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EMILIN-2 as a regulator of vascular efficiency

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1 ABSTRACT

Angiogenesis, the formation of the new blood vessels from pre-existing vasculature, is an important process occurring under normal as well as pathological conditions, such as cancer. This complex process is regulated by several cytokines, growth factors and by extracellular matrix components, in particular through the modulation of endothelial cell and pericyte function. In addition, the immune environment shapes the formation and remodeling of tumor associated vessels, despite the players in the interconnection between angiogenesis and inflammation are not completely unveiled. In this contest, we found that the extracellular matrix glycoprotein EMILIN-2 displays a prominent role. This study was prompted by the observations that tumor-associated vessels from *Emilin-2*$^{-/-}$ mice displayed lower pericyte coverage and were characterized by impaired vascular perfusion and reduced drug efficacy. These evidences suggested that EMILIN-2 could promote vessel maturation and stabilization affecting pericyte recruitment. In agreement with our hypothesis, we demonstrated that EMILIN-2 serves as an adhesion substrate and haptotactic stimulus for pericytes. These two functions rely on the engagement of integrin $\alpha_5\beta_1$ and $\alpha_6\beta_1$, highly expressed by pericytes. In addition, endothelial cells challenged with EMILIN-2 displayed higher production of PDGF-BB and HB-EGF, two key cytokines promoting pericyte recruitment. Furthermore, we found that EMILIN-2 contributes to vascular stabilization fostering the interconnection between endothelial cells and pericytes through the increase N-cadherin expression via the sphingosine-1-phosphate receptor as well as favoring the deposition of collagen type IV, the major basement membrane component. Finally, we provide evidences that EMILIN-2 may represent a molecular bridge in the interconnection between the angiogenesis and the immune response. Here we found that EMILIN-2 loss associates with increased PD-L1 expression which led to improved immunotherapy efficacy. Importantly, the immune checkpoint inhibition restored vascular stability thus resulting in decreased tumor hypoxia.

Taking into account the roles of EMILIN-2 reported in this study and the variable expression of the molecule in the tumor microenvironment, we envision that the analysis of EMILIN-2 expression may serve to predict the efficacy of cancer therapy. Hence, patients with high EMILIN-2 expression are characterized by a more stable vasculature and may better profit from the treatments due to improved drug delivery. On the other hand, patients displaying low EMILIN-2 levels not only would respond better to immunotherapy due to higher PD-L1 expression, but they would also benefit from the positive impact of the therapy in promoting vascular stability.

2 INTRODUCTION

2.1 Angiogenesis

The blood vessel network which consists of arteries, capillaries and veins enables the delivery of the oxygen and nutrients, as well as the removal of metabolic waste from all body tissues. Not surprisingly, the abnormalities that occur in this system lead to the onset of a number of diseases.

The cardiovascular system is the first functional organ system to develop in the vertebrate embryo and several processes take part in its development. In fact, blood vessels arise through two mechanisms namely: vasculogenesis and angiogenesis (Risau, 1997). Vasculogenesis refers to the *de novo* development of the blood vessels during the embryonic life, through the differentiation of endothelial precursor cells from the mesoderm. Whereas, the term angiogenesis applies to the biological process by which new vessels are formed from the pre-existing ones, and it is essential in many physiological (embryo development, ovulation and wound healing) and pathological conditions such as arthritis, diabetic retinopathy, and cancer (Risau, 1994).

During Angiogenesis, the primitive capillary plexus formed during vasculogenesis is remodeled and expanded through sprouting or intussusception of pre-existing vessels (Carmeliet, 2003). This complex process requires the interaction between different cell types, several cytokines and growth factors, and the extracellular matrix (ECM).

2.1.1 Endothelial Cells, Pericytes and their interplay

As the angiogenic process takes place, the vessels' lumen is covered by the endothelium, a continuous monolayer constituted by quiescent endothelial cells (ECs), which represents the inner layer of the vascular wall and establishes a barrier between the blood and the tissues.

In fact, ECs control the passage of fluids, substances, and also immune cells in and out of the bloodstream. Indeed, the permeability of the endothelium, which is tightly regulated by a plethora of molecular cues, significantly impacts not only immune cell, but also cancer cell extravasation (Claesson-Welsh, 2015). The regulation of vascular permeability mainly occurs through the modulation of the endothelial cell-cell junctions (Dejana, 2004; Matter & Balda, 2003; Potente et al., 2011)

In addition, the newly formed vessels require to be enwrapped by other cell types, which provide both physical and chemical support to ECs and are thus named periendothelial cells or

mural cells. Without a proper reinforcement by these cells, the vessels would be leaky, hyperdilated, and dysfunctional (Benjamin et al., 1998; Bergers & Song, 2005; Burri & Djonov, 2002). Periendothelial cells are characterized by different functionalities and can be identified by different molecular markers. While Smooth Muscle Cells (SMCs) are thought to provide mechanical cues to the vessels, pericytes directly interact and communicate with ECs (Armulik et al., 2005; Bergers & Song, 2005).

Indeed, pericytes exert an important role on EC proliferation, migration and stabilization and ECs stimulate expansion and activation of the pericytes' precursor cell population. The communication between the two cell types is tightly controlled by a series of signaling pathways operating in an autocrine and/or paracrine manner, and it is mediated by several molecules (Figure 1) (Ribatti et al., 2011).

The major regulators of the crosstalk between ECs and pericytes are the platelet-derived growth factors (PDGFs), a family of growth factors which drive cellular responses including proliferation, survival, migration and ECM deposition (Hoch & Soriano, 2003; Ross & Vogel, 1978). Among all the family members, ECs secrete PDGF-BB whereas pericytes express the platelet-derived growth factor receptor β (PDGFR-β), mediating the paracrine interaction between the two cell types (Betsholtz et al., 2001). The importance of these cytokines is highlighted by the fact that PDGF-BB- or PDGFR-β-depleted mice die during embryonic development due to microvascular defects characterized by drastically reduced pericyte coverage (Betsholtz, 2004).

Similarly, the heparin-binding EGF-like growth factor (HB-EGF) is crucial for pericyte motility, proliferation, and recruitment along the vessels. Stratman and colleagues demonstrated that the blockage of PDGF-BB and HB-EGF or their receptors compromised the pericyte coverage of the vessel tubes formed in a 3D matrix. Moreover, the same authors showed that both PDGF-BB and HB-EGF equally contribute to pericyte recruitment and vascular basement membrane (BM) assembly also *in vivo* (Stratman et al., 2010).

On the other side, the vascular endothelial growth factor receptor 2 (VEGFR2), a tyrosine kinase receptor expressed by the endothelium, is a strong inducer of EC proliferation, migration, permeability, and survival. VEGFR2 signals through the vascular endothelial growth factor A (VEGF-A), secreted as a soluble cytokine, which causes the autophosphorylation of the receptor (Watanabe et al., 1997).

The angiopoietin (Ang) family of growth factors plays an important role in endothelial sprouting, vessel wall remodeling and mural cell recruitment. In particular Ang-1 and Ang-2

display antagonistic effects through the engagement of the tyrosine kinase receptor Tie-2 expressed by ECs (Paik et al., 2004). Apart from the cytokines and receptors, cell adherence junctions play a prominent role in the interaction between the two cells. While VE-cadherin is exclusively expressed by ECs and maintains the EC-EC connection, N-cadherin is expressed by ECs and pericytes and highly contributes in the vessel maturation and stability (Angulo-Urarte et al., 2020; Bazzoni & Dejana, 2004).

2.1.2 Sprouting angiogenesis

In 1977, Ausprunk and Folkmann were the first to report that once there is a proangiogenic signal, the process of angiogenesis will take place and will disclose in few steps, leading to the establishment of a new and functionalized vasculature (Ausprunk & Folkman, 1977).

Sprouting angiogenesis is initiated in poorly perfused tissues, when oxygen sensing mechanisms detect hypoxia levels, thereby demanding the formation of new blood vessels to satisfy the metabolic requirements of the cells. Most types of parenchymal cells (myocytes, hepatocytes, neurons, astrocytes, etc.) respond to hypoxic environments by secreting the key proangiogenic growth factor VEGF-A (Gerhardt et al., 2003). The basic steps of sprouting angiogenesis include enzymatic degradation of capillary BM, EC proliferation, directed migration of ECs, tubuligenesis (EC tube formation), vessel fusion, vessel pruning, and pericyte stabilization (Figure 2).

As a first step, the proteolytic degradation of BM and the detachment of pericytes are essential for ECs liberation and sprouting. The BM degradation is mediated by the matrix metalloproteases (MMPs), mostly MMP1 (Srivastava et al., 2007). MMPs are involved also in the degradation of the surrounding matrix to ensure an easy branching of the growing vasculature, and this also leads to the release of the various angiogenic factors sequestered by ECM molecules, such as collagens (Ausprunk & Folkman, 1977). Upon proangiogenic stimuli the consequential detachment of the pericytes is facilitated by the activity of Ang-2 secreted by ECs (Eble & Niland, 2009; Huang et al., 2010).

Second, the loss of intercellular connections is required to allow ECs to migrate toward the angiogenic stimuli. ECs deputed to initiate new sprouts are named tip cells, and release MMPs to build the migratory path. In a rear position along the sprouting vessel, reside the stalk cells committed to proliferation in support of the sprout elongation (Augustin et al., 2009). The differentiation of EC into tip and stalk cells, as well as the establishment of the two distinct

phenotypes, are controlled by the interplay between the Notch and the VEGF-A/VEGFR2 signaling pathways (Eilken & Adams, 2010; Gerhardt et al., 2003; Geudens & Gerhardt, 2011). The stabilization, functionalization and maturation of the newly formed vessels occurs upon the recruitment of pericytes towards the PDGF-BB gradient provided by ECs (Phng & Gerhardt, 2009) and the deposition of the ECM constituting the BM.

Lastly, the oxygen and nutrients supply ensured by the onset of the blood flow within the vessel lumen, as well as the activation of the shear stress-responsive transcriptional factor Krüppel-like factor 2 (KLF2) prevent ECs from apoptosis and vessel regression leading to ECs quiescence (K et al., 2009).

