



Multimerin-2 orchestrates the cross-talk between endothelial cells and pericytes: A mechanism to maintain vascular stability



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Abstract

Tumor angiogenesis is vital for the growth and development of various solid cancers and as such is a valid and promising therapeutic target. Unfortunately, the use of the currently available anti-angiogenic drugs increases the progression-free survival by only a few months. Conversely, targeting angiogenesis to prompt both vessel reduction and normalization, has been recently viewed as a promising approach to improve therapeutic efficacy. As a double-edged sword, this line of attack may on one side halt tumor growth as a consequence of the reduction of nutrients and oxygen supplied to the tumor cells, and on the other side improve drug delivery and, hence, efficacy. Thus, it is of utmost importance to better characterize the mechanisms regulating vascular stability. In this context, recruitment of pericytes along the blood vessels is crucial to their maturation and stabilization. As the extracellular matrix molecule Multimerin-2 is secreted by endothelial cells and deposited also in juxtaposition between endothelial cells and pericytes, we explored Multimerin-2 role in the cross-talk between the two cell types. We discovered that Multimerin-2 is an adhesion substrate for pericytes. Interestingly, and consistent with the notion that Multimerin-2 is a homeostatic molecule deposited in the later stages of vessel formation, we found that the interaction between endothelial cells and pericytes promoted the expression of Multimerin-2. Furthermore, we found that Multimerin-2 modulated the expression of key cytokines both in endothelial cells and pericytes. Collectively, our findings posit Multimerin-2 as a key molecule in the cross-talk between endothelial cells and pericytes and suggest that the expression of this glycoprotein is required to maintain vascular stability.

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Introduction

Angiogenesis is the development of new blood vessels from pre-existing vasculature and this phenomenon occurs throughout both normal and

pathological conditions. In the last few decades particular interest has been dedicated to discern the role of this process in a number of diseases including retinopathy and cancer. In fact, the abnormal proliferation of cancer cells requires the

proximity of blood vessels to provide sufficient nutrients and oxygen [1]. Judah Folkman was the first to develop the idea that the blockage of the tumor-induced vasculature might be a promising therapeutic approach for cancer patients [2]. Since this groundbreaking hypothesis, considerable efforts have been devoted in the discovery of the molecular pathways regulating angiogenesis as possible therapeutic targets to hamper tumor growth, as well as in the identification of molecules displaying anti-angiogenic properties [3]. These efforts have led to the development of a new generation of molecules that entered the clinics mostly targeting the VEGFR2/VEGFA signaling pathway or other receptor tyrosine kinases [4–6]. However, anti-angiogenic therapies have so far deluded the expectations with only a small increase in progression free survival [7]. This is in part due to the establishment of mechanisms of resistance which is facilitated by the redundant molecular mechanisms regulating angiogenesis that include cytokines, receptors, and also extracellular matrix (ECM) molecules [8–26]. Indeed, the intricate complexity of the ECM [27,28] governs tumor growth and progression via several mechanisms [27,29–43]. Thus, a better understanding of the role of ECM may be key towards the development of more efficacious treatments. In this context, Multimerin-2 may represent a promising molecular target. Multimerin-2 belongs to the EDEN protein family [44], and like EMILIN-2, another member of this family involved in the regulation of angiogenesis and tumor growth [13,14,45–48], plays a strategic role in the tumor microenvironment. Multimerin-2 is expressed by endothelial cells and is virtually deposited along all blood vessels, including the tumor-associated vasculature [11,49]. Interestingly, Multimerin-2 halts the activation of the VEGFR2/VEGFA signaling axis through the sequestration of VEGFA [15,16], and the observation that its expression is lost in a number of tumor-associated vessels [14] suggests a possible role of Multimerin-2 as a prognostic marker. In fact, Multimerin-2 loss associates with increased vascular permeability resulting in impaired vascular efficiency and drug delivery [20]. In this study we further investigated on the role of Multimerin-2 as a gatekeeper of vascular stability. In particular, based on the fact that Multimerin-2 is located in the interface between endothelial cells and pericytes and its ablation results in a significant decrease of pericytes coverage in both normal and tumor-associated vessels [20], the aim of this study was to explore the role of this glycoprotein in the crosstalk between these two cell types.

Our results posit Multimerin-2 as key molecule in the interaction between endothelial cells and pericytes and corroborate the concept that the expression of this molecule is required for the maintenance of vascular stability.

Results

Multimerin-2 is an adhesion substrate for pericytes

It is well established that Multimerin-2 is specifically expressed by endothelial cells and is deposited adjacent to the cell membrane both in the ab-luminal and luminal sides [20,49]. Based on this strategic cellular distribution, we hypothesized that Multimerin-2 could play a critical role in the interaction between endothelial cells and pericytes. To this end, we first established if Multimerin-2 could act as an efficient adhesion substrate for pericytes and used human brain vascular pericytes (HBVP) for this purpose. First, we produced highly-pure recombinant human Multimerin-2 purified from media conditioned by stably-transfected E293-pCEP-Pu cells using the Ni-NTA resin (Fig. 1A). As previously shown [16], Multimerin-2 is visible as a double band in SDS-PAGE, possibly due to different glycosylation. Importantly, the detection of no additional bands confirmed the purity of the protein. Using this recombinant glycoprotein as substrate, we found that Multimerin-2 served as a suitable adhesion platform for pericytes, slightly more efficient than collagen type 1 (Fig. 1B,C). Similar results were obtained monitoring and quantifying pericytes adhesion over time with the xCELLigence real-time cell analysis system (Fig. 1D,E). Collectively, these results suggest that Multimerin-2 can function as a molecular bridge between endothelial cells and pericytes, as well as a possible crucial molecular cue mediating their crosstalk.

