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XXXVI CICLO DEL DOTTORATO DI RICERCA IN
BIOMEDICINA MOLECOLARE

**The role of Multimerin-2 in pericyte recruitment and
vascular stability**

Settore scientifico-disciplinare: BIO/11 BIOLOGIA MOLECOLARE

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1. ABSTRACT

The proper function of normal blood vessels is ensured by endothelial cells, which are tightly connected to one another by specific junctions, thus regulating vessel permeability and the intra- and extra-vascularization of immune cells. Additional stability regulators are pericytes and the vessels' basement membrane. Specifically, pericytes are mural cells embedded in the BM of blood microvessels, and envelope the vessels promoting vessel stabilization and maturation. In the context of vascular homeostasis, we demonstrated that Multimerin-2, an extracellular matrix glycoprotein secreted by endothelial cells and deposited along all blood vessels, is key in maintaining the integrity of endothelial cell junctions. Given its strategic localization in juxtaposition between the endothelium and pericytes and the finding that retinal vessels from *Multimerin-2^{-/-}* animals display impaired pericytes coverage, the aim of the present project was to investigate whether Multimerin-2 affected pericytes biology and if its loss impacted on the vascular efficiency and immune cell infiltration. We found that Multimerin-2 affected pericyte recruitment to the newly formed vessels both directly and indirectly. Indeed, we demonstrated that pericytes efficiently migrate towards a Multimerin-2 gradient and that endothelial cells challenged with Multimerin-2 displayed an increased expression of PDGF-BB and HB-EGF, two cytokines that foster pericyte recruitment. Moreover, we showed that Multimerin-2 functions as an adhesion substrate for pericytes, indicating that the expression of this molecule promotes both the recruitment and the pericyte-endothelial cell interaction. Once pericytes reach the endothelium, they need to proliferate to properly embrace the vasculature and we unveiled that Multimerin-2 increases pericyte viability and proliferation, hinging on IGF1R- β activation. Our *in vitro* results were further corroborated *in vivo* exploiting the *Multimerin-2^{-/-}* mouse model. Tumors developed in *Multimerin-2^{-/-}* mice following the injection of the syngeneic HG-SOC cells ID8 *Trp53^{-/-}* *Pten^{-/-}* and the colon carcinoma cells MC-38 showed impaired pericyte recruitment to the vessels and increased intratumoral hypoxia. Furthermore, in accordance with the notion that vascular inefficiency negatively affects drug delivery, following intraperitoneal injection of the drug, tumors grown in *Multimerin-2^{-/-}* mice displayed a reduced number of cisplatin-positive nuclei. Moreover, to verify if loss of Multimerin-2 impacted on the immune response we carried out a preliminary characterization of the tumor-infiltrating immune cells. Interestingly, we found that tumors from *Multimerin-2^{-/-}* mice display increased recruitment of macrophages. This finding was further supported by *in vitro* analyses showing that a higher number of THP-1 cells, monocytic-like cell line, transmigrated across the Multimerin-2-depleted endothelium. Taken together, these results suggest that Multimerin-2 expression is required for proper recruitment, proliferation, and adhesion of pericytes to

the endothelium. As a consequence, upon loss of Multimerin-2 the endothelium is destabilized leading to increased intratumoral hypoxia and poor drug delivery. Importantly, loss of Multimerin-2 also affects the immune response suggesting that the outcome of the patients may significantly be affected depending on the levels of Multimerin-2 loss in the tumor patients.

2. LIST OF ABBREVIATIONS

AJs: adherens junctions
Ang-1: Angiopoietin-1
Ang-2: Angiopoietin-2
BM: basement membrane
CAFs: cancer associated fibroblast
Col I: Collagen Type I
Cpt: cisplatin
CRC: colorectal cancer
CTLD: C-type lectin domain
DDRs: discoidin domain receptors
ECM: extracellular matrix
ECM: extracellular matrix
ECs: endothelial cells
EDEN: EMI domain endowed
EGFR: epidermal growth factor receptor
EMILIN-T: EMILIN-truncated
EMILIN1: Elastin microfibril interface–located protein 1
ESAM: endothelial cell selective adhesion molecule
FDA: Food and Drug Administration
FGF-2: fibroblast growth factor\
FGFs: fibroblast growth factors
gC1q C: terminal globular domain of C1q
HA: hyaluronic acid
HB-EGF: epidermal growth factor
HBVPs: human brain vascular pericytes
HDMEC: human dermal microvascular endothelial cells
HG-SOC: high grade serous ovarian cancer
HUVECs: human umbilical vein endothelial cells
ICAM-1: intercellular adhesion molecule
IGF1R-b: insulin like growth factor 1 receptor
IL-8: interleukin-8
IRS1: insulin receptor substrate 1
JAM: junctional adhesion molecule
MMPs: matrix metalloproteinases

NG2: neuron-glial 2
OS: overall survival
PDGF-BB: platelet-derived growth factor subunit B
PDGFR- β : platelet growth factor receptor- β
PECAM: platelet endothelial cell adhesion molecule
PFS: progression free survival
PGs: proteoglycans
PLC-g: phospholipase C-gamma 1
RGS-5: regulator of G protein signaling 5
shRNAs: short hairpin RNAs
SMCs: smooth muscle cells
TAF: tumor angiogenic factor
TAMs: tumor associated macrophages
TAMs: Tumor-associated macrophages
TGF- α transforming growth factor α
TGF- β transforming growth factor β
TJs: tight junctions
TME: tumour microenvironment
VCAM-1: vascular cell adhesion protein
VE-PTP vascular endothelial protein tyrosine phosphatase
VEGF: vascular endothelial growth factor
VEGFR-2: vascular endothelial growth factor receptor 2
VPF: vascular permeability factor
vSMC: vascular smooth muscles cells
wt: *wild type*
 α -SMA: alpha-smooth muscle actin

3. INTRODUCTION

2.1 Angiogenesis

The vascular system is a closed network of arteries, veins and interconnecting capillaries, organized into tree-like structures (Potente et al., 2011). Endothelial cells (ECs), the major components of the vessels, line the lumen of the blood vessels regulating the dynamic passage of oxygen, nutrients and cells, serving as the interface between blood and tissues (Krüger-Genge et al., 2019). The circulatory system begins to develop in the early embryonic stage once diffusion is not sufficient for the supply of oxygen and nutrients due to the ramping needs of the growing tissues and organs. The formation of new blood vessels is achieved by two fundamental processes: vasculogenesis and angiogenesis (Carmeliet, 2000). Vasculogenesis is defined as the differentiation of endothelial precursor cells, or angioblasts, and the *de novo* formation of a primitive vascular network (Risau, 1997). Following vasculogenesis, the preexisting blood vessels guide new blood vessel formation through angiogenesis, ensuring the expansion of the vascular network (Carmeliet and Jain, 2011). Angiogenesis occurs under physiological conditions (Carmeliet, 2005), such as embryo development, as well as under pathological conditions, such as chronic inflammation, immune disorders and cancer (Potente et al., 2011). Angiogenesis is finely regulated by a balance of pro- and anti-angiogenic molecules such as growth factors, cytokines, integrins and the extracellular matrix (ECM). By contrast, under pathological conditions, this subtly tuned balance is disrupted (Bergers and Benjamin, 2003). Vessel growth is a dynamic process, and the primary driver is hypoxia (Krock et al., 2011). Upon hypoxic condition, hypoxia inducible factor (HIF) is the main activated transcription factor, which in turn activates the transcription of angiogenic growth factors, among which one of the best studied is vascular endothelial growth factor A (VEGFA) (Ramakrishnan et al., 2014). ECs, normally in a quiescent state, sense VEGFA as a pro-angiogenic stimulus and become activated starting the sprouting process (Ferrara et al., 2003). Specifically, this event takes place thanks to activated and specialized ECs, called tip cells. These cells migrate and extend numerous long dynamic filopodia that create new connections between different sprouts to generate an interconnected and functional vascular network (Potente and Carmeliet, 2017; Suchting and Eichmann, 2009). Tip cells are followed by stalk cells that proliferate and form the trunk of the new blood vessels (Bussmann et al., 2011). Once the nutrients and oxygen supply meets the tissue demand, the secretion of pro-angiogenic factors is silenced and ECs return to their quiescent state. Subsequently, vessels are stabilized by the deposition of the basement

membrane (BM) and by the recruitment of pericytes and smooth muscle cells (SMCs) (Figure 1) (Carmeliet, 2000; Potente and Carmeliet, 2017).

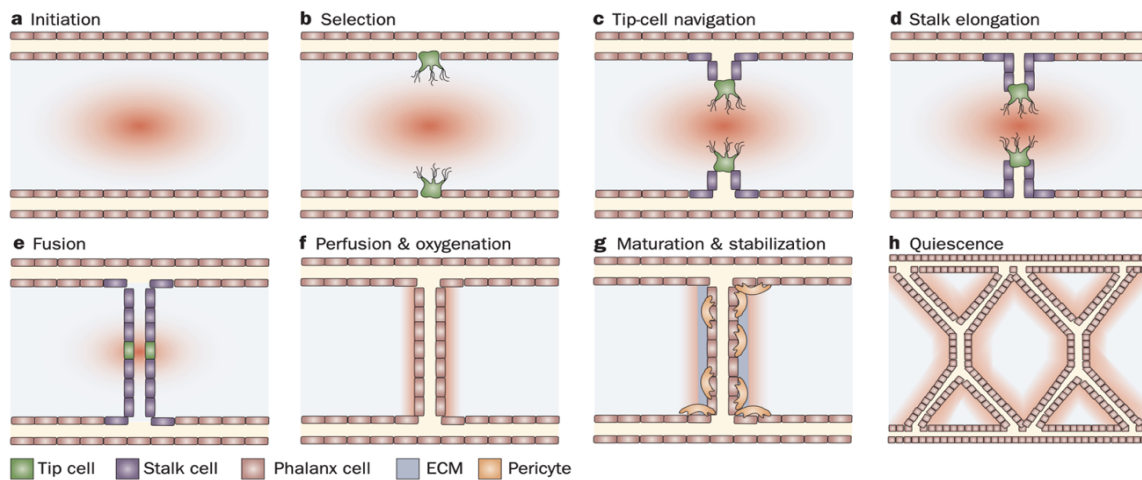


Figure 1. Formation of new blood vessels. *a)* In tissues, the need of oxygen induces the secretion of a VEGFA gradient (here indicated in red where the most intense color represents the highest concentration). *b)* The ECs exposed to the highest VEGFA concentration are selected to become a 'tip cells' (green). *c)* Tip cells sprout and through elongated filopodia create new connections. *d)* Tip cells are followed by stalk cells (violet) which proliferate and form the trunk of the new blood vessels. *e)* The new branch connects with another one via tip-cell fusion. *f)* Formation of a vascular lumen allows initiation of blood flow and, in turn, tissue oxygenation, which reduces VEGFA levels. *g)* Maturation and stabilization of the nascent plexus relies on the recruitment of pericytes (orange) and deposition of ECM (blue). *h)* In the established vasculature, ECs adapt a quiescent endothelial 'phalanx' (beige) phenotype. (Carmeliet et al., 2009)

2.2 Regulators of vascular stability

2.2.1 Endothelium and cell-cell junction

The endothelium represents the inner cellular lining of the blood vessels (Aird, 2007). It functions as a barrier which separates the blood from the surrounding tissues (Dejana, 2004), thus regulating vascular permeability and leukocyte trafficking (Vestweber et al., 2014). These functions are governed by two types of junctional complexes: the adherens junctions (AJs) and tight junctions (TJs), which tightly connect ECs (Figure 2) (Dejana, 2004). The establishment of AJs is mediated by cadherins (Dejana, 2004; Gumbiner, 2000), whereas TJs formation is assured by different protein families, such as members of the claudin family and junctional adhesion molecules (JAM), which are cell adhesion receptors (Furuse and Tsukita, 2006). ECs express cell type-specific transmembrane adhesion proteins such as VE-cadherin at AJs and claudin-5 at TJs (Nitta et al., 2003). VE-cadherin, one of the few endothelial-specific junctional molecules, is essential for the junctional stability, in fact

Carmeliet and colleagues showed that its genetic inactivation causes early embryo lethality at 9.5–10.0 days post gestation, due to ECs apoptosis and vascular insufficiency (Carmeliet et al., 1999). The importance of VE-cadherin in vascular function was further highlighted by the use of VE-cadherin blocking antibodies in the adult mouse, which led to a marked increase of vascular permeability (Corada et al., 1999).

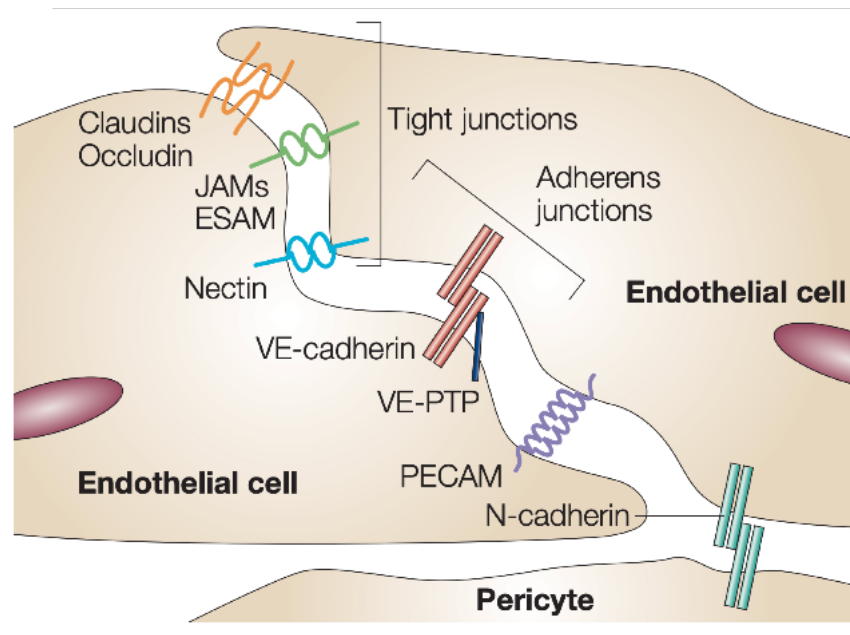


Figure 2. Endothelial junctional complexes. ECs are tightly connected by two types of junctional complexes: the AJs composed of claudins, JAMs and Nectin, while TJs are mainly characterized by VE-cadherin. The platelet endothelial cell adhesion molecule (PECAM) contributes to endothelial cell–cell adhesion, whereas neuronal cadherin (N-cadherin) is not concentrated at AJs and is instead responsible of the adhesion of ECs to pericytes and SMCs (Dejana, 2004).

2.2.2 Pericytes

Maturation and stabilization of the vasculature is not only achieved by ECs, on the contrary it is ensured by many other components. According to current theories, two are the major players modulating vascular homeostasis (Vontell et al., 2006):

- the perivascular ECM, in particular the vascular BM.
- the recruitment and integration of pericytes into the vessel wall.

Pericytes are mural cells initially described by Eberth and Rouget in the 1870s as spatially isolated cells with a bump-on-a-log morphology which wrap the blood capillaries (Figure 3A) (Armulik et al., 2011). Later, Zimmermann (1923) provided a detailed study of this cell type coining the name “pericytes” due to their anatomical perivascular position (Birbrair, 2018). Next, Sims described pericytes as cells embedded in the BM of the endothelium

belonging to the vascular smooth muscles cell (vSMC) lineage, despite their origin is still elusive (Sims, 1986). Since their origin and differentiation from vSMCs is still debated, to date an exclusive molecular marker specifically expressed by pericytes has not been identified. Nonetheless, few markers expressed by pericytes, albeit not exclusively, are frequently employed such as desmin, α -smooth muscle actin (α -SMA), regulator of G protein signaling 5 (RGS-5), neuron-gial 2 (NG2), platelet growth factor receptor- β (PDGFR- β) (Bergers and Song, 2005) and the CD248 receptor (Christian et al., 2008). Unlike vSMC which envelope large vessels, pericytes appear to be ubiquitously present along all the blood microvessels (Petrova et al., 2004). Pericytes morphology is highly plastic depending on their location (Su et al., 2021) and maturation stage. In general, these cells are characterized by a cell body with a prominent nucleus and a small cytoplasm with several long protrusions used to contact ECs through holes in the BM, which mediates most of the pericyte-EC interactions. These contacts have a peg-socket structure, where the pericyte cytoplasmic fingers (pegs) are inserted into EC invaginations (sockets). Additional structures promoting the interaction between ECs and pericytes are the adhesion plaques, microfilament bundles rich in fibronectin and attached to the pericyte plasma membrane which connect to ECs via electron-dense material present in the adjacent endothelial cytoplasm (Figure 3B) (Armulik et al., 2011; Harrell et al., 2018). The number and the size of pericyte-EC contacts vary between tissues, however at least 1,000 contacts have been described for a single EC (Armulik et al., 2011).

Once pericytes reach the endothelium they functionally provide structural support to blood vessels, regulating ECs proliferation, angiogenesis, blood vessel diameter and permeability, as well as leukocyte recruitment (Ayres-Sander et al., 2013; Folkman and D'Amore, 1996; Potente et al., 2011; Stark et al., 2013). Given this strategic role, pericyte density differs depending on the function of the vessels and organs in which they are found (Bergers and Song, 2005). The ratio of ECs and pericytes positively correlates with the endothelial barrier properties and ECs turnover, a large coverage associating with a lower ECs turnover (Armulik et al., 2011). Accordingly, the vasculature of the central nervous system is the most densely covered by pericytes, with a 1:1–3:1 ratio between ECs and pericytes (Mathiisen et al., 2010).

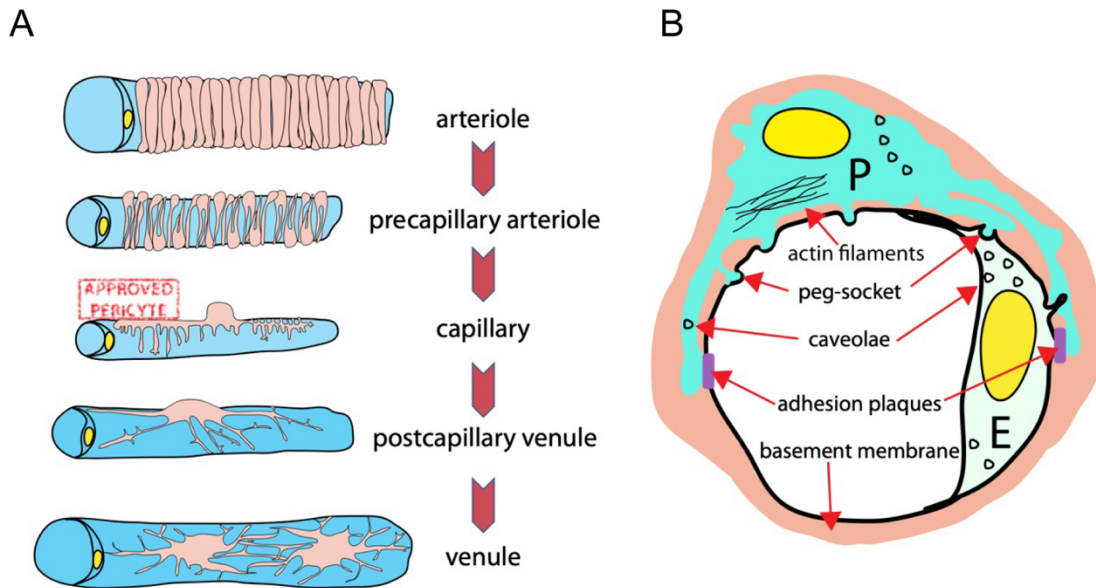


Figure 3. Pericyte biology. *A)* Different density of pericytes along different types of blood vessels. Pericytes are more abundant in small arterioles and capillaries. *B)* Mature pericytes envelope the endothelium sharing the BM. Despite being separated by the BM, pericytes and ECs establish numerous direct contacts such as peg-socket contacts and adhesion plaques (Armulik et al., 2011).

2.2.3 The ECs and pericyte crosstalk.

The spatial relationship between pericytes and ECs points to tight interactions involving paracrine or juxtacrine signaling (Armulik et al., 2011). Indeed, during the formation of new vessels, pericyte recruitment is driven by the platelet-derived growth factor subunit B (PDGF-BB) and its receptor (PDGFR- β) (Hellstrom et al., 1999). PDGF-BB is a soluble factor expressed by sprouting ECs, which acts as a mitogen and chemoattractant for mural cells, where it binds to PDGFR- β . PDGF-BB knockout mice show a significant loss in pericyte number in the microvascular beds, leading to vascular defects (Lindahl et al., 1997). The pericyte recruitment is mediated also by epidermal growth factor (HB-EGF), in fact Stratman and colleagues elegantly demonstrated that an efficient recruitment of pericytes to the vasculature requires the co-presence of PDGF-BB and HB-EGF (Stratman et al., 2010). Another important signaling complex in this context is the Angiopoietin/Tie system, a master regulator of ECs survival and vascular maturation (Eklund et al., 2017). In fact, Angiopoietins control many aspects of the vasculature including development, maintenance, and remodeling. This family comprises Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), and Angiopoietin-3/4 and they bind the tyrosine kinase receptors Tie1 and Tie2 (Thomas and Augustin, 2009). Ang-1 and Ang-2 are the better characterized and specifically engage the

Tie2 receptor. Ang-1 is expressed by SMCs and pericytes and promotes vascular stabilization activating Tie2 phosphorylation. On the other hand, Ang-2, is produced by ECs and acts as an autocrine antagonist of Ang-1-mediated Tie2 activation, inducing vascular destabilization at high concentration (Thomas and Augustin, 2009).

The crosstalk between ECs and pericytes is achieved not only by paracrine pathways, but also by physical contacts between the two cell types (Figure 4) (Bergers and Song, 2005; Díaz-Flores et al., 1991). Three types of EC-pericyte contacts have been reported (Rucker et al., 2000):

- gap junctions which provide a direct connection between the cytoplasm of pericytes and ECs, enabling the exchange of ions and small molecules,
- adhesion plaques which anchor pericytes to ECs,
- peg-and-socket contacts which enable the cells to penetrate through discontinuities in the vessel BM and touch each other.

N-cadherin is another mediator of the EC-pericyte interaction and these AJs are located in the peg–socket contacts. Unlike VE-cadherin, which is exclusively expressed by ECs and maintains the EC-EC connections, N-cadherin is expressed by both ECs and pericytes and it substantially contributes to vessel maturation and stability (Angulo-Urarte et al., 2020; Bazzoni & Dejana, 2004). The integrity of these EC-pericytes junction complexes is key in the maintenance of vascular integrity (Davis and Senger, 2005). Indeed, Gerhard and colleagues showed that the treatment of the developing chicken brain with N-cadherin function-blocking antibodies abrogated the EC-pericyte interactions, leading to vascular abnormalities and hemorrhage (Gerhardt et al., 2000).

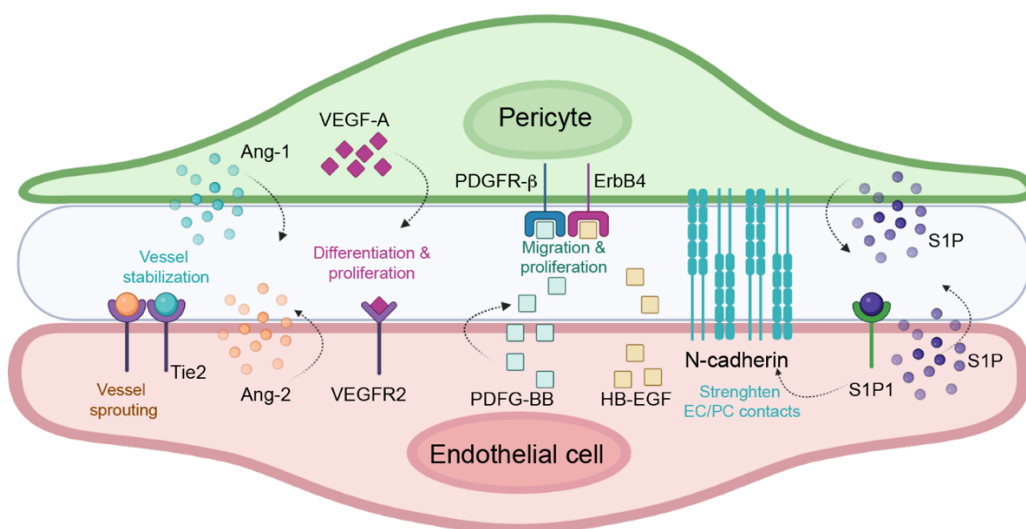


Figure 4. Crosstalk between ECs and pericytes. Schematic representation of the EC-pericyte interconnection governed by several ligand/receptor systems. Starting from left, the Ang-2/Tie2 signaling is responsible for vessel sprouting and destabilization, whereas the Ang-1/Tie2 signaling promotes vessel stabilization. The VEGF/VEGFR axis is implicated in ECs differentiation and proliferation. The EC-derived PDGF-BB engaging its receptor PDGFR- β triggers pericytes migration and proliferation. Sphingosine 1-phosphate (S1P) following engagement of its receptor S1P1 induces the migration of pericytes towards the endothelium and strengthens EC-pericyte interaction. Adapted from (Su et al., 2021) and created with Biorender .

2.3 Tumor angiogenesis

It is well established that tumor growth and progression is profoundly affected by microenvironmental components, among which the vasculature is a key player (Bergers and Benjamin, 2003). Like normal tissues, tumor cells require oxygen and nutrients for survival and proliferation (Lugano et al., 2020). The tumor vasculature plays an active role in promoting tumor growth and metastasis, not only providing nutrients and oxygen, but also impairing the proper immune cell infiltration to the tumor site (Sherwood et al., 1971). In 1971, Judah Folkman hypothesized that tumor growth beyond the size of 2–3 mm³ was dependent on the release of angiogenic factors and on the activation of angiogenesis (Sherwood et al., 1971). During tumor onset, the tissue hypoxia induces the activation of HIF, which regulates the expression of VEGFA and other pro-angiogenic genes. The exposure to unbalanced pro-angiogenic molecules leads to the activation of ECs, which are normally quiescent. Once ECs are activated, they secrete matrix metalloproteinases (MMPs) which degrade the ECM and form new sproutings. However, these newly formed tumor-associated vessels fail to reach a proper maturation (Lugano et al., 2020). Indeed, unlike the normal vasculature, tumor-associated vessels are immature, and they exhibit a typical aberrant structure, being characterized by abnormal size, i.e. smaller and/or dilated, disorganization and tortuosity. Accordingly, also vascular cells display extensive abnormalities; ECs are in fact characterized by widened interendothelial junctions and discontinuous or absent BM, whereas pericytes are abnormally shaped and poorly associated with ECs, thus jeopardizing the endothelial barrier function (Figure 5) (Carmeliet and Jain, 2011a; Morikawa et al., 2002). As a consequence, tumor-associated vessels are leaky and poorly functional (Fukumura and Jain, 2007), and these features first compromise the delivery of the drugs to the tumor, and second alter the infiltration of the immune cells to the tumor site (Carmeliet and Jain, 2011). Indeed, in tumor vessels, leakiness together with vessel compression cause a uniformly elevated interstitial fluid pressure that hinders delivery of blood-borne therapeutic agents, lowering the efficacy of chemo- and nano-therapies (Jain

et al., 2014). Importantly, it was recently reported that the inefficient and aberrant tumor vasculature plays a crucial role in generating an immune suppressive microenvironment (Zhao et al., 2021). For instance, ECs subjected to a hypoxic environment over-express the CLEVER-1/stabilin-1 receptor, which supports the recruitment and survival of immunosuppressive T cells. Furthermore, hypoxia-induced signaling also mediates the recruitment of tumor associated macrophages and their polarization towards the immunosuppressive M2 phenotype. In addition, increased VEGFA levels promote the clustering of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion protein (VCAM)-1, thus hampering the immune cell extravasation (Schaaf et al., 2018).

