAG (1:1 000) and anti-HA (1:1 000).

For endogenous Co-IP experiments using HUVECs, the cells were lysed using IP lysis buffer, supplemented with PMSF and protease and phosphatase inhibitors. After pre-clearing with Protein A/G agarose, 1 mg of

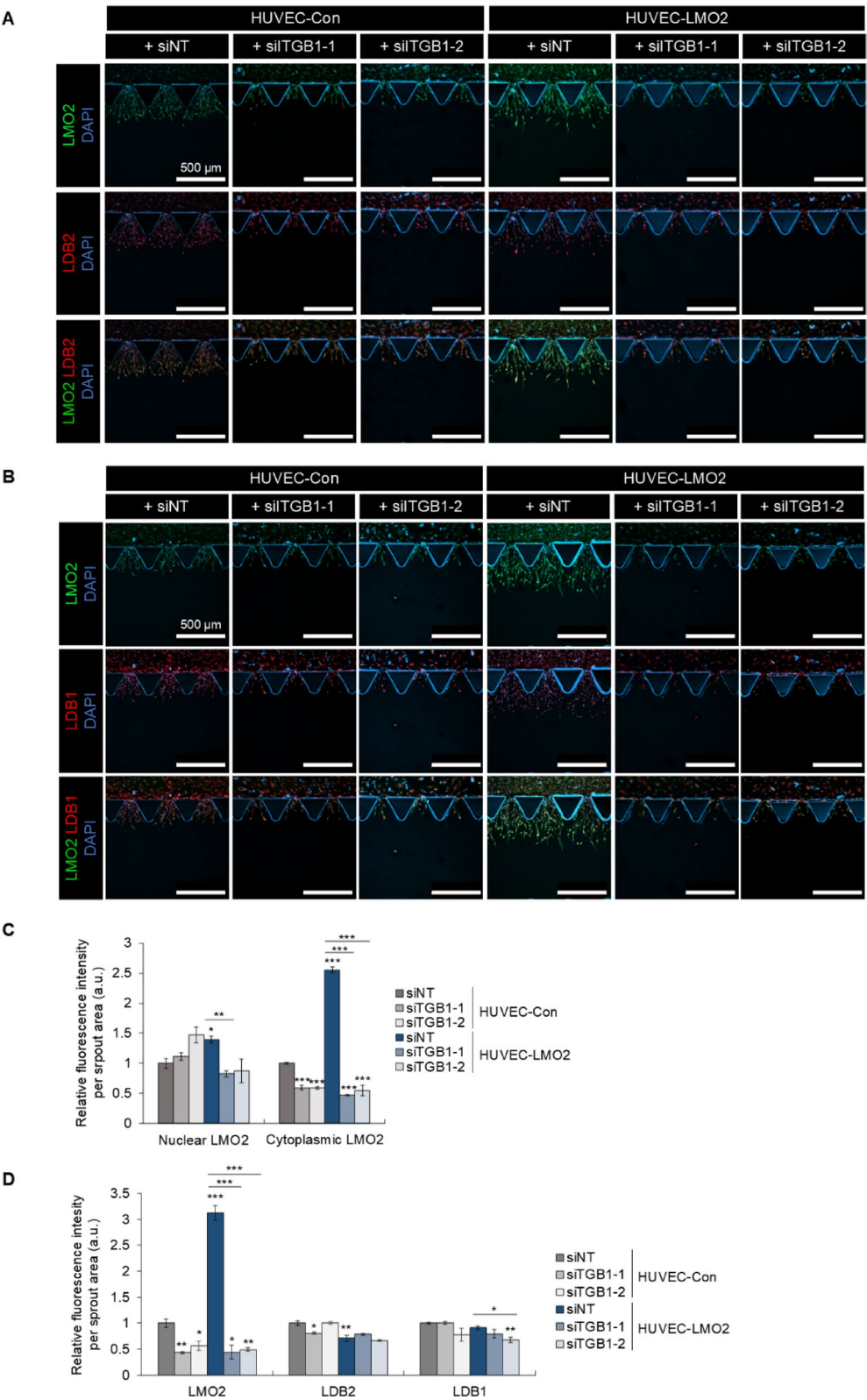


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Fig. 6 Cytoplasmic, but not nuclear, LMO2 mediates ITGB1-dependent phenotypes in endothelial cells (**A–B**) **A** and **B** Representative immunofluorescence images of siRNA-treated control or LMO2-overexpressing endothelial sprouts in a three-dimensional (3D) angiogenesis microchip model. Cells were stained with DAPI (blue), LMO2 (green), LDB2 (red) (**A**), or LDB1 (red) (**B**). Scale bar, 500 μ m. $n=3$ biological replicates. **C** Quantification of relative fluorescence intensity (arbitrary units [a.u.]) of nuclear and cytoplasmic LMO2 in siRNA-treated control or LMO2-overexpressing endothelial sprouts. Data are presented as mean $\pm$ SEM. Statistical significance was determined using two-sided unpaired *t*-tests (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). $n=3$ biological replicates. **D** Quantification of fluorescence intensity (arbitrary units [a.u.]) of LMO2, LDB1, and LDB2 in siRNA-treated control and LMO2-overexpressing endothelial sprouts. Data are presented as mean $\pm$ SEM. Statistical significance was determined using two-sided unpaired *t*-tests (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). $n=3$ biological replicates

protein was incubated with 4 μ g of anti-LMO2 antibody for immunoprecipitation. Beads were washed thoroughly with IP buffer and PBS containing 1 mM PMSF.

For subsequent immunoblotting, cell lysates were prepared in RIPA buffer (LPS Solution, Daejeon, Korea) containing β -glycerophosphate (1 mM), sodium pyrophosphate (2.5 mM), NaF (1 mM), Na_3VO_4 (1 mM), and protease inhibitors. Protein concentration was measured using the Bradford assay (Bio-Rad), and 10–50 μ g of total protein was subjected to SDS-PAGE, followed by western blot analysis. β -actin served as a loading control.