Multimerin-2 expression is enhanced by the recruitment of pericytes

We have previously demonstrated that Multimerin-2 is deposited in the late stages of vascular development and exerts homeostatic function stabilizing the newly formed vessels [12,20]. We hypothesized that pericyte recruitment occurring during vessel maturation could trigger the deposition of Multimerin-2, further contributing to vessel normalization. First, we evaluated if pericytes alone or in co-culture with endothelial cells expressed Multimerin-2. Notably, we found that pericytes did not express Multimerin-2 even when adjacent to endothelial cells (Fig. 2 A). Next, we carried out co-cultures of endothelial cells and pericytes and evaluated the expression of Multimerin-2 by immunofluorescence imaging. We found that the expression of Multimerin-2 increased significantly upon addition of pericytes to the endothelial cell cultures (Fig. 2 B). Interestingly, we found that the amount of Multimerin-2 deposition positively correlated with the number of pericytes used in the co-cultures (Fig. 2 C).

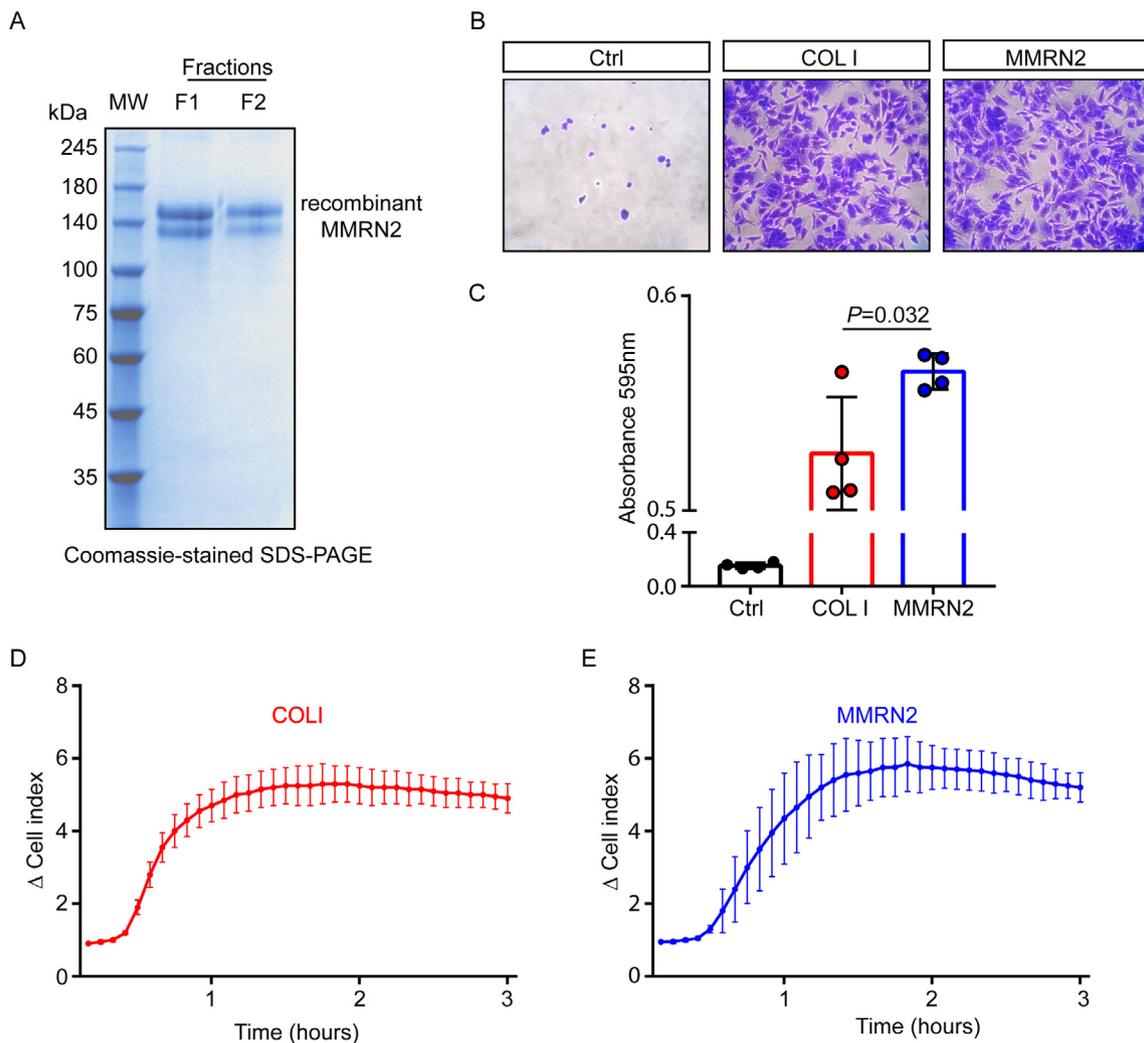


Fig. 1. Multimerin-2 is an adhesion substrate for pericytes. (A) SDS-PAGE analysis of fractions F1 and F2 obtained following the purification of Multimerin-2 from the conditioned media of E293-pCEP-Pu-transfected cells using the Ni-NTA resin. The molecular weight standards (MW) were included as a reference. (B) Representative images of adherent HBVP cells on Collagen I (COLI) and Multimerin-2 (MMRN2) substrates; BSA was used as a control; cells were stained with Crystal Violet. (C) Quantification graph of the adhesion assays reported in (B); extent of adhesion was assessed measuring absorbance of Crystal Violet dye at 595 nm; $n = 3$; P value was obtained using paired Student's t -test. (D) Evaluation of pericytes adhesion to Collagen I (Col I) by means of the xCELLigence system; cell adhesion was measured every 5 min for above 3 h; $n = 3$. (E) Evaluation of pericytes adhesion to Multimerin-2 (MMRN2) by means of the xCELLigence system; cell adhesion was measured every 5 min for above 3 h; $n = 3$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To verify if the increased expression of Multimerin-2 required a physical interaction between the two cell types or hinged on soluble factors, we cultured pericytes in transwells on the top side of 0.4 μm -pore membranes and endothelial cells at the bottom side, to allow cell-cell contacts (Fig. 3A). Under these conditions, we found a significant increase of Multimerin-2 expression when endothelial cells were in contact with pericytes (Fig. 3A,B). However, similar results were obtained when pericytes were plated on the top side of 0.4 μm -pored membranes and endothelial cells on the bottom of the transwell's well to allow only exchange of soluble factors

(Fig. 3C,D), suggesting that the effect is likely triggered by the secretion of soluble factors.

Multimerin-2 evokes the expression of key cytokines modulating the cross-talk between endothelial cells and pericytes

Accumulating evidences highlight the importance of a number of cytokines and cell surface receptors in the modulation of the crosstalk between endothelial cells and pericytes [50–56,19]. Thus, having established that the recruitment of pericytes elicits Multimerin-2 expression by endothelial cells, we next queried