Despite the fact that the key mechanisms and molecular players involved in angiogenesis are still being investigated, the major regulators of vessels sprouting, which include a substantial number of growth factors, have been well explored and established. For the development of a normal vasculature, it is of great importance that the balance between pro- and anti-angiogenic growth factors and proteins is maintained. Unfortunately, in diseases such as cancer this equilibrium is lost.

2.2 Tumor microenvironment

Tumor formation and progression is caused by two factors; First, the genetic and epigenetic modifications occurring in normal cells, which are responsible for the neoplastic transformation. Second, the intrinsic characteristics of the various components of tumor microenvironment (TME), as well as their modifications occurring during tumor progression, exert a profound influence on the tumor cell fate (Baghban et al., 2020). In fact, the TME represents the ecosystem of the tumor. Apart from cancer cells, the TME encompasses tumor-associated stromal cells including fibroblasts, ECs and immune cells, as well as non-cellular components such as growth factors and ECM components like collagen, fibronectin, hyaluronan, laminin, among others (Jahanban-Esfahlan et al., 2017)

Tumor cells guide all the cellular and non-cellular components into complex modifications and signaling in order to self-promote their restless growth and aggressiveness. The crosstalk between tumor cells and the TME not only fosters tumor progression and metastasis formation (Hanahan & Coussens, 2012), but can also contribute to drug-resistance. Despite the established importance of the TME in cancer development, much work is needed to better

discern the role of the different components and, hopefully, identify new tools targeting the complex crosstalk between cancer cells, host cells and the non-cellular components. Many studies have been directed to a better understanding of the role of angiogenesis and ECM remodeling in cancer progression (Dvorak, 2009; Folkman, 1974). However further studies are needed to better unveil the molecular mechanisms and the function of less studied components.

2.2.1 Tumor angiogenesis

Similar to normal tissues, tumors require to be nourished with nutrients and oxygen and to be freed from the metabolic waste. These needs are met through the production of pro-angiogenic molecules that stimulate the formation of new vessels mostly by the process of angiogenesis. Hence, tumor angiogenesis represents a pivotal step in tumor progression and is indeed considered as a hallmark of cancer (Hanahan & Weinberg, 2011). The difference between normal and tumor angiogenesis stands in the fact that vascular quiescence in physiological conditions is maintained by well-controlled balance of angiogenic inhibitors and stimuli. While in tumors, there is a shift of the relative balance between angiogenic and anti-angiogenic factors leading to aberrant vessel formation.

Spontaneously arising tumors are not vascularized and in this phase cancer cells survive by a simple diffusion of nutrients and oxygen from the surrounding tissues. When the tumor exceeds the size of 1-2mm^3, diffusion is not sufficient to nourish the core of the tumor and the hypoxic conditions lead to the so called "angiogenic switch", where tumor cells activate key mechanisms to promote vessel formation (Carmeliet et al., 1998). This event is chiefly fired up by the hypoxia-inducible factor (HIF)-1, which in turn up-regulates the expression of pro-angiogenic genes to restore the oxygen homeostasis (Semenza, 2010). In addition, oncogene activation in cancer cells stimulates the expression of several angiogenic factors, such as VEGF-A, PDGF-BB, and fibroblast growth factor 2 (FGF2) (Carmeliet, 2005). It must be pointed out that, under these conditions, the vascularization is poorly controlled and balanced, being characterized by an excess of pro-angiogenic molecules; this leads to the formation of an aberrant vasculature exhibiting structural and functional defects. These peculiar features are summarized in Figure 3.

As opposed to the normal vasculature, tumor-associated vessels are tortuous and display irregular branches and enlarged lumen. ECs fail to stay tightly anchored to one another, they

stack upon each other and give rise to a disrupted endothelium (Baluk et al., 2005). Also pericytes display an abnormal shape; they are loosely attached to the endothelium and they are fewer compared to normal vessels (Ozawa et al., 2005). Furthermore, the BM, which as previously mentioned, provides structural stability to the vessels, is characterized by an aberrant composition and distribution with a detrimental consequence for the interconnection between ECs and pericytes (Baluk et al., 2003). All these features cause an increase of vascular permeability which results in extensive leakage. This condition is unfavorable for therapy efficacy since drug delivery to the tumor is significantly impaired. Furthermore, the vascular inefficiency leads to increased hypoxia, which, in turn, favors the selection of tumors cells displaying a more aggressive phenotype. As a consequence, these cells can easily extravasate through the loose endothelium and reach the blood flow to establish metastasis at distant organs, thus leading to tumor progression (Goel et al., 2011).

2.2.1.1 Anti-angiogenic therapy and vessel normalization

Dr. Judah Folkman, one of the first researches to hypothesize that the tumor-associated vasculature could represent a valuable therapeutic target, in 1971 wrote: “If a tumor could be held indefinitely in the non-vascularized dormant state…it is possible that metastases will not arise.”

Since this groundbreaking hypothesis, a lot of effort was put forward to identify factors involved in angiogenesis. Dr. Ferrara and Henzel in 1989, were the first to identify and isolate the endothelial cell-specific mitogen VEGF. Although there are several related genes belonging to the VEGF family, including VEGF-B, VEGF-C, and placental growth factor (PlGF), most of the attention has been focused on VEGF-A, whose high expression has been reported to be a common feature in diverse cancers, and to correlate with tumor progression, vascular density, invasiveness, metastasis and recurrence (Apte et al., 2019). Based on these findings, VEGF-A was promptly considered as a suitable target for anti-angiogenic therapies. In this attempt, in Dr. Ferrara’s lab, an anti-VEGF-A neutralizing monoclonal antibody was developed and subsequently demonstrated to reduce tumor angiogenesis and tumor growth *in vivo (Kim et al., 1993)*. Ten years later, this humanized antibody, named bevacizumab, was approved for the treatment of metastatic colon cancer patients in combination with chemotherapy (Hurwitz et al., 2004). Currently, bevacizumab is applicable in several cancer types such as colorectal cancer, non-small cell lung cancer, glioblastoma, ovarian, and renal cancer. In the meanwhile,

a number of other drugs targeting the VEGF-A/VEGFR2 signaling pathway have been approved for cancer therapy (Zirlik & Duyster, 2018, p.).

However, the anti-angiogenic therapy deluded the expectations, with a modest increase in progression-free survival and no impact on the overall survival of the patients. This might be in part due to the fact that vascular disruption drastically reduces the blood flow leading to intratumoral hypoxia, which in turn, favors the development of more aggressive tumor phenotypes and metastatic spreading (Jain, 2003). Moreover, vascular disruption also significantly dampens the delivery of chemotherapy drugs to the tumor (Goel et al., 2011). To overcome this drawback, researchers have proposed on one side to reduce the vascularization of the tumors avoiding an extensive disruption, and on the other side, 'normalize' the remaining vessels. To simultaneously reduce the vasculature and readdress the vessels towards a normal phenotype would indeed lead to multiple effects. First, vascular pruning would lead to reduced tumor growth. Second, the normalized, more efficient vessels would alleviate tumor hypoxia avoiding the detrimental effects associated with this condition. Third, vascular normalization would also lead to improved drug delivery to the tumors, thus ameliorating the chemotherapy efficacy (Jain, 2001, 2005). Different strategies have been proposed to achieve this goal, including the tightening of the EC barrier through the inhibition of the hyperglycolytic metabolism and/or the promotion of pericyte recruitment (Cantelmo et al., 2016).

However, much work is needed to better understand the mechanisms regulating vascular stability and normalization and to develop new biomarkers involved in vascular stability that would be helpful stratifying the patients who might better benefit from anti-angiogenic and conventional chemotherapies (Figure 4).

2.2.2 Intersection between the tumor immune environment and angiogenesis

As previously mentioned, the TME also comprises a plethora of immune cells such as T cells, Tregs, Bregs, NK cells, neutrophils, and macrophages, which are recruited to the tumor site with the aim of eradicating the neoplastic cells. However, malignant cells are able not only to put in place strategic approaches in order to escape from the immune cell recognition and survive, but also shape the immune cells to respond to their needs, contributing to a large extent to cancer progression. Indeed, tumor cells establish two strategies to escape from immune cells. On one side, they promote the accumulation of immunosuppressive cells (Tregs and Bregs),

which release cytokines that suppress the T-lymphocyte activity. On the other side, they turn on the expression of immunosuppressive molecules, such as programmed death-ligand 1/programmed death-1 (PD-L1/PD-1), galectin-9/TIM-3, and CTLA-4, which, in turn, inactivate T-lymphocytes (Campbell & Koch, 2011; Jiang et al., 2019). Furthermore, the role of the immune environment in promoting tumor progression is highlighted by numerous studies showing that tumor cells are not the sole culprits in supporting. Indeed, also immune cells are educated by tumor cells to produce numerous angiogenic factors such as VEGF-A, TNF-alpha and IL-8 which in turn stimulate the development of blood vessels in support of the growing tumor (Murdoch et al., 2008; Owen & Mohamadzadeh, 2013; Riboldi et al., 2005; Yang et al., 2004).

However, the interaction is reciprocal, and the VEGF-driven angiogenesis can contribute to immune suppression in many ways. On one hand, VEGF-A was shown to inhibit the proliferation of T-cells (Ohm et al., 2003), and on the other hand to induce T-cell exhaustion by stimulating the expression of PD-L1, CTLA-4, TIM-3, and LAG3 (Ohm & Carbone, 2001). Furthermore, besides suppressing the function of anti-tumor immune cells, VEGF-A augments the expansion of immunosuppressive cells (Varney et al., 2005; Wada et al., 2009). Indeed, the use of bevacizumab was proven effective in restoring all the immune-suppressive effects of VEGF-A (Kusmartsev et al., 2008; Osada et al., 2008; Terme et al., 2013).