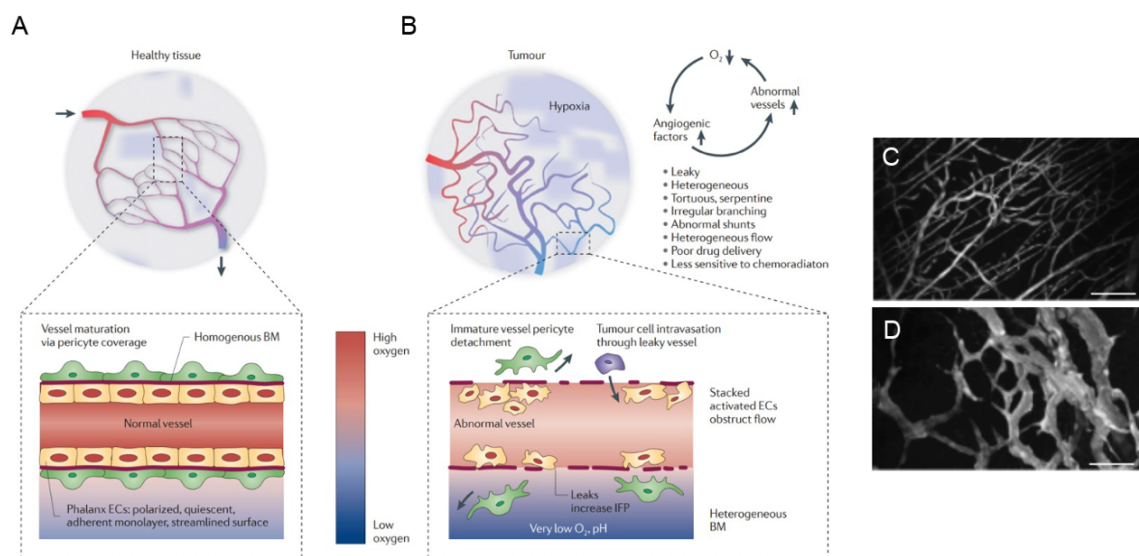


Figure 5. Healthy vessels VS tumor associated vessels. *A) Hierarchically well-organized healthy vasculature, formed by a quiescent monolayer of ECs, a homogenous BM and optimal pericyte coverage. B) Tumor-associated vessels structurally and functionally abnormal, characterized by discontinuous BM and pericyte detachment. Vascular inefficiency leads to poor oxygenation of tissues and consequently elevated intratumoral hypoxia (blue shading) (Carmeliet and Jain, 2011). C and D) Electron microscopy images of the architecture of normal and tumor-associated vessels, respectively. Adapted from (Munn, 2003).*

2.3.1 Anti-angiogenic therapies and vessels normalization

In 1971, Judah Folkman, in his renowned work published on the “*New England Journal of Medicine*” was the first to hypothesize that, since tumor blood vessel formation was dependent on a tumor angiogenic factor (TAF), the identification and blockade of pro-angiogenic factors and the consequent inhibition of the formation of new vessels formation, could have been a promising strategy to cause tumor growth arrest (Sherwood, Parris, and Folkman 1971). From this pioneer work, the targeting of tumor angiogenesis in order to

starve the tumors has become an attractive field of study. A massive effort has been made to characterize the pro- and anti-angiogenic factors and to identify new targetable molecules. VEGFA is the better characterized pro-angiogenic factor and was originally known as vascular permeability factor (VPF), given its capacity to increase the permeability of microvessels to plasma proteins (Ferrara and Henzel, 1989; Dvorak, 2000; Ferrara, 2005). VEGFA is up-regulated in many cancers such as breast (Yoshiji et al., 1996), lung (Alevizakos et al., 2013) and colorectal cancer (CRC), where increased VEGFA levels correlate with a more advanced disease (George et al., 2001). Thus, VEGFA represented an ideal target for the development of anti-angiogenic therapies. Indeed, in 2004, the first anti-angiogenic drug bevacizumab/Avastin, a monoclonal antibody able to sequester VEGFA, was approved by the Food and Drug Administration (FDA) for the treatment of metastatic colon cancer (Hurwitz et al., 2004). The clinical approval of this drug represented a novelty in the oncology treatments, since it was the first drug to target the tumor associated stroma instead of cancer cells (Augustin and Koh, 2022). However, antiangiogenic therapies did not meet the high expectations, since bevacizumab, as single agent, failed to improve the overall survival (OS) of the patients in different tumor types (Jain, 2005). Indeed, bevacizumab is used as monotherapy only to treat recurrent glioblastoma multiforme (Cohen et al., 2009). Nonetheless, bevacizumab is currently used in combination with chemotherapy for metastatic non-squamous non-small cell lung cancer and with interferon- α to manage metastatic renal cell carcinoma with improved patients' progression free survival (PFS) (Rini et al., 2008). Two phase III trials (GOG218, ICON 7) showed that bevacizumab in combination with carboplatin and paclitaxel prolonged the PFS of women with epithelial ovarian cancer leading to the approval of these combinations for women with advanced ovarian cancers. In 2006 FDA extended the approval of bevacizumab for the second-line treatment of metastatic colorectal cancer patients. Moreover, bevacizumab plus standard second-line chemotherapy beyond disease progression displayed clinical benefits in patients with metastatic colorectal cancer, according to a randomized phase III clinical trial (Bennouna et al., 2013). The limited clinical impact of bevacizumab as monotherapy is thought to be due to the fact that the blockage of tumor angiogenesis may induce the establishment of stromal and microenvironmental defense mechanisms. Indeed, prolonged VEGF inhibition leads to increased hypoxia which in turn leads to upregulation of other angiogenic cytokines and the possible selection of more aggressive and resistant cancer cells (Ribatti et al., 2019).

Given these drawbacks of antiangiogenic therapy, in the last years the “tumor vessels normalization” concept has been taking credit. The idea is on one side to reduce the vasculature and on the other to re-address the aberrant tumor-associated vasculature towards a normal phenotype. This would result in the establishment of a more functional vasculature and the consequent improvement of its efficiency and efficacy (Jain, 2001). The rational of this study was that a flawed delivery system leads to the concentration of the drugs and oxygen always in the same regions, impeding the proper homogeneous distribution of the drug within the tumor. On these grounds, the normalization of the vasculature would improve the intratumoral concentration of the drugs and oxygen (Jain, 1999).

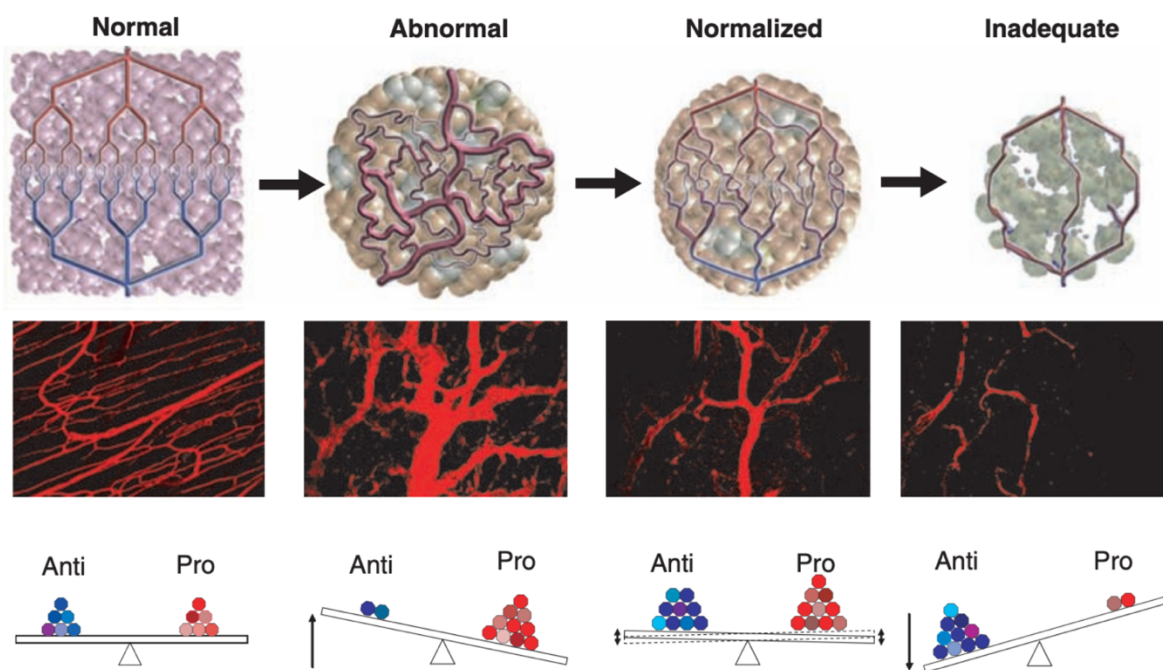


Figure 6. Tumor vessel normalization. From left, representation of the hierarchically well-organized normal vasculature due to the balanced presence of pro- and anti-angiogenic factors; an excess of anti-angiogenic factors leads to the formation of abnormal tortuous and disorganized vasculature; balanced anti-angiogenic treatments may revert the vasculature towards a normalized phenotype; prolonged or aggressive antiangiogenic regimens may lead to inadequate vascularization and increased hypoxia (Modified from Jain, 2005).

2.4 Overview of the Extracellular Matrix

The ECM is a three-dimensional non-cellular architectural scaffold, rich of fibrous proteins, glycosaminoglycans, which provides structural support to the surrounding cells (Naba et al., 2016; Vong and Kalluri, 2011). The ECM exerts multiple functions among which it can guide cell polarization, represent an adhesive substrate for cell migration and, per se or by binding morphogens and growth factors, can create concentration gradients functioning as an

haptotactic stimulus (Naba et al., 2016). The ECM is deposited in the intercellular spaces (interstitial matrix), or as a network of ECM molecules in which cells are embedded, or in specialized forms, such as the BM, a 50-nm-thick sheet of ECM molecules on which endothelial or epithelial and mural cells reside. The vascular BM, which is specifically deposited by endothelial and mural cells is crucial for vascular integrity and functions (Naba et al. 2012; Vong and Kalluri 2011).

The ECM can be categorized into major classes of macromolecules (Mouw et al., 2014):

- fibrous proteins (including collagens and elastin)
- glycoproteins (including fibronectin, proteoglycans (PGs) and laminin).

Collagens are the most abundant fibrous proteins of the body and their structural hallmark is a triple helix, a tight right-handed helix of three α -chains, each containing one or more regions characterized by the amino acid residue motif Gly-X-Y, where X and Y can be any amino acid residue (Brodsky and Persikov, 2005). Among the different collagens, type I, type IV, type XV and type XVIII collagens are those principally involved in angiogenesis (Mongiat et al., 2016). Type I collagen is the main structural protein of the interstitial ECM (Boot-Handford and Tuckwell, 2003), whereas types IV, XV and XVIII are the main components of the vascular BM (Kalluri, 2003). Among the glycoproteins, fibronectin plays an important role in the vascular system. Fibronectin is highly expressed along developing vessels during embryogenesis and, along with its splice variants, is important for endothelial-perivascular cell interactions during vascular tree morphogenesis (Astrof and Hynes, 2009).

2.4.1 Extracellular matrix remodeling in tumour

Cells sense biochemical and mechanical changes through specialized transmembrane receptors including integrins, discoidin domain receptors (DDR), and syndecans (Kai et al., 2019). The interaction of the cells with the ECM tunes their growth, survival, migration, invasion, and differentiation affecting their phenotype, tissue development and homeostasis. For these widespread effects the ECM deposition and modification, are tightly regulated (Lu et al., 2011). In tumors the ECM is extensively remodeled and these changes lead to altered ECM composition and increased stiffness. In fact, abnormal ECM composition and organization, an established hallmark of cancer (Socovich and Naba, 2019), alters the complex cellular signaling networks, leading to pathologic conditions such as inflammatory diseases, tissue fibrosis, and cancer (Kai et al., 2019). The main source of ECM molecules in the TME are cancer associated fibroblasts (CAFs). CAFs are recruited and activated by

cancer cells through the secretion of various pro-fibrotic and inflammatory growth factors, including transforming growth factor α and β (TGF- α and TGF- β), fibroblast growth factor 2 (FGF-2), PDGF-BB and EGF (Kalluri, 2016). Surprisingly, it has been demonstrated that cancer cells themselves represent critical source of the tumor ECM. Indeed, cancer cells synthesize collagen (Fang et al., 2019; Öhlund et al., 2013) as well as other ECM-associated proteins, such as fibronectin, syndecan, and laminin (Naba et al. 2012; Xiong and Xu 2016). Moreover, in many solid cancers different ECM proteins such as fibronectin, tenascin, elastin, collagens and hyaluronic acid (HA) and other proteoglycans are up-regulated (Acerbi et al., 2015; Laklai et al., 2016). The ECM remodeling profoundly affects the ECs behavior. For instance, LaValley et al demonstrated that ECM stiffness increases the internalization of vascular endothelial growth factor receptor 2 (VEGFR-2) in ECs thus affecting proliferation and stress fiber formation (LaValley et al., 2017).

2.5 Tumor-associated macrophages

The tumor microenvironment (TME) comprises a complex milieu of non-malignant cells and represents an essential niche for cancer initiation and progression. Indeed, the crosstalk between tumor cells and TME components is crucial during tumor progression (Choi and Moon, 2018). The immune cells are a key TME component able to profoundly affect tumor growth and capable to either suppress or promote tumor progression depending on the context (Anderson and Simon, 2020). Among the immune cells, tumor-associated macrophages (TAMs) represent the major components of the TME. Their origin is still elusive, although in most cancer models, they are recruited from bone marrow progenitors known as monocytes (Mantovani et al., 2022).

Generally, TAMs secrete growth factors, chemokines, and MMPs, which act directly on cancer cells or on other stromal cells, affecting tumor growth, angiogenesis, invasion, and metastasis (Condeelis and Pollard, 2006). Activated macrophages are classified into two main polarized phenotypes: classically activated macrophages (M1), which exert tumor-suppressive functions, and alternatively activated macrophages (M2), which promote tumor growth and progression (Lin et al., 2019).

The two subtypes are distinguished by the presence of specific markers such as CD86 (Stöger et al., 2012) and CD64 (Xu et al., 2017) for the M1 subtype, whereas the M2 macrophages typically express CD206 (mannose receptor-1), CD204 and CD163, also known as macrophage scavenger receptor (Röszer, 2015; Stifano and Christmann, 2016).

Generally, higher infiltration of TAMs can be detected in more advanced tumor stages, as observed in esophageal cancer, ovarian cancer (Yuan et al., 2017), breast cancer (Qiu et al., 2018) and pancreatic cancer (Yu et al., 2019). Importantly, high macrophage infiltration negatively impacts on patients' OS, as demonstrated in esophageal cancer (Li et al., 2019), pancreatic cancer (Yu et al., 2019), breast cancer (Zhao et al., 2017), lung cancer (Wu et al., 2016), gastric cancer (Wang et al., 2016) and CRC (Shibutani et al., 2017).

2.6 The EMILIN/multimerin family

The EMI domain endowed (EDEN) superfamily comprises ECM molecules characterized by the presence of a cysteine-rich sequence of approximately 80 amino acids named EMI domain (Braghetta, 2004). The molecules of this superfamily can be further clustered into three different subgroups:

- The first subgroup includes Emu1 and Emu2, which, except for the presence of the EMI domain, they do not share any other structural similarity with the other EDEN members (Leimeister et al., 2002).
- The second subgroup includes only one member i.e. EMILIN-truncated (EMILIN-T), which shares the structural features of the third family, except for the absence of the C-terminal C1q globular domain (gC1q).
- The third subgroup comprises four proteins: Emilin-1, Emilin-2, Multimerin-1 and Multimerin-2. Their structure is characterized by the presence of the N-terminal EMI domain, a central region of approximately 700 amino acid residues with high probability to form coiled-coil structures, followed by a distinct sequence characterizing each molecule that may confer specific structural and/or functional properties. At the C-terminus all these molecules display a gC1q-like domain typical of the gC1q/TNF superfamily members (Colombatti et al., 2012).

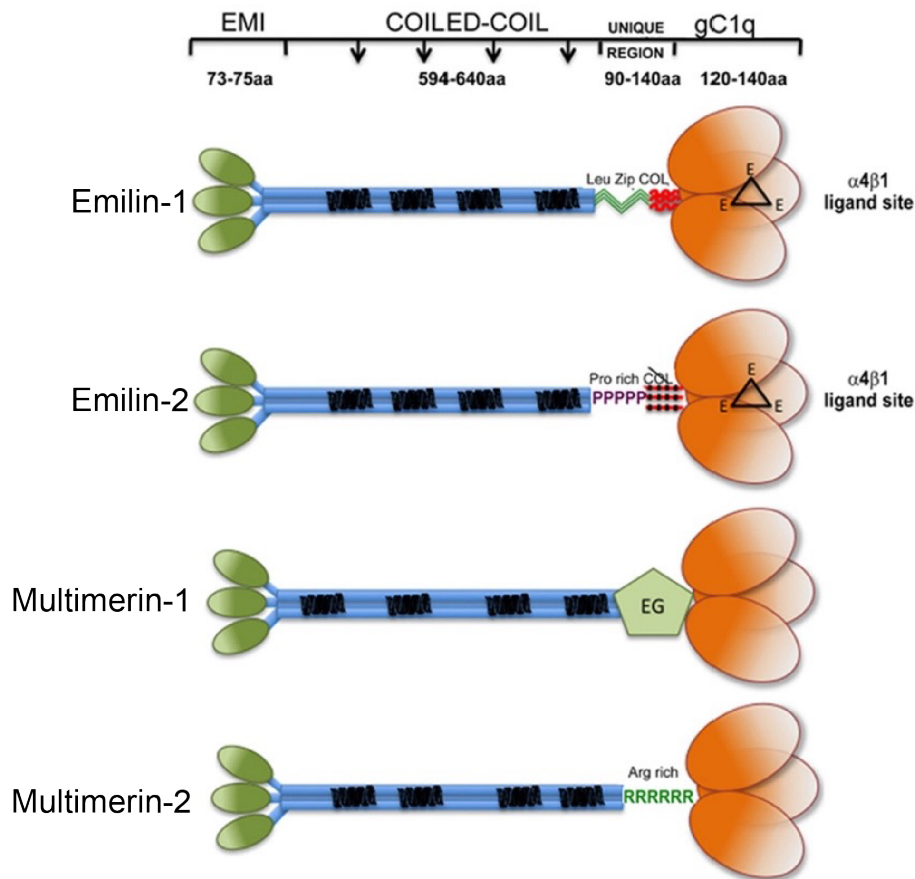


Figure 7. Schematic representation of the EMILIN/Multimerin family members. From left, (EMI) EMI domain in green, the coiled coil region highlighted in blue and black, the unique region is a specific sequence characterizing the members of the family, gC1q-like domain; (Pro rich) proline-rich domain; (EG) region with partial similarity with EGF domain; (RR) arginine-rich domain. Modified from (Colombatti et al., 2012).

2.6.1 Emilin-1

Elastin microfibril interface–located protein 1 (Emilin-1) is a glycosylated protein of 115 kDa isolated from chicken aortas in the late 1980s (Bressan et al., 1993). This molecule is strictly associated with elastic fibers and microfibrils in blood vessels, as well as in connective tissue of other organs (Colombatti et al., 2000). Bio-synthetic and immunodetection studies showed that SMCs, fibroblasts, and ECs, represent the major sources of Emilin-1 (Zanetti et al., 2004) which is synthesized and secreted into the ECM. Once secreted, Emilin-1 is expressed as a monomer within the cells, then it oligomerizes through the formation of disulfide bonds (Zanetti et al. 2004). Emilin-1 expression associates with elastic fibers and microfibrils in blood vessels (Colombatti et al. 1985; Colombatti et al. 2000). Emilin-1 is implicated in elastogenesis and in the maintenance of the blood vascular cell morphology (Zanetti et al., 2004). Indeed, *Emilin-1*^{-/-} mice display elevated

systemic blood pressure independent of the cardiac output without defects in vascular contractility or mechanical properties. Mechanistically, it has been shown that Emilin-1 also impinges on the maturation of TGF- β sequestering proTGF- β , thus regulating its bioavailability. In support of these findings *Emilin-1*^{-/-} mice display increased TGF- β signaling in the vascular wall, high blood pressure and systemic arterial hypertension (Zacchigna et al., 2006). The gC1q domain of Emilin-1 engages the $\alpha_4\beta_1$ and $\alpha_9\beta_1$ integrins promoting cell adhesion, migration, and halting proliferation. In fact, *Emilin1*^{-/-} mice display dermal and epidermal hyperproliferation because of the lack of interaction between Emilin-1 and the $\alpha_4\beta_1$ and $\alpha_9\beta_1$ integrins which normally upregulate the levels of pErk1/2 through PTEN (Danussi et al., 2011). Moreover, the interaction between gC1q-like domain and the $\alpha_9\beta_1$ integrin is relevant in the lymphatic system; unlike the other member of the family, Emilin-1 function is not only restricted to the blood vessels, but it is a structural modulator of the growth and maintenance of lymphatic vessels. Accordingly, *Emilin1*^{-/-} mice display lymphatic morphological alterations, thus supporting the direct role of Emilin-1 in the growth, maintenance, and integrity of the lymphatic vasculature (Capuano et al., 2019; Danussi et al., 2008).

2.6.2 Emilin-2

The Emilin-2 sequence was identified following a two-hybrid screening using the Emilin-1 gC1q-like domain as bait. Emilin-2 is structurally similar to Emilin-1 the distinctive trait being a proline-rich domain followed by a collagenous stalk (Mongiati et al., 2007).

Emilin-2 was found to be highly expressed in the cochlear MB of P8 murine pups, modest expression was detected in adult heart and lung and much lower expression levels in the forebrain, brainstem, cerebellum, and hypothalamus. The lowest expression was found in muscle, liver, kidney, and eye (Amma et al., 2003). More recent studies conducted in our laboratory highlighted Emilin-2 as an important component of the gastric mucosa, its expression being limited to the lamina propria (Andreuzzi et al., 2018).

Emilin-2 expression is epigenetically regulated by methylation being frequently downregulated in different solid tumors including breast, lung, prostate and colorectal cancers (Hill et al., 2010; Andreuzzi et al., 2022). From the functional point of view Emilin-2 was shown to display a tumor suppressive function acting directly on cancer cells, also indirectly affecting different components of the TME. In particular, Emilin-2 directly affects the viability of sarcoma cells triggering cell death through direct binding and activation of

death receptors DR4 and DR5 (Mongiat et al., 2007). Emilin-2 also negatively affects the proliferation of breast cancer cells, functioning as an extracellular modulator of the Wnt signaling pathway through the modulation of the availability of Wnt1 (Marastoni et al., 2014).

The indirect function of Emilin-2 in affecting tumor growth relies in its capability to promote tumor angiogenesis via the engagement of the epidermal growth factor receptor (EGFR) and the consequent increase of interleukin-8 (IL-8) production (Paulitti et al., 2018) (Figure 8). Accordingly, ablation of Emilin-2 significantly impairs vessels formation (Paulitti et al., 2018) and affects their stability and functionality halting pericytes recruitment (Fejza et al., 2023).

Emilin-2 also displays an immunomodulatory function; in fact, low Emilin-2 levels correlate with an increased presence of M2 macrophages, and with poor CRC patient prognosis (Andreuzzi et al., 2022).

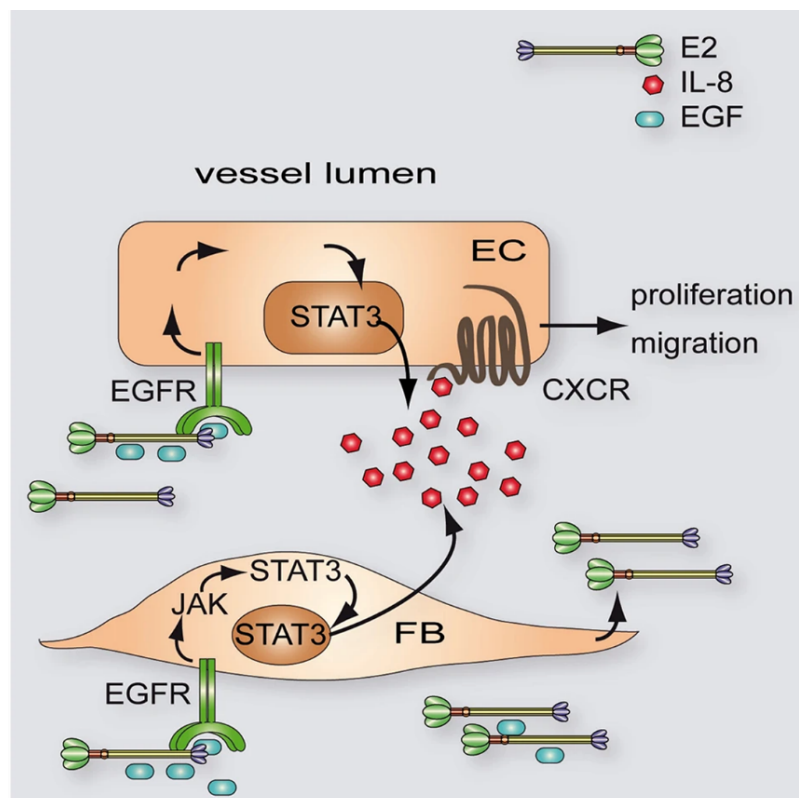


Figure 8. Emilin-2 stimulates angiogenesis via an EGFR-dependent IL-8 production. Emilin-2 directly engages the epithelial growth factor receptor (EGFR) leading to the activation of Jak2 and STAT3 and the over-production of IL-8. The mechanism occurs both in ECs (above), which do not express Emilin-2, and fibroblasts (below). In turn, IL-8 overexpression leads to increased EC activation following the engagement of the IL-8 receptor CXCR1 (Paulitti et al., 2018).

2.6.3 Multimerin-1

Multimerin-1 is one of the largest known proteins in the human body (Jeimy et al., 2008). It is a soluble, homopolymeric adhesive glycoprotein that is synthesized and stored in ECs, especially in the lungs (Uhlén et al., 2015), as well as in platelets and megakaryocytes (Adam et al., 2005; Hayward et al., 1998). Specifically, in resting platelets Multimerin-1 is stored in the Weibel-Palade body-like region α -granules (Hayward et al., 1993). Once released, Multimerin-1 binds and activates coagulation factor V (Jeimy et al., 2008). Multimerin-1 enhances platelet adhesion via shear-dependent mechanisms through the $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ integrins (Leatherdale et al., 2021). Lately, Multimerin-1 has been identified as a differentially expressed gene in various cancers and it has been proposed as a potential cancer biomarker (Posner, 2022) in both cervical cancer (Keeratichamroen et al., 2020) and in pediatric acute myeloid leukemia (Laszlo et al., 2015), where it is upregulated. Multimerin-1 has been found to be upregulated in pancreatic cancer (Bartha and Györffy, 2021), whereas it is downregulated in other tumor types and its downmodulation associates with chemoresistance in rectal cancer (Gantt et al., 2014) and radiosensitivity and clinical outcome in gastric cancer (Liang et al., 2020).

2.6.4 Multimerin-2

Multimerin-2, also known as EndoGlyx-1, is another member of the EMILIN/Multimerin family and shares all the structural features of the EMILIN/Multimerin family, except for the presence of arginine-rich region prior the gC1q-like domain (Colombatti et al. 2012). It is specifically deposited by ECs and it was discovered as a pan-endothelial ECM component in association with a high molecular weight glycoprotein complex (Christian et al., 2001).

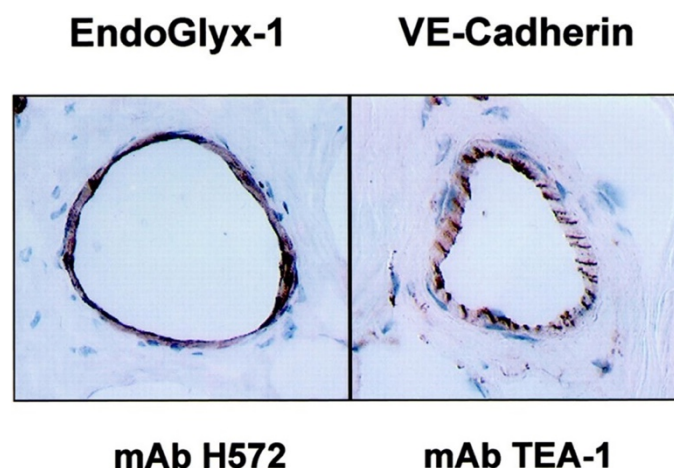


Figure 9. Immunohistochemical analysis of Multimerin-2 expression in human blood vessels. Sections of normal breast tissue stained with the anti-Multimerin-2 (EndoGlyx-1) H572 monoclonal antibody (mAb) or with the anti-VE-cadherin TEA-1 monoclonal antibody (mAb) (From Christian et al., 2001).

Immunohistochemical analyses showed Multimerin-2 expression in ECs of large, medium-sized, and small blood vessels, including capillaries, in all human organs tested; only hepatic and splenic sinusoids were found negative for Multimerin-2 (Sanz-Moncasi et al., 1994). Multimerin-2 is specifically deposited by the endothelium and is present both in the abluminal and luminal side of the vessels, thus suggesting an important role in vascular homeostasis (Galvagni et al., 2017). The function of Multimerin-2 was firstly revealed in a paper from our laboratory, in which the authors showed that the expression of the molecule significantly reduced ECs migration and the sprouting of new vessels *in vivo* (Lorenzon et al., 2012). It was also demonstrated that the overexpression of the molecule associated with impaired intra-tumoral angiogenesis and halted tumor growth. Mechanistically, Multimerin-2 interferes with the VEGF/VEGFR2 axis through a direct sequestration of VEGFA (Lorenzon et al., 2012), via the carbohydrate chains (Colladel et al., 2016). Consistently with our main hypothesis of Multimerin-2 as an angiostatic molecule, during sprouting angiogenesis, upon an angiogenic signal, Multimerin-2 is targeted for degradation mainly by MMP-9 and to a lower extent by MMP-2, to allow the sprouting of new vessels (Figure 10) (Andreuzzi et al., 2017). In line with these evidences, analyses conducted on CRC sections revealed that the presence of active MMP-9 correlated with extensive degradation of Multimerin-2 (Andreuzzi et al., 2017). Also the analyses of gastric cancer samples revealed that Multimerin-2 expression was significantly reduced in tumor-associated vessels (Andreuzzi et al., 2018).