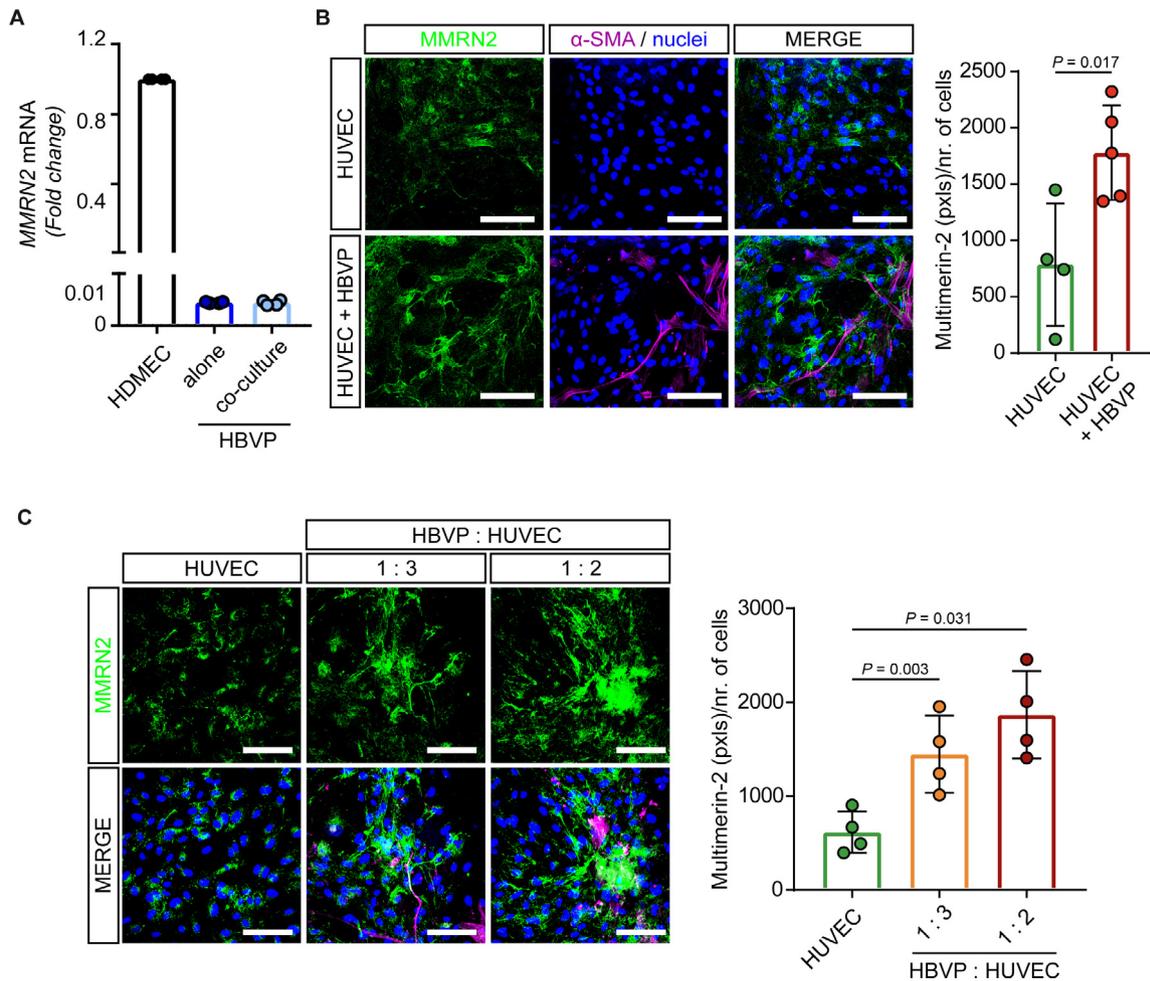


Fig. 2. Multimerin-2 deposition is enhanced by pericytes' recruitment. (A) Real-Time RT-PCR analysis of the Multimerin-2 (*MMRN2*) mRNA levels in HDMEC and HBVP cells, alone or in co-culture seeding HBVP cells on top of the transwell membrane and HDMEC cells on the bottom side; *GAPDH* was used as reference gene; $n = 3$; P value was obtained using paired Student's T -test. (B) On the left, representative immunofluorescence images of HUVEC cells alone (upper panel) or co-cultured with HBVP (lower panel); Multimerin-2 (MMRN2, green) was detected with an affinity purified polyclonal antibody, pericytes (red) were stained with α -SMA and nuclei (blue) with TO-PRO; scale bar $50 \mu\text{m}$. Quantification relative to the number of cells showing increased levels of Multimerin-2 (MMRN2) in the co-cultures is reported on the right graph; $n = 4$; P value was obtained using paired Student's t -test. (C) Left panels: representative immunofluorescence images of HUVEC cells alone or co-cultured with HBVP at different ratio; Multimerin-2 (MMRN2, green) was stained with an affinity purified polyclonal antibody; pericytes (red) were stained with α -SMA and nuclei (blue) with TO-PRO; scale bar $50 \mu\text{m}$; Quantification relative to the number of cells showing increased levels of Multimerin-2 (MMRN2) proportional to the increase of HPVP cells is reported on the right graph. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

if it could also stimulate the production of these vital molecules. To this end, we employed human dermal microvascular endothelial cells (HDMEC) and challenged them with pure recombinant Multimerin-2 (Fig. 1A). We found that treatment of HDMEC cells with Multimerin-2 induced the expression of platelet-derived growth factor (*PDGF*) (Fig. 4A) and heparin binding epidermal growth factor (*HB-EGF*) (Fig. 4B), potent cytokines mediating pericyte recruitment and proliferation [50–53]. On the contrary, and in line with

the concept that Multimerin-2 deposition promotes the establishment of a stable vasculature, the expression of the pro-angiogenic Apelin (*APLN*) (Fig. 4C) [54] and of Angiopoietin-2 (*ANGPT2*) (Fig. 4D) [55] were significantly reduced.

Moreover, we also analyzed the expression of a number of molecules in Multimerin-2-challenged pericytes. Unlike Notch-3-R and Angiopoietin-1, which did not significantly change (data not shown), we found that pericytes challenged with Multimerin-2 displayed an increased expression of