This well-orchestrated interaction between the inflammatory infiltrate and angiogenesis prompted the idea to target these two hallmarks of cancer simultaneously, with the aim to improve the efficacy of the treatments and to reduce the intrinsic and acquired resistance. Indeed, the combination of the anti-angiogenic and immunotherapies such as the use of bevacizumab with ipilimumab has given promising results and other combinations are still ongoing in clinical trials (M. Yi et al., 2019)

2.3 Extracellular Matrix (ECM)

The ECM is a three-dimensional, non-cellular structure which is present in all tissues and organs. The ECM not only provides the physical and mechanical support for the cells, but also initiates signaling cascades affecting cellular function and tissue homeostasis (Yue, 2014). The crucial role of ECM in tissue biology can be inferred by the fact that the genetic abnormalities of some of its components are associated with a wide range of pathological conditions (Bonnans et al., 2014). Based on the composition, the ECM macromolecules are divided into

two main classes: the proteoglycans, comprised of sulfated glycosaminoglycans that regulate many cellular processes (Iozzo & Schaefer, 2015), and the fibrous proteins such as collagens, elastins, fibronectins and laminins (Alberts et al., 2007). Collagens represent the most abundant fibrous proteins and constitute the main structural component of the ECM. (Rozario & DeSimone, 2010). These components closely interact with the adjacent cells providing a physical link between the cells and the surrounding stroma, but also modulating cell adhesion, migration, proliferation and differentiation (Hynes, 2009). The ECM modulates cell function mainly through the engagement of integrins, a family of cell surface receptors composed of two subunits, alpha and beta, which are arranged in a variety of combinations that confer different binding specificities and signaling properties (Alberts et al., 2002). Additionally, the ECM serves as a reservoir of growth factors, cytokines and bioactive fragments released upon limited proteolysis (Yue, 2014)

Notably, the ECM is constantly shaped and remodeled. However, in tumors the changes are often directed towards extensive alterations that can even promote the early steps of tumorigenesis, as well as cancer progression and metastasis formation (Kai et al., 2019).

2.3.1 The role of ECM in tumor angiogenesis

The role of ECM and its degradation products in angiogenesis is complex and most of the studies focus the attention on their role in affecting EC function. Cancer cells can alter the ECM reshaping fibroblasts towards a tumor-promoting phenotype; the cancer-associated fibroblasts (CAFs) (Kai et al., 2019). CAFs represent one of the main source of collagen whose aberrant deposition of fibrils increases the tumor stoma stiffness (Winkler et al., 2020). Besides collagen, several other ECM proteins are up-regulated during cancer, such as fibronectin which can exert strong pro-angiogenic properties (W. Yi et al., 2016). Similarly, other ECM proteins, like perlecan, biglycan, hyaluronan and laminins are up-regulated in cancers (Andreuzzi, Capuano, et al., 2020) and these changes can affect the function of many cell types including ECs. For instance, increased ECM stiffness triggers VEGFR2 expression thus promoting angiogenesis (Mammoto et al., 2009). Besides the ECM stiffness, the tumor stroma is characterized by an increased expression of catalytic enzymes such as MMPs. In turn, MMPs promote ECM remodeling further inducing angiogenesis by freeing growth factors and active fragments (Mohan et al., 2020). VEGFs and FGFs are among the cytokines that are released

during the ECM processing, and as discussed previously, these molecules play a pivotal role in angiogenesis, lymphangiogenesis and, hence, tumor progression (Fejza et al., 2021).

2.3.2 The EMILIN protein family

EMILINs are a family of ECM glycoproteins distinguished by the presence of a cysteine-rich EMI domain at the N-terminus and, for some of them, also the gC1q-like domain at C-terminus (Colombatti et al., 2012). Based on the arrangement of the major protein domains, EMILINs are clustered in three groups:

- The first group is characterized by the presence of both the EMI domain at the N-terminus and the gC1q domain in the C-terminus. This group includes the major family members that are: Multimerin-1 (Hayward et al., 1991), Multimerin-2 (Sanz-Moncasi et al., 1994), EMILIN-1 (Colombatti et al., 1985) and EMILIN-2 (Doliana et al., 2001)
- The second group consists of only one protein, EMILIN-3, which displays a molecular structure similar to that of the first group, except for the absence of the gC1q domain (Leimeister et al., 2002).
- The third group differs from the first two in terms of molecular structure; the only similarity is the presence of the EMI domain at the N-terminus. This cluster of molecules includes Emu1 and Emu2 (Leimeister et al., 2002; Schiavinato et al., 2012).

The structures and the domains of all four family members of the first group are shown in Figure 5.

2.3.2.1 Multimerin-1

Multimerin-1 is a soluble S-S linked homopolymer stored in the platelets, megakaryocytes and ECs, and it is deposited in the ECM of ECs forming fibrillar structures (Hayward et al., 1991). Multimerin-1 mediates the adhesion of platelets and neutrophiles via the binding to integrin $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ (Adam et al., 2005). Multimerin-1 binds to collagen and von Willebrand factor inducing platelet adhesion and promoting the thrombus formation (Adam et al., 2005). Taken together Multimerin-1 plays an important homeostatic role in controlling platelet aggregation and its consequences.

2.3.2.2 Multimerin-2

Multimerin-2, first named EndoGlyx-1, was identified during a screening for new specific markers of the vascular endothelium, using a monoclonal antibody (mAb H572) that was produced against human umbilical vein ECs (HUVECs) (Sanz-Moncasi et al., 1994). Multimerin-2 is exclusively deposited along all the blood vessels, large and small, of all organs except from hepatic and splenic sinusoids. Likewise, immunohistochemical assays have revealed the presence of Multimerin-2 in the tumor tissues as well, including the so-called "hot spots" areas of angiogenesis (Sanz-Moncasi et al., 1994). The functions and the molecular mechanisms by which Multimerin-2 affects ECs were first described in our laboratory. We in fact demonstrated that Multimerin-2 inhibits ECs migration and counteracts sprouting angiogenesis *in vivo* (Lorenzon et al., 2012). These effects rely on the capability of the protein to sequester VEGF-A, thus preventing the binding of the cytokine to the VEGFR2 receptor and halting its activation (Colladel et al., 2016). Multimerin-2 or its active fragments, once ectopically overexpressed in the microenvironment, impair tumor growth most likely halting its vascularization (Colladel et al., 2016; Lorenzon et al., 2012). Indeed, to allow efficient EC sprouting, Multimerin-2 must be depleted and this occurs through two distinct mechanisms: 1) reduced expression: EC challenged with angiogenic cytokines display decreased Multimerin-2 mRNA levels; 2) increased degradation: during active angiogenesis Multimerin-2 is targeted for degradation by MMP-9 and MMP-2, two metalloproteinases highly activated during vascular sprouting (Andreuzzi et al. 2017). Indeed, immunofluorescent analyses performed on human colorectal cancers display extensive Multimerin-2 degradation in juxtaposition with active MMP-9 (Andreuzzi et al., 2017). Interestingly, tumor associated vessels often lose the expression of Multimerin-2, as observed in gastric cancer (Figure 6) (Andreuzzi et al., 2018), and this may correlate with a decreased vascular efficiency.

Recently, Multimerin-2 was shown to interact with several C-type lectin transmembrane receptors such as CLEC14A, CD93 and CD248 affecting EC and pericyte behavior (Galvagni et al., 2017; Khan et al., 2017; Noy et al., 2015). Indeed, Multimerin-2 is co-expressed with CD93 in the tumor associated vessels and the disruption of the Multimerin-2/CD93 interaction reduces the adhesion and migration of ECs, thus affecting their sprouting capabilities (Galvagni et al., 2017; Tosi et al., 2020).

Altogether, these information about Multimerin-2 could provide new insights in how to effectively target pathological angiogenesis

2.3.2.3 EMILIN-1

EMILIN-1, is a 115 kDa glycoprotein located specifically at the interface between the amorphous elastin surface and microfibrils (Elastin Microfibrils Interface Located proteIN) and was first identified while attempting to isolate elastic tissue-specific glycoproteins (Bressan et al., 1993). EMILIN-1 is highly expressed in the wall of the large vessels and within the connective tissue of several organs (Colombatti et al., 1985). EMILIN-1 mRNA was detected in the blood vessels wall and in the perineuronal mesenchyme at day 8.5 during the mouse embryo development. In addition, EMILIN-1 is expressed in the mesenchyme of lungs and liver and, in the late gestation, in the interstitial connective tissue of smooth muscle-rich tissues (Braghetta et al., 2002). EMILIN-1 expression is important for the regulation of blood pressure. In fact, *Emilin-1*$^{-/-}$ mice are hypertensive, displaying increased blood pressure, augmented peripheral resistance and reduced vessels size. The molecular mechanism underlying these effects involve the blockage of TGF-β maturation and availability (Zacchigna et al., 2006). Additionally, EMILIN-1 displays adhesive and migratory properties for different cell types through the engagement of integrins. More precisely, through the gC1q domain it serves as a ligand for integrin α4β1 and α9β1 (Spessotto et al., 2003). Furthermore, *Emilin-1*$^{-/-}$ mice show defective lymphatic vessels, characterized by hyperplasia, enlargement and irregular pattern (Capuano et al., 2019; Danussi et al., 2008). Likewise, the defective drainage capability of *Emilin-1*$^{-/-}$ mice, impaired the inflammatory resolution during colon carcinogenesis by lymphatic vessels, thus promoting tumor outgrowth (Capuano et al., 2019)

2.3.2.4 EMILIN-2

EMILIN-2 was originally isolated as a novel protein while searching for EMILIN-1 interactors using the globular C1q domain as a bait in a two-hybrid system screening (Doliana et al., 2001). The molecular structure is very similar with that of EMILIN-1, except for the presence of a prolin-rich sequence between the coiled coil region and the collagenous stalk in EMILIN-2 instead of a leucine zipper motif present in EMILIN-1. EMILIN-2 is expressed during the

embryonic development as well as in the adulthood (Braghetta et al., 2004; Doliana et al., 2001). Moreover, EMILIN-2 was found to be a major component of the BM of the cochlea (Amma et al., 2003; Russell et al., 2020). Despite much work needs to be undertaken to better clarify the role of EMILIN-2 in cancer, in our laboratory we identified a prominent role of this molecule in tumor onset and progression. In a sarcoma model, EMILIN-2 was found to exert a tumor suppressive function by triggering the extrinsic apoptotic pathway through the engagement of the death receptors DDR4 and DDR5 (Mongiat et al., 2007). Furthermore, in a breast cancer cell model, EMILIN-2 halted cancer cell growth and migration by down-modulating the Wnt signaling pathway. This occurs through a direct interaction with the Wnt1 ligand, leading to decreased LRP6 phosphorylation and consequently the down-regulation of β-catenin activation resulting in halted viability and migration of breast cancer cells (Marastoni et al., 2014). Importantly, the *Emilin-2* gene is inactivated by methylation in several types of cancer, such as breast, lung and colon suggesting an important role during cancer progression (Hill et al., 2010).