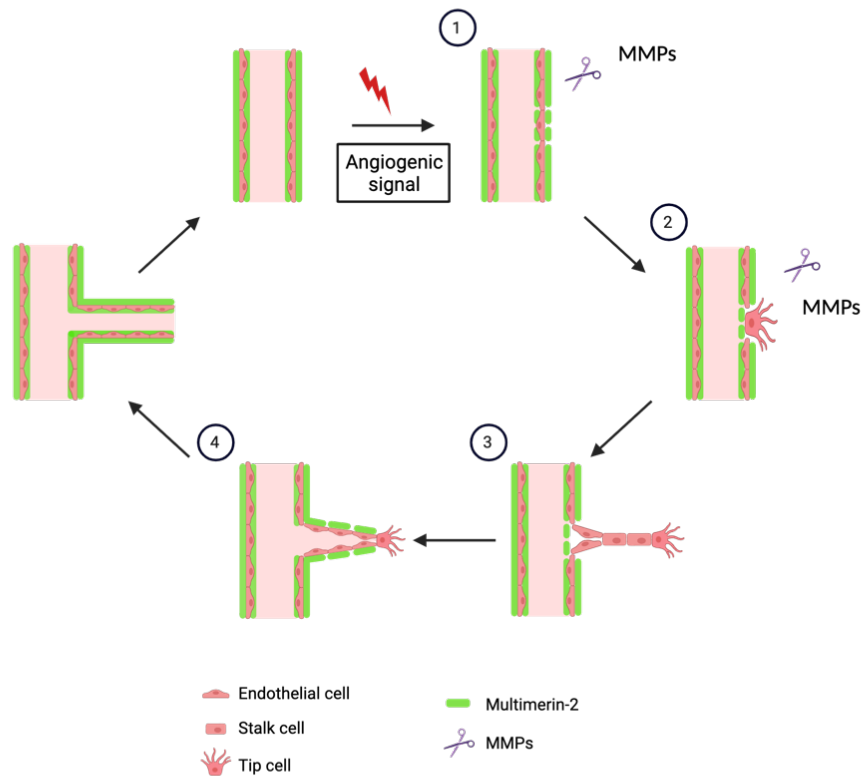


Figure 10. Schematic representation of Multimerin-2 expression during sprouting angiogenesis. (1) Upon angiogenic stimulus, Multimerin-2 is degraded by MMPs; (2) Tip cells selection; (3) Stalk cells proliferate and the new vessels are formed; (4) Multimerin-2 is secreted by ECs to stabilize the vessels; (5) The new vessel is formed and ECs return in their quiescent state (Adapted from (Mongiat et al., 2016) and created with Biorender).

We recently demonstrated that loss of Multimerin-2, caused by metalloproteinases degradation, strongly impacts on the homeostasis of the endothelium. In fact, the down-regulation of Multimerin-2 induces the dismantlement of the ECs junctions, increasing permeability of the vessels and hindering their efficiency (Pellicani et al., 2020). Multimerin-2 represents a ligand for two C-type lectin domain (CTLD) containing receptors CD93 and CLEC14A, expressed by ECs as well as by immune cells (Khan et al. 2017). Interestingly, Multimerin-2 was also shown to be a possible ligand of CD248, a transmembrane protein of the CTLD group 14 family that is expressed by pericytes but not by ECs. The CD248 region involved in the interaction with Multimerin-2 is distinct from that of CLEC14A and CD93, suggesting that Multimerin-2 may simultaneously interact with CLEC14A and CD93 expressed on the ECs surface and with CD248 expressed by pericytes, suggesting that Multimerin-2 may function as a bridge between ECs and pericytes (Khan et al., 2019).

4. AIMS OF THE STUDY

The maintenance of blood vessel homeostasis and functionality is crucial to allow proper tissue oxygenation and nourishment. In fact, abnormal vasculature is a common feature in different pathologies, including cancer. Hence, the study of the mechanisms regulating vascular functionality and the identification of strategies to circumvent this problem are solicited clinical needs.

In this context, we focused our study on the ECM glycoprotein Multimerin-2, which plays a key role in the maintenance of inter-endothelial cell junctions, thus promoting vascular homeostasis. Multimerin-2 is secreted by ECs in juxtaposition with the adjacent pericytes, thus we hypothesized that it could also affect the recruitment of pericytes during vascular maturation, a process required for the stabilization of the vasculature. In support of this hypothesis, our previous observations indicated that retinal vessels from *Multimerin-2*^{-/-} animals displayed impaired pericyte coverage. Based on this evidence, the main purpose of this project was to verify if and how Multimerin-2 could influence pericyte biology and behavior, and to evaluate if its loss impacted on vascular efficiency and the infiltration of immune cells in ovarian and colon cancer.

More specifically the aim of the present PhD project was to:

- Verify if Multimerin-2 affects pericyte biology and behavior.
- Unveil the molecular mechanisms through which Multimerin-2 putatively regulates pericyte behavior and vessel stability.
- Assess if the loss of Multimerin-2 affects pericyte recruitment and vascular efficiency *in vivo*.
- Verify if low Multimerin-2 expression alters the infiltration of immune cells in colon and ovarian tumors.

5. MATERIALS AND METHODS

5.1 Cell culture

Human brain vascular pericytes (HBVP, Cat. #1200) were purchased/provided from ScienCell (Carlsbad, CA, USA). Cells were cultured in Pericyte Medium (PM, Cat. #1201) supplemented with 2% Fetal Bovine Serum (FBS), 1% Pericyte Growth Supplement (PGS) and 100 I.U./mL of penicillin/streptomycin (P/S) (ScienCell, Carlsbad, CA, USA) in a 37 °C, 5% CO₂ incubator. Human umbilical vein endothelial cells (HUVEC) were isolated from freshly obtained umbilical cords through collagenase A digestion of the interior of the umbilical vein. HUVECs were collected in Endothelial Cell Medium (ECM, Cat. #1001) from ScienCell (Carlsbad, CA, USA), enriched with 5% FBS, 1% of Endothelial Cell Growth Supplement (ECGS) and 100 I.U./mL of P/S (ScienCell, Carlsbad, CA, USA). The 293-EBNA (E293) cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were stably transfected by electroporation with the pCEP-Pu Multimerin-2 construct. Cells were cultured in DMEM (Gibco, Milan, Italy) in the presence of 0.5 µg/ml of puromycin and 250 µg/ml of G418. Positive clones were isolated, and Multimerin-2 expression analyzed by Western blotting. 293FT cells (Thermo Fisher Scientific, Massachusetts, USA) were used for lentiviral production and were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, sodium-pyruvate (NaPir) and nonessential amino acids. The human leukemia monocytic cell line THP-1 was provided by ATCC and cultured in RPMI medium 1640 (Gibco, Milan, Italy) supplemented with 10% fetal bovine serum (Gibco, Milan, Italy), 10 mM Hepes, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate (Gibco), and 100 I.U./mL P/S (Gibco, Milan, Italy). The MC-38 colon carcinoma cell line was kept in DMEM (Gibco, Milan), with 10% fetal bovine serum (Gibco, Milan, Italy), 2mM glutamine, 0.1 mM non-essential amino acids (Gibco, Milan, Italy), 1 mM sodium pyruvate (Gibco), 10 mM Hepes and 100 I.U./mL of P/S (Gibco, Milan, Italy). The ID8 *Trp53*^{-/-} *Pten*^{-/-} high grade serous ovarian cancer (HG-SOC) cells were kindly provided by the McNeish laboratory, and cultured in DMEM medium (Gibco, Milan, Italy) supplemented with 4% FBS (Gibco, Milan, Italy), 1% insulin-transferrin-selenium (ITS, Gibco, Milan, Italy) and NaPir (Gibco, Milan, Italy). All cells were maintained in humidified incubator at 37°C and 5% CO₂, and regularly checked for mycoplasma contamination using MycoAlertTM Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

5.2 Cell transfection and transduction

Endogenous downregulation of Multimerin-2 in HUVEC cells was obtained using the Knock-out RNAi System (Clontech); specifically, HUVEC cells were transduced with adenoviruses carrying this siRNA sequence: 5'-GCAGACAGTGAAGTTCAACACCACATACA-3' at the multiplicity of infection (MOI) of 500. Transduction was performed for 72 hours. To down-regulate CD248 endogenous expression in HBVP cells, five short hairpin RNAs (shRNAs) targeting human CD248 mRNA were obtained from Merck (Darmstadt, Germany). The shRNA sequences are reported below (Table 1).

Table 1.

ShCD248 #1 TRCN0000053457	5'-CCCTATGATCTCAACCAAATA-3'
ShCD248#2 TRCN0000053453	5'-CACCACCTCATTTGCCTGGAAT-3'
ShCD248 #3 TRCN0000443679	5'-CCAACCTATCCAGATCTGCCTT-3'
ShCD248 #4 TRCN0000053454	5'-AGCGCATCACTGACTGCTATC-3'
ShCD248 #5 TRCN0000437821	5'-TCACCCATCAGCCCTACACAT-3'

4x10⁶ 293FT cells were plated in 10 cm dishes and the next day transfected using the calcium phosphate method employing 19,8 µg of vector, 6 µg of pCMV-VSVG and 10 µg psPAX2 (Addgene, Massachusetts, USA). Media were changed 12 hours post-transfection, and the viral supernatant was collected 24 or 48 hours later, filtered through 0.45 µm membranes, pooled and dispensed on HBVP cells in the presence of 8 µg/ml polybrene (Sigma Aldrich, St. Louis, MO, USA). The viral supernatant was replaced by fresh medium 12–24 hours later. After 72 hours from the transduction, knockdown efficiency of the CD248 shRNA lentiviruses was confirmed by Western blot.

5.3 Antibodies and reagents

The polyclonal anti-Multimerin-2 antibody was produced in our laboratory as previously described (Colladel et al., 2016b). Anti-IGF1R- β , anti-phosphoIGF1R- β , anti-AKT, anti-phospho-Akt, anti-PLC- γ , anti-phospho-PLC- γ , anti-IRS1, anti-phospho-IRS1 were purchased by Cell Signaling Technology Antibodies (Massachusetts, USA). The antibody against the murine CD31 was from BD Bioscience (New Jersey, USA). The antibody against the murine GLUT1 and Cpt/DNA adducts were purchased by Merck Millipore (Massachusetts, USA). Secondary antibodies used for immunofluorescence and conjugated with Alexa Fluor 488, 546 and 633 were provided by Invitrogen (Massachusetts, USA), as well as the TO-PRO-3 fluorescent dye used for nuclear counterstain. Secondary antibodies used for immunohistochemistry, conjugated with horse radish peroxidase (HRP) or alkaline phosphatase were from Vector Laboratories (California, USA). The secondary antibodies used for Western blot were HRP-conjugated (Amersham, Amersham, UK) or IRDye[®]-conjugated (Li-COR Biosciences, Nebraska, USA). CellTrace[™] CFSE Cell proliferation kit (Invitrogen, Massachusetts, USA) was used to label HBVP or THP-1 cells for detection. The IGFR1 inhibitor NVP-AEW541 was resuspended in DMSO at a final concentration of 5 mM and was purchased from Sigma-Aldrich (Missouri, USA). The broad analysis of phosphorylated proteins was performed using the RayBio[®] C-Series Human Phosphorylation Multi-Pathway Profiling Array C55 (RayBiotech, Georgia, USA), samples were prepared according to the manufacture's protocol and membranes were acquired using the ChemiDoc Touch Imaging System (Bio-Rad, California, USA).

5.4 RNA extraction, retrotranscription and Real-time PCR

Total RNA was extracted from cells using the TRIzol lysis reagent (Roche Applied Science, Mannheim, Germany) according to manufacturer protocol. RNA quantification was obtained using the Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA) and its quality assessed by agarose gel electrophoresis. Retrotranscription was performed with the AMV-RT enzyme (Promega, Wisconsin, USA) in the presence of the Random Primer mix (Promega, Wisconsin, USA), according to manufacturer instructions.

After determination of primer specificity and efficiency, quantitative Real-Time PCR was carried out with the iQTM SYBR[®] Green Supermix (Bio-Rad, California, USA) using the

BIORAD CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, California, USA).. Data normalization was performed using ACTIN and/or GAPDH as housekeeping genes. All the primers for gene expression analyses were purchased from Sigma-Aldrich. All the primer sequences are reported below (Table 2).

Table 2.

Target gene	Sequence (from 5' to 3')
h-GAPDH	Fw: GAGAGACC CTCACTGCTG
	Rev: GATGGTACAT GACAAGGTGC
h-PDGF-BB	Fw: CCTTGGCTCTGCTGGAATGA
	Rev: GCTGGTTCTTCCCTCACGTT
h-HB-EGF	Fw: TTATCCTCCAAGCCACAAGCA
	Rev: AGCCCCTTGCCTTTCTTCTTT
h-ANGPT2	Fw: TGGCTGGGAAAT GAGTTTGT
	Rev: GGTTGGCTGATGCTGCTTAT
h-Apelin	Fw: GCTGCTCTGGCTCTCCTTGA
	Rev: ATTCCTTGACCCTCTGGGCT
h-VCAN	Fw: CAAGACCACCCACTGTGGAA
	Rev: CAAATCACTCATTCGACCTGTCTT
h-WNT5A	Fw: GGGTGGGAACCAAGAAAAA
	Rev: TGGAACCTACCCATCCCATA
h-OAS1	Fw: CTGGATTCTGCTGGCTGAAAG
	Rev: CTGGAGTGTGCTGGGTCTATG
h-CDH11	Fw: TCAAGGGCCCCAGAAATCAC
	Rev: TTGAGCTCATCACGTCAGGG
h-PDGFC	Fw: AAATGAGCCTCTTCGGGCTT
	Rev: CTGAGGATCTTGTA CTCCGTTCT
h-STAT1	Fw: AGAGTCTGCAGCAAGTTCGG
	Rev: CCACCACAAACGAGCTCTGAA
h-TGFBI	Fw: GAAACTCTGGGCGGCAAAAA
	Rev: GCTACCAGCATGCTAAAGCG
h-NRP2	Fw: ACGACATGGAGTACCAGATTGT
	Rev: AAATTCTCACCTGCAAAAGCCG

Since the primer efficiency was ~100%, the 2- $\Delta\Delta C_t$ method was used for analysis.

5.5 Nanostring analyses

RNA was extracted from HBVP cells treated or not with recombinant Multimerin-2. RNA was extracted using TRIzol lysis reagent (Roche Applied Science, Penzberg, Germany), according to manufacturer's protocol. RNA quantification was obtained using the Nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA) and the quality

assessed by agarose gel electrophoresis. To remove possible DNA contaminations, RNA was purified using TURBO™ DNase *free* (Thermo fisher Scientific, Massachusetts, USA). Finally, RNA was cleaned and concentrated by means of RNA Clean & Concentrator™ -5 kit (Zymo research, California, USA).

100 ng of RNA were used as input for the nCounter Assay using the nCounter® PanCancer Progression Panel (NanoString Technologies, Washington, USA) which included 770 genes. Briefly, the NanoString nCounter® technology is based on two probes that hybridize directly to an RNA target in solution. The reporter probe carries a fluorescent barcode specific for a single RNA target and the capture probe contains a biotin moiety that immobilizes the hybridized complex. After 16 hours of hybridization, samples were transferred to the nCounter® Prep Station instrument to remove the excess of probes. The purified target-probe complexes were then immobilized and aligned on the imaging surface of the nCounter® cartridges, transferred to the nCounter® Digital Analyzer, where they were scanned. Data collection was performed using nCounter® Digital Analyzer (NanoString Technologies, Washington, USA) following the manufacturer's instructions to count individual fluorescent barcodes and quantify target RNA molecules present in each sample. The run was carried out at high density scan (600 fields of view).

5.6 Purification of recombinant Multimerin-2

The E293-pCEP-Pu Multimerin-2 cells were expanded, and upon confluency, cultivated in serum-free DMEM for 48 hours. The conditioned media were collected and equilibrated with a buffer containing 50 mM NaH₂PO₄, 150 mM NaCl and 10 mM imidazole at pH=8. Recombinant Multimerin-2 was purified by high affinity using the Ni-NTA resin and eluted with the elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole). The different collected fractions were analyzed by SDS-PAGE followed by Coomassie blue staining. Positive fractions for Multimerin-2 were pulled and dialyzed against PBS using the dialysis membranes Spectra/Por® (FisherScientific, New Hampshire, USA) at the molecular weight cut off of 1 kDa and concentrated using 8 kDa polyethylene glycol (PEG) (Sigma-Aldrich, Missouri, USA). The thus obtained recombinant molecule was quantified by means of the Bradford assay (Biorad, California, USA), aliquoted and stored at – 80° C.

5.7 Adhesion assays

Cell adhesion assays were carried out in 96 well plates coated overnight at 4° C with 10 µg/mL of Multimerin-2 or Collagen Type I (Col I). Plates were airdried at room temperature under the tissue culture hood and 2×10^4 starved HBPV cells were added to serum free media supplemented with 0,1% BSA. Adhesion was performed in a humidified incubator at 37° C for 1 hour. Following Crystal Violet staining, the extent of cell adhesion was measured assessing the absorbance at 595 nm at the Infinite M1000 TECAN spectrophotometer (TECAN, Männedorf, Switzerland). To quantitatively monitor cell behavior over time, we adopted the Real-Time Cell Analyzer dual plate instrument (Roche, Penzberg, Germany), which measures the electrical impedance caused by cell attachment and proliferation, expressed as the cell index, an arbitrary measurement defined as $(R_n - R_b)/15$, where R_b represents the background impedance of the well measured with medium alone, and R_n the impedance of the well in the presence of cells. Thus, the cell index reflects the overall cell number, attachment quality, and cell morphology that change over time. The Real-Time Cell Analyzer dual plate instrument was placed in a humidified incubator maintained at 37° C in the presence of 5% CO₂. For the adhesion experiments, the 96-well e-plates were pre-coated over night at 4°C with the indicated molecules (10 µg/ml); cells were then seeded at the number of 5×10^4 cells/well in FBS-free medium and monitored every 5 minutes for 3 hours. Data analysis was performed using Real-Time Cell Analyzer software (version 1.2) supplied with the instrument.

5.8 Western blot analyses

Cell lysates were prepared using cold RIPA lysis buffer (150mM NaCl, 50mM Tris HCl [ph8], 1% Igepal, 0,5% sodium deoxycholate, 0,1% SDS) in the presence of a protease inhibitor cocktail (Roche, Penzberg, Germany), 1mM sodium orthovanadate, and 1 mM dithiothreitol. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, California, USA). For the electrophoresis analyses protein extracts were prepared with Laemmli Sample Buffer and the samples boiled for 5 minutes at 95°C. Samples were loaded in 4-20% Criterion Precast Gels (Bio-Rad, California, USA) and transferred into Hybond-ECL nitrocellulose membranes (Amersham, Amersham, UK). Membrane saturation was performed for 1 hour at room temperature with EveryBlot Blocking Buffer (Bio-Rad,

Caloformia, USA) diluted 1:1 with TBST (100mM Tris-HCl pH 7.5, 0,9% NaCl, 0,1% Tween 20). Primary antibodies were diluted in EveryBlot Blocking Buffer/TBST and incubated overnight at 4°C under constant shaking. After incubation with the secondary antibodies the membranes were developed using the ChemiDoc Touch Imaging System (Bio-Rad, California, USA) or the Odyssey infrared imaging system (Li-COR Biosciences, Nebraska, USA).

5.9 Immunofluorescence and immunohistochemistry

Cells were seeded and grown on cover glass slides placed in 24-well plates. Once reached confluency, cells were fixed for 15 minutes at room temperature with 4% paraformaldehyde diluted in PBS. After 3 washes with PBS, permeabilization was conducted for 5 minutes with 0,1% Triton X-100 followed by washes with PBS. Cells were next incubated 1 hour at room temperature with 2% BSA to block aspecific binding, followed by overnight incubation at 4°C with the primary antibodies. Secondary antibodies were added at the dilution 1:200 together with TO-PRO-3 at the dilution of 1:500 to stain the nuclei and Alexa Fluor 546-conjugated phalloidin 1:200, to stain the actin cytoskeleton. The cells were then incubated for 1 hour at room temperature, washed and incubated with the secondary antibodies for 1 hour at room temperature, followed by 3 washes with PBS. Immunofluorescence was acquired with a Leica TCS SP8 Confocal System (Leica, Wetzlar, Germany) and quantifications were obtained by means of the Volocity software (Volocity, Ontario, USA). Immunofluorescence on tumor samples was carried out on serial cryostat section of 5-7 µm obtained from OCT embedded tissues. Slices were collected on positively charged glass slides (Super-Frost Plus) (Thermo fisher scientific, Massachusetts, USA), air dried, washed in PBS and then fixed with 4% PFA for 15 minutes at room temperature. Following 3 washes with PBS, sections were permeabilized with 0,3% Triton X-100 for 3-5 minutes and blocked with 2% BSA for 1 hour at room temperature. Primary antibodies were incubated overnight at 4°C (anti-CD31 1:20; anti-αSMA 1:100; anti-Cpt DNA adducts 1:250; anti-Glut1 1:100, CD68 1:100). Secondary antibodies were added at the dilution 1:200 together with TO-PRO-3 at the dilution of 1:5000 and incubated for 1 hour at room temperature. Following 3 washes with PBS, sections were mounted with Mowiol supplemented with 2,5% 1,4 diazabicyclo-(2,2,2)-octane (DABCO) to preserve fluorescence. Immunofluorescences were acquired with a Leica TCS SP8 Confocal System

(Leica, Wetzlar, Germany) and quantifications were obtained with the Volocity software (Volocity, Ontario, USA).

Immunohistochemistry was performed on paraffin-embedded tumor samples from the MITO16A clinical trial or kindly provided by the Pathology Unit of CRO-IRCCS Institute of Aviano (Italy). Briefly, sections were deparaffinated with xylene and progressively rehydrated. Antigen retrieval was achieved following incubation with boiling citrate buffer pH=6 for 20 minutes. Next, endogenous peroxidase and alkaline phosphatase were saturated with the Bloxall solution (Vector Laboratories, California, USA). Blocking was performed with 1% BSA/2% FBS in PBS for 1 hour at room temperature and primary antibody incubation (anti-Multimerin-2 at the dilution of 1:400; anti-CD34 at the dilution of 1:3000 in 0,2% BSA/PBS) was carried out overnight at 4°C. Following 3 washes with PBS for 5 minutes, incubation with secondary antibodies was performed according to manufacturer instructions. Signals were then developed with the DAB Substrate Kit (Vector Laboratories, California, USA) and ImmPACT™ Vector® Red Substrate Kit (Vector Laboratories, California, USA). After hematoxylin counterstaining, sections were mounted using Bio-Mount HM (Bio-Optica, Milan, Italy). Images were acquired with light microscope Leica DM 750 (Leica, Wetzlar, Germany)

5.10 Ki-67 assay

4x10⁵ HBVP cells were seeded and grown on 1% gelatin-coated cover glass slides, placed in 24-well plates. HBVP cells were stimulated with recombinant Multimerin-2 or PBS for 24 and hours and fixed with 4% PFA. Glasses were washed 3 with PBS for 5 minutes and incubated with PBS 0.4% Triton X-100 for 10 minutes at RT followed by three washes with PBS for 5 minutes. Glasses were blocked with 10% normal goat serum (NGS) in PBS for 1 hour at room temperature, followed by overnight incubation at room temperature with the anti-Ki 67 antibody diluted 1:100 in 1% BSA. Following 3 washes with PBS cells were incubated at room temperature for 1 hour with the Alexa Fluor 488 conjugated rabbit secondary antibody diluted 1:200 in PBS. After three washes, glasses were mounted with Mowiol containing 2.5% DABCO and image acquisition was carried out with the Leica TCS SP8 Confocal System (Leica, Wetzlar, Germany).

5.11 Transmigration assay

For the transmigration of the monocytic cell line THP-1 through the endothelium, we employed transwells characterized by 3 μm pore-sized membranes (Corning Incorp., Corning, NY). 8×10^5 HUVEC cells silenced or not for Multimerin-2 were plated on the upper surface of the insert membranes previously coated in a 37°C/5% CO₂ incubator with a 1% gelatin solution for 1 hour. THP-1 cells were labeled with CellTrace™ CFSE Cell proliferation Kit (Invitrogen by Thermo Fisher Scientific) next 10^4 cells in serum-free RPMI 1640 were seeded on the upper compartment of the transwell chamber and transmigration was conducted for 8 hours. The membranes were then fixed with 4% PFA for 15 minutes followed by two washes with PBS. Images and evaluation of the number of cells transmigrated through the manipulated endothelium were obtained with the Eclipse Ti microscope (Nikon, Tokyo, Japan) and counted manually.

5.12 Haptotaxis assay

For the haptotaxis assays we employed transwells® containing 8 μm -pored membranes (Costar, NY, USA). Membranes were coated on the upper side with 1 $\mu\text{g}/\text{ml}$ recombinant Multimerin-2, BSA or fibronectin, used respectively as negative and positive controls. Next, the underside of the membranes was coated with 5 $\mu\text{g}/\text{ml}$ of the aforementioned molecules, and the bottom of the wells 25 $\mu\text{g}/\text{ml}$. After 1.5 hours at 37°C, membranes were blocked with 2% BSA. Pericytes were labeled with the CellTrace™ CFSE Cell proliferation Kit (Invitrogen by Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer instructions. Cells were then starved for 2 hours in serum free PM and 1×10^5 cells/well were seeded on top of the membranes. Cells were let migrate overnight and the cells on top of the membrane were removed with a cotton swab. Cells migrated on the bottom of the membranes were fixed with 4% PFA for 15 minutes. Images and evaluation of the number of cells transmigrated through the manipulated endothelium were obtained with the Eclipse Ti microscope (Nikon, Tokyo, Japan).

5.13 *In vivo* tumor growth

Eight weeks old *wild type* (wt) and *Multimerin-2*^{-/-} C57BL/6N mice were subcutaneously injected in both flanks (3×10^5 cells/flank) with MC-38 colon cancer cells in 100 μL of PBS.

Similarly, ID8 *Trp53*^{-/-} *Pten*^{-/-} HG-SOC cells were subcutaneously injected in both the flanks (7x10⁶ cells/flank). Tumors were let grow for four weeks; tumors length and width measured with the caliper and the tumor volume calculated applying the following formula: volume=(π x length x width²)/6. For drug delivery, after the tumor reached the size of about 100 mm³, mice were intraperitoneally injected with 10mg/kg of Cisplatin and sacrificed 6 hours later. At the end of the experiment, tumors were excised and included in OCT for subsequent immunofluorescence analyses. For the quantifications of the Cpt DNA adducts we analyzed three fields per tumor section from the central part of each tumor.

5.14 Bioinformatic analyses

Gene expression analyses were performed using the nSolver software provided by NanoString and by Dseq2. As a first step, we run a quality check pipeline, to ensure sufficient quality of the data. Six positive controls were included to measure the efficiency of the hybridization reaction. Six negative control probes, designed against engineered RNA sequences not present in biological samples, were used to set background thresholds. Finally, normalization was performed on at least 8 housekeeping genes. In parallel, we also performed bioinformatic analyses using the package for differential gene expression analysis Dseq2 and the two analyses were finally merged.

5.15 Statistical analysis

Statistical analyses were performed using GraphPad Prism software. All values were represented as the mean $\pm$ standard deviation, obtained from at least 3 independent experiments. Statistical significance was determined using unpaired Student's t-test. One-way ANOVA test was used for multiple comparisons. Differences were considered statistically significant when $p \leq 0,05$.

6. RESULTS

6.1 Multimerin-2 affects pericyte recruitment both directly and indirectly

Our previous studies demonstrated that the deposition of Multimerin-2 regulates the integrity of inter-endothelial cell junctions and promotes vascular stability (Riedemann and Macaulay, 2006). Interestingly, analyses performed on retinas from P5 *Multimerin-2*^{-/-} pups indicated that the vessels were poorly covered by pericytes (Pellicani et al., 2020). Given this compelling published evidence and the strategic localization of Multimerin-2 in the interface between ECs and pericytes we hypothesized that the deposition of Multimerin-2 could aid pericyte recruitment to the vessels. To verify our hypothesis, using the Ni-NTA resin we first obtained highly pure recombinant human Multimerin-2 from conditioned media of 293-EBNA cells stably transfected with the pCEP-Pu-Multimerin-2 construct as previously described (Lorenzon et al. 2012). As expected, the SDS-PAGE analysis highlights the presence of a double band following Coomassie blue staining, possibly resulting from different extents of glycosylation (Figure 1A). The lack of additional low molecular weight bands indicated the absence of fragmentation products thus proving the integrity of the molecule.

We first evaluated if, upon deposition, Multimerin-2 could directly affect pericyte recruitment, assessing the capability to function as a haptotactic stimulus. To this end, we created a Multimerin-2 gradient coating the transwells carrying 8µm-pored membranes with increasing concentrations of purified recombinant Multimerin-2 or BSA, used as negative control (Figure 1B). Next, CFSE-labeled human brain vascular pericytes (HBVP) were plated on top of the transwells and let migrate overnight towards the putative ECM stimulus. As reported in figure 1B, the representative images obtained by fluorescent microscopy revealed that pericytes migrated very efficiently towards the Multimerin-2 stimulus, unlike what observed with the control, thus indicating that Multimerin-2 directly promoted pericyte recruitment functioning as a haptotactic stimulus for these cells.