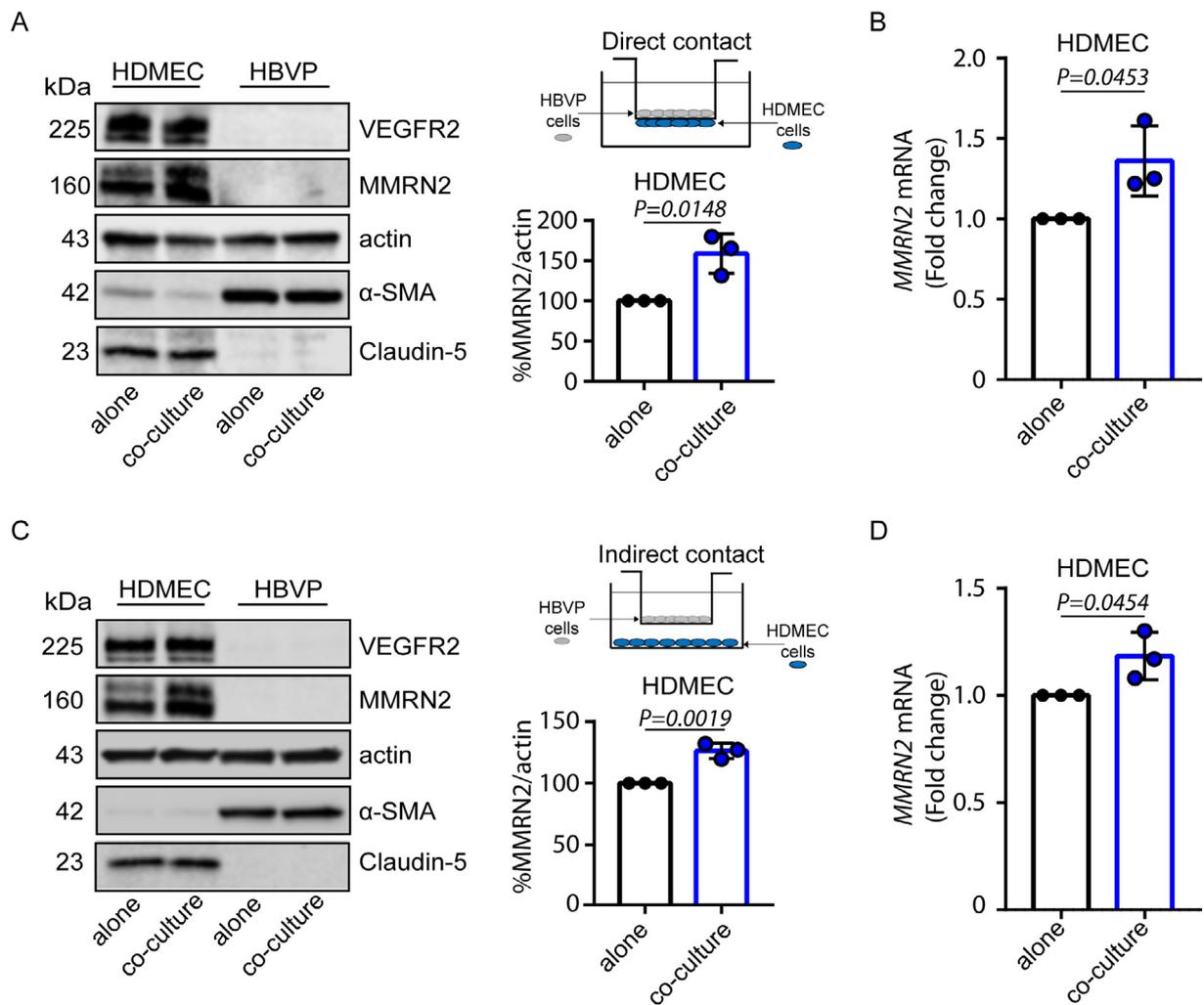


Fig. 3. Pericytes stimulate Multimerin-2 expression in endothelial cells without the need of cell–cell contact. (A) Left panel, Western blot images of HDMEC and HBVP cells alone or co-cultured in both sides of a transwell membrane; VEGFR2 and Claudin-5 were used as markers of endothelial cells, whereas α -SMA as a marker of pericytes; actin was used as protein loading control; the relative quantification of the Multimerin-2 (MMRN2) levels normalized to actin is reported in the middle graph on the bottom; top right panel: schematic representation of the experimental procedure; $n=3$; P value was obtained using paired Student's t -test. (B) Real-Time RT-PCR analyses of the Multimerin-2 (MMRN2) mRNA levels in HDMEC cells co-cultured with HBVP cells as in A; $GAPDH$ was used as reference gene; P values were obtained using paired Student's t -test. (C) Left panel, Western blot images of HDMEC and HBVP cells alone or co-cultured without cell–cell contact (HDMEC in the bottom of the well and HBVP on the top side of the transwell); VEGFR2 and Claudin-5 were used as markers of endothelial cells, whereas α -SMA as a marker of pericytes; actin was used as protein loading control; the relative quantification of the Multimerin-2 (MMRN2) levels normalized to actin is reported in the middle graph on the bottom; top right panel: schematic representation of the experimental procedure; $n=3$; P value was obtained using paired Student's t -test. (D) Real-Time RT-PCR analyses of the Multimerin-2 (MMRN2) mRNA levels in HDMEC cells co-cultured with HBVP cells as in C; $GAPDH$ was used as reference gene; P values were obtained using paired Student's t -test.

Tie-2 (*TIE2*) (Fig. 5A), known to regulate pericyte motility [56]. Interestingly, treatment of pericytes with Multimerin-2 led also to increased expression of its putative receptor *CD248* (Fig. 5B) [19]. Collectively, our findings demonstrate that Multimerin-2 is a key component in the cross-talk between endothelial cells and pericytes, and that its deposition prompts the establishment of vascular quiescence.

Discussion

In this study, we explored the function of Multimerin-2 in the cross-talk between endothelial cells and pericytes. Our results demonstrate that Multimerin-2 functions as a key molecular bridge for the interaction between these two cell types. During angiogenesis, following the formation of the endothelial tube network, pericytes are