Interestingly, EMILIN-2 exerts contrasting effects in the TME since, while reducing tumor growth *in vivo*, the injection of recombinant EMILIN-2 simultaneously enhanced the vascularization of the tumors growth in nude mice (Mongiat et al., 2010). Mechanistically, EMILIN-2 directly binds the epidermal growth factor receptor (EGFR) expressed by fibroblasts and EC stimulating the production of IL-8 through the activation of the Jak2/STAT3 pathway. In turn, IL-8 enhances the proliferation and migration of ECs. In fact, tumors grown in *Emilin2*$^{-/-}$ animals display reduced vascular density, poor perfusion and increased vascular leakage, negatively impacting on drug delivery and chemotherapy efficacy (Figure 7) (Paulitti et al., 2018). Furthermore, EMILIN-2 exerts an important function also in other tumor models. In fact, we have recently demonstrated that the expression of EMILIN-2 is down-regulated in gastric cancer patients, and this correlates with an altered vascularization of the tumors (Andreuzzi, Capuano, et al., 2020; Andreuzzi et al., 2018).

3 AIMS OF STUDY

Over the past decade, researchers have provided unprecedent insight on different mechanisms and molecular components that switch on angiogenesis to nourish cancer cell growth, with a considerable effort towards the development of effective anti-angiogenic therapies. However, despite the fact that anti-angiogenic therapy has entered the clinics and has been proven valuable in certain settings, the clinical outcome of the patients did not meet the expectations.

Since vascular disruption leads to tumor hypoxia and poor drug delivery, researcher have speculated on the possibility on one side, to reduce the vasculature, and on the other, to readdress the abnormal vessels associated with the tumors towards a normal phenotype. This approach could reduce tumor growth and simultaneously improve the delivery of drugs to the tumor.

Much attention has also been focused on the role of the immune response in affecting formation and remodeling the tumor associated vessels despite the players in the interplay between angiogenesis and inflammation are not completely understood.

Thus, there is an awaited clinical need to better understand the mechanisms regulating angiogenesis to develop new predictive biomarkers to stratify the patients that would benefit from the therapies.

In this context, we have provided both *in vitro* and *in vivo* evidences indicating that the extracellular molecule EMILIN-2 exerts a pro-angiogenic function. However, further investigations were needed in order to shed light on the mechanisms by which EMILIN-2 regulates vascular stability and function and its potential role as a biomarker.

Therefore, the aim of this PhD project was to:

1. Assess the role of EMILIN-2 in the regulation of vascular stability particularly focusing on its function in pericyte recruitment and the interplay between ECs and pericytes.
2. Verify the role of EMILIN-2 in the interplay between the immune environment and angiogenesis.

These two aspects will be separately dealt with under the Results section.

4 RESULTS- Part I

4.1 EMILIN-2 loss affects pericyte coverage in tumor associated vessels

In a recent publication, our group reported that EMILIN-2 induces angiogenesis via the EGF/EGFR pathway. In the same study we demonstrated that the vessels formed in absence of EMILIN-2 are less efficient displaying increased vascular leakage and impaired perfusion (Paulitti et al., 2018). Given that pericytes exert an important function during vessel maturation, we focused our attention on this cell type and, taking advantage of our *Emilin-2*$^{-/-}$ mouse model, we verified the presence of pericytes along the blood vessels. To this end, B16F10 syngenic melanoma tumors grown in *wild type* and *Emilin-2*$^{-/-}$ mice were stained with an anti-a-SMA antibody. These analyses indicated that tumor vessels grown in *Emilin-2*$^{-/-}$ animals were significantly less covered by pericytes compared to those grown in *wild type* mice (Figure 1). Next, to verify the possible involvement of EGF/EGFR pathway in the impaired pericyte coverage, we analyzed the section of tumors treated with the EGFR blocking agent (AG1478). These analyses demonstrated that the loss of pericytes in tumor vessels developed in *Emilin-2*$^{-/-}$ mice did not hinge on EGFR activation since the use of the blocking agent did not exert any significant effect (Figure 1). These evidences prompted us to verify the molecular mechanisms by which Emilin-2 affected pericyte recruitment and vessels stability.

4.2 EMILIN-2 represents a substrate for pericytes' adhesion

Considering that EMILIN-2 is broadly expressed in a variety of tissues and that we have recently found that it is often deposited in close association with tumor vessels (Figure 2), we first hypothesized that it may represent an adhesive substrate for pericytes. Indeed, the adhesion assays indicated that EMILIN-2 represented a good substrate for pericyte adhesion (Figure 3A). Since the main mediators of cell adhesion are integrins, to verify which integrin was involved in the interaction we employed specific blocking antibodies directed towards the main subunits. Out of six blocking antibodies used, the anti-α_5 and anti-α_6 significantly impaired the adhesion of pericytes to EMILIN-2 (Figure 3B, C). Furthermore, the employment of an anti-β_1 subunit blocking antibody indicated that the integrins mediating the adhesion were integrins $\alpha_5\beta_1$ and $\alpha_6\beta_1$, since the adhesion was almost completely abolished (Figure 3D).

The engagement of the integrins leads to activation of intracellular signaling pathways profoundly affecting cell function (Alberts et al., 2002; Silva et al., 2008). To verify if EMILIN-2 triggered integrin activation we analyzed the phosphorylation of the dual kinase complex FAK and Src, which, upon adhesion of pericytes to EMILIN-2, were strongly activated similarly to what occurred upon adhesion to fibronectin, used as positive control (Figure 4). Taken together, these results demonstrated that the adhesion of pericytes to EMILIN-2 is mediated by integrins $\alpha_5\beta_1$ and $\alpha_6\beta_1$ and leads to the activation of the down-stream signaling pathways.

4.3 EMILIN-2 acts as a migratory stimulus for pericytes

Given the prominent role of EMILIN-2 as an adhesive substrate for pericytes, we asked if EMILIN-2 could function as a haptotactic stimulus guiding pericytes towards the vessels. To verify this possibility EMILIN-2 was coated on the underside of transwell's membranes and the cells were let to migrate towards the ECM stimulus (Figure 5A). This experiment demonstrated that EMILIN-2 represented a strong haptotactic stimulus significantly increasing the migration of pericytes (Figure 4B). Furthermore, this effect was integrin $\alpha_5\beta_1$- and $\alpha_6\beta_1$-dependent since the migration of the cells was almost completely abolished by the anti-α_5, anti-α_6 and anti-β_1 blocking antibodies (Figure 5B, C)

4.4 EMILIN-2 elicits the production of cytokines fundamental for pericyte recruitment

During vessel maturation ECs switch on the expression of important cytokines such as, PDGF BB and HB-EGF in order to recruit pericytes which express their cognate receptors on the cell surface (Hoch & Soriano, 2003; Iivanainen et al., 2003; Stratman et al., 2010). The engagement of these receptors ultimately leads to increased pericyte proliferation and migration towards the cytokine stimulus. Thus, we next queried if EMILIN-2 was able to trigger the expression of these cytokines in ECs. To address this point, we treated the HUVECs with recombinant EMILIN-2 and checked the expression of PDGF BB and HB-EGF. We verified that, when challenged with EMILIN-2, ECs increased the production of PDGF-BB and HB-EGF both at the mRNA and the protein level (Figure 6A-C). Since cancer cells shape the TME according to their needs, and are an important source of the above-mentioned cytokines, we next wondered if EMILIN-2 could trigger the expression of these cytokines also in cancer cells. To this end, the gastric cancer cell line AGS was challenged with recombinant EMILIN-2, and, to our surprise we detected higher PDGF-BB and HB-EGF mRNA levels in tumor cells treated with EMILIN-2 (Figure 6E-F). This suggests that the levels of EMILIN-2 within the TME may deeply impact on the functionality of the tumor associated vessels.

4.5 EMILIN-2 promotes the recruitment of pericytes to the vessel sprouts.

Having established the direct and indirect molecular mechanisms by which EMILIN-2 elicits the recruitment of pericytes, we next wanted to determine if an EMILIN-2-rich microenvironment could in truth promote the coverage of new sprouting vessels by this mural cell type. To this end, we exploited a 3D *in vitro* model which closely mimics the vasculature formation and consists of a co-culture of ECs and pericytes embedded in a collagen type I matrix. When placed in culture under this condition ECs would form tubes branching in three dimensions followed by the recruitment of pericytes which eventually promote tube stabilization. 3D cultures were challenged with EMILIN-2 or vehicle and we could detect that, tubes formed in the presence of EMILIN-2 were more regular and formed better branches compared to the vehicle control (Figure 7A). Having recognized the effect of EMILIN-2 in the tube formation, we next aimed to better evaluate its role in the pericyte recruitment by staining the 3D tubes with anti-α- SMA antibody. Indeed, the immunofluorescence analyses indicated

that EMILIN-2 sustained pericyte recruitment along the tubes; the coverage was significantly higher than the vehicle control and comparable to that induced by PDGF-BB (Figure 7B).

4.6 The EC-Pericytes' interconnection is strengthen by EMILIN-2

During angiogenesis, the interaction between ECs and pericytes is fundamental for the formation of functional and mature blood vessels, and the ECM components strongly aids this interaction (Stratman & Davis, 2012). Hence, having established the effect of EMILIN-2 on pericyte recruitment, we aimed to further investigate its role in maintaining the EC-pericyte interconnection. To begin with, we focused our attention on cell-adhesion molecules. As described in the introduction section, N-cadherin represents a key molecule creating an adhesive "zipper" between ECs and pericytes (Dejana, 2004). To verify if EMILIN-2 affected the expression of this molecule, we treated human umbilical vein endothelial cells (HUVECS) and human brain vascular pericytes (HBVP), both separately and in co-culture with recombinant EMILIN-2. Interestingly, in HUVEC cells we observed a significant increase of the N-cadherin expression when challenged with EMILIN-2, compared to PBS control. Similar effects were observed under co-culture conditions (Figure 8).