During vessel maturation ECs secrete PDGF-BB and HB-EGF two important cytokines that stimulate the recruitment of pericytes to the vessels (Stratman et al., 2010). To verify if the deposition of Multimerin-2 could prompt the expression of these two key cytokines, human dermal microvascular endothelial cells (HDMEC) were challenged with recombinant Multimerin-2 for 3-6 and 12 hours (Figure 1C). In support of an indirect function of Multimerin-2 in stabilizing the vasculature, treatment of HDMEC cells with the recombinant molecule significantly correlated with the up-regulation of the PDGF-BB and HB-EGF mRNA levels respect to the control, as assessed by qPCR (Fejza et al., 2021).

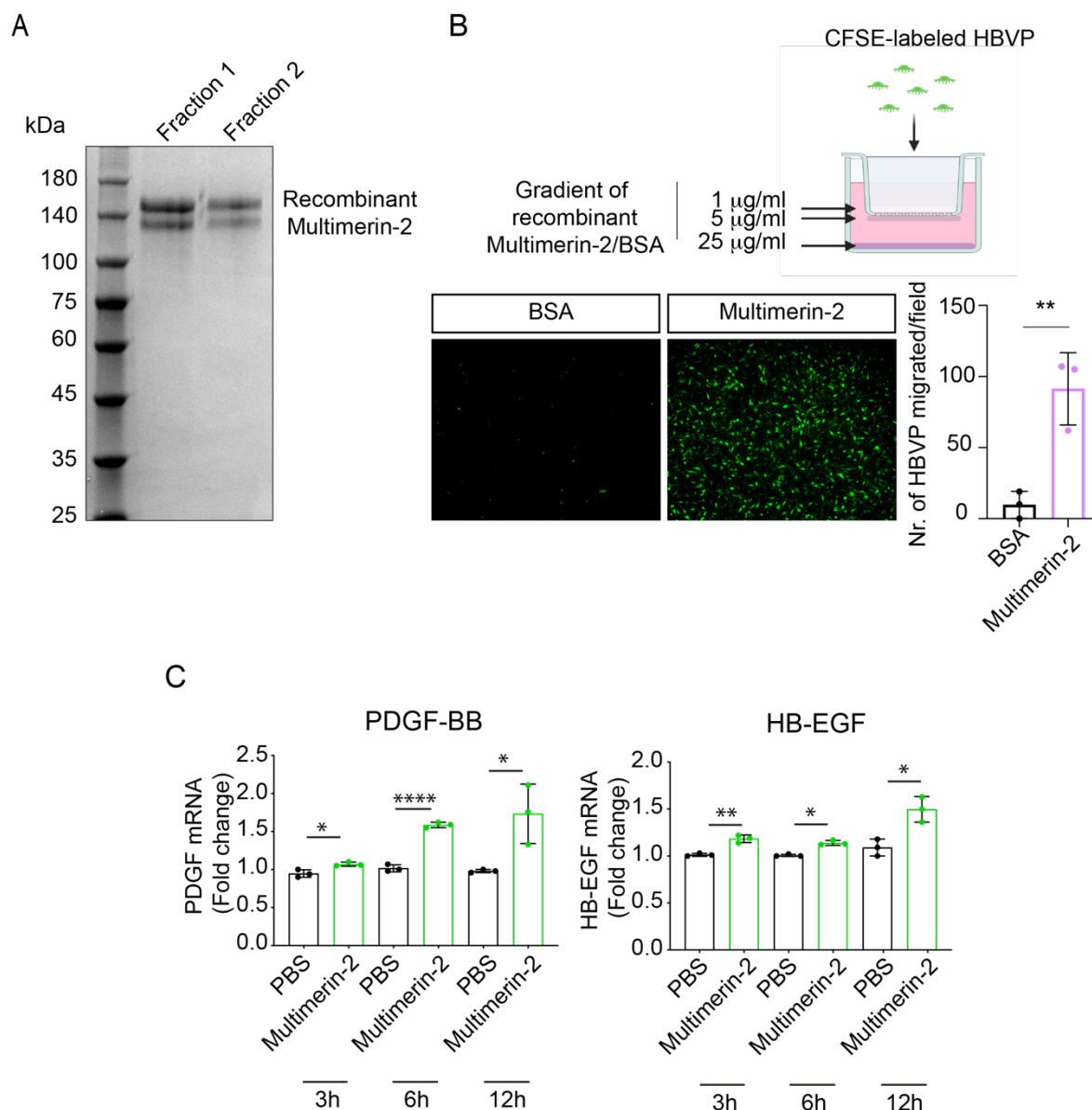
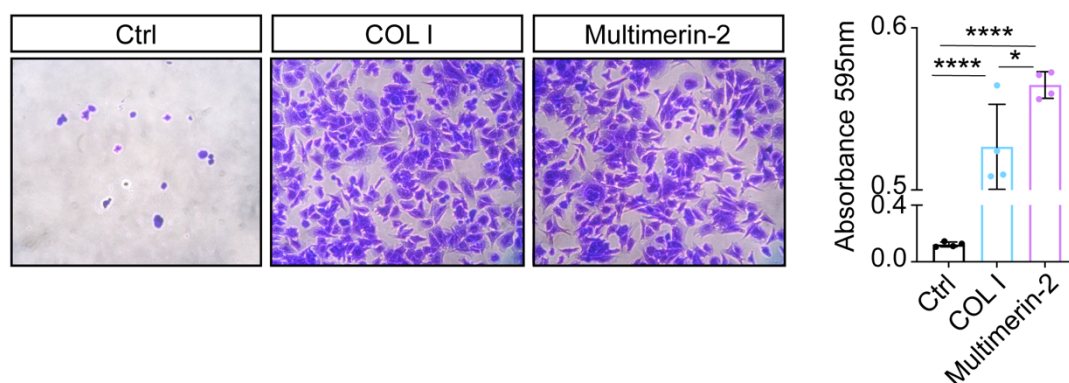


Figure 1. Multimerin-2 prompts pericyte recruitment both directly and indirectly. *A)* Representative image of the SDS-PAGE analysis of fraction 1 and 2 obtained during the purification of Multimerin-2 from the media collected from 293-EBNA cells transfected with the p-CEP-Pu-Multimerin-2 construct using the Ni-NTA resin. The molecular weights are reported on the left and the gel was stained with Coomassie blue. *B)* Top: Schematic representation of the haptotaxis assay. The transwell's membrane was coated on top with 1 µg/ml of Multimerin-2, on the underside with 5 µg/ml and the well with 25 µg/ml. CellTrace™ CFSE-labeled HBVP cells were plated on top of the transwell. Bottom: Representative images of CellTrace™ CFSE labeled HBVP cells migrated through 8 µm pored transwells' membranes, coated with increasing concentrations of recombinant Multimerin-2, to mimic the ECM gradient, or BSA used as a negative control. Right, quantification of HBVP cells migration as assessed by counting; **p=0.0065. *C)* Real-Time PCR analyses of PDGF-BB (on the left) and HB-EGF (on the right) by HDMEC cells upon stimulation with recombinant Multimerin-2 or PBS as control for 3, 6 and 12 h; n = 3; *p<0.05, **p<0.005, ****p<0.0001. P values were obtained using paired Student's t-test.

6.2 Multimerin-2 functions as an adhesive substrate for pericytes

Considering that Multimerin-2 is expressed in juxtaposition between ECs and pericytes, we hypothesised that the deposition of Multimerin-2 by ECs could aid the adhesion of pericytes to the endothelium. To verify this possibility, we tested the capability of Multimerin-2 to function as an adhesion substrate for pericytes. To this end, HBVP cells were seeded on 96-well plates coated with 10 μ g/ml of recombinant Multimerin-2, type I collagen or BSA, used as positive or negative controls, respectively. In line with our hypothesis, HBVP cells adhered very efficiently to the Multimerin-2 coated wells, similarly to what observed with type I collagen. These results were further corroborated with dynamic measurements performed over time exploiting the xCELLigence real-time cell analysis system. This device employs electric cell-substrate impedance sensing to assess and record the bio-impedance values generated by the adhesion and spreading of the cells. The dynamic analyses of pericyte adhesion showed that HBVP cells promptly adhered to Multimerin-2 slightly, but not significantly, more efficiently than type I collagen, reaching a plateau after about 6 hours of adhesion (Fejza et al., 2021).

A



B

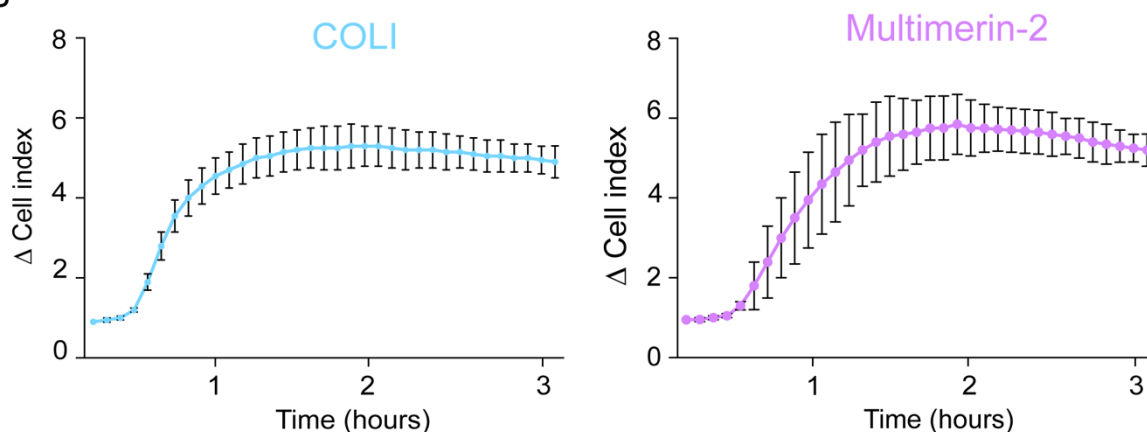


Figure 2. Multimerin-2 functions as an adhesion substrate for pericytes. *A) Representative images of the adhesion of HBVP on type I collagen (COL1) and Multimerin-2; cells were stained with Crystal Violet and BSA was used as a negative control (Ctrl). Relative quantification of the Crystal violet signal was assessed at the spectrophotometer measuring the absorbance at 595 nm; n=3; *p=0.032, ***p<0,0001. The p value was obtained using paired Student's t-test. B) Evaluation of pericyte adhesion to type I Collagen (Col I) (left) or Multimerin-2 (right) by means of the xCELLigence system; cell adhesion was measured every 5 min for 3 hours; n=3.*

6.3 Multimerin-2 promotes pericyte viability and proliferation

Pericyte migration toward the endothelium is followed by their proliferation, the two processes being precisely synchronized during the formation of the vascular network (Payne et al., 2021). In fact, upon recruitment to the endothelium, pericytes need to proliferate to efficiently embrace the newly formed vessel. Thus, we next enquired if the interaction of these cells with Multimerin-2 could affect their viability and proliferation. To verify these possibilities, we first assessed the pericyte viability and performed MTT assays using HBVP cells challenged with recombinant Multimerin-2 in the presence or not of recombinant PDGF-BB for 24 and 48 hours. As reported in Figure 3A, at 48 hours the viability of HBVP cells was significantly increased upon treatment with recombinant Multimerin-2. Interestingly, an additive effect was observed when the cells were simultaneously challenged with Multimerin-2 and PDGF-BB. To verify if Multimerin-2 promoted the proliferation of pericytes, HBVP cells were treated for 24 and 48 hours with recombinant Multimerin-2 in combination or not with PDGF-BB. Consistent with the viability assays, following 48 hours of treatment, the staining with Ki-67 highlighted a significantly increased proliferation of HBVP cells when challenged with Multimerin-2.

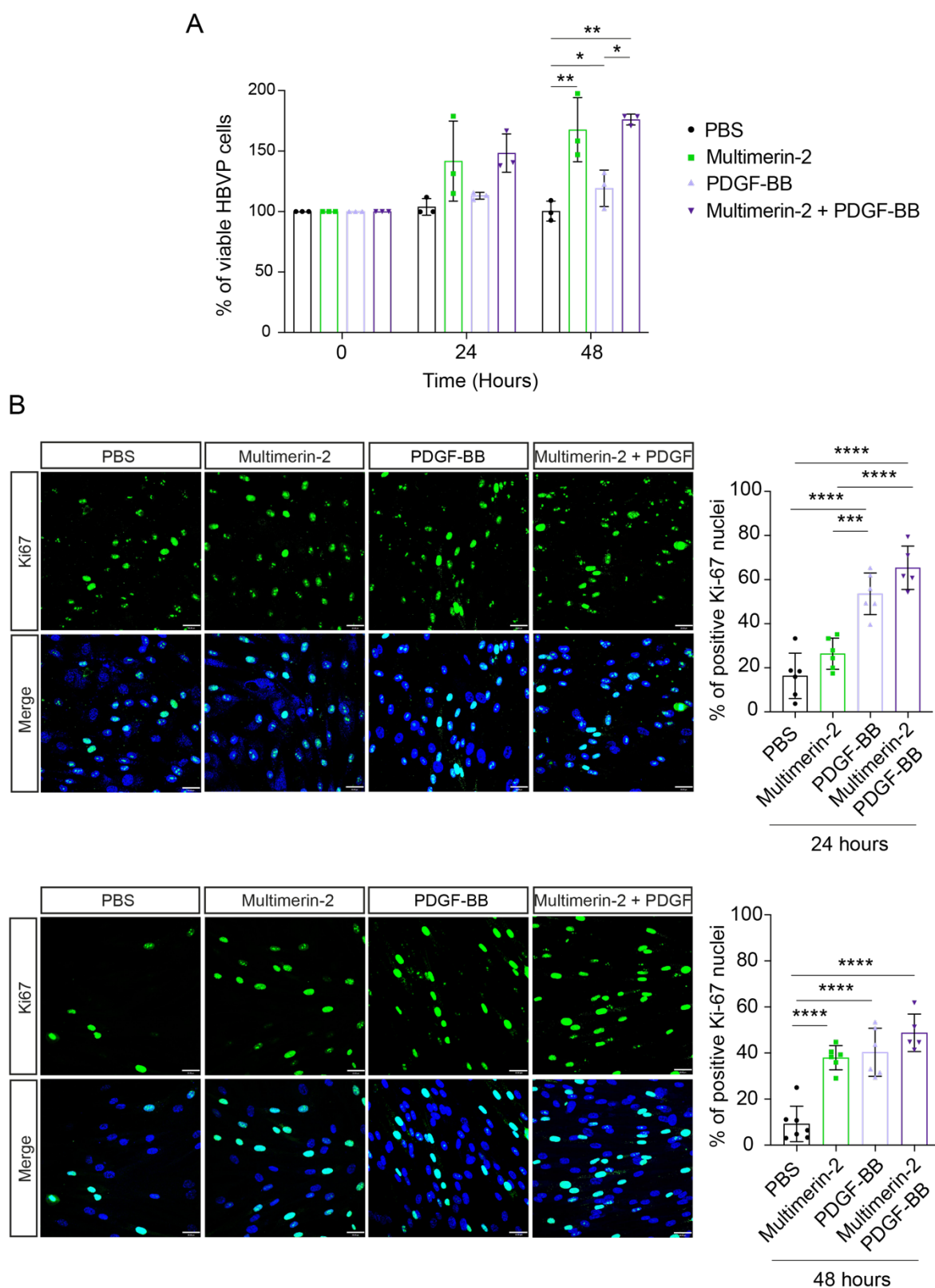


Figure 3. Multimerin-2 promotes pericyte viability and proliferation. *A)* MTT assay performed on HBVP cells challenged with Multimerin-2 and/or PDGF-BB; vehicle (PBS) was used as control. Statistical significance was determined by one-way ANOVA (* $p < 0.05$; ** $p < 0.005$). *B)* Representative images of the Ki-67 staining performed with HBVP cells following 24 (above) of 48 hours (below) of treatment with Multimerin-2 alone or in combination with PDGF-BB or vehicle (PBS).

*Scale bar=32 μ m. The relative quantifications of the percentage of Ki-67 positive nuclei/total cells are reported on the right graphs. Statistical significance was determined by one-way ANOVA (** $p<0.0003$; **** $p<0.0001$).*

6.4 Multimerin-2 triggers pericyte activation via Akt

Prompted by the results obtained from functional assays, we next aimed to explore the molecular mechanisms by which Multimerin-2 affected pericyte functions, a previously unexplored terrain. To broadly scrutinize the possible signaling pathways influenced by Multimerin-2, we first took advantage of a wide approach employing a human phosphorylation pathway profiling array. HBVP cells were starved for 2 hours and then stimulated for 15 minutes with recombinant Multimerin-2 or vehicle. Under these experimental conditions, treatment of HBVP cells with Multimerin-2 induced the phosphorylation of a number of proteins including PDK1, CREB, STAT3 and EGFR. However, the highest increase of the phosphorylation levels was observed for Akt (Figure 4A). The results were next validated by Western blot analyses. To this end, starved HBVP cells were challenged with recombinant Multimerin-2 for 5, 10 and 15 minutes. With these analyses, among the putatively Multimerin-2-activated proteins, we were only able to confirm the activation of Akt, which was detected at all analyzed time points (Figure 4B). Importantly, this finding was consistent with the higher viability and proliferation rates observed upon treatment with Multimerin-2 (Figure 3).

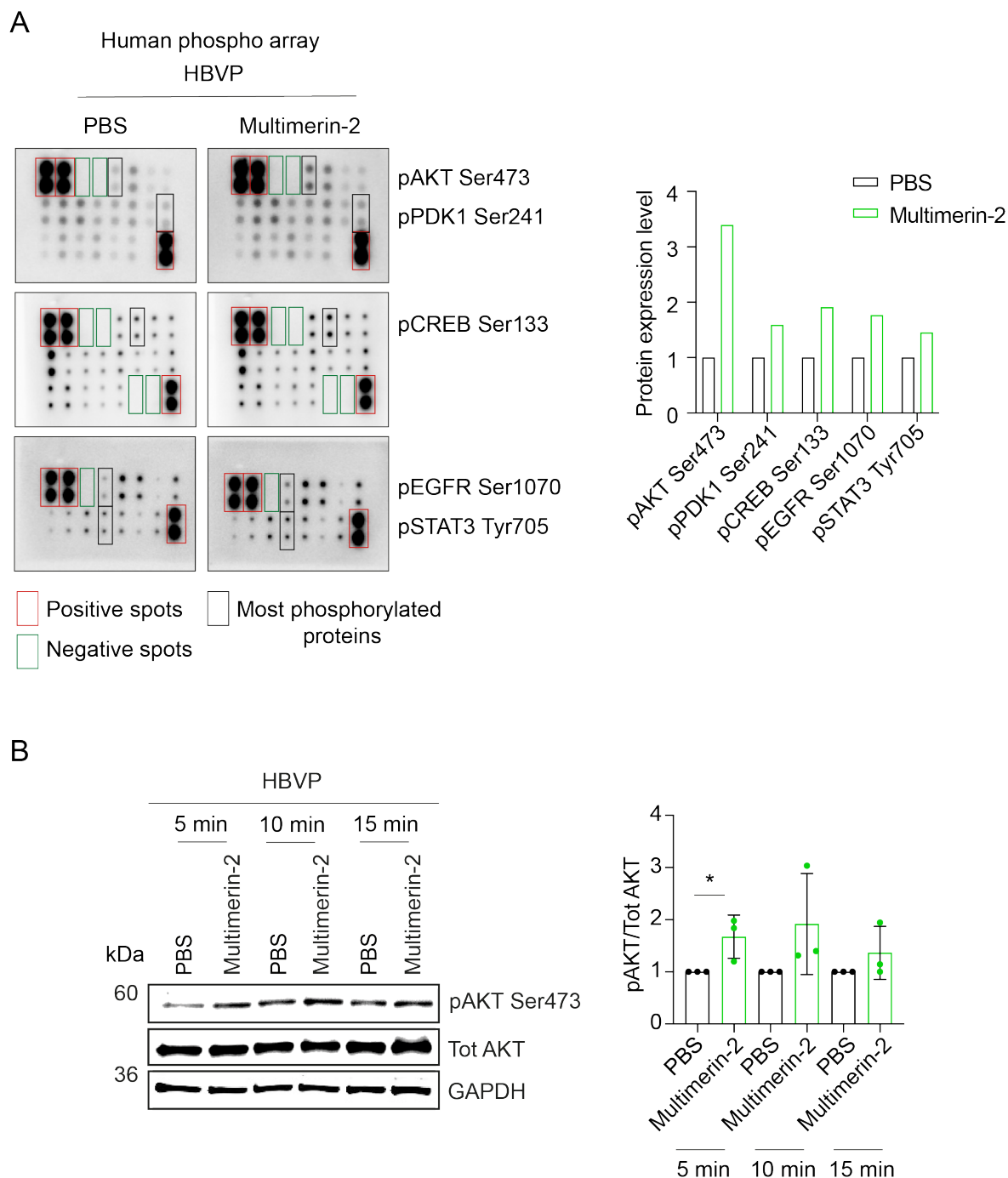


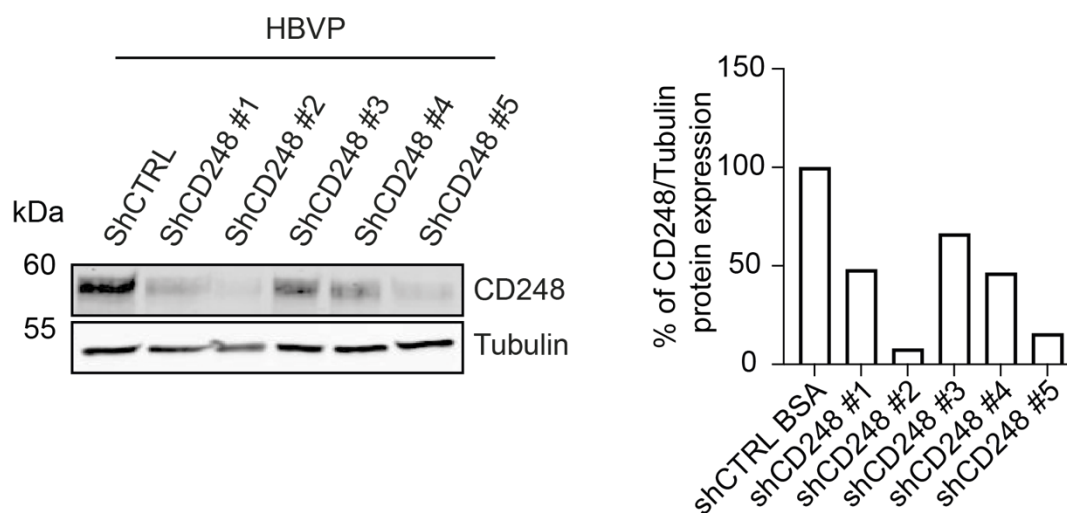
Figure 4. Multimerin-2 activates pericyte function through Akt. A) On the left, representative images of the phospho-array; the five most phosphorylated proteins are highlighted by black rectangles. Red and green rectangles indicate positive and negative references, respectively. The relative quantification of the most phosphorylated proteins is reported on the right graphs. **B)** Western blot analysis showing Akt activation at Ser473 upon stimulation for 5, 10 and 15 minutes with Multimerin-2 or vehicle (PBS); GAPDH was used as a protein loading control. * $p < 0.05$. Statistical significance was determined using paired Student's *t*-test.

6.5 The Multimerin-2-driven pericyte viability and haptotactic function are not mediated by CD248

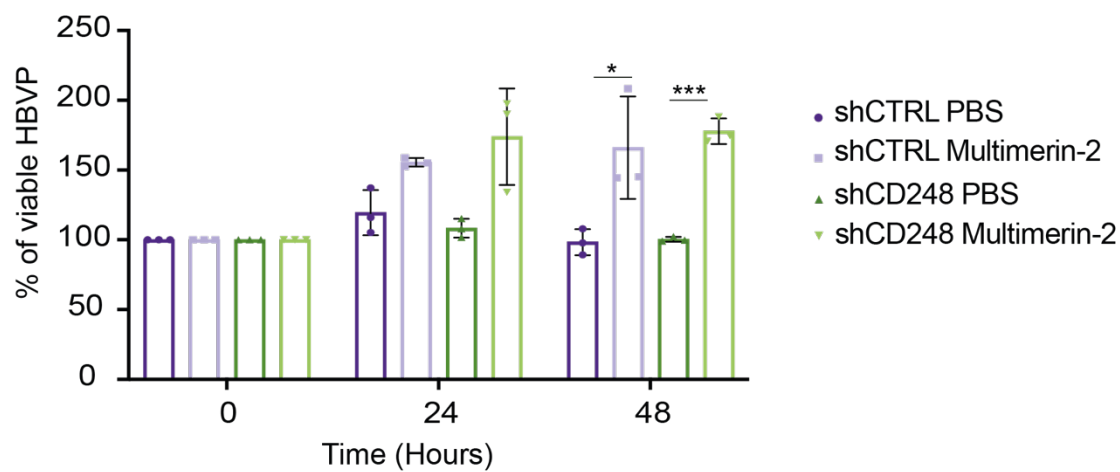
We next enquired which transmembrane protein could mediate Akt activation upon Multimerin-2 stimulation. First, we hypothesized that this effect could be mediated by CD248, a type 1 transmembrane glycoprotein found on the plasma membrane of activated mesenchymal cells. As previously mentioned, CD248 is a putative binding partner of Multimerin-2 (Khan et al., 2017), it is highly expressed in pericytes and was shown to affect their proliferation via the PDGF-BB signaling pathway (Tomkiewicz et al., 2010). To verify if the putative Multimerin-2/CD248 interaction could mediate the Multimerin-2-driven proliferative effects, by means of shRNA lentiviral particles we transiently knockdown CD248 expression in HBVP cells. To this end, we produced 5 lentiviral shRNAs specifically targeting CD248 (shCD248#1, shCD248#2, shCD248#3, shCD248#4, shCD248#5), and we assessed the efficiency of CD248 knockdown by Western blot (Figure 5A). Among the five tested shRNAs, we selected shRNA #2 which displayed the highest efficiency reducing the CD248 expression by 92%. We next evaluated the implication of CD248 in HBVP cells viability. To this end, HBVP cells were transduced with shCD248 #2 or the control shRNA lentiviral vector and after 72 hours cells were seeded and treated with recombinant Multimerin-2 or vehicle for 24 and 48 hours. As reported in Figure 5B, the knock-down of CD248 did not affect the Multimerin-2-driven cell viability, suggesting that Multimerin-2 does not influence HBVP viability through the engagement of CD248.

We next verified if CD248 could mediate the haptotactic function of Multimerin-2. To this end, we carried out haptotaxis assays creating a Multimerin-2 gradient as previously described and using labeled HBVP cells following or not the knockdown of CD248. Cells were let migrate overnight and the extent of migration towards the ECM stimulus was analyzed by fluorescent microscopy. However, as reported in Figure 5C, the knockdown of CD248 did not affect the capability of HBVP cells to migrate towards the Multimerin-2 gradient. Taken together these results indicated that CD248 does not mediate Multimerin-2-driven viability or haptotaxis of pericytes.