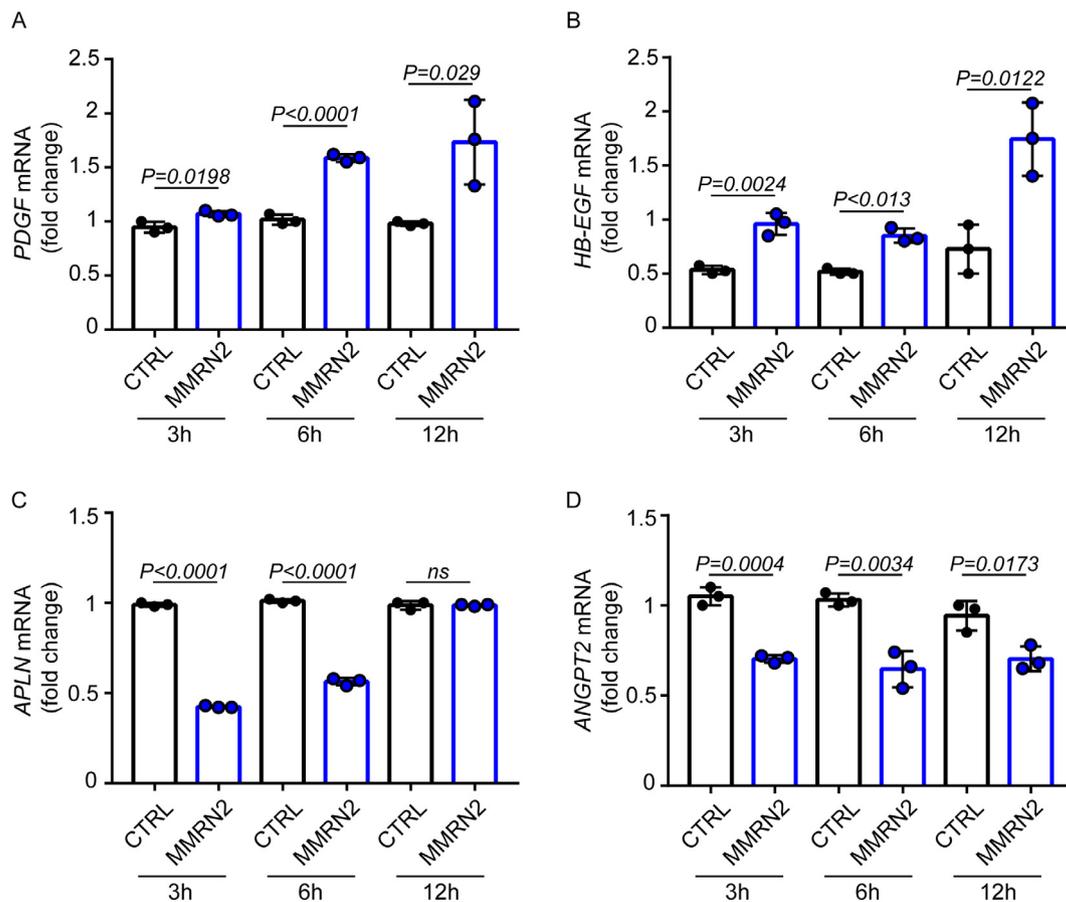


Fig. 4. Multimerin-2 affects the expression of endothelial cell-derived cytokines relevant for pericytes' behavior. (A) and (B) Real-Time RT-PCR analyses of the *PDGF* and *HB-EGF* mRNA levels; *GAPDH* was used as reference gene; HDMEC cells were treated with recombinant Multimerin-2 (MMRN2) or PBS as a control (CTRL) for 3, 6 and 12 h; $n = 3$; P values were obtained using paired Student's t -test. (C) and (D) Real-Time RT-PCR analyses of the Apelin (*APLN*) and Angiopoietin-2 (*ANGPT2*) mRNA levels; *GAPDH* was used as reference gene; HDMEC cells were treated with recombinant Multimerin-2 (MMRN2) or PBS as a control (CTRL) for 3, 6 and 12 h; $n = 3$; P values were obtained using paired Student's t -test.

recruited to the abluminal surface prompting basement membrane deposition, vascular maturation and stabilization [57]. However, despite its strategic location in juxtaposition with the endothelial cell membrane, the role of Multimerin-2 in this context has remained unexplored prior to this investigation. Here we demonstrate that Multimerin-2 represents an adhesion substrate for pericytes. Thus, based on our results it is conceivable to assume that the initial deposition of Multimerin-2 supports the formation of a stable vasculature through direct and indirect mechanisms. On one side, Multimerin-2 favors pericyte recruitment by stimulating the secretion of key cytokines involved in pericyte recruitment, such as PDGF and HB-EGF [58]. Further analyses will be required to determine if the altered expression of these cytokines hinges on the activation of VEGFR2 [16] or the Multimerin-2 receptor CD93 [11], or if other receptors mediate these changes once engaged by Multimerin-2. On the other side, following recruitment, Multimerin-2 exerts a direct effect through its