Further on, to shed light on the molecular mechanisms by which EMILIN-2 affected N-cadherin expression, we focused the attention on the sphingosine-1-phosphate receptor ($S1P_1$); a G-protein-coupled receptor for the bioactive lipid S1P, the role of which was highlighted as pivotal in N-cadherin trafficking and vessel maturation (Paik et al., 2004) (Figure 9A). By treating the HUVEC cells with recombinant EMILIN-2, we checked the expression of this receptor by RT_PCR analysis. Indeed, EMILIN-2 challenged cells expressed higher mRNA levels of $S1P_1$ overtime compared to PBS control (Figure 9B).

Of critical importance during the maturation and stabilization of the newly formed vessels is the synthesis and deposition of the BM. Although the BM represents an integral structure underlying ECs, recently a handful of evidences highlighted the requirement of a heterotypic cell-cell contact for a proper deposition and assembly of the BM (Davis & Senger, 2005; Stratman & Davis, 2012). Taking into account these facts, we aimed to assess if EMILIN-2 could alter the expression of type IV collagen, one of the most abundant BM component. The expression was verified in HUVEC and HBVP cells alone or in co-culture, and, as expected the expression of type IV collagen was higher under the co-culture condition (Figure 9). Interestingly, the presence of EMILIN-2 increased type IV collagen only in ECs when the two cell types were cultured alone; and the expression was higher under the condition where cells were co-cultured in a ratio of 1:2 HBVP/HUVEC, suggesting that EMILIN-2 strengthens the cross-talk between ECs and pericytes in terms of BM deposition (Figure 10)

4.7 The *Emilin-2*$^{-/-}$ phenotype is rescued by 3PO treatment *in vivo*

Having established the role of EMILIN-2 in pericyte recruitment, we aimed to rescue the phenotype *in vivo.* To this end, the B16F10 syngenic melanoma cells were subcutaneously injected in *wild-type* and *Emilin-2*$^{-/-}$ mice followed by the treatment with 3PO, a compound known to stimulate the recruitment of pericytes to the vessels. Following staining of the tumor sections with the anti-α-SMA antibody, the immunofluorescence analyses confirmed a prominent lack of pericytes covering tumor vessels from *Emilin-2*$^{-/-}$ mice (Figure 11). The 3PO treatment slightly improved the coverage of the tumor-associated vessels with pericytes in *wild type* animals, despite the difference was not significant; whereas it successfully ameliorated knockout phenotype (Figure 11).

4.8 Restoring pericyte coverage in *Emilin-2* $^{-/-}$ animals ameliorates drug delivery.

We had previously demonstrated that drug delivery was significantly impaired in *Emilin-2* $^{-/-}$ animals (Paulitti et al). To verify if this flaw could depend at least in part on a poor vascular efficiency due to the aberrant pericyte coverage, we injected tumor-bearing *wild-type* and *Emilin-2*$^{-/-}$ mice with cisplatin 6 hours prior sacrificing the animals. Drug delivery was assessed counting the positive nuclei following staining of the tumor sections with an anti-DNA/cisplatin adducts antibody, which recognizes the guanine-guanine DNA intra-strand crosslinks caused by the drug. As expected, tumors from *Emilin-2*$^{-/-}$ mice were characterized by significantly lower number of cells reached by the drug, whereas treatment with 3PO, slightly, despite not significantly, improved drug delivery in wild type animals, whereas it associated with a significantly higher number of DNA-cisplatin adducts in *Emilin-2*$^{-/-}$ mice (Figure 12). These results suggested that the poor drug delivery in *Emilin-2*$^{-/-}$ mice primarily hinged on an impaired coverage of the vessels by pericytes.

5 RESULTS - Part II

5.1 Loss of EMILIN-2 associates with increased PD-L1 expression

As discussed in the introduction section, in the TME a well-orchestrated interaction between angiogenesis and immune environment occurs. The two processes are intermingled and reciprocally contribute to one another in many ways (Rahma & Hodi, 2019). Recently, unpublished evidences done by our group show that EMILIN-2 not only affects angiogenesis but also contributes in the shaping of the immune environment as well. In particular, we have observed an increased expression of the immunosuppressive molecule (PD-L1) in different tumor models grown in *Emilin-2*$^{-/-}$ mice. Prompted by this information, we wanted to verify if the effects of EMILIN-2 in the immune environment could impact also tumor vasculature. To this end, we subcutaneously injected *wild-type* and *Emilin-2*$^{-/-}$ animals with B16F10 syngenic melanoma cells and treated them with an anti-PD-L1 blocking antibody or the isotype control. As previously observed, possibly due the pro-angiogenic role of EMILIN-2 (Paulitti et al., 2018), tumors grew more efficiently in *wild type* than in *Emilin-2* $^{-/-}$ animals (Figure 13A). Interestingly, the administration of the anti-PD-L1 antibody reduced tumor growth by 20.3% in wild type mice compared to the control (Figure 13B), whereas the efficacy of the treatment was considerably superior in *Emilin-2* $^{-/-}$ mice, accounting for a 72.7% reduction in the tumor growth (Figure 13B).

Next, the PD-L1 mRNA levels were assessed in tumors grown in *wild-type* and *Emilin-2*$^{-/-}$ animals and we found significantly higher levels in tumors from *Emilin-2*$^{-/-}$ mice (Figure 14A). This finding was further confirmed by immunofluorescence analyses which indicated that the PD-L1 protein levels were 25-fold higher in tumors developed in *Emilin-2*$^{-/-}$ mice compared to wild type-derived tumors (Figure 14B).

5.2 The Blockage of PD-L1 Promotes Tumor Angiogenesis and Pericytes Recruitment Rescuing the Vascular Defects in Emilin-2$^{-/-}$ Mice

It is prominent that vessel normalization can improve the efficacy of immune check- point blockade; however, less is known about the impact of PD-1/PD-L1 inhibition in tumor vascularization. Taking in consideration our previous results that the abolishment of EMILIN-2 leads to altered vascularization, lower numbers of vessels, poor perfusion and impaired pericyte coverage we investigated the effect of the anti-PD-L1 treatment on tumor vasculature.

Strikingly, the anti-PD-L1 treatment in *Emilin-2*$^{-/-}$ mice, triggered tumor vascularization, as assessed by CD31 staining (Figure 15A, B). Notably, PD-L1 inhibition not only promoted tumor vascularization, but was also associated with increased recruitment of pericytes to levels comparable with those observed in *wild type* animals, as assessed by α-SMA staining (Figure 15C, D)

Furthermore, tumor hypoxia arises from inadequate oxygenation due to a poor aberrant and unfunctional vascularization. To verify the levels of tumor hypoxia under these experimental conditions, the tumor sections were stained with GLUT1. The immunofluorescence analyses indicated that tumors from *Emilin-2*$^{-/-}$ mice were characterized by higher hypoxic levels compared to those from *wild type* animals. However, in accordance with the previous observations, the anti-PD-L1 blockage was efficient in ameliorating the oxygenation of the tumors in *Emilin-2*$^{-/-}$ mice (Figure 16A, B).

Taken together the results presented in this thesis suggest the possible use of EMILIN-2 as a biomarker. On one side, EMILIN-2 regulates the vessels stability, thus improving drug delivery. On the other hand, the loss of EMILIN-2 correlates to a better response to immune-check inhibitors due to higher expression of PD-L1. Importantly, the exceedingly abnormal vasculature associated with low EMILIN-2 -expressing tumors was improved by the immune check point inhibition which represents an additional benefit in these patients.

6 DISCUSSION

In this thesis work, we highlighted some of the multiple roles exerted by EMILIN-2 in the TME affecting vessel maturation and efficiency. Based on the results presented in this study and on the pivotal importance of vascular efficiency in affecting the delivery of therapeutic drugs, we hypothesize that EMILIN-2 may represent a valuable biomarker to stratify oncologic patients who would benefit from conventional therapies.

During angiogenesis, the formation of the vessels is followed by the recruitment of pericytes, prompting basement membrane deposition and vascular maturation and stabilization (Stratman et al., 2009). In this context, the ECM components influence the ECs and pericyte behavior through different mechanisms (Jain, 2003). Since we observed that tumor-associated vessels grown in the absence of EMILIN-2 displayed aberrant pericyte coverage, the first part of this thesis was focused in studying the mechanisms by which EMILIN-2 affected pericyte recruitment and the crosstalk between ECs and pericytes.

Indeed, the hypothesis that EMILIN-2 might influence vascular stability and efficiency was set after our previous observation where a significant impairment of vessel perfusion and increased vascular leakage were reported in tumor-bearing *Emilin-2* $^{-/-}$ mice. Interestingly, the analyses carried out on human colorectal cancer samples indicated that its deposition is often adjacent to the blood vessels further underpinning our hypothesis that EMILIN-2 plays a pivotal role in supporting the maturation and stabilization of vasculature. Importantly, the Emilin-2 expression was variable among patients, suggesting that vascular efficiency could be highly compromised in tumors expressing low levels of EMILIN-2.

Indeed, we have previously found that EMILIN-2 plays an important role during vascularization overall exerting a pro-angiogenic function (Marastoni et al., 2014). We have in fact demonstrated that EMILIN-2 enhances EC proliferation and migration through the activation of the EGF/EGFR pathway, one of the main drivers of angiogenesis (Paulitti et al., 2018). On the contrary, in this study we found that the EMILIN-2 -dependent recruitment of pericytes does not hinge on the EGF/EGFR signaling pathway, since the use of an EGFR blocking agent in the murine melanoma model did not affect the coverage of the vessels by pericytes. This, suggested that EMILIN-2 impacted on pericyte recruitment through a different cell-specific mechanism.