HUVEC spheroid sprouting assay

HUVECs (1×10^3 cells) were suspended in a solution containing 0.25% methylcellulose (Cat. M0512; Sigma-Aldrich) and EBM-2 basal medium (CC-3156; Lonza). Next, 20 μ L of this cell suspension was dispensed as hanging drops and incubated overnight at 37 °C in a humidified 5% CO_2 incubator to allow spheroid formation. Collagen I (4.07 mg/mL stock; 354236; Corning, Massachusetts, USA) was added to attain a final volume of 100 μ L. The spheroids were gently collected using PBS, centrifuged at 500 rpm for 5 min using a swing-bucket rotor, and resuspended in 60 μ L of 0.25% methylcellulose. This suspension was mixed with the prepared collagen solution, and a total of 150 μ L of the final mixture was transferred into 48-well plates, followed by polymerization at 37 °C for 30 min.

After gel polymerization, 500 μ L of EGM-2 medium, supplemented with 100 μ g/mL recombinant VEGF (293-VE-050; R&D Systems, Minneapolis, Minnesota, USA), was overlaid. Endothelial sprouting was assessed five days post-incubation. Sprouts were fixed in 4% PFA and processed for immunofluorescence staining. Quantitative image analysis was performed using ImageJ software (v1.52a), with the Angiogenesis Analyzer and Analyze Particles plugins (<http://image.nih.gov/ij/>) [55].

Wound healing assay

Briefly, HUVECs (1.5×10^5 cells) were seeded in a 6-well plate pre-coated with 0.2% gelatin. The medium was replaced daily until confluent monolayers were obtained. Subsequently, the cells were treated with 50 μ g/mL mitomycin C (M4287; Sigma-Aldrich) for 2 h to inhibit cell proliferation. The cell monolayer was manually scratched using a 1 000- μ L plastic pipette tip, washed twice with

PBS, and incubated with EGM-2. Images were captured every 24 h using an inverted microscope, and the distance of the wound was quantified using ImageJ (v1.52a; <http://imagej.nih.gov/ij/>).