adhesive properties, functioning as a docking site for pericytes. Interestingly, and in agreement with the concept that Multimerin-2 functions as a homeostatic molecule, its expression is enhanced by the interaction between the two cell types. Our data further demonstrate that a physical interaction between endothelial cells and pericytes is not required to boost Multimerin-2 expression, suggesting that it could be triggered by pericyte-derived soluble factors, yet to be discovered. Further studies will be necessary to identify these factors and thoroughly uncover the transcriptional and post-transcriptional mechanisms regulating Multimerin-2 gene expression profiles. Since PDGF exerts potent effects also on vascular smooth muscle cells (VSMCs) [59], it cannot be excluded that the expression of Multimerin-2 may also impact on the recruitment of this cell type, and/or function as an adhesive substrate for VSMCs. Given the putative influence on different vascular cells, altered expression of Multimerin-2 may reflect not only in angiogenesis but also in other vascular diseases.

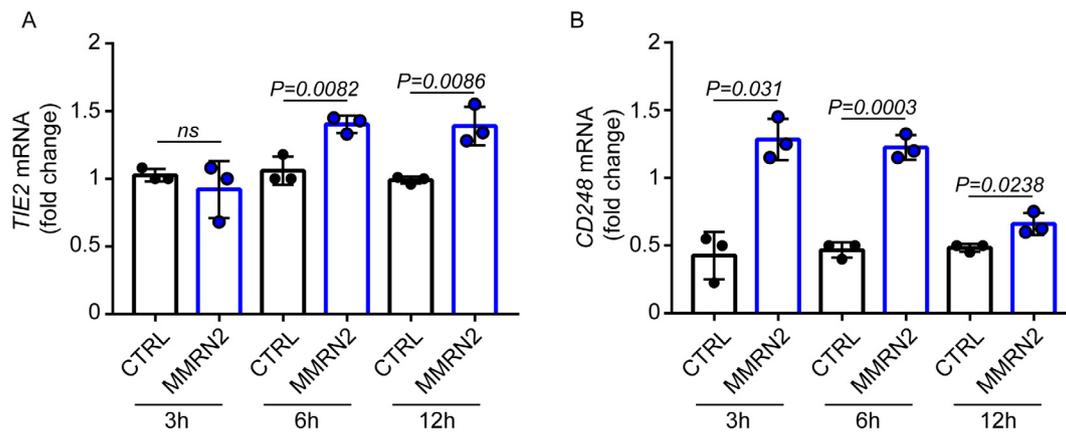


Fig. 5. Multimerin-2 affects the expression of key receptors in pericytes. A) Real-Time RT-PCR analyses of the *Tie2* (*TIE2*) mRNA levels; HBVP cells were stimulated with recombinant Multimerin-2 (MMRN2) or PBS as a control (CTRL) for 3, 6 and 12 h; *GAPDH* was used as reference gene; n = 3; P values were obtained using paired Student's *t*-test. (B) Real-Time RT-PCR analyses of the *CD248* mRNA levels; HBVP cells were stimulated with recombinant Multimerin-2 (MMRN2) or PBS as a control (CTRL) for 3, 6 and 12 h; *GAPDH* was used as reference gene; n = 3; P values were obtained using paired Student's *t*-test.