Pericyte adhesion and migration through the ECM allows the proper recruitment of these cells along the newly formed blood vessel. In this view, we investigated if EMILIN-2 may influence the pericyte recruitment through its adhesive properties. Indeed, EMILIN-2 was shown to act as an adhesive substrate. Importantly, we demonstrated that this role of EMILIN-2 is exerted

through the engagement of integrin α5β1 and α6β1 and the subsequent activation of the downstream molecules FAK and Src kinases (Schaller & Parsons, 1994). The integrins $\alpha_5\beta_1$ and $\alpha_6\beta_1$ are two crucial integrins involved in pericyte physiology. In fact, integrin subunit α_6 is already known to play a fundamental role in the envelopment of the blood vessels with pericytes, as demonstrated upon its depletion, which leads to prominent vascular instability (Reynolds et al., 2017). The role of the α_5 subunit is less characterized in this context, since the genetic ablation of integrin $\alpha_5\beta_1$ is lethal due to embryonic vascular defects. However, its role in pericytes is highlighted by its peculiar expression during the differentiation of mesenchymal cells into pericytes (Silva et al., 2008). Considering the fact that EMILIN-2 expression is in association with the vasculature we can speculate that it might represent a path towards which pericytes migrate, and that its absence in the TME may hinder this process. In fact, in accordance with this hypothesis, our results indicate that EMILIN-2 represents a haptotactic stimulus that pericytes sense through integrin $\alpha_5\beta_1$ and $\alpha_6\beta_1$.

An important stimulus for the proper recruitment of pericytes is represented by soluble factors secreted by ECs (Aguilera & Brekken, 2014). EMILIN-2 affects the expression of a number of angiogenic cytokines (Andreuzzi, Fejza, et al., 2020), however prior to this investigation, the influence on chemokines affecting pericyte behavior has never been investigated. In this work, we found that ECs challenged with EMILIN-2 increase the expression of important cytokines in this context, such as PDGF-BB and HB-EGF. In fact, these two cytokines display a crucial role in the recruitment of mural cells, the effect of which is mediated through the signaling via the PDGFRβ and the ErbB1/ErbB2 receptors, respectively, expressed by mural cells (Iivanainen et al., 2003; Zhang et al., 2007). Importantly, PDGFRβ pathway was shown to be interconnected with integrin signaling (Heldin, 2013). More precisely, the genetic ablation of pericyte integrin $\alpha_6\beta_1$ was correlated with a reduced expression of PDGFRβ and, therefore, with a diminished response to PDGF-BB (Reynolds et al., 2017). In another study, it was reported that the PDGF-BB-induced phosphorylation of PDGFRβ occurs in an $\alpha_5\beta_1$-integrin-dependent manner and that the interaction between the two receptors is pivotal for mesenchymal cells at sites of vascular remodeling (Veevers-Lowe et al., 2011). Thus, it can be inferred that a similar mechanism could be engaged by pericytes. Given all these observations, we can speculate that EMILIN-2 may represent a key ECM cue promoting the synergism between the two signaling pathways through the engagement of integrin $\alpha_6\beta_1$ and $\alpha_5\beta_1$ thus triggering the expression and activation of the PDGFRβ. Yet more investigation is needed to further confirm this hypothesis.

Once pericytes have reached the newly formed vessels a close interconnection with ECs is engaged to stabilize the vasculature (Potente et al., 2011) and in this study we have demonstrated that EMILIN-2 plays a role also in this context. During vessel maturation the adhesion molecule N-cadherin plays a key role in creating a tight junction between ECs and pericytes (Dejana, 2004) and its expression on the EC surface and trafficking are regulated by the sphingosine 1-phosphate receptor ($S1P_1$) (Paik et al., 2004). EMILIN-2 was shown not only to enhance N-cadherin expression in ECs cultured alone or with pericytes, but also to induce an increase of $S1P_1$ expression, suggesting that the EMILIN-2 -dependent increase of N-cadherin expression is mediated in a $S1P_1$-dependet manner. These results suggest that the expression of EMILIN-2 in the TME is important not only for the recruitment of pericytes but also for the stabilization of the EC-pericyte interaction.

Following pericyte recruitment and intercellular interactions, another important step in vessel maturation is the synthesis and assembly of vascular BM components which is promoted by a close contact between ECs and pericytes (Davis & Senger, 2005; Stratman et al., 2009; Stratman & Davis, 2012) and strongly affected by microenvironmental components. Among these components we found that also EMILIN-2 affects the BM deposition. In fact ECs challenged with EMILIN-2 enhance the expression of type IV collagen, the main BM component (Tanjore & Kalluri, 2006). Importantly, although more experiments are needed to confirm, we observed an increase of type IV collagen by EMILIN-2 when the cells were co-cultured in a ratio of 1:2 HBVP/HUVECs.

These results further highlighted the role of EMILIN-2 as an important regulator of vascular stability and efficiency. Indeed, restoring pericyte coverage in the *Emilin-2*$^{-/-}$ animals through the use of 3PO improved the drug delivery in these animals, suggesting that patients displaying low EMILIN-2 expression could suffer from poor drug delivery and fail to successfully respond to the treatments.

Furthermore, ample evidences have underlined the importance of vessel normalization upon immunotherapies, yet little is known how immunosuppressive checkpoint inhibitors affect tumor vascularization. Previously, our group has observed an increased expression of the immunosuppressive molecule PD-L1 in absence of EMILIN-2 in different tumor types (unpublished data), suggesting that in tumors characterized by low EMILIN-2 levels, the altered vascularization can be accompanied by a more immunosuppressive microenvironment. Indeed, the anti-PD-L1 treatment of syngeneic melanoma tumors was highly effective in *Emilin-2*$^{-/-}$ compared to *wild type* animals, possibly due to higher expression of PD-L1. Strikingly, the anti-PD-L1 inhibitor led to tumor vessels normalization, with a great

improvement of the exceedingly abnormal vasculature characterizing *Emilin-2*$^{-/-}$ derived tumors. In fact, we observed not only an increased number of vessels but, importantly, also a higher pericyte coverage that reduced the intra-tumor hypoxia overall. To our knowledge, this is the first time that this effect is observed in melanoma, despite few sources describe the role of PD-L1 inhibition in tumor vessels normalization (Tian et al., 2017). From the literature it is known that the inhibition of checkpoint molecules results in an improved activation of Th1 cells and to the consequent release of IFN-γ that mediates the anti-tumorigenic immune response and may also act towards vessel normalization. Thus, it is possible to speculate that the altered angiogenesis due to EMILIN-2 loss could be overtaken by the activation of Th1 lymphocytes which, in turn, among other cytokines, secrete IFN-γ, with a consequent impact on melanoma-associated vascular normalization, as previously demonstrated (Tian et al., 2017).These observations further support the notion that the immune and vascular systems are tightly interconnected and that combining anti-angiogenic and immune therapies may represent a promising approach to improve the survival of the patients (Rahma & Hodi, 2019). However, the identification of subgroups of patients that will most likely benefit from immunotherapy is an ongoing challenge, as indicated by the great deal of effort that has been made to draw expression-based signatures with predictive value.

Considering that the expression of EMILIN-2 is variable in tumors, as documented in different cohorts of gastric (Andreuzzi et al., 2018; Andreuzzi, Fejza, et al., 2020) and colon cancer patients (unpublished results), and taking into account the roles of EMILIN-2 reported in this thesis, we can speculate that EMILIN-2 could represent a potential biomarker to predict the efficacy of conventional and targeted therapies, as well as immunotherapy.

In conclusion, taken together these results suggest that the efficacy of the treatments could be superior in patients characterized by high EMILIN-2, due to optimal drug delivery. On the other hand, patients displaying low EMILIN-2 levels not only would respond better to immunotherapy due to higher PD-L1 expression, but they would also benefit from the positive impact of the therapy in promoting vascular stability.

7 MATERIALS AND METHODS

7.1 Cell Culture

Human Umbilical Vein Endothelial Cells (HUVEC) were isolated from umbilical cord vein as previously described (Jaffe et al., 1973). Cells were then cultured in ECM medium (Science Cell, Carlsbad, CA, USA), enriched with 5% Fetal Bovine Serum (FBS), 1% of Endothelial Cell Growth Supplement (ECGS) and 100 I.U./mL of penicillin/streptomycin (PS) (Science Cell). Human brain vascular pericytes (HBVP) were purchased from Science Cell. They were cultured in Pericyte Medium with 2% FBS, 1% Pericyte Growth Supplement and 100 I.U./mL of PS (Science Cell). The AGS**,** 293-EBNA (E293) and B16F10 cells were ere obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). AGS cells were cultured in F-12 medium (Gibco, Milan, Italy) containing 10% fetal bovine serum (FBS; Gibco, Milan, Italy). B16F10 and 293-EBNA cells were cultured in Dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco, Milan, Italy); for 293-EBNA cells 250μg/ml G418 were added.

All cells were maintained in humified incubator at 37°C and 5% CO_2, and regularly checked for mycoplasma contamination using MycoAlertTM Mycoplasma Detection Kit (Lonza).

7.2 Antibodies and reagents

The monoclonal anti-human CD31 antibody was from Invitrogen (Milan, Italy) whereas the anti-mouse CD31 was purchased from Abcam (Cambrige, UK). The anti-αSMA antibody, anti-integrin blocking anti-α6, anti-PDGF-BB, N-cadherin antibody and anti-collagen type IV were purchased from Abcam (Cambrige, UK). The Ni-NTA agarose was purchased from QIAGEN (Milan, Italy). The secondary Alexa Fluor 488- and 546-conjugated antibodies, TO-PRO- 3 and HB-EGF antibody were from Invitrogen (Milan, Italy). Secondary HRP-conjugated antibodies were from Amersham (Milan, Italy). The anti-phospho FAK (Y397), anti- FAK, anti-phospho Src and anti-Src antibodies were from Cell Signaling Technologies (Danvers, USA). The anti-GLUT1, anti-Cisplatin DNA adducts antibody, the anti-integrin blocking anti-α1, -α2, -α4, and -α5, anti-GLUT1 antibody and PFKFB3 inhibitor- 3PO were from Merck Millipore (Milan, Italy). The Fibronectin, Collagen type I and Poly-L-lysine were purchased from Sigma Aldrich (Missouri, USA). The anti-integrin β1 antibody was from Beckman Coulter (Miami,USA). The FuGene6 reagent was purchased from Promega (Milan, Italy). The in vivo anti-mouse PD-L1 antibody and the rat IgG2b isotype control were from BioXcell

(Lebanon, NH, USA). Anti-vinculine was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Tyrphostin AG-1478 was from Sigma-Aldrich (Milan, Italy).