A



B



C

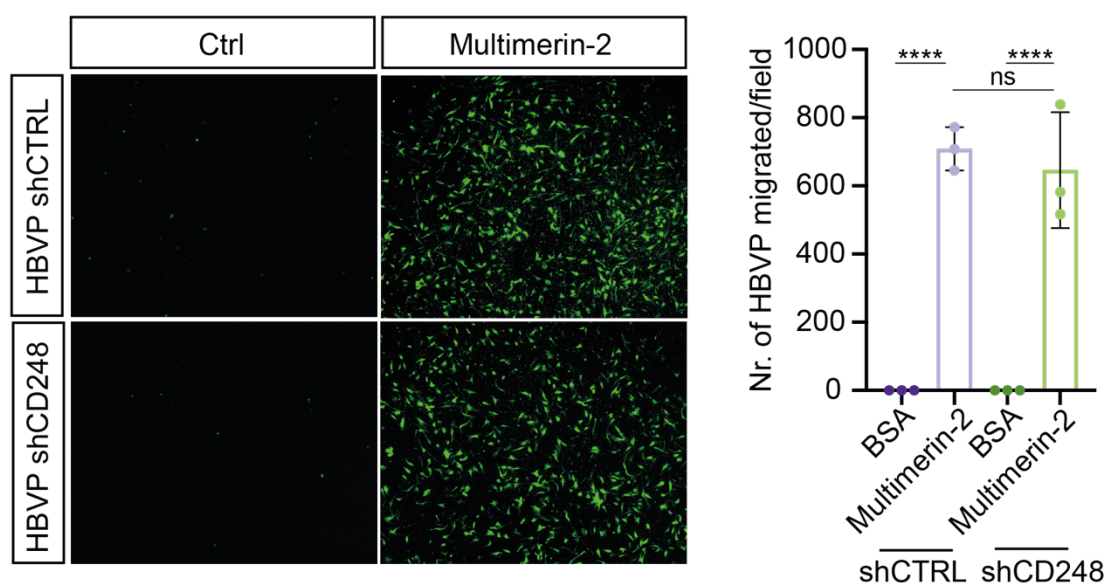
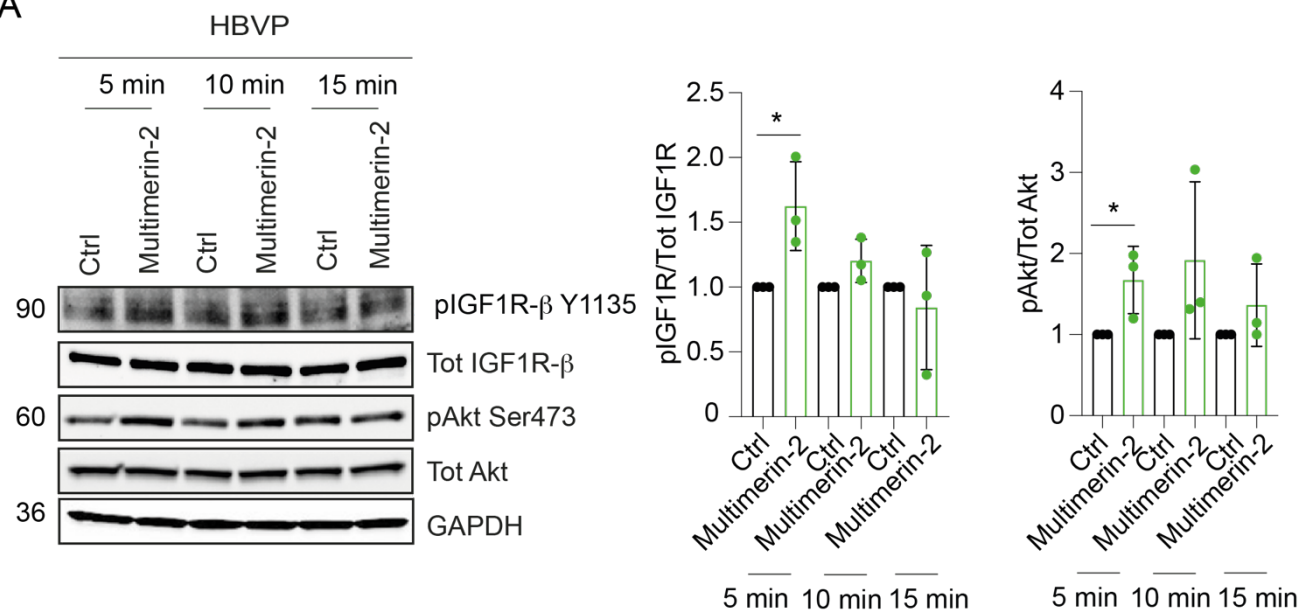


Figure 5. Multimerin-2 does not affect pericytes viability and haptotaxis via CD248. *A)* Representative images of the Western blot analyses of CD248 expression following transduction of HBVP cells with the CD248 specific shRNA (shCD248 #1, #2, #3, #4, #5) or control (shCTRL) lentiviral vectors; tubulin was used for protein loading control. On the right, relative quantification *B)* MTT assay performed on HBVP cells silenced (shCD248) or not (shCTRL) for CD248 challenged with Multimerin-2 or vehicle (PBS), as control. Statistical significance was determined by one-way ANOVA test * $p < 0.05$ and *** $p < 0.005$. *C)* Left; representative images of CellTrace™ CFSE-labeled HBVP cells, silenced (shCD248) or not (shCTRL) for CD248, migrated through transwells coated with increasing concentration of recombinant Multimerin-2, or vehicle (PBS) used as a negative control. The number of migrated HBVP cells are reported on the right. Statistical significance was determined by unpaired Student's *t* test **** $p < 0.0001$).

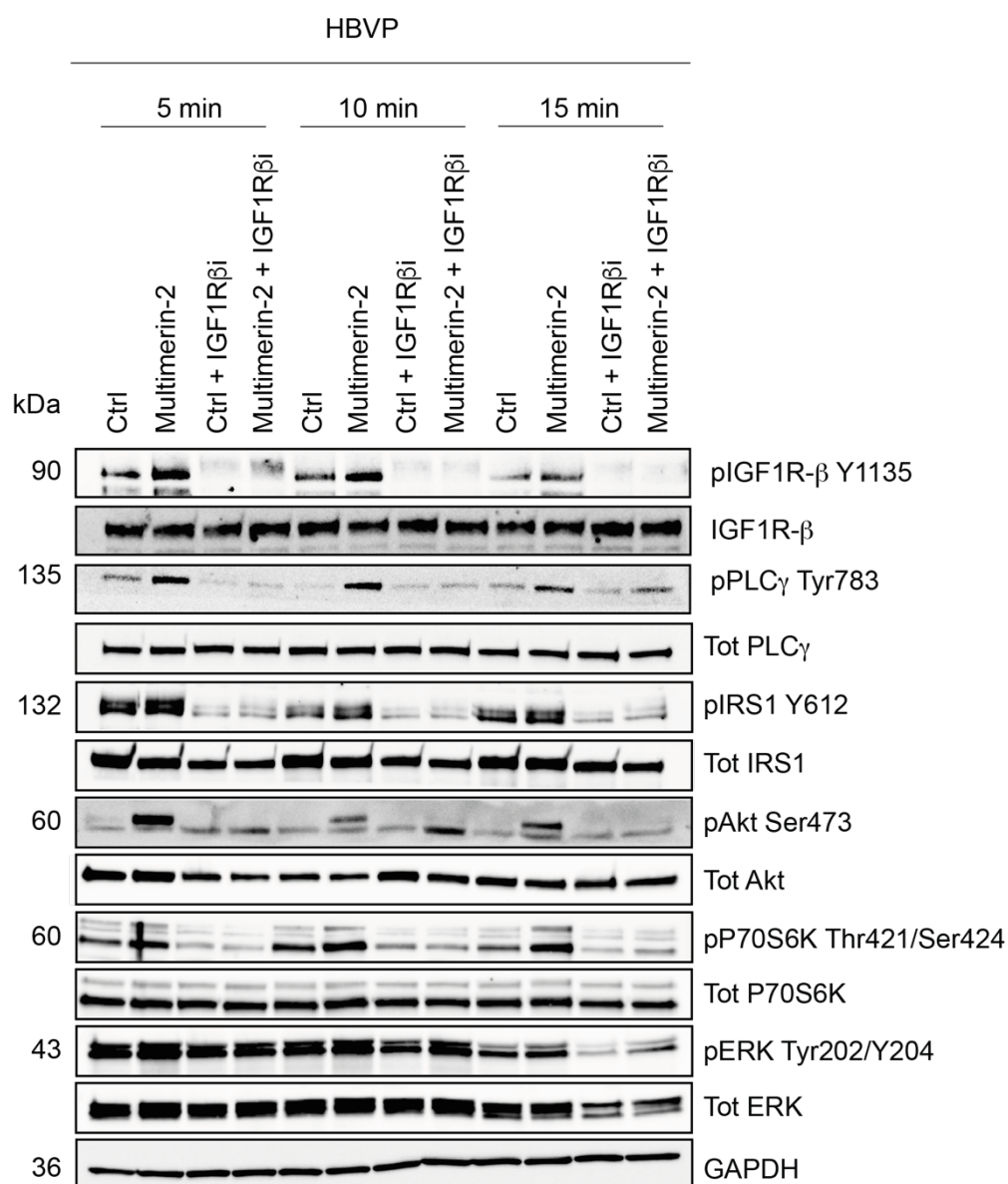
6.6 Multimerin-2 activates IGF1R- β signaling pathway

Excluded the possible involvement of CD248 in this context, we searched for other molecular mediators of Multimerin-2 function. Since Akt is the central hub of many different pathways, we enquired which other receptor could mediate its activation. We decided to focus on IGF1R- β for different reasons; first it is markedly expressed by pericytes (Delafontaine et al., 2004), second it is a well-known regulator of Akt activation (Riedemann and Macaulay, 2006), third, and most importantly, it was shown to mediate the proliferation of mural cells like vSMCs (Shuang et al., 2018). To verify whether Multimerin-2 could trigger the activation of this receptor, starved HBVP cells were challenged or not with Multimerin-2 for 5, 10 and 15 minutes. Interestingly, treatment with Multimerin-2 induced a strong activation of the IGF1R- β signaling pathway, as evidenced by the increased phosphorylation of IGF1R- β at Tyr 1135 and of Akt at Ser473 (Figure 6). To further corroborate these results, we carried out a similar experiment blocking IGF1R- β with 20 μ M of the IGF1R inhibitor NVP-AEW541. Indeed, the pre-treatment of HBVP cells with the IGF1R inhibitor led to a complete abrogation of the signaling pathway evidenced by the downregulation of the activation of PLC- γ , IRS1, Akt, P70S6K, and, to a lesser extent, ERK. (Figure 6B).

A



B



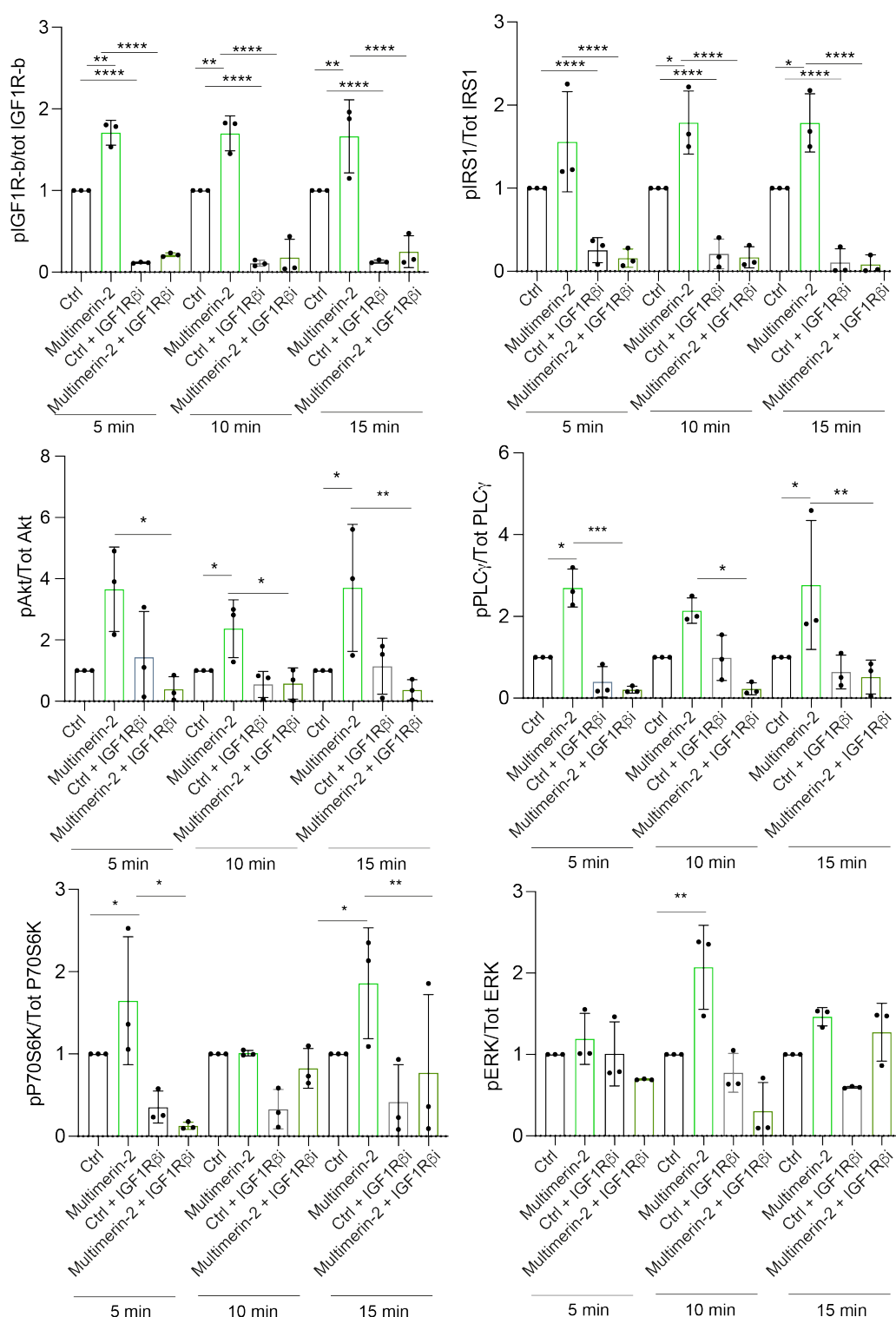


Figure 6. Multimerin-2 activates the IGF1R- β signaling pathway in pericytes. A) Western blot analysis of HBVP lysates showing increased phosphorylation of IGF1R- β at Tyr1135 and Akt at Ser473 upon stimulation with Multimerin-2 or vehicle (PBS) for 5, 10 and 15 minutes. **B)** Above, Western blot analysis of HBVP cells stimulated with Multimerin-2 with or without IGF1R inhibition using inhibitor NVP-AEW541. HBVP cells were starved and pretreated with the IGF1R- β inhibitor (20 μ M) for 3 hours, then stimulated with Multimerin-2 for 5, 10 and 15 minutes or vehicle (DMSO) as ctrl. Below, relative quantifications are reported. * $p < 0,05$; ** $p < 0,005$; *** $p < 0,0005$, **** $p < 0,0001$. Statistical significance was determined by one-way ANOVA.

6.7 Multimerin-2 regulates the expression of genes involved in pericyte proliferation and vessels stabilization

To have a broader view regarding the role of Multimerin-2 in our context, we performed a transcriptomic analysis, exploiting the NanoString technology. To this end, starved HBVP cells were challenged with Multimerin-2 or vehicle for 6 hours and the mRNA levels assessed. The results indicate that the interplay between Multimerin-2 and pericytes does not lead to extensive changes in gene expression. In accordance with our findings, among the few genes whose expression was altered by the treatment with Multimerin-2 there was an enrichment of genes involved in the regulation of junctional stability, as assessed by the GSEA bioinformatic analyses (<https://www.gsea-msigdb.org/gsea/index.jsp>) (Figure 7A,B). Nonetheless, we analyzed and validated the most significantly altered genes and all of these genes are involved in the regulation of cell proliferation or vascular stability. In particular, we found increased expression of Neuropilin-2 (NRP), cadherin-11 (CDH11), TGFBI, Versican (VCAN), WNT5A. Ang-2 was found to be down-regulated, whereas the expression of PDGFC and STAT1 did not change significantly. Despite we detected an increase of OAS1 expression in Multimerin-2-challenged cells, this gene was poorly expressed by these cells (data not shown).

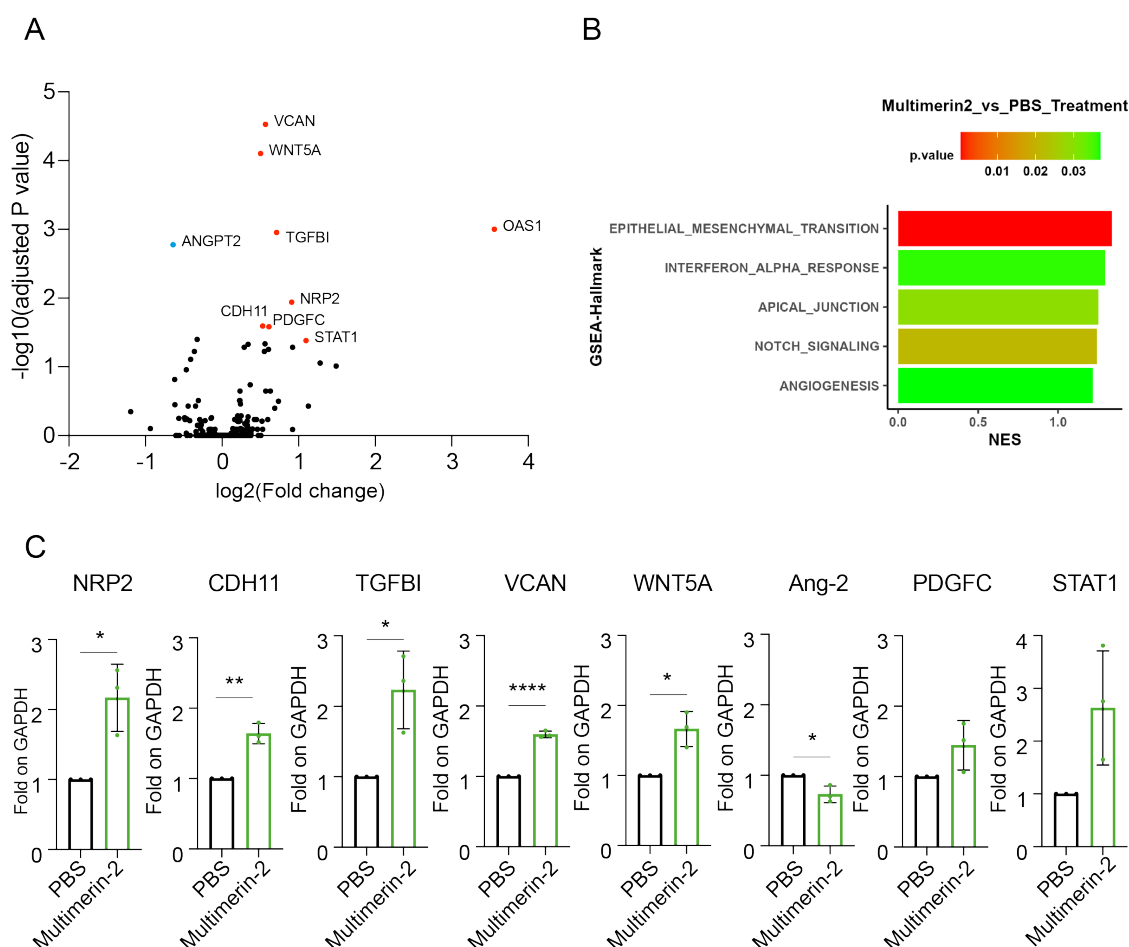


Figure 7. Multimerin-2 alters the expression of genes involved in pericyte proliferation and vascular homeostasis. A) Volcano plot including the most significantly differentially expressed genes retrieved by NanoString analysis. The most significantly upregulated genes are highlighted in red, while the downregulated genes are shown in light blue **B)** Bioinformatic analysis of the most enriched pathways obtained using GSEA (Gene Set Enrichment Analysis) normalized enrichment score (NES). **C)** Graphs of the Real-time PCR analyses of the most up or downregulated genes in HBVP cells stimulated for 6 hours with Multimerin-2; vehicle (PBS) was used as control. Statistical significance was determined by unpaired Student's *t* test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

6.8 Multimerin-2 expression is frequently down-regulated in tumor-associated vessels

Given the impact of Multimerin-2 in vessel maturation, we next evaluated the expression of the molecule in tumor associated vessels. To this end we employed formalin fixed and paraffin-embedded ovarian cancer samples from the MITO16A clinical trial (400 samples) aimed at evaluating the efficacy of carboplatin-paclitaxel in combination with bevacizumab as a first line treatment. We also performed preliminary analyses on paraffin-embedded CRC samples kindly provided by the Pathology Unit of CRO-IRCCS Institute of Aviano (10

samples). Multimerin-2 was stained with a specific anti-human Multimerin-2 affinity-purified polyclonal antibody, whereas blood vessels were identified using an antibody against CD34, a pan-endothelial cell marker. As expected, the tumor tissues were highly vascularized as highlighted by the anti-CD34 antibody. As previously described Multimerin-2 was widely expressed also in tumor-associated vessels (Christian et al., 2001), however we could observe that some of the vessels displayed discontinuous deposition of the molecule, and other vessels were characterized by low or barely detectable Multimerin-2 deposition, as shown by the representative images (Figure 8A). These results were further corroborated by *in silico* analyses interrogating the online database TNMplot (<https://tnmplot.com/analysis/>). In agreement with the immunohistochemistry analyses, as shown in Figure 8B, the box plots show differential expression of Multimerin-2 in normal and neoplastic tissues, with significant downregulation of the molecule in both ovarian and colon cancers.

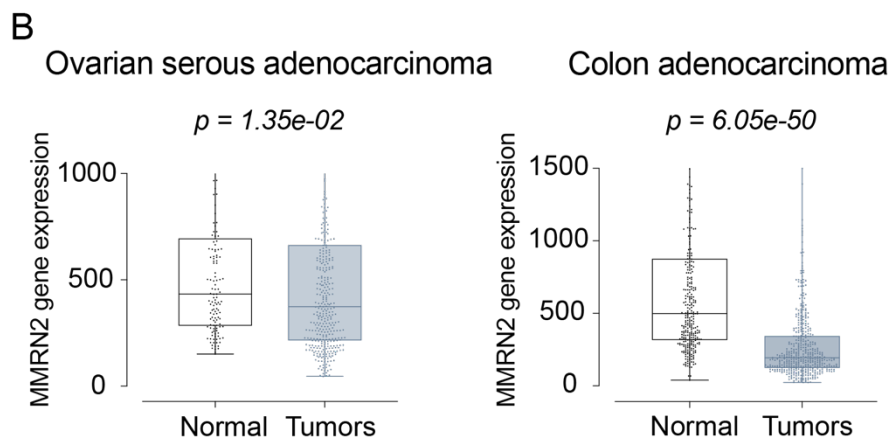
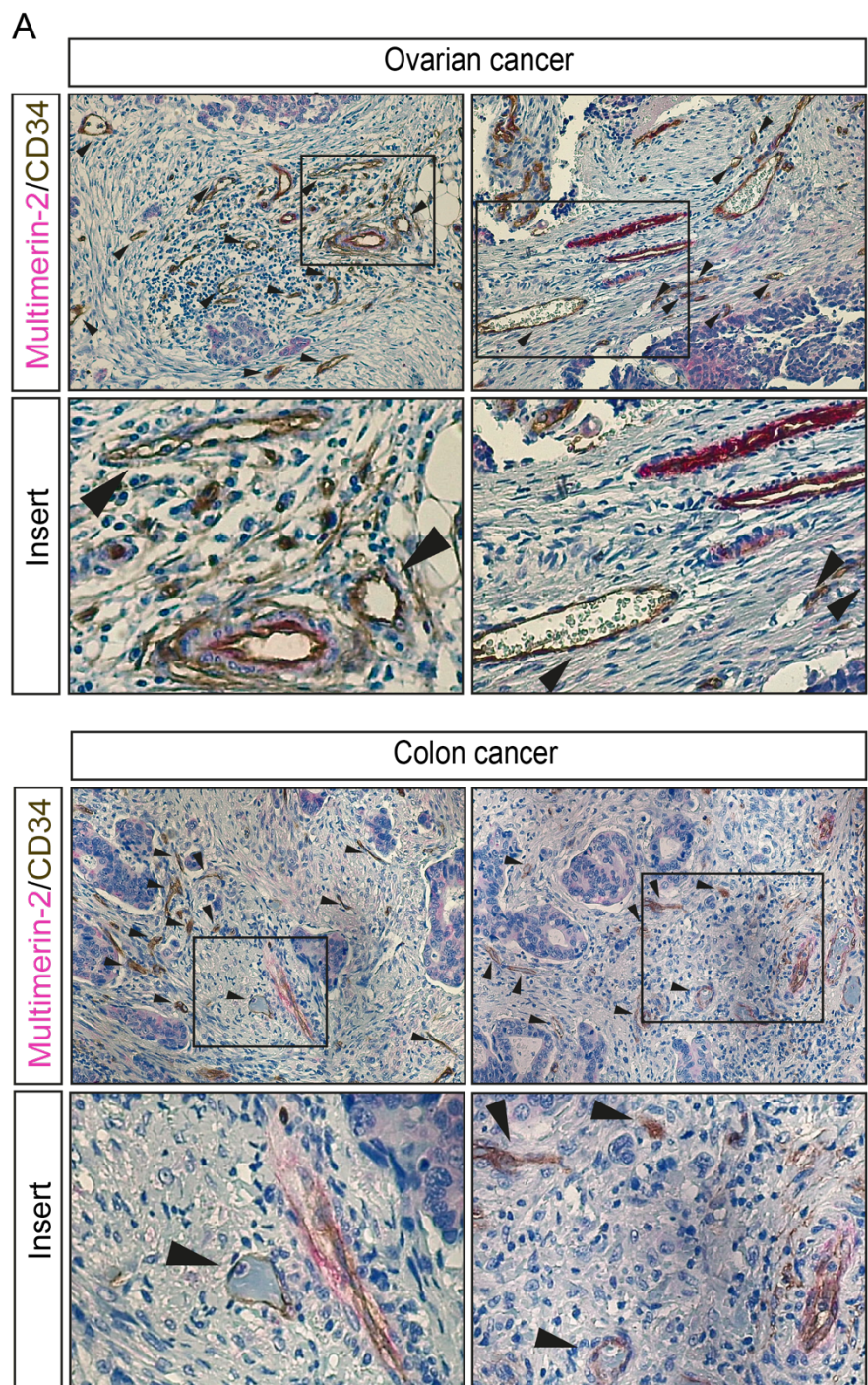


Figure 8. Multimerin-2 expression is downregulated in tumor associated vessels. **A)** Representative images of immunohistochemistry analyses performed on human samples from ovarian (left) and colon (right) cancer patients. Tumor sections were stained for Multimerin-2 (purple); CD34-antibody (brown) was used to identify vessels. Vessels displaying low Multimerin-2 expression are highlighted by black arrows. **B)** Box plots reporting the bioinformatic analyses of the Multimerin-2 mRNA expression in normal and neoplastic samples from ovarian (left) and colon (right) cancer patients, as assessed from the RNA-seq analyses available on the TNMplot database (<https://tnmplot.com/analysis>).

6.9 Multimerin-2 loss associates with impaired pericyte recruitment and vascular efficiency in preclinical settings

To verify these results *in vivo* we took advantage of the *Multimerin-2*^{-/-} mouse model. To this end *wt* and *Multimerin-2*^{-/-} C57BL/6 mice were subcutaneously injected with two syngeneic tumor cell lines: the HG-SOC ID8 cell line carrying the *Trp53*^{-/-} *Pten*^{-/-} mutations and colon carcinoma cell line MC-38. A slight yet not significant decrease of tumor growth was observed only in *Multimerin-2*^{-/-} mice injected with ovarian cancer cells (Figure 9).

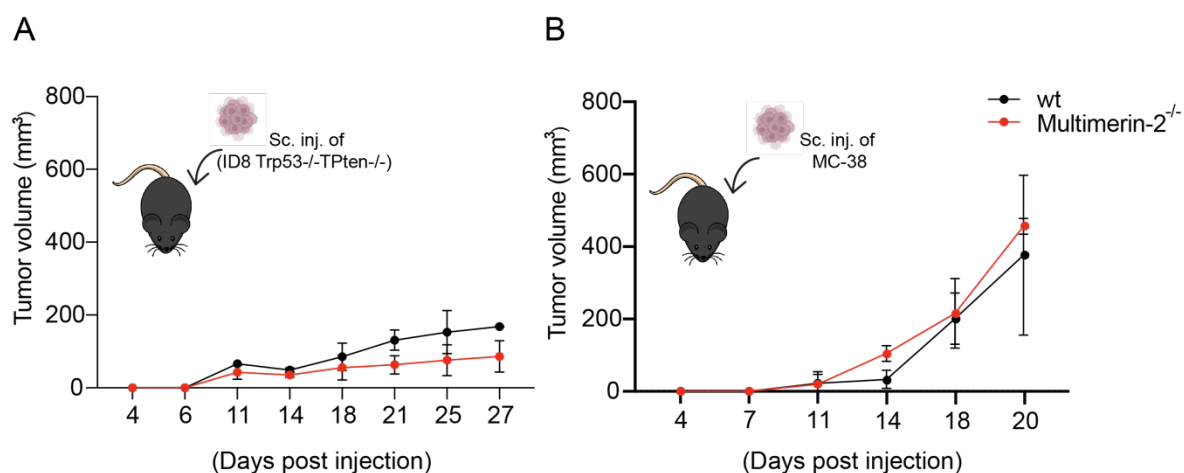
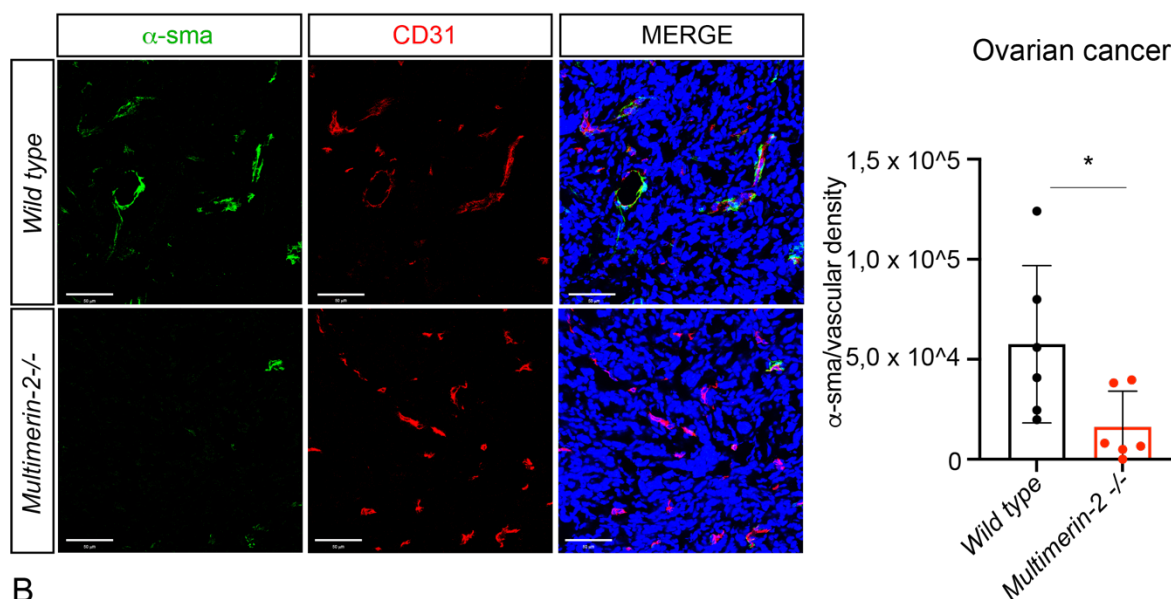


Figure 9. Multimerin-2 loss does not significantly affect the growth of ovarian and colon cancer cells in mice. **A)** Tumor growth following subcutaneous injection (sc. inj.) of the syngeneic ID8 *Trp53*^{-/-} *Pten*^{-/-} ovarian cancer cells in *wt* and *Multimerin-2*^{-/-} mice. **B)** Tumor growth following subcutaneous injection (sc. inj.) of the syngeneic MC-38 colon cancer cells in *wt* and *Multimerin-2*^{-/-} mice.