Besides having a role in the recruitment of pericytes through the stimulation of PDGF and HB-EGF expression in endothelial cells, the treatment of these cells with Multimerin-2 induces down-regulation of Apelin and Angiopoietin-2 expression. Apelin is known to drive endothelial cells towards a pro-angiogenic state and its expression is regulated by Notch signaling which, in conjunction with VEGFA, orchestrates the formation of tip and stalk cells during angiogenic sprouting [54,60]. Since Multimerin-2 sequesters VEGFA and down-modulates the VEGFA/VEGFR2 signaling axis, it is possible that it might affect apelin expression modulating the interplay between the VEGFR2 and the Notch signaling pathways. A strong reduction of Apelin expression in endothelial cells treated with Multimerin-2 was not protracted, suggesting that Apelin may be predominantly active during the early stages of vascular development. Multimerin-2-challenged endothelial cells also displayed a decreased expression of Ang-2, a growth factor known to destabilize the vascular network, despite its role in angiogenesis seems to be context-dependent [55,61]. It is well established that Ang-2 expression is regulated by hypoxia [62] and Multimerin-2 affects HIF-1 α expression [15]. However, the hypoxia-driven regulation of Ang-2 expression mainly hinges on cyclooxygenase-2 (COX-2)-dependent prostanoids [62]. Further studies will be necessary to determine if Multimerin-2 affect also this pathway.

We have demonstrated that not only does Multimerin-2 alter the expression pattern in endothelial cells but also in pericytes, highlighting the importance of this molecule in the communication between the two cell types. When challenged with Multimerin-2, pericytes express increased levels of Tie-2, whose silencing is known to associate with increased cell motility

[56]. Thus, the higher expression of the receptor when cells are challenged with Multimerin-2 is in agreement with the homeostatic role of this glycoprotein in the late steps of vascular sprouting. Remarkably, Multimerin-2 stimulates the expression of CD248, a group of 14c-type lectin proteins recently shown to interact with Multimerin-2 and to function as its putative receptor [63]. This finding suggests that Multimerin-2 may engage a positive feedback loop to promote the adhesion of pericytes to the endothelium. Following recruitment, pericytes and endothelial cells together contribute to the synthesis and assembly of vascular basement membranes [57,64] and our data hint that Multimerin-2 may represent an important player in these contexts. Taken together, our results suggest that the deposition of Multimerin-2 is a key step during vascular maturation, acting not only as a substrate for pericyte adhesion, but also as a central modifier of the expression pattern of important molecules regulating the cross-talk between ECs and pericytes and vascular stability.

Materials and methods

Cell cultures

The 293-EBNA (E293) cell line was obtained from ATCC (Manassas, VA, USA), transfected as previously described [16], and cultured in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco, Milan, Italy) supplemented with 1% Penicillin/Streptomycin (Gibco, Milan, Italy), 250 μ g/ml G418 and 0,5 μ g/ml puromycin. Human Brain Vascular Pericytes (HBVP) and Human Dermal Microvascular Endothelial Cells (HDMEC) were purchased from ScienCell (San Diego, CA, USA) and cultured in Pericyte Medium (PM) and in Endothelial Cell Med-

ium ECM medium (ScienceCell, San Diego, CA, USA), respectively. Instead, Human Umbilical Vein Endothelial Cells (HUVECs) were obtained as previously described [65] and cultivated in ECM medium. All cell lines were maintained at 37 °C under a humidified atmosphere containing 5% CO₂ and routinely tested for mycoplasma contamination using MycoAlert™ Mycoplasma Detection kit (LONZA, Stein Switzerland).

Antibodies and other reagents

The polyclonal antibody for Multimerin-2 was produced in our laboratory as previously described [16]. The anti-VEGFR2 and anti-actin antibodies were purchased from Cell Signalling Technologies, whereas the anti- α SMA antibody was from Abcam. The antibody against Claudin-5 was from Santa Cruz Biotechnologies. Secondary antibodies for immunofluorescence were conjugated with Alexa Fluor 488 or 546 (Invitrogen) and nuclei were stained with TO-PRO 3 (Invitrogen). HRP secondary antibodies were purchased from Bethyl. Recombinant Multimerin-2 was produced in E293 cells and purified with Ni-NTA agarose resin from QUIAGEN; the purity was assessed through Blue Coomassie staining. Collagen I was from BD Biosciences (San Jose, CA, USA)

Immunofluorescence

For immunofluorescence analyses, HUVEC and HBVP cells were cultured on glass coverslips and fixed with PFA for 15 min. After washing with phosphate-buffered saline (PBS), the cells were incubated with 0.5% Triton X-100 in PBS for 5 min at room temperature, saturated with 1% BSA, 10% normal goat serum (DAKO) in PBS for 1 hr at room temperature, and stained over night at 4 °C with the appropriate antibodies. Next, the samples were washed with PBS and incubated for 1 h at room temperature with the appropriate secondary antibodies and TO-PRO3 to stain the nuclei. After washing with PBS, glasses were mounted in Mowiol containing 2.5% (w/v) of 1,4-diaxszabicy clo-(2,2,2)-octane (DABCO). Images were acquired with a Leica TCS SP8 Confocal system (Leica Microsystems Heidelberg, Mannheim, Germany), using the Leica Confocal Software (LCS). Fluorescence intensity and quantification was evaluated by means of the Volocity software (PerkinElmer Inc., Waltham, MA, USA).