7.3 RNA extraction, retrotranscription and Real-Time PCR

RNA extraction from cells was performed using TRIzol lysis reagent (Invitrogen) followed by chloroform phase separation and isopropanol precipitation. RNA quantification was obtained using the Nanodrop spectrophotometer and its quality assessed by agarose gel electrophoresis. Retrotranscription was performed with the AMV-RT enzyme (Promega) in the presence of the Random Primer mix (Promega), according to manufacturer instructions. After determination of primer specificity and efficiency, quantitative Real-Time PCR was carried out with iQTM SYBR® Green Supermix (Bio-Rad) and BIORAD CFX96 TouchTM Real-Time PCR Detection System. Primer sequences are listed in Table 1.

Target gene	**Oligo sequences**
h_PDGF-BB	Fw: AAGTGTGAGACAGTGGCAG
	Rev: GCTTGAATTTCCGGTGCTTG
h_HB-EGF	Fw: TTATCCTCCAAGCCACAAGCA
	Rev: AGCCCCTTGCCTTTCTTCTTT
h_S1P1 receptor	Fw: TTGAGCGAGGCTGCGGT
	Rev: TCCAGACGAACGCTAGAGGG
h_GAPDH	Fw: GAGAGACCCTCACTGCTG
	Rev: GATGGTACATGACAAGGTGC
m_PD-L1	Fw: GGAATTGTCTCAGAATGGTC
	Rev: GTAGTTGCTTCTAGGAAGGAG
m_β-Actin	Fw: CTGTCGAGTCGCGTCCACC
	Rev: ATCGTCATCCATGGCGAACTG

Table 1. *List of primers used for Real-Time PCR. For each target genes are reported the corresponding sequences of forward (Fw) and reverse (Rev) primers.*

Since the primer efficiency was ~100%, the $2^{-\Delta\Delta Ct}$ method was used for analysis.

7.4 Western Blot

Cells were lysed in ice-cold lysis buffer (1 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% TrytonX100, 0,1% SDS, 0,1% Na Deoxycholate) supplemented with 25 mM NaF, 1 mM DTT, 1 mM Na_3VO_4 and the protease inhibitors cocktail (Roche). Protein extracts were prepared for electrophoresis with Laemmli Sample Buffer and the samples boiled 10 minutes at 95°C. Samples were loaded in 4-20% Criterion Precast Gels (Bio-Rad) for molecular weight separation and transferred into Hybond-ECL nitrocellulose membranes (Amersham, GE-Healthcare). Membrane saturation was performed with 5% BSA in TBST (100mM Tris-HCl pH 7.5, 0,9% NaCl, 0,1% Tween 20) for 1 hour at room temperature. Primary antibodies were prepared in 5% BSA in TBST and incubated overnight at 4°C. The anti-PDGF-BB antibody was used at a concentration of 0.5µg/mL; the anti-HB-EGF, anti-vinculin, anti-phospho-FAK (Y397), anti-FAK and anti-Src were used at the final dilution of 1:1000; anti-phospho Src (Y461) was used at 1:500 final dilution. After incubation with the secondary antibodies the membranes were developed using the ChemiDoc Touch Imaging System (Bio-Rad) or the Odyssey infrared imaging system (Li-COR Biosciences).

7.5 Haptotaxis

For the haptotaxis assays, 8 µm ® membranes of the transwells (Costar, NY, USA) were coated on the underside with 10 µg/ml recombinant EMILIN-2 or BSA as a control. After 1.5 h at 37°C, membranes were blocked with BSA. Pericytes were starved for 3 h and placed on top of the membrane (7.5 x 10^4 cells/well) in serum free DMEM. For α integrin subunits screening, 5 µg/ml of the anti-α1, -α2, -α3, -α4, -α5 or -α6 blocking antibodies were used. Whereas for β integrin subunit 1 µg/ml anti-integrin β1antiboy was used. Following an overnight migration, cells on top of the membrane were removed with a cotton swab, and the cells on the bottom were fixed, stained with crystal violet and counted under the microscope.

7.6 Cell adhesion and impedance measurements

Cell adhesion assays were carried out in 96 well plates coated overnight at 4°C with 10µg/mL of EMILIN-2 or Collagen Type I. Plates were air-died at room temperature under the tissue culture hood and $2x10^4$ serum starved HBPV cells were plated in serum free media supplemented with 0,1% BSA. Adhesion was performed in humidified incubator at 37°C for 1

hour. Following Crystal Violet staining, cells were counted under microscope. To quantitatively monitor cell behavior in real-time, we adopted the xCELLigence Real-Time Cell Analyzer dual plate instrument (Roche) which measures the electrical impedance caused by cell attachment and proliferation and expressed as the cell index, an arbitrary measurement defined as (Rn−Rb)/15, in which Rb is the background impedance of the well measured with medium alone, and Rn is the impedance of the well measured at any time (t) in the presence of cells. Thus, the cell index is a reflection of overall cell number, attachment quality, and cell morphology that change as a function of time. The Real-Time Cell Analyzer dual plate instrument was placed in a humidified incubator maintained at 37°C and 5% CO2. For adhesion experiments, the E-plates 96 were precoated over night at 4°C with recombinant EMILIN-2(10 μg/ml) or BSA, and cells were then seeded at 50,000 cells/well in FCS-free medium. For α integrin subunits screening, 5 μg/ml of the anti-α1, -α2, -α3, -α4, -α5 or -α6 blocking antibodies were used. Whereas for β subunit, 1 μg/ml of anti-integrin β1 was used. Cells were monitored once every 5 min for 2 h. Data analysis was performed using Real-Time Cell Analyzer software (version 1.2) supplied with the instrument.

7.7 Adhesion assay for the integrin pathway analysis

For the integrin pathways analysis, plates were coated with 3,5 μg/well of EMILIN-2, Fibronectin, Collagen type I and Poly-L-lysine as control, and incubated at 37°C and 5% CO_2 for 1 hour. 300.000 HBVP cells for each condition were detached with EDTA 0.5mM and were serum starved for 3 hours in 37°C and 5% CO_2 incubator. Cell were then incubated for 10 minutes with 5 μg/ml of the anti-α5 and -α6 blocking antibodies or 1 μg/ml of anti-integrin β1 antibody prior to plating into the coated wells. The plate was briefly spined down to allow the contact of the cells with the substrates and after 30 minutes the cells were lysated and Western Blot analyses was performed.

7.8 Protein Purification

E293 cells were transfected using FuGene6 reagent (Promega, Milan, Italy). E293 cells transfected with the pCEP-Pu-EMILIN-2 constructs were then selected with 250 g/ml G418 and 0.5 g/ml puromycin. Confluent E293 cells were then incubated in serum-free medium for 48 hours to further collect the media and the proteins were purified with Ni-NTA beads; the purity was assessed through Blue Coomassie staining.

7.9 Co-culture experiments and cell stimulation

For the evaluation of N-cadherin expression, 300.000 HUVEC and HBVP cells were plated alone or 100.000 HBVP cells and 200.000 HUVEC cells were co-cultured on cover glass slides and treated with 3 μg/ml of recombinant EMILIN-2 or PBS as a control for 24 hours. Glasses were then fixed and proceeded with immunofluorescence staining.

For Collagen type IV expression, 300.000 HUVEC and HBVP cells were plated alone or 100.000 HBVP cells and 200.000 HUVEC cells were co-cultured in 6 well plates. After reaching confluency, cells were serum starved for 3 hours and treated with 3 μg/ml of recombinant EMILIN-2 or PBS as a control for 24 hours. Cells were then lysated and Western blot analyses was performed.

7.10 3D tube formation model

The formation of the tubes in a 3D model was done following the protocol from ScienCell. Briefly, for each condition one 75 μl of gel dot was required. Each gel dot comprises of Collagen type I, water and specific buffers provided in the kit. The cells were added to the mixture of 75 μl gel to the final proportion of 7,5 x 10^4 HUVEC/ 1,5 x 10^4 HBVP. The gel dots were plated in 24 well from ibidi GmbH (Germany). Once the gel dots have been plated, they were left for 5 minutes under the hood undisturbed for a better polymerization and then incubated at 37°C and 5% CO_2 for 1 hour. After the polymerization, 700 μl of 3D medium provided with the kit was gently added to each well. The medium was changed every other day for 5 days until the end of the experiment when the gels were fixed and proceeded with the IF.

7.11 Immunofluorescence

Treated cells were seeded and grown on cover glass slides placed in 6 well plates. Once reached confluency, cells were fixed in 4% paraformaldehyde (PFA) (in PBS) for 15 minutes at room temperature. After 3 washes with PBS (5 min each), permeabilization was performed with 0,1% Triton X-100 for 5 minutes and rinsed with PBS. Cells were next incubated with 2% BSA for 1 hour at room temperature to block aspecific binding followed by overnight incubation at 4°C with primary antibodies (anti-N-cadherin 1:100). Secondary antibodies were added at the dilution 1:200 together with TO-PRO-3 1:500 and phalloidin conjugated with Alexa Fluor 546, to stain actin cytoskeleton in cells, and incubated for 1 hour at room temperature.

Immunofluorescence was acquired with a Leica TCS SP8 Confocal System. Fluorescence intensity and quantification were evaluated by means of the Volocity software.