We next verified if the vessels associated with these tumors displayed an altered pericyte coverage, as suggested by the *in vitro* analyses. Indeed, the use of the anti- α SMA antibody on the tumor sections from both ovarian and colon tumors, highlighted a significantly decreased pericyte coverage in tumor vessels grown in *Multimerin-2*^{-/-} animals compared to that observed in *wt* mice (Figure 10 A,B).

A



B

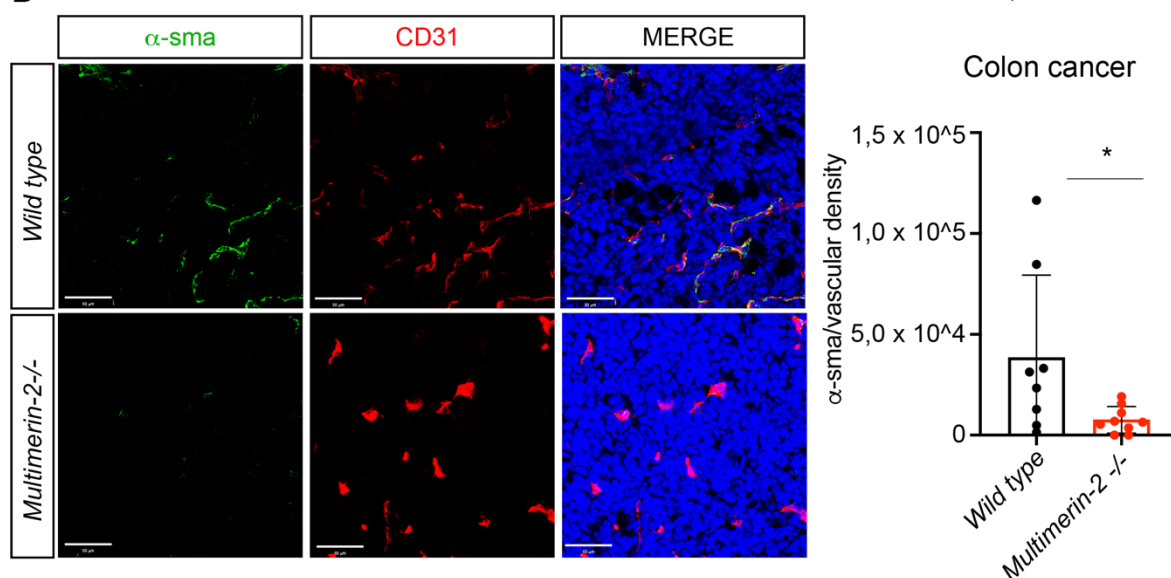
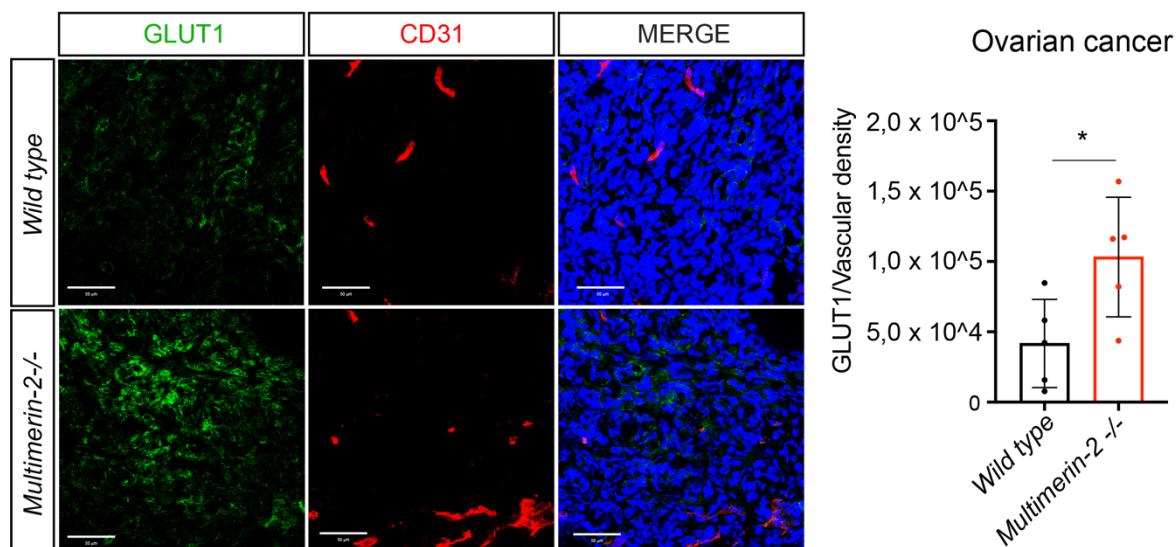


Figure 10. Multimerin-2 loss affects pericyte coverage in vessels associated with ovarian and colon tumors. A) Representative immunofluorescence images of the pericyte coverage in tumors from wt or Multimerin-2^{-/-} mice following subcutaneous injection of HG-SOC ID8 Trp53^{-/-} Pten^{-/-} cells. Vessels were stained with an anti-CD31 antibody (red), pericytes with an anti-α-SMA antibody (green), and nuclei with TO-PRO (blue). Scale bar=50 μm. Quantification of α-SMA intensity was normalized on the vascular density. The relative quantification is reported in the graph on the right; *p=0,0410. **B)** Representative immunofluorescence images of the pericyte coverage in tumors from wt or Multimerin-2^{-/-} mice following subcutaneous injection of the MC-38 colon cancer cells. Staining was performed as in panel A. The relative quantification of the α-SMA/vascular density is reported in the graph on the right. *p=0,0400. The p values were obtained using unpaired Student's t-test.

Since in tumors a highly disorganized and inefficient vasculature is known to hinder the oxygenation of the tissues, we next evaluated the hypoxic levels within these tumors. Accordingly, both ovarian and colon tumors from Multimerin-2^{-/-} mice displayed a

significant increased hypoxia, as assessed with the hypoxic marker GLUT1 (Chung et al., 2009) (Figure 11 A,B).

A



B

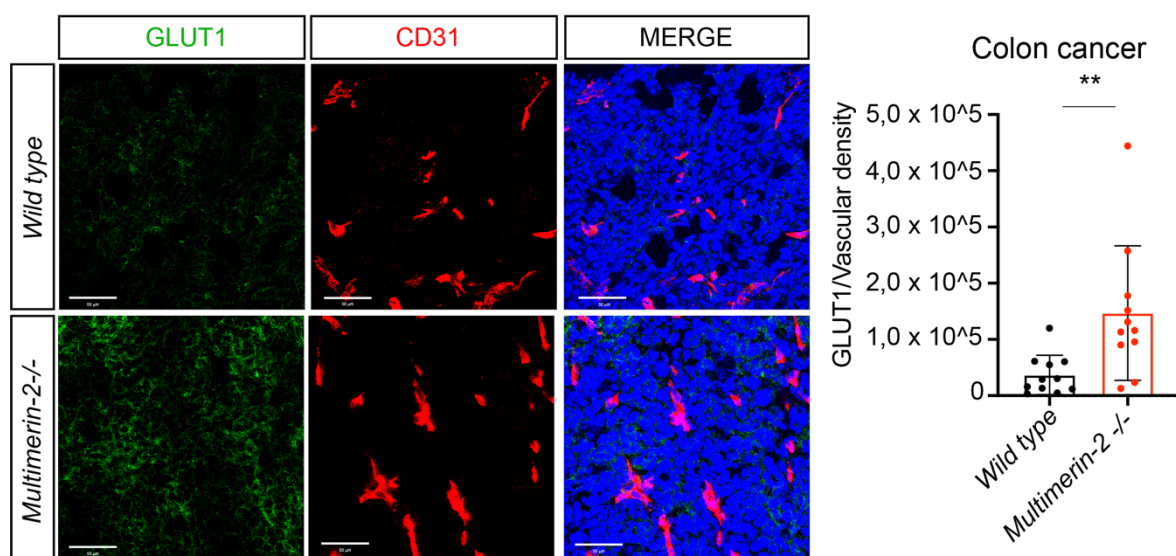
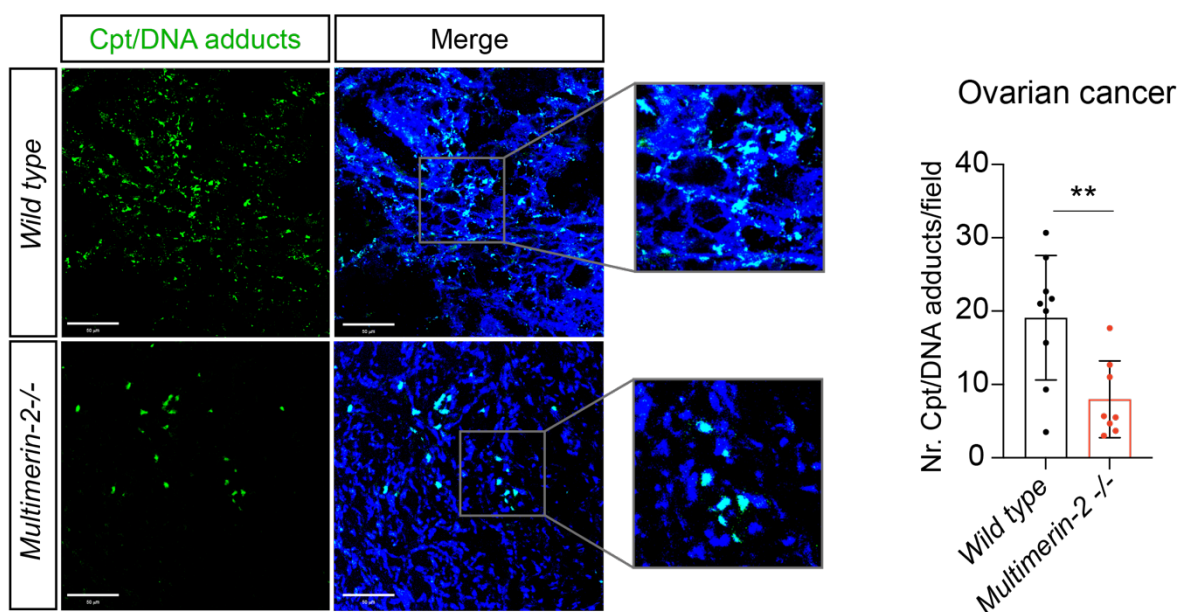


Figure 11. Tumors from Multimerin-2^{-/-} mice display increased intratumoral hypoxia. **A)** Representative immunofluorescence images of GLUT1 expression in tumor sections from wt and Multimerin-2^{-/-} mice subcutaneously injected with the HG-SOC ID8 Trp53^{-/-} Pten^{-/-} cells. Hypoxic areas are highlighted with an anti-GLUT1 antibody (green), vessels are stained with an anti-CD31 antibody (red), and nuclei with TO-PRO (blue). Scale bar=50 μm. Quantification of GLUT1 was normalized on the vascular density. The relative quantification is reported in the graph on the right; **p*=0,0316. **B)** Representative immunofluorescence images of GLUT1 expression in tumor sections from wt and Multimerin-2^{-/-} mice subcutaneously injected with the MC-38 colon cancer cells. Stainings were performed as reported above in panel (A). Scale bar=50 μm. Quantification of GLUT1 was normalized on the vascular density. The relative quantification is reported in the graph on the right; **p*=0,0084. The *p* values were obtained using the unpaired Student's *t*-test.

Prompted by these promising results, we next verified whether the vascular inefficiency associated with the loss of Multimerin-2 could impact on drug delivery. To verify this hypothesis, *wt* and *Multimerin-2^{-/-}* mice carrying the ID8 *Trp53^{-/-}Pten^{-/-}* and MC-38 cells were intraperitoneally injected with Cisplatin (Cpt) six hours before sacrifice and the presence of Cpt/DNA adducts was assessed in the tumor samples. In accordance with our hypothesis, tumors from *Multimerin-2^{-/-}* mice displayed a significantly lower number of Cpt/DNA adducts in the tumor cell nuclei respect to what observed in tumors from *wt* animals (Figure 12 A,B).

A



B

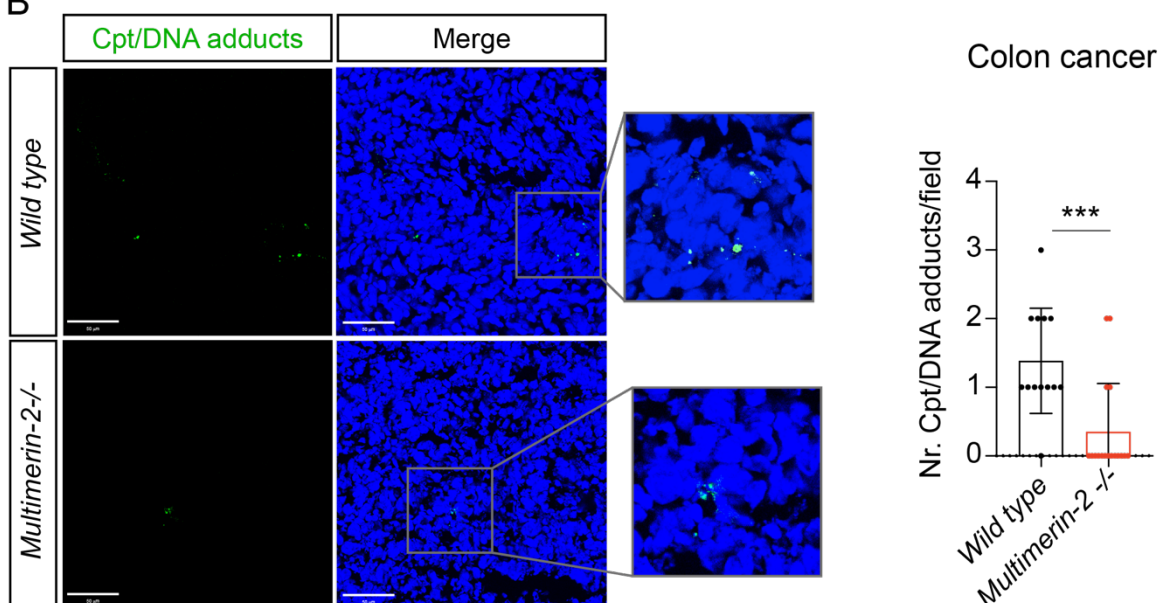
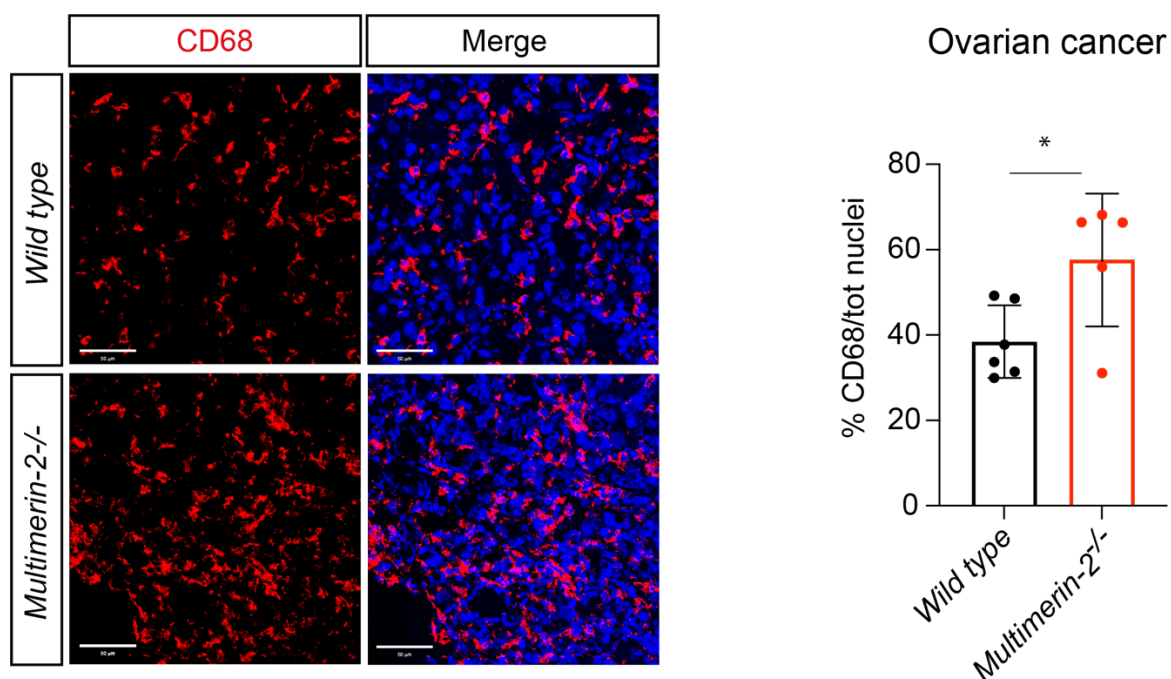


Figure 12. Multimerin-2 loss impairs the drug delivery in vivo. *A)* Representative immunofluorescent images of Cisplatin (Cpt) DNA adducts in tumor sections derived from wt and Multimerin-2^{-/-} mice subcutaneously injected with the ID8 Trp53^{-/-} Pten^{-/-} cells. Mice were intraperitoneally injected with Cpt to evaluate drug delivery. Cpt/DNA adducts are stained in green and nuclei with TO-PRO (blue). *B)* Representative immunofluorescent images of Cpt/DNA adducts in tumor sections derived from wt and Multimerin-2^{-/-} mice subcutaneously injected with the MC-38 colon cancer cells. Mice were intraperitoneally injected with Cpt as indicated above. Cpt/DNA adducts are stained in green and nuclei with TO-PRO (blue). Cpt/DNA adducts were counted in the nuclei of tumor cells. Scale bars= 50 μ m. Quantification of the number of Cpt/DNA adducts per field is reported in the graph on the right; magnifications are reported in the inserts on the right ** $p=0.0060$, *** $p=0.0007$. P values were calculated by the unpaired Student's t-test.

6.10 Loss of Multimerin-2 alters the macrophage infiltration within the tumors

Since it is well established that vessel dysfunction impacts on the intra and extravasation processes of immune cells (Amersfoort et al., 2022) , we next enquired whether the loss of Multimerin-2 could affect also the infiltration of immune cells and started with a preliminary characterization of the macrophage infiltrate. The use of an antibody recognizing CD68, a pan macrophage marker, allowed to detect a higher number of macrophages in both ovarian and colon tumors developed in Multimerin-2^{-/-} mice (Figure 13 A,B).

A



B

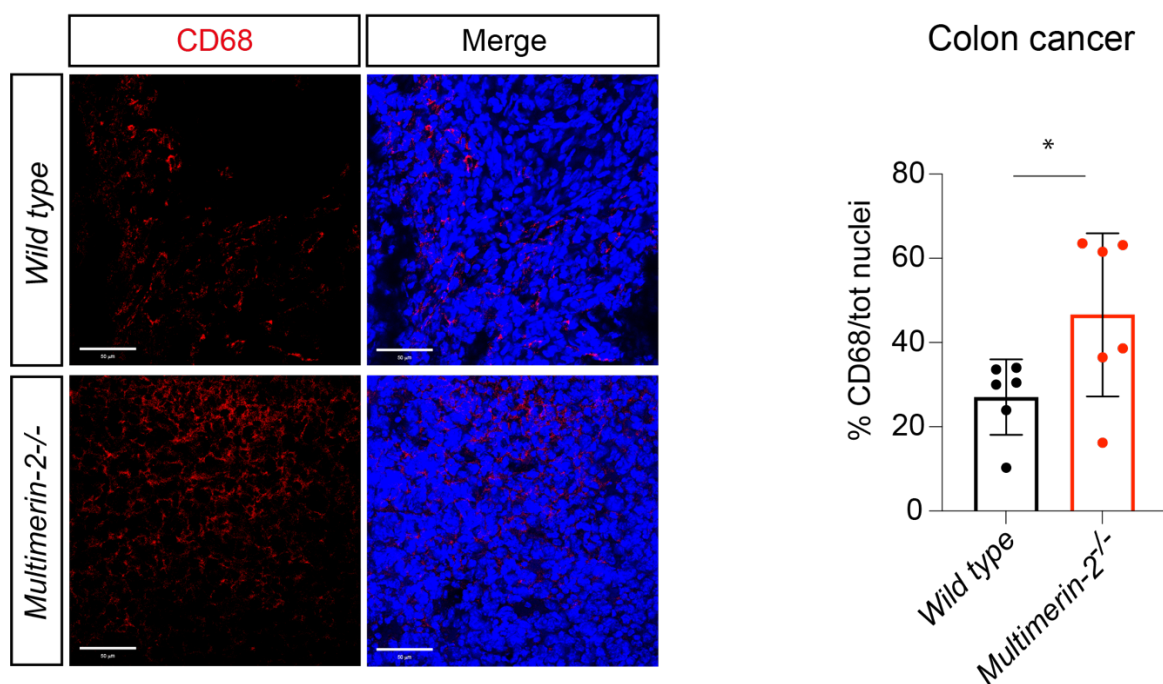


Figure 13. Loss of Multimerin-2 affects the macrophage infiltration in the tumors. **A)** Representative images of CD68, a pan-macrophage marker, in tumor sections derived from wt and Multimerin-2^{-/-} mice subcutaneously injected with HG-SOC ID8 Trp53^{-/-} Pten^{-/-} cells. Macrophages are stained in red with an anti-CD68 antibody and nuclei with TO-PRO (blue). The relative quantification is reported in the graph on the right; *p=0.0289. **B)** Representative images of CD68, in tumor sections derived from wt and Multimerin-2^{-/-} mice subcutaneously injected with MC-38 cells. Macrophages are stained in

red with an anti-CD68 antibody and nuclei with TO-PRO (blue). $*p=0,0487$. The p values were obtained using the unpaired Student's t -test.

The *in vivo* preliminary results were further supported by *in vitro* analyses. Indeed, we found that THP-1 cells, a monocytic-like cell line, transmigrated more efficiently through a Multimerin-2-depleted endothelium developed in co-culture with HBVP cells (Figure 14).

Taken together these results demonstrate that the deposition of Multimerin-2 is required to aid pericyte recruitment and blood vessel maturation, and that loss of Multimerin-2 impacts of vascular functionality and the tumor immune response.

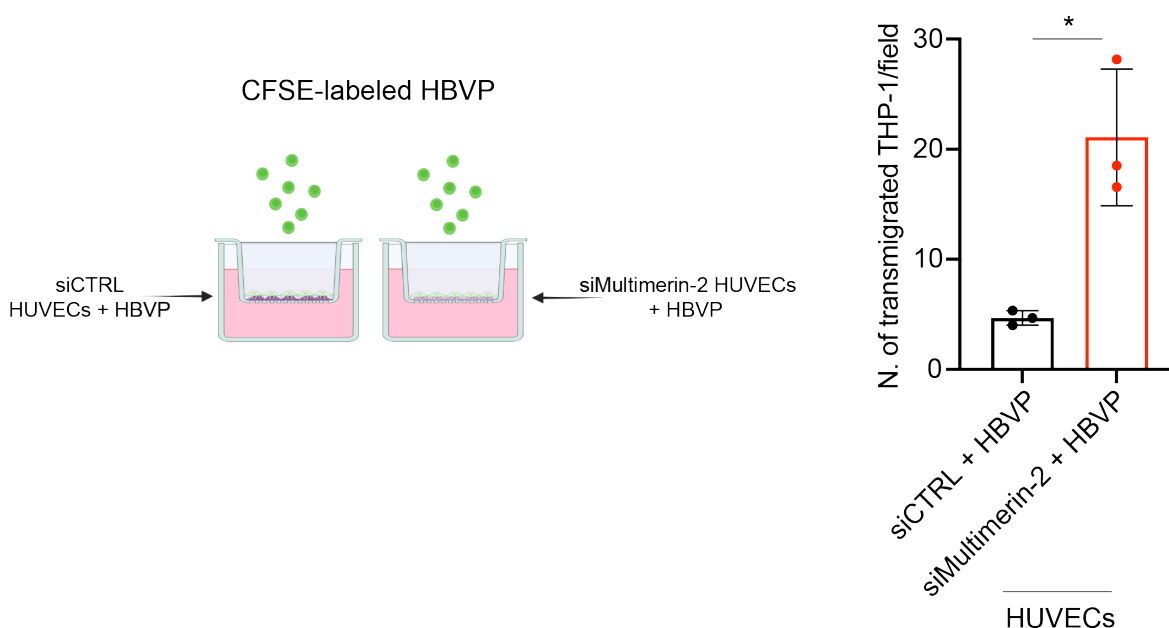


Figure 14. Loss of Multimerin-2 favors the transmigration of THP-1 cells through the endothelium. Left, representative scheme of the transmigration assay. CellTrace™ CFSE labeled THP-1 cells were let migrate across the HUVEC cells monolayer following transduction with the siCTRL or siMultimerin-2 adenoviral constructs. The monolayer was co-cultured with HBVP cells to better mimic the physiological condition. Below, graph representing the number of THP-1 cells transmigrated through the endothelium covered by pericytes. $*p=0.01$. The statistical significance was determined by unpaired Student's t test.

7. DISCUSSION

In the current thesis work, we further explored the role of Multimerin-2 in vascular stabilization. In our previous investigations we determined that Multimerin-2 is key in maintaining the integrity of inter-endothelial junctions, however a possible involvement of Multimerin-2 in the recruitment of pericytes, a vital process during vascular stabilization (Vontell et al., 2006), was not explored prior to this study.

This study was prompted by two main reasons. First, the molecule is deposited by ECs and present both at the luminal and abluminal side of the vasculature (Galvagni et al., 2017), where it could likely interact with pericytes. Thus, we envisioned that Multimerin-2 could function as a molecular bridge between EC and pericytes. Second, we observed that retinal vessels from *Multimerin-2^{-/-}* pups displayed a severely impaired pericyte coverage (Pellicani et al., 2020).

In line with our hypothesis, we first demonstrated that Multimerin-2 functions as a haptotactic stimulus during the recruitment of pericyte to the endothelium. Little is known about the function of ECM in this context, nonetheless we have found that another member of the family, i.e. Emilin-2 displays similar effects (Fejza et al., 2023). The two molecules are characterized by a similar molecular structure; however, their distribution is dissimilar. In fact, Emilin-2 is not deposited by ECs and is widely distributed in tissues, despite it has been observed in proximity to the vessels (Fejza et al., 2023). It is thus possible that the two molecules create a gradient adjacent to the vessels to facilitate the recruitment of pericytes, possibly via the same molecular domain, yet these aspects still need to be fully elucidated. Since pericytes are known to be primarily recruited upon the expression of specific soluble factors secreted by ECs (Aguilera and Brekken, 2014), we also considered this possibility and found that Multimerin-2-challenged ECs increase the expression of PDGF-BB and HB-EGF, two key cytokines promoting pericyte recruitment (Stratman et al., 2010). Once pericytes are recruited to the vasculature, they wrap ECs and we have established that Multimerin-2 is an optimal substrate for the adhesion of these cells, thus representing vehicle for the EC-pericyte crosstalk. Taken together, and in accordance with observations of the *Multimerin-2^{-/-}* retinal vessels (Pellicani et al., 2020), these findings suggest that the deposition of Multimerin-2 by ECs represents a crucial step for the proper recruitment and adhesion of pericytes to the endothelium and for the establishment of a mature functional vasculature. Pericytes undergo profound cytoskeletal rearrangements during vascular maturation (Sugihara et al., 2020), as to whether Multimerin-2 impinges also in

this process, still needs to be determined. Multimerin-2 was also found to increase pericyte proliferation and at first, we hypothesized that this could be mediated by CD248, a putative Multimerin-2 binding partner (Khan et al., 2017), highly expressed by pericytes, and known to regulate their proliferation (Tomkiewicz et al., 2010). However, the knockdown of the receptor did not affect the proliferation and migration of pericytes. Since ECs express CD93, a cell surface lectin receptor belonging to the same family of CD248, and also a binding partner of Multimerin-2 (Khan et al., 2017), it is possible that the function of CD248 in this context is simply to anchor pericytes to ECs through the interaction with Multimerin-2. Instead, we demonstrated that the Multimerin-2-arbitrated proliferation of pericytes is mediated by IGF1R- β , a receptor known to affect pericyte proliferation via the activation of Akt (Riedemann and Macaulay, 2006). As to whether this receptor also mediates the migration and/or adhesion of pericytes, still needs to be determined. Interestingly, it has been shown that IGF1R- β mediates adhesion through integrins (Takada et al., 2017). We have previously demonstrated that Emilin-2 mediates pericyte adhesion through integrins $\alpha_5\beta_1$ and $\alpha_6\beta_1$ (Fejza et al., 2023) and we cannot exclude the involvement of a similar pathway for Multimerin-2, possibly through the formation of a molecular complex with IGF1R.