qRT-PCR

Total RNA was isolated from cell lines with Trizol and reverse transcribed using AMV-RT (Promega, Milan, Italy). After determination of the primer specificity and efficiency, Real-time RT-PCRs were performed with iQ™ SYBR® Green Supermix (Bio-Rad, Milan, Italy) and BIORAD CFX96 Touch™ Real Time PCR Detection System. The specificity

of the Real-time RT-PCR reactions was determined by analyzing the melting curve of the amplified products, which were evaluated by agarose gel electrophoresis, and the 2^{- $\Delta\Delta$ Ct} method could be applied for the analysis. The oligonucleotide sequences were: *PDGF-BB* forward: 5'-AAGTGTGAGACAGTGGCAG-3', reverse: 5'-GCTTGATTTCCGGTGCTTG-3'; *HB-EGF* forward: 5'-TTATCCTCCAAGCCACAAGCA-3', reverse: 5'-AGCCCTTGCCCTTTCTTTCTTT-3'; *APLN* forward: 5'-GCTGCTCTGGCTCTCCTTGA-3', reverse: 5'-ATTCCTTGACCCTCTGGGCT-3'; *CD248* forward: 5'-GGTGCCAACGTGTGTCTTTT-3', reverse: 5'-GCATGGATGACCCAGCGATA-3'; *TIE2* forward: 5'-CCTTGGCTCTGCTGGAATG-3', reverse: 5'-GCTGGTTCTTCCCTCACGTT-3'; *ANGPT2* forward: 5'-TGGCTGGGAAATGAGTTTGT-3', reverse: 5'-GGTTGGCTGATGCTGCTTAT-3'; *GAPDH* forward: 5'-GAGAGACCCTCAAGTGC-3', reverse: 5'-GATGGTACATCAGAAAGACCTGGAAGCTCC-3', reverse: 5'-GAAAAGCTGGCATAGAAGGC-3'.

Cell transfection and immunoblotting

E293 cells were transfected using FuGene6 reagent (Promega, Milan, Italy). Cells transfected with the pCEP-Pu-Multimerin-2 were selected with 250 μ g/ml G418 and 0.5 μ g/ml puromycin. Confluent cells were then incubated in serum-free medium for 48 h and the conditioned medium collected. For Western immunoblotting, cells were lysed in cold RIPA buffer (150 mM NaCl, 10 mM Tris, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA) containing a protease inhibitor cocktail (Roche Diagnostics S.p.a., Milan, Italy). Proteins were resolved in 4% to 20% Criterion Precast Gels (Bio-Rad, Milan, Italy) and transferred onto Hybond-ECL nitrocellulose membranes, blocked with 5% BSA in TBS-T buffer, probed with the appropriate antibodies, and developed using enhanced chemiluminescence (Amersham, Milan, Italy).

Cell adhesion and impedance measurements

Cell adhesion assays were carried out in 96 well plates coated overnight at 4 °C with 10 μ g/mL of Multimerin-2 or Collagen Type I. Plates were air-dried at room temperature under the tissue culture hood and 2x10⁴ starved HBPV cells were added to serum free media supplemented with 0,1% BSA. Adhesion was performed in humidified incubator at 37 °C for 1 h. Following Crystal Violet staining, the extent of cell adhesion was measured assessing the absorbance at 595 nm.

To quantitatively monitor cell behavior in real-time, we adopted the Real-Time Cell Analyzer dual plate instrument (Roche) which measures the

electrical impedance caused by cell attachment and proliferation and expressed as the cell index, an arbitrary measurement defined as $(R_n - R_b)/15$, in which R_b is the background impedance of the well measured with medium alone, and R_n is the impedance of the well measured at any time (t) in the presence of cells. Thus, the cell index is a reflection of overall cell number, attachment quality, and cell morphology that change as a function of time. The Real-Time Cell Analyzer dual plate instrument was placed in a humidified incubator maintained at 37 °C 5% CO₂. For adhesion experiments, the E-plates 96 were precoated over night at 4 °C with the indicated molecules (10 µg/ml), and cells were then seeded at 5x10⁴ cells/well in FBS-free medium. Cells were monitored every 5 min for 3 h. Data analysis was performed using Real-Time Cell Analyzer software (version 1.2) supplied with the instrument.

Co-culture experiments and cell stimulation

For immunofluorescence analyses 2x10⁵ HUVEC cells and different ratios of HBVP cells (from 4x10⁴ up to 1x10⁵ cells) were co-seeded on glass coverslips placed in a 6 well plates. Co-cultures were carried out for 2,5 days. For Western blot and qRT-PCR analyses 3x10⁵ HDMEC and HBVP cells were plated on gelatin coated 0,4µm transwell supports, in a 1:1 proportion. HDMEC were seeded on the bottom side of the transwell membrane (for direct contact) or on the bottom of the wells (for indirect contact). After 3 h, HBVP cells were placed on the top of the transwell and interaction was allowed for 4 h. For qRT-PCR analyses HDMEC and HBVP cells were stimulated with 3 µg/mL of recombinant Multimerin-2 or PBS as a control for 3, 6 and 12 h.

Statistical analyses

Statistical analyses were performed with the SigmaPlot and the GraphPad Prism software and the values represent the mean ± SD. The statistical significance of the differences was determined by the two-sided Student's *t* test for the comparisons between two groups. For all the evaluations reported in the manuscript the investigators were blinded. All the measurements were included for the statistical analyses and differences were considered statistically significant when $P \leq 0.05$.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations:

Ang-2, Angiopoietin-2; CD93, cluster of differentiation 93; CD248, cluster of differentiation 248; ECM, extracellular matrix; EDEN, EMI Domain ENdowed; HB-EGF, heparin binding epidermal growth factor; HBVP, human brain vascular pericytes; HDMEC, human dermal vascular endothelial cells; HUVEC, human umbilical vein endothelial cells; Notch-3-R, Notch Receptor 3; PDGF, platelet-derived growth factor; VEGFA, vascular endothelial growth factor A; VEGFR2, vascular endothelial growth factor receptor 2; VSMCs, vascular smooth muscle cells

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