3D microfluidic angiogenesis assay

Microfluidic devices (DAX-1; AIM Biotech, MA, USA) were used to perform the 3D angiogenesis assays. Fibrinogen solution (11.11 mg/mL) (F8630; Sigma-Aldrich) was incubated overnight at 37 °C in a water bath to ensure complete dissolution. After filtration through a 0.22- μ m membrane, the fibrinogen was mixed with 100 U/mL thrombin (T7201; Sigma-Aldrich), 3 U/mL aprotinin (A3428; Sigma-Aldrich), and EGM-2 medium on ice. Two separate mixtures were prepared: Vial 1 containing 90 μ L of fibrinogen and 10 μ L of aprotinin, and Vial 2 containing 100 μ L of EGM-2 and 1 μ L of thrombin. Equal volumes of Vial 1 and Vial 2 were combined, and 10 μ L of the final mixture was injected into the central gel region of the chip. The gel was allowed to polymerize for 15 min in a 37 °C incubator.

After gelation, the media channel was coated with 15 μ L of 50 μ g/mL fibronectin (F0895; Sigma-Aldrich) diluted in EGM-2 for 1 h at 37 °C. The coating solution was flushed by adding 70 μ L of EGM-2 to one port and 50 μ L to the opposite port of the same media channel. Subsequently, 65 μ L of EGM-2 was added to both ends of the media channel, and the device was tilted vertically (90°). For cell seeding, 20 μ L of EGM-2 was added to one inlet, and 10 μ L of a HUVEC suspension (8×10^4 cells/10 μ L) was loaded using a 10- μ L pipette. After a 2-min incubation to allow cell attachment, the opposite inlet was subjected to the same procedure. The chip was then maintained in a vertical position at 37 °C for 1 h and subsequently returned to a horizontal position.

For the induction of angiogenic sprouting, media containing 40 ng/mL VEGF-A (293-VE; R&D Systems) and 125 nM sphingosine-1-phosphate (860492P; Millipore) were added and refreshed every 12 h during the culture period.

Proximity ligation assay (PLA)

HUVECs were seeded onto 0.2% gelatin-coated 48-well chamber slides or within 3D microfluidic platforms and then fixed with 4% PFA. Thereafter, cells were permeabilized with 0.1% Triton X-100 for 15 min at room

temperature (RT). The PLA was performed using primary antibodies followed by Duolink® in situ PLA probes—anti-rabbit PLUS (DUO92002; Sigma-Aldrich), anti-mouse MINUS (DUO92004; Sigma-Aldrich)—and Red detection reagents (DUO92008; Sigma-Aldrich), according to the manufacturer's guidelines. Cells were incubated with the following primary antibodies overnight at 4 °C: anti-LMO2, anti-LDB2, and anti-ITGB1 (all 1:200). Following PLA probe hybridization and ligation, nuclei were counterstained with DAPI (1 µg/mL; Sigma-Aldrich) for 5 min, and confocal images were acquired using a laser scanning microscope (LSM800; Carl Zeiss, Oberkochen, Germany).

Immunofluorescence analysis

HUVECs or endothelial sprouts cultured on 3D microfluidic platforms were fixed in 4% PFA for 15 min at RT. The samples were then rinsed with PBS three times, permeabilized with 0.1–0.3% Triton X-100 for 15–30 min at RT, and blocked in 3% bovine serum albumin (BSA) (82–100-6; Merck) in PBS for 1–2 h. For immunostaining, specimens were incubated overnight at 4 °C with primary antibodies (diluted 1:200), followed by incubation with Alexa Fluor 488- or 647-conjugated secondary antibodies (1:400) (A10042, A21202; Thermo Fisher Scientific) for 2 h at RT. Nuclei were counterstained with 1 µg/mL DAPI (D9542; Sigma-Aldrich) for 5–10 min. For F-actin visualization, HUVEC sprouts were stained with phalloidin. The sprouts were fixed in 4% PFA for 15 min, permeabilized with 0.3% Triton X-100 for 10 min, and blocked with 3% BSA for 1 h at RT. Subsequently, the samples were incubated with 6.6 µM Alexa Fluor-conjugated phalloidin (A12379; Invitrogen) for 2 h, followed by DAPI staining for 5 min.