Immunofluorescence on tumor samples was carried out on serial cryostat section of 7μM or 10μM obtained from OCT embedded tissues. Slices were collected on positively charged glass slides (BDH 56 Super-Frost Plus), air dried, washed in PBS and then fixed with 4% PFA (in PBS) for 15 minutes at room temperature. Following 3 washes with PBS, sections were permeabilized with 0,3% Triton X- 100 for 5 minutes and blocked with 2 % BSA for 1 hour at room temperature. Primary antibodies were incubated overnight at 4°C (anti- EMILIN-2 1:20; anti-CD31 1:20; anti-αSMA 1:100; anti-Cisplatin DNA adducts 1:250; anti- PD-L1 1:100 and anti-Glut1 1:100). Secondary antibodies were added at the dilution 1:200 together with TO-PRO-3 1:5000 and incubated for 1 hour at room temperature. Following 3 washes with PBS cover glasses and sections were mounted with Mowiol supplemented with 2,5% 1,4 diazabicyclo-(2,2,2)-octane (DABCO) to preserve fluorescence. Immunofluorescence was acquired with a Leica TCS SP8 Confocal System. Fluorescence intensity and quantification were evaluated by means of the Volocity software.

For the 3D immunofluorescence, the 3D gel dots were fixed in the multi well with 2% PFA for 20 minutes at room temperature. Washes were made with 100 mM glycine 10-15 min each. The gel dots were permeabilizated with 0.5% Triton X-100 for 10 minutes at 4°C and blocked with 5% BSA in IF buffer (1X PBS containing 0.1% BSA, 0.2% triton X-100, 0.05 % Tween-20) for 1 hour at RT. The primary antibodies (anti-CD31 1:20, anti-SMA 1:100) were diluted in IF buffer added of 5% BSA overnight at 4°C. Secondary antibodies were used at a dilution of 1:200 and incubated for 45 min at RT. The 3D gel dots were then washed with IF buffer and put in PBS. For the visualization of the nuclei the dots were incubated with DAPI for 10 minutes at RT before the acquisition with EVOS FL auto 2 Cell Imaging System. The quantification and intensity were evaluated with ImageJ 2 software.

7.12 In vivo tumor growth.

Six weeks old wild type and *Emilin-2*$^{-/-}$ C57BL/6N mice were subcutaneously injected in both flanks (5×10^5 cells/flank) with B16F10 melanoma cells resuspended in 100 μL of DMEM without phenol red (Gibco, Milan, Italy). At a size of ~30 mm^3, mice were injected intraperitoneally with 10 mg/kg AG-1478 or vehicle and sacrificed after 12 days. For rescue experiments, *wild type* and *Emilin-2*$^{-/-}$ C57BL/6NCrl mice were injected with B16F10 melanoma cells (5×10^5 cells/flank). In the third day following the injection 3PO (25mg/kg)

or DMSO as control were intraperitoneally injected. The injection was repeated every other day until the end of the experiment. For drug delivery, after the tumor reached ~100 mm^3 size, mice were intraperitoneally injected with 10mg/kg of Cisplatin and sacrificed 6 hours later. For the anti-PD-L1 treatment, six weeks old *wild-type* and *Emilin-2*$^{-/-}$ C57BL/6N mice were subcutaneously injected in both flanks (5×10^5 cells/flank) with B16F10 melanoma cells. The treatment with 9 mg/kg of anti-PD-L1 and anti-IgG2b isotype control antibodies started 7 days following the cell injection and was performed twice a week for 20 days. Tumor growth was measured every 2 days using caliper. At the end of the experiment, tumors were excised and included in OCT for subsequent immunofluorescence analyses.

The animals were housed in the dedicated facility and kept at a constant temperature (23 °C) and humidity (~ 50%) on a 12-h light/dark cycle with ad libitum access to water and food.

7.13 Study approval

For this study 19 patients with colorectal cancer were consecutively enrolled. Written informed consent was obtained from each patient on the day of the procedure. The methodologies conformed to the standards set by the Declaration of Helsinki. This study was approved by the Institutional Board of CRO-IRCCS, National Cancer Institute of Aviano (PN), Italy (IRB no. CRO-2014-03). Laboratory and pathological results were collected by means of the Hospital database.

All the in vivo studies were approved by the Institutional Ethics Committee of the CRO of Aviano and the Italian Ministry of Health and the principle of the '3 Rs' (Reduction, Refinement and Replacement) according to the European guidelines were adopted.

7.14 Statistical analysis

Statistical analyses were performed using SigmaPlot or GraphPad Prism software. All values were represented as the mean ± standard deviation, obtained from at least 3 independent experiments. The number of experimental replicates used are reported in corresponding figure legends. Statistical significance was determined using the two tails Student's t- test. One way ANOVA test was used for multiple comparisons. Differences were considered statistically significant when $P \leq 0{,}05$.

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9 PUBLICATIONS

1. **Deregulated expression of Elastin Microfibril Interfacer 2 (EMILIN2) in gastric cancer affects tumor growth and angiogenesis.**
   Eva Andreuzzi, Albina Fejza, Alessandra Capuano, Evelina Poletto, Eliana Pivetta, Roberto Doliana, Rosanna Pellicani, Andrea Favero, Stefania Maiero, Mara Fornasarig, Renato Cannizzaro, Renato V Iozzo, Paola Spessotto, Maurizio Mongiat. -Matrix Biol Plus. 2020 Feb 19;6-7:100029. doi: 10.1016/j.mbplus.2020.100029. eCollection 2020 May.

2. **Role of Extracellular Matrix in Gastrointestinal Cancer-Associated Angiogenesis.**
   Eva Andreuzzi, Alessandra Capuano, Evelina Poletto, Eliana Pivetta, Albina Fejza, Andrea Favero, Roberto Doliana, Renato Cannizzaro, Paola Spessotto, Maurizio Mongiat. -Int J Mol Sci. 2020 May 23;21(10):3686. doi: 10.3390/ijms21103686.

3. **Multimerin-2 orchestrates the cross-talk between endothelial cells and pericytes: A mechanism to maintain vascular stability.**
   Albina Fejza, Evelina Poletto, Greta Carobolante, Lucrezia Camicia, Eva Andreuzzi, Alessandra Capuano, Eliana Pivetta, Rosanna Pellicani, Roberta Colladel, Stefano Marastoni, Roberto Doliana, Renato V Iozzo, Paola Spessotto, Maurizio Mongiat. -Matrix Biol Plus. 2021 May 28; 11:100068. doi: 10.1016/j.mbplus.2021.100068. eCollection 2021 Aug.

4. **ECM Remodeling in Squamous Cell Carcinoma of the Aerodigestive Tract: Pathways for Cancer Dissemination and Emerging Biomarkers.**
   Albina Fejza, Lucrezia Camicia, Evelina Poletto, Greta Carobolante, Maurizio Mongiat, Eva Andreuzzi. Cancers. 2021 Jun 2;13(11):2759. doi: 10.3390/cancers13112759.

5. **The Efficacy of Anti-PD-L1 Treatment in Melanoma Is Associated with the Expression of the ECM Molecule EMILIN2.**
   Albina Fejza, Maurizio Polano, Lucrezia Camicia, Evelina Poletto, Greta Carobolante, Giuseppe Toffoli, Maurizio Mongiat, Eva Andreuzzi. Int J Mol Sci. 2021 Jul 13;22(14):7511. doi: 10.3390/ijms22147511.

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Estratto per riassunto della tesi di dottorato

Studente: Albina Fejza matricola: 956462

Dottorato: in Science and Technology of Bio and Nanomaterials

Ciclo: XXXIV

Titolo della tesi[1] : **EMILIN-2 as a regulator of vascular efficiency**

Abstract (English):

Angiogenesis, the formation of the new blood vessels from pre-existing vasculature, is a hallmark of cancer and is finely regulated by several cytokines, growth factors and by extracellular matrix components. Moreover, the immune environment shapes the formation and remodeling of tumor associated vessels, despite the players are not completely unveiled. In this contest, we found that the extracellular matrix glycoprotein EMILIN-2 displays a prominent role in promoting vessel maturation and stabilization by serving as an adhesion substrate and haptotactic stimulus for pericytes, inducing the production of PDGF- PDGF-BB and HB-EGF on endothelial cells, and by promoting the interconnection between endothelial cells and pericytes through the increase of N-cadherin expression and favoring the deposition of collagen type IV. Finally, we provide evidences that EMILIN-2 may represent a molecular bridge between the angiogenesis and the immune response, since EMILIN-2 loss associates with increased PD-L1 expression which leads to improved immunotherapy efficacy. Importantly, the therapy restored vascular stability thus resulting in decreased tumor hypoxia.

Taking into account the roles of Emilin-2 reported in this study, we envision that the analysis of EMILIN-2 expression may serve to predict the efficacy of cancer therapy.

[1] Il titolo deve essere quello definitivo, uguale a quello che risulta stampato sulla copertina dell'elaborato consegnato.

Abstract (Italian):

L'angiogenesi, la formazione di nuovi vasi sanguigni dal sistema vascolare preesistente, è una caratteristica del cancro, finemente regolata da diverse citochine, fattori di crescita e componenti della matrice extracellulare. Inoltre, l'ambiente immunitario influenza la formazione e il rimodellamento dei vasi associati al tumore, sebbene i fattori eziologici non siano del tutto noti. In questo contesto, abbiamo scoperto che la glicoproteina della matrice extracellulare EMILIN-2 ricopre un ruolo importante nel promuovere la maturazione e la stabilizzazione dei vasi, fungendo da substrato di adesione e stimolo aptotattico per i periciti.Inoltre, EMILIN-2 induce la produzione di PDGF-PDGF-BB e HB-EGF dalle cellule endoteliali, promuove l'interconnessione tra cellule endoteliali e periciti attraverso l'aumento dell'espressione di N-caderina e favorisce la deposizione di collagene di tipo IV. Infine, abbiamo dimostrato che l'EMILIN-2 può rappresentare un ponte molecolare tra l'angiogenesi e la risposta immunitaria, poiché la perdita di EMILIN-2 è associata ad una maggiore espressione di PD-L1 che porta a una migliore efficacia dell'immunoterapia. È importante sottolineare che la terapia ha ripristinato la stabilità vascolare con conseguente diminuzione dell'ipossia tumorale.

Tenendo conto dei ruoli dell'EMILIN-2 riportati in questo studio, riteniamo che l'analisi dell'espressione dell'EMILIN-2 possa servire a predire l'efficacia della terapia oncologica.

Firma dello studente

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