The finding that Multimerin-2 affects different aspects of the pericyte biology suggests that it might trigger different molecular pathways in these cells. This possibility was in fact confirmed by the NanoString transcriptomic analyses, which indicated that Multimerin-2 affects the expression of a number of genes in pericytes. Among these molecules, Ang-2 is significantly downregulated in Multimerin-2-treated pericytes. Ang-2 overexpression correlates with pericyte detachment, vasculature leakiness (Akwii et al., 2019), and this is in agreement with the role of Multimerin-2 in maintaining vascular stability. Furthermore, the up-regulation of Ang-2 also affects the trans-endothelial migration of immune and cancer cells; we cannot exclude that the different infiltration of immune cells detected in tumors from *Multimerin-2*^{-/-} animals may also hinge on this pathway.

These results gain additional interest in light of the fact that, during cancer growth and progression, Multimerin-2 expression is frequently lost in tumor-associated vessels, as demonstrated by the immunohistochemistry analyses performed on a number of human tumor sections. In this perspective, patients displaying low Multimerin-2 expression may be characterized by poor pericyte recruitment and exacerbated vascular instability. To gain pre-clinical insights in this viewpoint we exploited the *Multimerin-2*^{-/-} model. The

growth of ovarian cancer cells was slightly but not significantly impaired in *Multimerin-2^{-/-}* mice, as observed in the melanoma model (Pellicani et al., 2020). This could be due to the fact that the utterly unfunctional vasculature prevents the proper release of oxygen and nutrients to the tumor cells. However, an improper supplying does not significantly affect the growth of these aggressive cells, and, in fact, this slight effect was even not detected in colon cancer cells. Nonetheless, an unfunctional vasculature also affects the delivery of drugs to the tumor cells, and this may strongly affect the outcome upon anti-cancer therapy. Indeed, we found less Cpt/DNA adducts in tumor cells grown in *Multimerin-2^{-/-}* animals, thus suggesting that loss of Multimerin-2 negatively affects the distribution of the drugs within the tumor. Of note, our results demonstrate that this phenomenon is not tumor type dependent, on the contrary it is transversal since we obtained consistent results both in colon and in ovarian cancer, as previously observed in melanoma (Pellicani et al., 2020). A deeper analysis of both the tumor models grown in the absence of Multimerin-2 indicated that the tumor-associated vessels displayed impaired pericyte coverage and enhanced intratumoral hypoxia. These findings are in agreement with the concept/viewpoint that the deposition of Multimerin-2 is important to trigger pericyte recruitment and vascular stability/efficiency and that the loss of its expression further exacerbates the abnormal features of tumor-associated vessels. It has been reported that in breast cancer low pericyte coverage correlates with the presence of distant metastasis, with a shorter disease-free survival and with poor overall survival (Cooke et al., 2012). Thus, we can hypothesize that vessels devoid of Multimerin-2 may also represent the preferential route for the metastatic dissemination. And this is conceivable not only considering the abnormal morphological features of the vessels lacking Multimerin-2, but also in the light that tumors grown in *Multimerin-2^{-/-}* animals are characterized by pronounced hypoxia, a leading feature of tumor progression and dissemination (Qi et al., 2020).

Vascular abnormalities not only affect the transmigration of tumor cells across the vessels but also the infiltration of immune cells. Accordingly, our preliminary results show a higher number of macrophages within the tumors grown in *Multimerin-2^{-/-}* animals. Similar results were obtained *in vitro*, where the Multimerin-2-depleted endothelium favoured the transmigration of monocytes. It is likely that the discontinuous inter-endothelial junctions upon loss of Multimerin-2 may be the primary culprit of this phenomenon. However, it still needs to be determined if loss of pericyte coverage also contributes to the altered immune cell infiltration in this context (Török et al., 2021).

Also, we cannot exclude a possible contribution of CD248, which is highly expressed by pericytes and it has been shown to induce macrophage activation in inflammatory processes (Cheng et al., 2021) .

A rich immune infiltrate has been associated with a favourable outcome, despite this theory is controversial (Wang et al., 2023). To verify if the altered immune response associated to the loss of Multimerin-2 correlates with a better or worse outcome, it will be necessary to characterize the polarization/activation of macrophages, as well as the presence of other inflammatory sub-populations.

Taken together these results suggest that Multimerin-2 is required for the maintenance of vessel stability and functionality. Our findings are relevant from the translational point of view and suggest that Multimerin-2 could be used as a biomarker to predict the therapy efficacy. In fact, given the ample variability, the patients could be potentially stratified based on the levels of Multimerin-2 expression. In fact, our results suggest that patients displaying extensive loss of Multimerin-2 could be characterized by poor prognosis and/or reduced benefits from the chemo/immune-therapies.

8. BIBLIOGRAPHY

- Acerbi, I., Cassereau, L., Dean, I., Shi, Q., Au, A., Park, C., Chen, Y.Y., Liphardt, J., Hwang, E.S., Weaver, V.M., 2015. Human breast cancer invasion and aggression correlates with ECM stiffening and immune cell infiltration. *Integrative Biology* 7, 1120–1134. <https://doi.org/10.1039/c5ib00040h>
- Adam, F., Zheng, S., Joshi, N., Kelton, D., Sandhu, A., Suehiro, Y., Jeimy, S., Santos, A., Massé, J.-M., Kelton, J., Cramer, E., Hayward, C., 2005. Analyses of cellular multimerin 1 receptors: in vitro evidence of binding mediated by α Ib β 3 and α v β 3. *Thromb Haemost* 94, 1004–1011. <https://doi.org/10.1160/TH05-02-0140>
- Aguilera, K.Y., Brekken, R.A., 2014. Recruitment and retention: factors that affect pericyte migration. *Cell. Mol. Life Sci.* 71, 299–309. <https://doi.org/10.1007/s00018-013-1432-z>
- Aird, W.C., 2007. Phenotypic Heterogeneity of the Endothelium: I. Structure, Function, and Mechanisms. *Circulation Research* 100, 158–173. <https://doi.org/10.1161/01.RES.0000255691.76142.4a>
- Akwii, R.G., Sajib, M.S., Zahra, F.T., Mikelis, C.M., 2019. Role of Angiopoietin-2 in Vascular Physiology and Pathophysiology. *Cells* 8, 471. <https://doi.org/10.3390/cells8050471>
- Alevizakos, M., Kaltsas, S., Syrigos, K.N., 2013. The VEGF pathway in lung cancer. *Cancer Chemother Pharmacol* 72, 1169–1181. <https://doi.org/10.1007/s00280-013-2298-3>
- Amersfoort, J., Eelen, G., Carmeliet, P., 2022. Immunomodulation by endothelial cells — partnering up with the immune system? *Nat Rev Immunol* 22, 576–588. <https://doi.org/10.1038/s41577-022-00694-4>
- Amma, L.L., Goodyear, R., Faris, J.S., Jones, I., Ng, L., Richardson, G., Forrest, D., 2003. An emilin family extracellular matrix protein identified in the cochlear basilar membrane. *Molecular and Cellular Neuroscience* 23, 460–472. [https://doi.org/10.1016/S1044-7431\(03\)00075-7](https://doi.org/10.1016/S1044-7431(03)00075-7)
- Anderson, N.M., Simon, M.C., 2020. The tumor microenvironment. *Current Biology* 30, R921–R925. <https://doi.org/10.1016/j.cub.2020.06.081>
- Andreuzzi, E., Capuano, A., Pellicani, R., Poletto, E., Doliana, R., Maiero, S., Fornasarig, M., Magris, R., Colombatti, A., Cannizzaro, R., Spessotto, P., Mongiat, M., 2018. Loss of Multimerin-2 and EMILIN-2 Expression in Gastric Cancer Associate with Altered Angiogenesis. *IJMS* 19, 3983. <https://doi.org/10.3390/ijms19123983>
- Andreuzzi, E., Colladel, R., Pellicani, R., Tarticchio, G., Cannizzaro, R., Spessotto, P., Bussolati, B., Brossa, A., De Paoli, P., Canzonieri, V., Iozzo, R.V., Colombatti, A., Mongiat, M., 2017. The angiostatic molecule Multimerin 2 is processed by MMP-9 to allow sprouting

- angiogenesis. *Matrix Biology* 64, 40–53. <https://doi.org/10.1016/j.matbio.2017.04.002>
- Andreuzzi, E., Fejza, A., Polano, M., Poletto, E., Camicia, L., Carobolante, G., Tarticchio, G., Todaro, F., Di Carlo, E., Scarpa, Melania, Scarpa, Marco, Paulitti, A., Capuano, A., Canzonieri, V., Maiero, S., Fornasarig, M., Cannizzaro, R., Doliana, R., Colombatti, A., Spessotto, P., Mongiat, M., 2022. Colorectal cancer development is affected by the ECM molecule EMILIN-2 hinging on macrophage polarization via the TLR-4/MyD88 pathway. *J Exp Clin Cancer Res* 41, 60. <https://doi.org/10.1186/s13046-022-02271-y>
- Armulik, A., Genové, G., Betsholtz, C., 2011. Pericytes: Developmental, Physiological, and Pathological Perspectives, Problems, and Promises. *Developmental Cell* 21, 193–215. <https://doi.org/10.1016/j.devcel.2011.07.001>
- Astrof, S., Hynes, R.O., 2009. Fibronectins in vascular morphogenesis. *Angiogenesis* 12, 165–175. <https://doi.org/10.1007/s10456-009-9136-6>
- Augustin, H.G., Koh, G.Y., 2022. Antiangiogenesis: Vessel Regression, Vessel Normalization, or Both? *Cancer Research* 82, 15–17. <https://doi.org/10.1158/0008-5472.CAN-21-3515>
- Ayres-Sander, C.E., Lauridsen, H., Maier, C.L., Sava, P., Pober, J.S., Gonzalez, A.L., 2013. Transendothelial Migration Enables Subsequent Transmigration of Neutrophils through Underlying Pericytes. *PLoS ONE* 8, e60025. <https://doi.org/10.1371/journal.pone.0060025>
- Bartha, Á., Györfy, B., 2021. TNMplot.com: A Web Tool for the Comparison of Gene Expression in Normal, Tumor and Metastatic Tissues. *IJMS* 22, 2622. <https://doi.org/10.3390/ijms22052622>
- Bennouna, J., Sastre, J., Arnold, D., Österlund, P., Greil, R., Van Cutsem, E., Von Moos, R., Viéitez, J.M., Bouché, O., Borg, C., Steffens, C.-C., Alonso-Orduña, V., Schlichting, C., Reyes-Rivera, I., Bendahmane, B., André, T., Kubicka, S., 2013. Continuation of bevacizumab after first progression in metastatic colorectal cancer (ML18147): a randomised phase 3 trial. *The Lancet Oncology* 14, 29–37. [https://doi.org/10.1016/S1470-2045\(12\)70477-1](https://doi.org/10.1016/S1470-2045(12)70477-1)
- Bergers, G., Benjamin, L.E., 2003. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 3, 401–410. <https://doi.org/10.1038/nrc1093>
- Bergers, G., Song, S., 2005a. The role of pericytes in blood-vessel formation and maintenance. *Neuro-Oncology* 7, 452–464. <https://doi.org/10.1215/S1152851705000232>
- Bergers, G., Song, S., 2005b. The role of pericytes in blood-vessel formation and maintenance. *Neuro-Oncology* 7, 452–464. <https://doi.org/10.1215/S1152851705000232>
- Birbrair, A., 2018. Pericyte Biology: Development, Homeostasis, and Disease, in: Birbrair,

- A. (Ed.), *Pericyte Biology - Novel Concepts*, *Advances in Experimental Medicine and Biology*. Springer International Publishing, Cham, pp. 1–3. https://doi.org/10.1007/978-3-030-02601-1_1
- Boot-Handford, R.P., Tuckwell, D.S., 2003. Fibrillar collagen: The key to vertebrate evolution? A tale of molecular incest. *Bioessays* 25, 142–151. <https://doi.org/10.1002/bies.10230>
- Braghetta, P., 2004. Overlapping, complementary and site-specific expression pattern of genes of the EMILIN/Multimerin family. *Matrix Biology* 22, 549–556. <https://doi.org/10.1016/j.matbio.2003.10.005>
- Bressan, G.M., Daga-Gordini, D., Colombatti, A., Castellani, I., Marigo, V., Volpin, D., 1993. Emilin, a component of elastic fibers preferentially located at the elastin-microfibrils interface. *The Journal of cell biology* 121, 201–212. <https://doi.org/10.1083/jcb.121.1.201>
- Brodsky, B., Persikov, A.V., 2005. Molecular Structure of the Collagen Triple Helix, in: *Advances in Protein Chemistry*. Elsevier, pp. 301–339. [https://doi.org/10.1016/S0065-3233\(05\)70009-7](https://doi.org/10.1016/S0065-3233(05)70009-7)
- Bussmann, J., Wolfe, S.A., Siekmann, A.F., 2011. Arterial-venous network formation during brain vascularization involves hemodynamic regulation of chemokine signaling. *Development* 138, 1717–1726. <https://doi.org/10.1242/dev.059881>
- Capuano, A., Pivetta, E., Baldissera, F., Bosisio, G., Wassermann, B., Bucciotti, F., Colombatti, A., Sabatelli, P., Doliana, R., Spessotto, P., 2019. Integrin binding site within the gC1q domain orchestrates EMILIN-1-induced lymphangiogenesis. *Matrix Biology* 81, 34–49. <https://doi.org/10.1016/j.matbio.2018.10.006>
- Carmeliet, P., 2005. Angiogenesis in life, disease and medicine. *Nature* 438, 932–936. <https://doi.org/10.1038/nature04478>
- Carmeliet, P., 2000. Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 6, 389–395. <https://doi.org/10.1038/74651>
- Carmeliet, P., Jain, R.K., 2011a. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat Rev Drug Discov* 10, 417–427. <https://doi.org/10.1038/nrd3455>
- Carmeliet, P., Jain, R.K., 2011b. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat Rev Drug Discov* 10, 417–427. <https://doi.org/10.1038/nrd3455>
- Carmeliet, P., Lampugnani, M.-G., Moons, L., Breviario, F., Compernelle, V., Bono, F., Balconi, G., Spagnuolo, R., Oosthuysen, B., Dewerchin, M., Zanetti, A., Angellilo, A.,

- Mattot, V., Nuyens, D., Lutgens, E., Clotman, F., De Ruiter, M.C., Gittenberger-de Groot, A., Poelmann, R., Lupu, F., Herbert, J.-M., Collen, D., Dejana, E., 1999. Targeted Deficiency or Cytosolic Truncation of the VE-cadherin Gene in Mice Impairs VEGF-Mediated Endothelial Survival and Angiogenesis. *Cell* 98, 147–157. [https://doi.org/10.1016/S0092-8674\(00\)81010-7](https://doi.org/10.1016/S0092-8674(00)81010-7)
- Cheng, T.-L., Lin, Y.-S., Hong, Y.-K., Ma, C.-Y., Tsai, H.-W., Shi, G.-Y., Wu, H.-L., Lai, C.-H., 2021. Role of tumor endothelial marker 1 (Endosialin/CD248) lectin-like domain in lipopolysaccharide-induced macrophage activation and sepsis in mice. *Translational Research* 232, 150–162. <https://doi.org/10.1016/j.trsl.2021.03.009>
- Choi, H., Moon, A., 2018. Crosstalk between cancer cells and endothelial cells: implications for tumor progression and intervention. *Arch. Pharm. Res.* 41, 711–724. <https://doi.org/10.1007/s12272-018-1051-1>
- Christian, S., Ahorn, H., Novatchkova, M., Garin-Chesa, P., Park, J.E., Weber, G., Eisenhaber, F., Rettig, W.J., Lenter, M.C., 2001a. Molecular Cloning and Characterization of EndoGlyx-1, an EMILIN-like Multisubunit Glycoprotein of Vascular Endothelium. *Journal of Biological Chemistry* 276, 48588–48595. <https://doi.org/10.1074/jbc.M106152200>
- Christian, S., Ahorn, H., Novatchkova, M., Garin-Chesa, P., Park, J.E., Weber, G., Eisenhaber, F., Rettig, W.J., Lenter, M.C., 2001b. Molecular Cloning and Characterization of EndoGlyx-1, an EMILIN-like Multisubunit Glycoprotein of Vascular Endothelium. *J. Biol. Chem.* 276, 48588–48595. <https://doi.org/10.1074/jbc.M106152200>
- Christian, S., Winkler, R., Helfrich, I., Boos, A.M., Besemfelder, E., Schadendorf, D., Augustin, H.G., 2008. Endosialin (Tem1) Is a Marker of Tumor-Associated Myofibroblasts and Tumor Vessel-Associated Mural Cells. *The American Journal of Pathology* 172, 486–494. <https://doi.org/10.2353/ajpath.2008.070623>
- Chung, F.-Y., Huang, M.-Y., Yeh, C.-S., Chang, H.-J., Cheng, T.-L., Yen, L.-C., Wang, J.-Y., Lin, S.-R., 2009. GLUT1 gene is a potential hypoxic marker in colorectal cancer patients. *BMC Cancer* 9, 241. <https://doi.org/10.1186/1471-2407-9-241>
- Cohen, M.H., Shen, Y.L., Keegan, P., Pazdur, R., 2009. FDA Drug Approval Summary: Bevacizumab (Avastin®) as Treatment of Recurrent Glioblastoma Multiforme. *The Oncologist* 14, 1131–1138. <https://doi.org/10.1634/theoncologist.2009-0121>
- Colladel, R., Pellicani, R., Andreuzzi, E., Paulitti, A., Tarticchio, G., Todaro, F., Colombatti, A., Mongiat, M., 2016a. MULTIMERIN2 binds VEGF-A primarily via the carbohydrate chains exerting an angiostatic function and impairing tumor growth. *Oncotarget* 7, 2022–

2037. <https://doi.org/10.18632/oncotarget.6515>

Colladel, R., Pellicani, R., Andreuzzi, E., Paulitti, A., Tarticchio, G., Todaro, F., Colombatti, A., Mongiat, M., 2016b. MULTIMERIN2 binds VEGF-A primarily via the carbohydrate chains exerting an angiostatic function and impairing tumor growth. *Oncotarget* 7, 2022–2037. <https://doi.org/10.18632/oncotarget.6515>

Colombatti, A., Bressan, G.M., Castellani, I., Volpin, D., 1985. Glycoprotein 115, a glycoprotein isolated from chick blood vessels, is widely distributed in connective tissue. *The Journal of cell biology* 100, 18–26. <https://doi.org/10.1083/jcb.100.1.18>

Colombatti, A., Doliana, R., Bot, S., Canton, A., Mongiat, M., Mungiguerra, G., Paron-Cilli, S., Spessotto, P., 2000. The EMILIN protein family. *Matrix Biology* 19, 289–301. [https://doi.org/10.1016/S0945-053X\(00\)00074-3](https://doi.org/10.1016/S0945-053X(00)00074-3)

Colombatti, A., Spessotto, P., Doliana, R., Mongiat, M., Bressan, G.M., Esposito, G., 2012. The EMILIN/Multimerin Family. *Front. Immun.* 2. <https://doi.org/10.3389/fimmu.2011.00093>

Condeelis, J., Pollard, J.W., 2006. Macrophages: Obligate Partners for Tumor Cell Migration, Invasion, and Metastasis. *Cell* 124, 263–266. <https://doi.org/10.1016/j.cell.2006.01.007>

Cooke, V.G., LeBleu, V.S., Keskin, D., Khan, Z., O’Connell, J.T., Teng, Y., Duncan, M.B., Xie, L., Maeda, G., Vong, S., Sugimoto, H., Rocha, R.M., Damascena, A., Brentani, R.R., Kalluri, R., 2012. Pericyte Depletion Results in Hypoxia-Associated Epithelial-to-Mesenchymal Transition and Metastasis Mediated by Met Signaling Pathway. *Cancer Cell* 21, 66–81. <https://doi.org/10.1016/j.ccr.2011.11.024>

Corada, M., Mariotti, M., Thurston, G., Smith, K., Kunkel, R., Brockhaus, M., Lampugnani, M.G., Martin-Padura, I., Stoppacciaro, A., Ruco, L., McDonald, D.M., Ward, P.A., Dejana, E., 1999. Vascular endothelial–cadherin is an important determinant of microvascular integrity *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 96, 9815–9820. <https://doi.org/10.1073/pnas.96.17.9815>

Danussi, C., Petrucco, A., Wassermann, B., Pivetta, E., Modica, T.M.E., Del Bel Belluz, L., Colombatti, A., Spessotto, P., 2011. EMILIN1- $\alpha 4/\alpha 9$ integrin interaction inhibits dermal fibroblast and keratinocyte proliferation. *J Cell Biol* 195, 131–145. <https://doi.org/10.1083/jcb.201008013>

Danussi, C., Spessotto, P., Petrucco, A., Wassermann, B., Sabatelli, P., Montesi, M., Doliana, R., Bressan, G.M., Colombatti, A., 2008. *Emilin1* Deficiency Causes Structural and Functional Defects of Lymphatic Vasculature. *Molecular and Cellular Biology* 28, 4026–

4039. <https://doi.org/10.1128/MCB.02062-07>

Davis, G.E., Senger, D.R., 2005. Endothelial Extracellular Matrix: Biosynthesis, Remodeling, and Functions During Vascular Morphogenesis and Neovessel Stabilization. *Circulation Research* 97, 1093–1107.

<https://doi.org/10.1161/01.RES.0000191547.64391.e3>

Dejana, E., 2004. Endothelial cell–cell junctions: happy together. *Nat Rev Mol Cell Biol* 5, 261–270. <https://doi.org/10.1038/nrm1357>

Delafontaine, P., Song, Y.-H., Li, Y., 2004. Expression, Regulation, and Function of IGF-1, IGF-1R, and IGF-1 Binding Proteins in Blood Vessels. *ATVB* 24, 435–444. <https://doi.org/10.1161/01.ATV.0000105902.89459.09>

Díaz-Flores, L., Gutiérrez, R., Varela, H., Rancel, N., Valladares, F., 1991. Microvascular pericytes: a review of their morphological and functional characteristics. *Histol Histopathol* 6, 269–286.

Dvorak, H.F., 2000. VPF/VEGF and the angiogenic response. *Seminars in Perinatology* 24, 75–78. [https://doi.org/10.1016/S0146-0005\(00\)80061-0](https://doi.org/10.1016/S0146-0005(00)80061-0)

Eklund, L., Kangas, J., Saharinen, P., 2017. Angiopoietin–Tie signalling in the cardiovascular and lymphatic systems. *Clinical Science* 131, 87–103. <https://doi.org/10.1042/CS20160129>

Fang, S., Dai, Y., Mei, Y., Yang, M., Hu, L., Yang, H., Guan, X., Li, J., 2019. Clinical significance and biological role of cancer-derived Type I collagen in lung and esophageal cancers: Type I collagen in NSCLC and ESCC. *Thorac Cancer* 10, 277–288. <https://doi.org/10.1111/1759-7714.12947>

Fejza, A., Camicia, L., Carobolante, G., Poletto, E., Paulitti, A., Schinello, G., Di Siena, E., Cannizzaro, R., Iozzo, R.V., Baldassarre, G., Andreuzzi, E., Spessotto, P., Mongiat, M., 2023. Emilin2 fosters vascular stability by promoting pericyte recruitment. *Matrix Biology* S0945053X23000835. <https://doi.org/10.1016/j.matbio.2023.08.002>

Fejza, A., Poletto, E., Carobolante, G., Camicia, L., Andreuzzi, E., Capuano, A., Pivetta, E., Pellicani, R., Colladel, R., Marastoni, S., Doliana, R., Iozzo, R.V., Spessotto, P., Mongiat, M., 2021. Multimerin-2 orchestrates the cross-talk between endothelial cells and pericytes: A mechanism to maintain vascular stability. *Matrix Biology Plus* 100068. <https://doi.org/10.1016/j.mbplus.2021.100068>

Ferrara, N., 2005. The role of VEGF in the regulation of physiological and pathological angiogenesis, in: Clauss, M., Breier, G. (Eds.), *Mechanisms of Angiogenesis*. Birkhäuser Basel, Basel, pp. 209–231. https://doi.org/10.1007/3-7643-7311-3_15

- Ferrara, N., Gerber, H.-P., LeCouter, J., 2003. The biology of VEGF and its receptors. *Nat Med* 9, 669–676. <https://doi.org/10.1038/nm0603-669>
- Ferrara, N., Henzel, W.J., 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochemical and Biophysical Research Communications* 161, 851–858. [https://doi.org/10.1016/0006-291X\(89\)92678-8](https://doi.org/10.1016/0006-291X(89)92678-8)
- Folkman, J., D'Amore, P.A., 1996. Blood Vessel Formation: What Is Its Molecular Basis? *Cell* 87, 1153–1155. [https://doi.org/10.1016/S0092-8674\(00\)81810-3](https://doi.org/10.1016/S0092-8674(00)81810-3)
- Fukumura, D., Jain, R.K., 2007. Tumor microvasculature and microenvironment: Targets for anti-angiogenesis and normalization. *Microvascular Research* 74, 72–84. <https://doi.org/10.1016/j.mvr.2007.05.003>
- Furuse, M., Tsukita, S., 2006. Claudins in occluding junctions of humans and flies. *Trends in Cell Biology* 16, 181–188. <https://doi.org/10.1016/j.tcb.2006.02.006>
- Galvagni, F., Nardi, F., Spiga, O., Trezza, A., Tarticchio, G., Pellicani, R., Andreuzzi, E., Caldi, E., Toti, P., Tosi, G.M., Santucci, A., Iozzo, R.V., Mongiat, M., Orlandini, M., 2017. Dissecting the CD93-Multimerin 2 interaction involved in cell adhesion and migration of the activated endothelium. *Matrix Biology* 64, 112–127. <https://doi.org/10.1016/j.matbio.2017.08.003>
- Gantt, G.A., Chen, Y., DeJulius, K., Mace, A.G., Barnholtz-Sloan, J., Kalady, M.F., 2014. Gene expression profile is associated with chemoradiation resistance in rectal cancer. *Colorectal Dis* 16, 57–66. <https://doi.org/10.1111/codi.12395>
- George, M.L., Tutton, M.G., Janssen, F., Arnaout, A., Abulafi, A.M., Eccles, S.A., Swift, R.I., 2001. VEGF-A, VEGF-C, and VEGF-D in colorectal cancer progression. *Neoplasia* 3, 420–427. <https://doi.org/10.1038/sj.neo.7900186>
- Gerhardt, H., Wolburg, H., Redies, C., 2000. N-cadherin mediates pericytic-endothelial interaction during brain angiogenesis in the chicken. *Developmental Dynamics* 218, 472–479. [https://doi.org/10.1002/1097-0177\(200007\)218:3<472::AID-DVDY1008>3.0.CO;2-#](https://doi.org/10.1002/1097-0177(200007)218:3<472::AID-DVDY1008>3.0.CO;2-#)
- Ginhoux, F., Jung, S., 2014. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol* 14, 392–404. <https://doi.org/10.1038/nri3671>
- Gumbiner, B.M., 2000. Regulation of Cadherin Adhesive Activity. *The Journal of Cell Biology* 148, 399–404. <https://doi.org/10.1083/jcb.148.3.399>
- Harrell, C.R., Simovic Markovic, B., Fellabaum, C., Arsenijevic, A., Djonov, V., Volarevic, V., 2018. Molecular mechanisms underlying therapeutic potential of pericytes. *J Biomed Sci* 25, 21. <https://doi.org/10.1186/s12929-018-0423-7>
- Hayward, C.P., Bainton, D.F., Smith, J.W., Horsewood, P., Stead, R.H., Podor, T.J.,