For histological analysis, the tissues were embedded in the tissue freezing medium (Cat. 3801480; Leica, Wetzlar, Germany) for 3 h at –25 °C. Frozen tissue blocks were cut into 40-µm-thick sections and blocked overnight with 3% BSA containing 0.3% Triton X-100 in PBS at 4 °C. The sections were then incubated at 4 °C with the following primary antibodies for 12 h: anti-CD31 (1:200) (MAB1398Z; Merck), anti-LMO2 (1:200) (NB110-78626; Novus Biologicals), anti-VEGFR2 (1:200) (9585; Cell Signaling Technology), and anti-VEGFR2 (1:200) (2479S; Cell Signaling Technology). After washing four times with PBS containing 0.1% Tween-20 (PBS-T), the sections were incubated with the Alexa Fluor 488-conjugated or Alexa Fluor 647-conjugated secondary antibodies (1:400) (127-605-160; Jackson Lab) for 12 h at 4 °C. Nuclei were stained with DAPI (1 µg/mL) for 10 min at RT. Samples were mounted using ProLong™ Gold Antifade Mountant (p36930; Invitrogen). The area of the blood vessels was determined by measuring the CD31⁺ area within randomly selected 0.4 mm² regions. The fluorescence

intensities of LMO2 and VEGFR2 were measured in the CD31⁺ area within randomly selected tumor regions of 0.4 mm². Images were analyzed using ImageJ (version 1.52a; <http://imagej.nih.gov/ij/>).

Images were acquired at RT using a confocal laser scanning microscope (LSM800; Carl Zeiss) equipped with ZEN software (version 2018; blue edition) using a 10× objective lens. Quantification of the fluorescence signal intensity was performed using ImageJ. For colocalization analysis, the merged regions of the two fluorescent signals were analyzed using a consistent intensity threshold across all samples. To quantify the subcellular distribution of LMO2, the nuclear LMO2 signal was defined as the overlapping region between the DAPI and LMO2 signals and measured accordingly. Cytoplasmic LMO2 signal intensity was calculated by subtracting the DAPI⁺LMO2⁺ co-localized signal area from the total LMO2 signal intensity per unit area (µm²) of the whole cell or sprout region. All the quantifications were performed using identical threshold settings to ensure consistency.

Cell viability assay

Cell viability was assessed using the Cyto-X assay kit (CYT3000; LPS Solution, Daejeon, Korea), which measures metabolic activity as an indicator of cell health. In brief, cells were plated in 96-well plates at a density of 5×10⁴ cells/well. After adding the Cyto-X reagent, the plates were incubated for 3 h at 37 °C. Absorbance was measured at 450 nm using a multimode microplate reader (Sense 425-301; Hidex, Turku, Finland).

Cell fractionation assay

Subcellular fractionation was performed using a commercial cell fractionation kit (ab109719; Abcam), according to the manufacturer's instructions. Briefly, cell pellets (6.6×10⁶ cells/mL) were resuspended in Buffer A and treated with Detergent I to selectively permeabilize the plasma membrane. After centrifugation at 10 000×g for 2 min, the supernatant was collected as the cytoplasmic fraction. The remaining pellets were resuspended in Buffer A treated with Detergent II for further solubilization. After a second centrifugation step under the same conditions, the residual pellets were collected as the nuclear fraction. The mitochondrial fraction was not used in subsequent analyses.

Fraction-specific protein expression was assessed by immunoblotting using organelle marker proteins: Lamin B1 (1:3 000) (ab16048; Abcam) for the nucleus; Calnexin (1:2 000) (GTX109669; GeneTex) for the endoplasmic reticulum; and α-tubulin (1:10 000) (T6199; Sigma-Aldrich) for the cytoplasm.

ECM adhesion assay

Endothelial adhesion to extracellular matrix (ECM) substrates was assessed using HUVEC-Con and HUVEC-LMO2 cells. Briefly, 48-well tissue culture plates were coated with human collagen type IV (10 µg/mL; C5533; Sigma-Aldrich, Missoula, MO, USA), fibronectin (10 µg/mL; F0895; Sigma-Aldrich), or laminin (10 µg/mL; 23017-015; Invitrogen, Carlsbad, CA, USA) and rinsed with PBS immediately before use. HUVEC-Con and HUVEC-LMO2 cells were harvested, resuspended in serum-free medium, and seeded onto the coated wells at a density of 1×10^5 cells in 200 µL per well. After 1.5 h incubation at 37 °C, non-adherent cells were gently removed by washing with pre-warmed PBS, and adherent cells were fixed with 4% paraformaldehyde. Fixed cells were stained with crystal violet, washed, and air-dried. For quantification, stained cells were imaged with an inverted microscope, and adherent cell confluency was measured using ImageJ.