- Warkentin, T.E., Kelton, J.G., 1993. Multimerin is found in the alpha-granules of resting platelets and is synthesized by a megakaryocytic cell line. *J. Clin. Invest.* 91, 2630–2639. <https://doi.org/10.1172/JCI116502>
- Hayward, C.P.M., Cramer, E.M., Song, Z., Zheng, S., Fung, R., Massé, J.-M., Stead, R.H., Podor, T.J., 1998. Studies of Multimerin in Human Endothelial Cells. *Blood* 91, 1304–1317. <https://doi.org/10.1182/blood.V91.4.1304>
- Hellstrom, M., Kal n, M., Lindahl, P., Abramsson, A., Betsholtz, C., 1999. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* 126, 3047–3055. <https://doi.org/10.1242/dev.126.14.3047>
- Hill, V.K., Hesson, L.B., Dansranjav, T., Dallol, A., Bieche, I., Vacher, S., Tommasi, S., Dobbins, T., Gentle, D., Euhus, D., Lewis, C., Dammann, R., Ward, R.L., Minna, J., Maher, E.R., Pfeifer, G.P., Latif, F., 2010. Identification of 5 novel genes methylated in breast and other epithelial cancers. *Mol Cancer* 9, 51. <https://doi.org/10.1186/1476-4598-9-51>
- Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., Rogers, B., Ross, R., Kabbinavar, F., 2004. Bevacizumab plus Irinotecan, Fluorouracil, and Leucovorin for Metastatic Colorectal Cancer. *N Engl J Med* 350, 2335–2342. <https://doi.org/10.1056/NEJMoa032691>
- Jain, R.K., 2005. Normalization of Tumor Vasculature: An Emerging Concept in Antiangiogenic Therapy. *Science* 307, 58–62. <https://doi.org/10.1126/science.1104819>
- Jain, R.K., 2001. Normalizing tumor vasculature with anti-angiogenic therapy: A new paradigm for combination therapy. *Nat Med* 7, 987–989. <https://doi.org/10.1038/nm0901-987>
- Jain, R.K., 1999. Understanding barriers to drug delivery: high resolution in vivo imaging is key. *Clin Cancer Res* 5, 1605–1606.
- Jain, R.K., Martin, J.D., Stylianopoulos, T., 2014. The Role of Mechanical Forces in Tumor Growth and Therapy. *Annu. Rev. Biomed. Eng.* 16, 321–346. <https://doi.org/10.1146/annurev-bioeng-071813-105259>
- Jeimy, S.B., Fuller, N., Tasneem, S., Segers, K., Stafford, A.R., Weitz, J.I., Camire, R.M., Nicolaes, G.A.F., Hayward, C.P.M., 2008a. Multimerin 1 binds factor V and activated factor V with high affinity and inhibits thrombin generation. *Thromb Haemost* 100, 1058–1067.
- Jeimy, S.B., Tasneem, S., Cramer, E.M., Hayward, C.P.M., 2008b. Multimerin 1. *Platelets* 19, 83–95. <https://doi.org/10.1080/09537100701832157>

- Kai, F., Drain, A.P., Weaver, V.M., 2019. The Extracellular Matrix Modulates the Metastatic Journey. *Developmental Cell* 49, 332–346. <https://doi.org/10.1016/j.devcel.2019.03.026>
- Kalluri, R., 2016. The biology and function of fibroblasts in cancer. *Nat Rev Cancer* 16, 582–598. <https://doi.org/10.1038/nrc.2016.73>
- Kalluri, R., 2003. Basement membranes: structure, assembly and role in tumour angiogenesis. *Nat Rev Cancer* 3, 422–433. <https://doi.org/10.1038/nrc1094>
- Keeratichamroen, S., Subhasitanont, P., Chokchaichamnankit, D., Weeraphan, C., Saharat, K., Sritana, N., Kantathavorn, N., Wiriyaukaradecha, K., Sricharunrat, T., Paricharttanakul, N.M., Auewarakul, C., Svasti, J., Srisomsap, C., 2020. Identification of potential cervical cancer serum biomarkers in Thai patients. *Oncol Lett*. <https://doi.org/10.3892/ol.2020.11519>
- Khan, K.A., McMurray, J.L., Mohammed, F., Bicknell, R., 2019. C-type lectin domain group 14 proteins in vascular biology, cancer and inflammation. *FEBS J* 286, 3299–3332. <https://doi.org/10.1111/febs.14985>
- Khan, K.A., Naylor, A.J., Khan, A., Noy, P.J., Mambretti, M., Lodhia, P., Athwal, J., Korzystka, A., Buckley, C.D., Willcox, B.E., Mohammed, F., Bicknell, R., 2017. Multimerin-2 is a ligand for group 14 family C-type lectins CLEC14A, CD93 and CD248 spanning the endothelial pericyte interface. *Oncogene* 36, 6097–6108. <https://doi.org/10.1038/onc.2017.214>
- Krock, B.L., Skuli, N., Simon, M.C., 2011. Hypoxia-Induced Angiogenesis: Good and Evil. *Genes & Cancer* 2, 1117–1133. <https://doi.org/10.1177/1947601911423654>
- Krüger-Genge, Blocki, Franke, Jung, 2019. Vascular Endothelial Cell Biology: An Update. *IJMS* 20, 4411. <https://doi.org/10.3390/ijms20184411>
- Laklai, H., Miroshnikova, Y.A., Pickup, M.W., Collisson, E.A., Kim, G.E., Barrett, A.S., Hill, R.C., Lakins, J.N., Schlaepfer, D.D., Mouw, J.K., LeBleu, V.S., Roy, N., Novitskiy, S.V., Johansen, J.S., Poli, V., Kalluri, R., Iacobuzio-Donahue, C.A., Wood, L.D., Hebrok, M., Hansen, K., Moses, H.L., Weaver, V.M., 2016. Genotype tunes pancreatic ductal adenocarcinoma tissue tension to induce matricellular fibrosis and tumor progression. *Nat Med* 22, 497–505. <https://doi.org/10.1038/nm.4082>
- Laszlo, G.S., Alonzo, T.A., Gudgeon, C.J., Harrington, K.H., Gerbing, R.B., Wang, Y.-C., Ries, R.E., Raimondi, S.C., Hirsch, B.A., Gamis, A.S., Meshinchi, S., Walter, R.B., 2015. Multimerin-1 (*MMRNI*) as Novel Adverse Marker in Pediatric Acute Myeloid Leukemia: A Report from the Children’s Oncology Group. *Clinical Cancer Research* 21, 3187–3195. <https://doi.org/10.1158/1078-0432.CCR-14-2684>
- LaValley, D.J., Zanutelli, M.R., Bordeleau, F., Wang, W., Schwager, S.C., Reinhart-King,

- C.A., 2017. Matrix stiffness enhances VEGFR-2 internalization, signaling, and proliferation in endothelial cells. *Converg. Sci. Phys. Oncol.* 3, 044001. <https://doi.org/10.1088/2057-1739/aa9263>
- Leatherdale, A., Parker, D., Tasneem, S., Wang, Y., Bihan, D., Bonna, A., Hamaia, S.W., Gross, P.L., Ni, H., Doble, B.W., Lillicrap, D., Farndale, R.W., Hayward, C.P.M., 2021. Multimerin 1 supports platelet function in vivo and binds to specific GPAGPOGPX motifs in fibrillar collagens that enhance platelet adhesion. *Journal of Thrombosis and Haemostasis* 19, 547–561. <https://doi.org/10.1111/jth.15171>
- Leimeister, C., Steidl, C., Schumacher, N., Erhard, S., Gessler, M., 2002. Developmental Expression and Biochemical Characterization of Emu Family Members. *Developmental Biology* 249, 204–218. <https://doi.org/10.1006/dbio.2002.0764>
- Liang, L.-B., Huang, X.-Y., He, H., Liu, J.-Y., 2020. Prognostic Values of Radiosensitivity Genes and CD19 Status in Gastric Cancer: A Retrospective Study Using TCGA Database. *PGPM Volume 13*, 365–373. <https://doi.org/10.2147/PGPM.S265121>
- Lin, Y., Xu, J., Lan, H., 2019. Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications. *J Hematol Oncol* 12, 76. <https://doi.org/10.1186/s13045-019-0760-3>
- Lindahl, P., Johansson, B.R., Levéen, P., Betsholtz, C., 1997. Pericyte Loss and Microaneurysm Formation in PDGF-B-Deficient Mice. *Science* 277, 242–245. <https://doi.org/10.1126/science.277.5323.242>
- Lorenzon, E., Colladel, R., Andreuzzi, E., Marastoni, S., Todaro, F., Schiappacassi, M., Ligresti, G., Colombatti, A., Mongiat, M., 2012. MULTIMERIN2 impairs tumor angiogenesis and growth by interfering with VEGF-A/VEGFR2 pathway. *Oncogene* 31, 3136–3147. <https://doi.org/10.1038/onc.2011.487>
- Lu, P., Takai, K., Weaver, V.M., Werb, Z., 2011. Extracellular Matrix Degradation and Remodeling in Development and Disease. *Cold Spring Harbor Perspectives in Biology* 3, a005058–a005058. <https://doi.org/10.1101/cshperspect.a005058>
- Lugano, R., Ramachandran, M., Dimberg, A., 2020. Tumor angiogenesis: causes, consequences, challenges and opportunities. *Cell. Mol. Life Sci.* 77, 1745–1770. <https://doi.org/10.1007/s00018-019-03351-7>
- Mantovani, A., Allavena, P., Marchesi, F., Garlanda, C., 2022. Macrophages as tools and targets in cancer therapy. *Nat Rev Drug Discov* 21, 799–820. <https://doi.org/10.1038/s41573-022-00520-5>
- Marastoni, S., Andreuzzi, E., Paulitti, A., Colladel, R., Pellicani, R., Todaro, F., Schiavinato,

- A., Bonaldo, P., Colombatti, A., Mongiat, M., 2014. EMILIN2 down-modulates the Wnt signalling pathway and suppresses breast cancer cell growth and migration: EMILIN2 binds Wnt1 impairing tumour progression. *J. Pathol.* 232, 391–404. <https://doi.org/10.1002/path.4316>
- Mathiisen, T.M., Lehre, K.P., Danbolt, N.C., Ottersen, O.P., 2010. The perivascular astroglial sheath provides a complete covering of the brain microvessels: An electron microscopic 3D reconstruction. *Glia* 58, 1094–1103. <https://doi.org/10.1002/glia.20990>
- Mongiat, M., Andreuzzi, E., Tarticchio, G., Paulitti, A., 2016. Extracellular Matrix, a Hard Player in Angiogenesis. *IJMS* 17, 1822. <https://doi.org/10.3390/ijms17111822>
- Mongiat, M., Ligresti, G., Marastoni, S., Lorenzon, E., Doliana, R., Colombatti, A., 2007. Regulation of the Extrinsic Apoptotic Pathway by the Extracellular Matrix Glycoprotein EMILIN2. *Molecular and Cellular Biology* 27, 7176–7187. <https://doi.org/10.1128/MCB.00696-07>
- Morikawa, S., Baluk, P., Kaidoh, T., Haskell, A., Jain, R.K., McDonald, D.M., 2002. Abnormalities in Pericytes on Blood Vessels and Endothelial Sprouts in Tumors. *The American Journal of Pathology* 160, 985–1000. [https://doi.org/10.1016/S0002-9440\(10\)64920-6](https://doi.org/10.1016/S0002-9440(10)64920-6)
- Mouw, J.K., Ou, G., Weaver, V.M., 2014. Extracellular matrix assembly: a multiscale deconstruction. *Nat Rev Mol Cell Biol* 15, 771–785. <https://doi.org/10.1038/nrm3902>
- Munn, L., 2003. Aberrant vascular architecture in tumors and its importance in drug-based therapies. *Drug Discovery Today* 8, 396–403. [https://doi.org/10.1016/S1359-6446\(03\)02686-2](https://doi.org/10.1016/S1359-6446(03)02686-2)
- Naba, A., Clauser, K.R., Ding, H., Whittaker, C.A., Carr, S.A., Hynes, R.O., 2016. The extracellular matrix: Tools and insights for the “omics” era. *Matrix Biology* 49, 10–24. <https://doi.org/10.1016/j.matbio.2015.06.003>
- Naba, A., Clauser, K.R., Hoersch, S., Liu, H., Carr, S.A., Hynes, R.O., 2012a. The Matrisome: In Silico Definition and In Vivo Characterization by Proteomics of Normal and Tumor Extracellular Matrices. *Molecular & Cellular Proteomics* 11, M111.014647. <https://doi.org/10.1074/mcp.M111.014647>
- Naba, A., Clauser, K.R., Hoersch, S., Liu, H., Carr, S.A., Hynes, R.O., 2012b. The Matrisome: In Silico Definition and In Vivo Characterization by Proteomics of Normal and Tumor Extracellular Matrices. *Molecular & Cellular Proteomics* 11, M111.014647. <https://doi.org/10.1074/mcp.M111.014647>
- Nitta, T., Hata, M., Gotoh, S., Seo, Y., Sasaki, H., Hashimoto, N., Furuse, M., Tsukita, S.,

2003. Size-selective loosening of the blood-brain barrier in claudin-5–deficient mice. *The Journal of Cell Biology* 161, 653–660. <https://doi.org/10.1083/jcb.200302070>
- Öhlund, D., Franklin, O., Lundberg, E., Lundin, C., Sund, M., 2013. Type IV collagen stimulates pancreatic cancer cell proliferation, migration, and inhibits apoptosis through an autocrine loop. *BMC Cancer* 13, 154. <https://doi.org/10.1186/1471-2407-13-154>
- Paulitti, A., Andreuzzi, E., Bizzotto, D., Pellicani, R., Tarticchio, G., Marastoni, S., Pastrello, C., Jurisica, I., Ligresti, G., Bucciotti, F., Doliana, R., Colladel, R., Braghetta, P., Poletto, E., Di Silvestre, A., Bressan, G., Colombatti, A., Bonaldo, P., Mongiat, M., 2018. The ablation of the matricellular protein EMILIN2 causes defective vascularization due to impaired EGFR-dependent IL-8 production affecting tumor growth. *Oncogene* 37, 3399–3414. <https://doi.org/10.1038/s41388-017-0107-x>
- Payne, L.B., Darden, J., Suarez-Martinez, A.D., Zhao, H., Hendricks, A., Hartland, C., Chong, D., Kushner, E.J., Murfee, W.L., Chappell, J.C., 2021. Pericyte migration and proliferation are tightly synchronized to endothelial cell sprouting dynamics. *Integrative Biology* 13, 31–43. <https://doi.org/10.1093/intbio/zyaa027>
- Pellicani, R., Poletto, E., Andreuzzi, E., Paulitti, A., Doliana, R., Bizzotto, D., Braghetta, P., Colladel, R., Tarticchio, G., Sabatelli, P., Bucciotti, F., Bressan, G., Iozzo, R.V., Colombatti, A., Bonaldo, P., Mongiat, M., 2020. Multimerin-2 maintains vascular stability and permeability. *Matrix Biology* 87, 11–25. <https://doi.org/10.1016/j.matbio.2019.08.002>
- Petrova, T.V., Karpanen, T., Norrmén, C., Mellor, R., Tamakoshi, T., Finegold, D., Ferrell, R., Kerjaschki, D., Mortimer, P., Ylä-Herttuala, S., Miura, N., Alitalo, K., 2004. Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis. *Nat Med* 10, 974–981. <https://doi.org/10.1038/nm1094>
- Posner, M.G., 2022. Multimerin-1 and cancer: a review. *Bioscience Reports* 42, BSR20211248. <https://doi.org/10.1042/BSR20211248>
- Potente, M., Carmeliet, P., 2017. The Link Between Angiogenesis and Endothelial Metabolism. *Annu. Rev. Physiol.* 79, 43–66. <https://doi.org/10.1146/annurev-physiol-021115-105134>
- Potente, M., Gerhardt, H., Carmeliet, P., 2011. Basic and Therapeutic Aspects of Angiogenesis. *Cell* 146, 873–887. <https://doi.org/10.1016/j.cell.2011.08.039>
- Qi, L., Chen, J., Yang, Y., Hu, W., 2020. Hypoxia Correlates With Poor Survival and M2 Macrophage Infiltration in Colorectal Cancer. *Front. Oncol.* 10, 566430. <https://doi.org/10.3389/fonc.2020.566430>
- Qiu, S.-Q., Waaijer, S.J.H., Zwager, M.C., De Vries, E.G.E., Van Der Vegt, B., Schröder,

- C.P., 2018. Tumor-associated macrophages in breast cancer: Innocent bystander or important player? *Cancer Treatment Reviews* 70, 178–189. <https://doi.org/10.1016/j.ctrv.2018.08.010>
- Ramakrishnan, S., Anand, V., Roy, S., 2014. Vascular Endothelial Growth Factor Signaling in Hypoxia and Inflammation. *J Neuroimmune Pharmacol* 9, 142–160. <https://doi.org/10.1007/s11481-014-9531-7>
- Ribatti, D., Annese, T., Ruggieri, S., Tamma, R., Crivellato, E., 2019. Limitations of Anti-Angiogenic Treatment of Tumors. *Translational Oncology* 12, 981–986. <https://doi.org/10.1016/j.tranon.2019.04.022>
- Riedemann, J., Macaulay, V.M., 2006. IGF1R signalling and its inhibition. *Endocr Relat Cancer* 13, S33–S43. <https://doi.org/10.1677/erc.1.01280>
- Rini, B.I., Halabi, S., Rosenberg, J.E., Stadler, W.M., Vaena, D.A., Ou, S.-S., Archer, L., Atkins, J.N., Picus, J., Czaykowski, P., Dutcher, J., Small, E.J., 2008. Bevacizumab Plus Interferon Alfa Compared With Interferon Alfa Monotherapy in Patients With Metastatic Renal Cell Carcinoma: CALGB 90206. *JCO* 26, 5422–5428. <https://doi.org/10.1200/JCO.2008.16.9847>
- Risau, W., 1997. Mechanisms of angiogenesis. *Nature* 386, 671–674. <https://doi.org/10.1038/386671a0>
- Röszer, T., 2015. Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators of Inflammation* 2015, 1–16. <https://doi.org/10.1155/2015/816460>
- Rucker, H.K., Wynder, H.J., Thomas, W.E., 2000. Cellular mechanisms of CNS pericytes. *Brain Research Bulletin* 51, 363–369. [https://doi.org/10.1016/S0361-9230\(99\)00260-9](https://doi.org/10.1016/S0361-9230(99)00260-9)
- Sanz-Moncasi, M.P., Garin-Chesa, P., Stockert, E., Jaffe, E.A., Old, L.J., Rettig, W.J., 1994. Identification of a high molecular weight endothelial cell surface glycoprotein, endoGlyx-1, in normal and tumor blood vessels. *Lab Invest* 71, 366–373.
- Schaaf, M.B., Garg, A.D., Agostinis, P., 2018. Defining the role of the tumor vasculature in antitumor immunity and immunotherapy. *Cell Death Dis* 9, 115. <https://doi.org/10.1038/s41419-017-0061-0>
- Sherwood, L.M., Parris, E.E., Folkman, J., 1971a. Tumor Angiogenesis: Therapeutic Implications. *N Engl J Med* 285, 1182–1186. <https://doi.org/10.1056/NEJM197111182852108>
- Sherwood, L.M., Parris, E.E., Folkman, J., 1971b. Tumor Angiogenesis: Therapeutic Implications. *N Engl J Med* 285, 1182–1186.

<https://doi.org/10.1056/NEJM197111182852108>

Shibutani, M., Maeda, K., Nagahara, H., Fukuoka, T., Nakao, S., Matsutani, S., Hirakawa, K., Ohira, M., 2017. The peripheral monocyte count is associated with the density of tumor-associated macrophages in the tumor microenvironment of colorectal cancer: a retrospective study. *BMC Cancer* 17, 404. <https://doi.org/10.1186/s12885-017-3395-1>

Shuang, T., Fu, M., Yang, G., Wu, L., Wang, R., 2018. The interaction of IGF-1/IGF-1R and hydrogen sulfide on the proliferation of mouse primary vascular smooth muscle cells. *Biochemical Pharmacology* 149, 143–152. <https://doi.org/10.1016/j.bcp.2017.12.009>

Sims, D.E., 1986. The pericyte—A review. *Tissue and Cell* 18, 153–174. [https://doi.org/10.1016/0040-8166\(86\)90026-1](https://doi.org/10.1016/0040-8166(86)90026-1)

Socovich, A.M., Naba, A., 2019. The cancer matrisome: From comprehensive characterization to biomarker discovery. *Seminars in Cell & Developmental Biology* 89, 157–166. <https://doi.org/10.1016/j.semcdb.2018.06.005>

Stark, K., Eckart, A., Haidari, S., Tirniceriu, A., Lorenz, M., Von Brühl, M.-L., Gärtner, F., Khandoga, A.G., Legate, K.R., Pless, R., Hepper, I., Lauber, K., Walzog, B., Massberg, S., 2013. Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and “instruct” them with pattern-recognition and motility programs. *Nat Immunol* 14, 41–51. <https://doi.org/10.1038/ni.2477>

Stifano, G., Christmann, R.B., 2016. Macrophage Involvement in Systemic Sclerosis: Do We Need More Evidence? *Curr Rheumatol Rep* 18, 2. <https://doi.org/10.1007/s11926-015-0554-8>

Stöger, J.L., Gijbels, M.J.J., Van Der Velden, S., Manca, M., Van Der Loos, C.M., Biessen, E.A.L., Daemen, M.J.A.P., Lutgens, E., De Winther, M.P.J., 2012. Distribution of macrophage polarization markers in human atherosclerosis. *Atherosclerosis* 225, 461–468. <https://doi.org/10.1016/j.atherosclerosis.2012.09.013>

Stratman, A.N., Schwindt, A.E., Malotte, K.M., Davis, G.E., 2010. Endothelial-derived PDGF-BB and HB-EGF coordinately regulate pericyte recruitment during vasculogenic tube assembly and stabilization. *Blood* 116, 4720–4730. <https://doi.org/10.1182/blood-2010-05-286872>

Su, H., Cantrell, A.C., Zeng, H., Zhu, S.-H., Chen, J.-X., 2021. Emerging Role of Pericytes and Their Secretome in the Heart. *Cells* 10, 548. <https://doi.org/10.3390/cells10030548>

Suchting, S., Eichmann, A., 2009. Jagged Gives Endothelial Tip Cells an Edge. *Cell* 137, 988–990. <https://doi.org/10.1016/j.cell.2009.05.024>

Sugihara, K., Sasaki, S., Uemura, A., Kidoaki, S., Miura, T., 2020. Mechanisms of

- endothelial cell coverage by pericytes: computational modelling of cell wrapping and *in vitro* experiments. *J. R. Soc. Interface.* 17, 20190739. <https://doi.org/10.1098/rsif.2019.0739>
- Takada, Y., Takada, Y.K., Fujita, M., 2017. Crosstalk between insulin-like growth factor (IGF) receptor and integrins through direct integrin binding to IGF1. *Cytokine & Growth Factor Reviews* 34, 67–72. <https://doi.org/10.1016/j.cytogfr.2017.01.003>
- Thomas, M., Augustin, H.G., 2009. The role of the Angiopoietins in vascular morphogenesis. *Angiogenesis* 12, 125–137. <https://doi.org/10.1007/s10456-009-9147-3>
- Tomkowicz, B., Rybinski, K., Sebeck, D., Sass, P., Nicolaides, N.C., Grasso, L., Zhou, Y., 2010. Endosialin/TEM-1/CD248 regulates pericyte proliferation through PDGF receptor signaling. *Cancer Biology & Therapy* 9, 908–915. <https://doi.org/10.4161/cbt.9.11.11731>
- Török, O., Schreiner, B., Schaffenrath, J., Tsai, H.-C., Maheshwari, U., Stifter, S.A., Welsh, C., Amorim, A., Sridhar, S., Utz, S.G., Mildenerger, W., Nassiri, S., Delorenzi, M., Aguzzi, A., Han, M.H., Greter, M., Becher, B., Keller, A., 2021. Pericytes regulate vascular immune homeostasis in the CNS. *Proc. Natl. Acad. Sci. U.S.A.* 118, e2016587118. <https://doi.org/10.1073/pnas.2016587118>
- Uhlén, M., Fagerberg, L., Hallström, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szgyarto, C.A.-K., Odeberg, J., Djureinovic, D., Takanen, J.O., Hober, S., Alm, T., Edqvist, P.-H., Berling, H., Tegel, H., Mulder, J., Rockberg, J., Nilsson, P., Schwenk, J.M., Hamsten, M., Von Feilitzen, K., Forsberg, M., Persson, L., Johansson, F., Zwahlen, M., Von Heijne, G., Nielsen, J., Pontén, F., 2015. Tissue-based map of the human proteome. *Science* 347, 1260419. <https://doi.org/10.1126/science.1260419>
- Vestweber, D., Wessel, F., Nottebaum, A.F., 2014. Similarities and differences in the regulation of leukocyte extravasation and vascular permeability. *Semin Immunopathol* 36, 177–192. <https://doi.org/10.1007/s00281-014-0419-7>
- Vong, S., Kalluri, R., 2011. The Role of Stromal Myofibroblast and Extracellular Matrix in Tumor Angiogenesis. *Genes & Cancer* 2, 1139–1145. <https://doi.org/10.1177/1947601911423940>
- Vontell, D., Armulik, A., Betsholtz, C., 2006. Pericytes and vascular stability. *Experimental Cell Research* 312, 623–629. <https://doi.org/10.1016/j.yexcr.2005.10.019>
- Wang, L., Geng, H., Liu, Y., Liu, L., Chen, Y., Wu, F., Liu, Z., Ling, S., Wang, Y., Zhou, L., 2023. Hot and cold tumors: Immunological features and the therapeutic strategies. *MedComm* 4, e343. <https://doi.org/10.1002/mco2.343>
- Wang, X.L., Jiang, J.T., Wu, C.P., 2016. Prognostic significance of tumor-associated

- macrophage infiltration in gastric cancer: a meta-analysis. *Genet. Mol. Res.* 15. <https://doi.org/10.4238/gmr15049040>
- Wu, P., Wu, D., Zhao, L., Huang, L., Chen, G., Shen, G., Huang, J., Chai, Y., 2016. Inverse role of distinct subsets and distribution of macrophage in lung cancer prognosis: a meta-analysis. *Oncotarget* 7, 40451–40460. <https://doi.org/10.18632/oncotarget.9625>
- Xiong, G.-F., Xu, R., 2016. Function of cancer cell-derived extracellular matrix in tumor progression. *JCMT* 2, 357. <https://doi.org/10.20517/2394-4722.2016.08>
- Xu, N., Tang, X.-H., Pan, W., Xie, Z.-M., Zhang, G.-F., Ji, M.-H., Yang, J.-J., Zhou, M.-T., Zhou, Z.-Q., 2017. Spared Nerve Injury Increases the Expression of Microglia M1 Markers in the Prefrontal Cortex of Rats and Provokes Depression-Like Behaviors. *Front. Neurosci.* 11. <https://doi.org/10.3389/fnins.2017.00209>
- Yoshiji, H., Gomez, D.E., Shibuya, M., Thorgeirsson, U.P., 1996. Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. *Cancer Res* 56, 2013–2016.
- Yu, M., Guan, R., Hong, W., Zhou, Y., Lin, Y., Jin, H., Hou, B., Jian, Z., 2019. Prognostic value of tumor-associated macrophages in pancreatic cancer: a meta-analysis. *CMAR* Volume 11, 4041–4058. <https://doi.org/10.2147/CMAR.S196951>
- Zacchigna, L., Vecchione, C., Notte, A., Cordenonsi, M., Dupont, S., Maretto, S., Cifelli, G., Ferrari, A., Maffei, A., Fabbro, C., Braghetta, P., Marino, G., Selvetella, G., Aretini, A., Colonnese, C., Bettarini, U., Russo, G., Soligo, S., Adorno, M., Bonaldo, P., Volpin, D., Piccolo, S., Lembo, G., Bressan, G.M., 2006. Emilin1 Links TGF- β Maturation to Blood Pressure Homeostasis. *Cell* 124, 929–942. <https://doi.org/10.1016/j.cell.2005.12.035>
- Zanetti, M., Braghetta, P., Sabatelli, P., Mura, I., Doliana, R., Colombatti, A., Volpin, D., Bonaldo, P., Bressan, G.M., 2004. EMILIN-1 Deficiency Induces Elastogenesis and Vascular Cell Defects. *Molecular and Cellular Biology* 24, 638–650. <https://doi.org/10.1128/MCB.24.2.638-650.2004>
- Zhao, X., Qu, J., Sun, Y., Wang, J., Liu, X., Wang, F., Zhang, H., Wang, W., Ma, X., Gao, X., Zhang, S., 2017. Prognostic significance of tumor-associated macrophages in breast cancer: a meta-analysis of the literature. *Oncotarget* 8, 30576–30586. <https://doi.org/10.18632/oncotarget.15736>
- Zhao, Y., Ting, K.K., Coleman, P., Qi, Y., Chen, J., Vadas, M., Gamble, J., 2021. The Tumour Vasculature as a Target to Modulate Leucocyte Trafficking. *Cancers* 13, 1724. <https://doi.org/10.3390/cancers13071724>

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