In silico analysis

To analyze the region-specific transcriptome profiles of patients with GBM, we obtained fragments per kilobase of transcripts per million mapped reads data from the IvyGAP website (<http://glioblastoma.alleninstitute.org/>) [15]. Regional information was categorized into seven types: hyperplastic blood vessels in the cellular tumor ($n=22$); microvascular proliferation ($n=28$); leading edge ($n=19$); infiltrating tumor ($n=24$); cellular tumor ($n=111$); perinecrotic zone ($n=26$); and pseudopalisading cells around necrosis ($n=40$). The relative genes were converted to z-scores, and the mean $\pm$ SD value of the z-score was calculated.

UMAP visualization was performed using publicly available scRNA-seq data from human GBM tissues (EGAS00001005300), originally published by Johnson et al. (2021) [12]. The UMAP plot was generated based on the preprocessed dataset provided by the authors, using R scripts publicly shared on GitHub (<https://github.com/TheJacksonLaboratory/singlecellglioma-verhaaklab>). This analysis focused specifically on EC populations, and the expression distributions of LMO2 and LDB1 were visualized using the existing UMAP embedding.

To explore the expression patterns of LMO2, LDB1, and LDB2 across diverse cancer types and associated cell populations, we used publicly available scRNA-seq datasets (EGAS00001002543) from the Curated Cancer Cell Atlas (<https://www.weizmann.ac.il/sites/3CA/>) [16]. Dot plots were generated to illustrate both the average expression levels and percentage of cells expressing each gene across the most prevalent cell types within each cancer type. Cell types were included in the analysis only if they constituted at least 1% of the total cell population in ≥ 5 individual datasets. Gene expression values were defined as $\log_2(\text{TPM}/10)$.

To examine the expression patterns of LMO2, KDR (VEGFR2), and ITGB1 across different cell types, we used publicly available scRNA-seq datasets [28, 29, 33]. For each dataset, we created dot plots that display both the average expression and the proportion of cells expressing each gene within the major cell populations. Only cell types accounting for at least 1% of all cells in five or more datasets were included in the analysis. Gene expression was measured as $\log_2(\text{TPM}/10)$.

To visualize the distribution of *ITGB1* expression across different cell populations in GBM, we analyzed GBMseq database (GSE84465) (www.gbmseq.org) [27], and other brain tumor scRNA-seq data. The t-SNE plots were generated using cell-type annotations provided in the original datasets without additional filtering. *ITGB1* expression levels were overlaid onto t-SNE projections to assess their enrichment in the tumor, endothelial, and stromal compartments.

For GO analysis, raw count data from the GSE162631 dataset were processed by applying the following quality control filters: cells expressing fewer than 200 genes and genes expressed in fewer than 30 cells were excluded [33]. Gene expression values were normalized using the transcripts per million (TPM) method and subsequently transformed to a $\log_2(\text{TPM}+1)$ scale. EC clusters were identified based on original annotations, and LMO2-positive EC-enriched clusters were isolated for downstream analysis. GO analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resources 6.8 (<http://david.ncifcrf.gov/home.jsp>) [56]. GO enrichment analysis of the molecular functions was performed using a list of genes specifically enriched in these clusters. Functional enrichment results were visualized using the STRING database (<https://string-db.org/>), and gene networks were plotted accordingly [57]. A false discovery rate threshold of < 0.05 was used to define statistically significant GO terms.

To identify the putative LMO2-interacting partners involved in endothelial focal adhesion signaling, we analyzed LMO2 pull-down proteomic data from a previously published dataset (ProteomeXchange ID: PXD028254) [8]. The resulting protein list was cross-referenced with subcellular localization annotations classified as “focal adhesion” in the Human Protein Atlas (<https://www.proteinatlas.org/>) to prioritize candidates with potential functional relevance in endothelial adhesion dynamics [34].

Structural models of the LMO2–ITGB1–TLN1 complex were generated using AlphaFold3 (Google DeepMind/Isomorphic Labs) [39]. Amino acid sequences for full-length human LMO2, the cytoplasmic tail of integrin $\beta 1$ (ITGB1; residues 752–798), and the N-terminal head domain of talin-1 (TLN1; residues 1–402) were provided as three separate chains in multimer mode. Five models were generated with default parameters,

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